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14. ABSTRACT

Stromal-epithelial interactions regulate breast cell fate via integrin-growth factor receptor (GFR) interactions that activate tyrosine kinases that are tempered by protein tyrosine phosphatases (PTP). Using a series of molecular screening approaches we profiled PTP expression in normal, transformed and phenotypically-reverted breast tissue and identified the Band 4.1 PTPs MEG1 and D1 as candidate PTP metastasis suppressors. Our studies have implicated two Band 4.1 PTPs MEG1 and D1 as important regulators of adhesion-dependent mammary morphogenesis that are consistently altered in tumors by aberrant tumor-generated mechanical force. Specifically, we implicated PTP MEG1 as a key regulator of adherens junction assembly/integrity and found that matrix force and tumor-generated contractility chronically increase PTP expression. Importantly, ectopic elevated expression of MEG1 in nonmalignant MECs enhanced their integrin-dependent cell adhesion, disrupted tissue polarity and altered cell growth and survival. Studies are in progress to identify MEG1-specific effector proteins in normal, transformed and phenotypically-reverted MECs and to dissect out the mechanism whereby force regulates PTP expression.

15. SUBJECT TERMS
protein tyrosine phosphatase, tyrosine kinase, PTP-Meg 1, PTP-D1, Band 4.1 PTP, integrin, architecture, adherens junction, ECM, 3D

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INTRODUCTION:

Stromal-epithelial interactions and integrins regulate mammary gland development and homeostasis, and modulate tumor progression and treatment responsiveness (Unger and Weaver, 2003; Zahir and Weaver, 2004). Although it is not known how the stroma exerts such a profound effect on mammary epithelial cell (MEC) function, studies in culture have demonstrated that the basement membrane (BM) extracellular matrix (ECM); the insoluble protein component of the stroma surrounding MECs in vivo) regulates MEC growth, survival, migration and differentiation through transmembrane receptors called integrins (Miranti and Brugge, 2002). Integrins mediate their biological effects by initiating biochemical and biophysical signaling that involves protein tyrosine phosphorylation events and that also rely upon cooperative interactions with growth factor receptors and cytoskeletal interactor/modifier molecules (Moghal, 1999, Assoian and Schwartz 2002). The level of tyrosine phosphorylation induced by integrin and growth factor receptor activation is dynamically regulated by the concerted action of protein tyrosine kinases and protein tyrosine phosphatases (PTPs). Several lines of evidence suggest that both transmembrane and intracellular PTPs play an important role in mammary morphogenesis (Aoki, 1999, Schaapveld, 1997). Indeed, culture studies have implicated PTPs as critical regulators of cell-cell interactions (Aberle, 1996), integrin-mediated signaling (Angers-Loustau, 1999, Angers-Loustau, 1999), and focal adhesion assembly (Vadlamudi, 2002), and as modifiers of integrin-growth factor receptor interactions (Moghal, 1999, Angers-Loustau, 1999). However, very little is known about the molecular functions and substrate targets of PTPs in adhesion-dependent MEC behavior. Thus the overall goal of our studies is to delineate the importance of and identify plausible mechanisms whereby PTPs regulate, and are themselves regulated by ECM-integrin-growth factor receptor signaling, and thereafter to determine the role of PTPs in breast cancer progression.

To achieve our goal we have been studying normal and perturbed morphogenesis of MECs using a human breast cancer model called HMT-3522, which progresses from non-malignant S-1 through pre-malignant S-2 to tumorigenic T4-2 MECs (Rizki and Weaver et al., In Revision), as well as using a non-malignant immortalized MEC line established from a fibrocystic breast lesion called MCF10A (Muthaswamy et al., 2001). When used in conjunction with a reconstituted BM assay these MECs recapitulate the morphological and behavior of MECs as they transit from a non-transformed, through to pre-malignant and finally to malignant, invasion breast tissue phenotypes. Using these MEC lines our studies have clearly established that the progressive malignant behavior of the cells in this series is linked to alterations in integrin-growth factor expression and activity and perturbed MEC-ECM responsiveness because we are able to phenotypically revert our mammary tumor cells in culture, and in vivo by inhibiting the activity of either β1 integrin and/or the epidermal growth factor receptor (Weaver et al., 1997; reviewed in Unger and Weaver, 2003). Alternately, we can drive the malignant behavior of non-malignant and the pre-malignant MECs by altering the expression of specific integrin heterodimers such as α5β1 and α6β4 integrin (Zahir et al., 2003; Rozenberg et al., Submitted; Friedland et al., Accepted, unpublished observations).

We successfully identified 15 PTPs whose mRNA levels were regulated during adhesion-directed morphogenesis in MECs and that were modified in transformed MECs. Specifically, we noted that the Band 4.1 PTPs PTP Meg1, D1, H1 and FAP were decreased in differentiated 3D mammary tissues and that their expression was elevated in transformed cells. Data showed that PTP Meg 1 and D1 were significantly increased 24 hours following the interaction of nonmalignant MECs with a reconstituted BM, and prior to differentiation whereas the tumors did not appropriately modulate PTP expression in response to ECM cues. Studies have implicated MEG1 and D1 in the regulation of cell adhesion, formation of focal adhesion and cytoskeletal organization, and their dysregulation has been implicated in uncontrolled proliferation and altered cell-ECM interactions (Gutmann, 2001; Ogata, 1999, Ogata, 2999, Wadham, 2000) and we and others showed that they are robustly regulated during

normal mammary morphogenesis in the developing mammary gland in vivo. Accordingly, our working hypothesis is that: **PTP Meg1 and D1 play pivotal roles in ECM directed MEC morphogenesis and that their dysregulation contributes to malignant transformation and acquisition of an invasive, metastatic breast tumor phenotype.**

To test this hypothesis our studies have been designed to complete the following specific aims:

Specific Aim 1: Generate PTP-Meg 1 and D1 antibodies and then characterize the expression, activity and localization of these endogenous PTPs in normal and dyregulated BM-directed MEC morphogenesis, and identify the morphological parameters linked to their regulation.

Specific Aim 2: Analyze the cellular and molecular consequences of over expression of PTP-Meg 1 and D1 wild type and dominant negative mutants during tumor progression and following phenotypic reversion.

Specific Aim 3: Identify and characterize known and/or novel PTP-Meg 1 and D1 substrates/binding porteins and/or cellular targets that may lie on pathways implicated in Specific Aims 1 and 2.

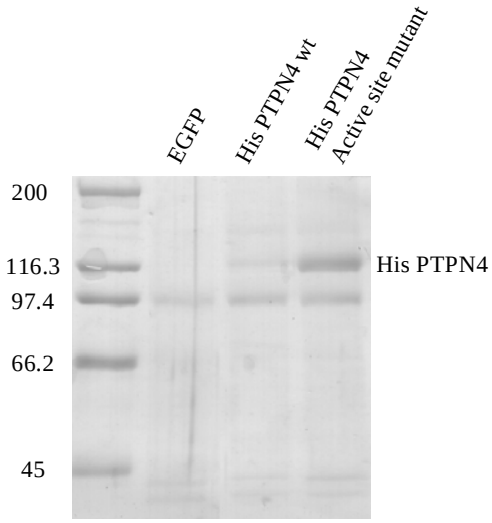
Summary of Achievements - Proposal Body:

To generate molecular tools that will permit a comprehensive characterization of molecular changes in PTP-Meg1 and D1 during malignant transformation and tumor of the breast

In our earlier reports we demonstrated that we had set up methods for quantifying mRNA expression using RT-PCR and RNAse protection assays. We also had obtained an antibody to PTP-MEG1 and conducted comprehensive studies using this antibody. We presented data using RT-PCR and RNAse protection and immunoblotting regarding the expression levels of PTPMeg1 and D1 in nontransformed, transformed and phenotypically-reverted mammary tissues using both two dimensional and three dimensional culture conditions (see previous progress reports). The data have been summarized and are have been submitted for publication. However, because the antibody that we obtained from a colleague was useful under quite limited conditions (see figures in earlier progress report; i.e. was not amenable to immunostaining and not specific enough for immunoprecipitation experiments) in the past few years we have been attempting to generate our own panel of PTP specific antibodies (polyclonal and monoclonal), and also have generated EGFP and triple HA-tagged expression constructs to track PTP Meg1 and D1 expression in live cells.

We chose to focus on initial studies on developing reagents to study the band 4.1 PTP N4 or Meg1. Given the large size of PTPN4 (116 kDa) we chose to use a baculoviral expression system to prepare a full length recombinant protein in Sf9 insect cells. We reasoned that expression of such a large protein in bacteria would be less likely to produce native functional PTPN4 than use of this proven eukaryotic expression system. The preparation of native functional PTPN4 would more likely yield for us antibodies, polyclonal and ultimately monoclonal, that would be useful for a range of immunological techniques including western blot, immunocytochemistry and immunoprecipitation. The latter, particularly if immunoprecipitating antibodies are obtained to native epitopes opens the way to coimmunoprecipitation of interacting, presumptive substrate, proteins. Additionally immunoprecipitating antibodies could be used as necessary reagents to explore the enzymology and regulation of PTPN4 when combined with standard molecular biology approaches of protein expression and structural manipulation. Successful expression of full length functional PTPN4 should also provide additional and complementary approaches to study of these aspects of protein function. We used the pFastBacHTA system from Life Technologies to prepare recombinant baculovirus expressing PTPN4

wild type and active site mutants as N terminally six histidine tagged proteins in Sf9 cells to facilitate rapid one step affinity purification on nickel agarose. We decided to express the active site mutant as well as the wild type given our earlier results from mammary epithelial cells that the active site mutant may express at a higher level in some cell types, and may provide a more rapid route to the desired immunogen (except for the active site substitution we expect the mutant to be structurally identical and therefore an equally effective immunogen) Additionally, preparation of large amounts of active site mutant would allow the preparation of it on a solid support that could be used for the affinity purification of putative substrate proteins. As can be seen in Figure 1, in this trial experiment predominately full length PTPN4 could be prepared from infected Sf9 cells from both wild type and active site mutant (band at ~116kDa present only in cells infected with PTPN4 recombinant baculovirus and not EGFP control) under mild conditions of lysis and elution (250 mM imidazole step elution). We expect that most of the additional contaminating bands will be removed by using a linear gradient of imidazol as these are likely to be histidine containing proteins that elute at lower to intermediate concentrations. If this does not work conventional chromatography by ion exchange should allow a higher degree of purity. Interestingly, as observed in human mammary epithelial cells under identical conditions of infection and purification it appeared that the active site mutant expressed better than the wild type in Sf9 cells as well supporting the conjecture that ther may indeed be some active site dependent feedback regulation of PTPN4 expression in some cell types. Presently we are expanding the production of the recombinant baculovirus for the purpose of scaling up recombinant protein preparation.



60 mm dish of Sf9 cells infected with recombinant baculovirus expressing EGFP or 6 His tagged PTPN4 wild type or active site mutant lysed and purified under native conditions on nickel agarose. Proteins eluted with 250 mM imidazole buffer were separated on a 7.5% SDS polyacrylamide gel and stained with Coomassie Blue. Note higher level of expression of active site mutant suggesting the possibility of a negative feedback loop of phosphatase activity on expression level.

Figure 1. Successful construction and recombinant baculoviral expression of EGFP or 6 His tagged PTPN4 or MEG1 wild type or active site mutant protein for the preparation of PTP-specific antibodies.

Preparation of Expression constructs and manipulation of Band 4.1 PTPs in nonmalignant MECs

One of our major objectives has been to examine the effect of ectopic expression of Band 4.1 type PTPs on normal and transformed MEC behavior. To this end we have been working diligently to generate tractable constitutive and inducible retroviral expression systems that can be used to manipulate PTPN4 (MEG1) expression in nonmalignant MECs. Because HMT-3522 MECs are quite challenging to work with, they grow quite slowly and it takes months to prepare cell lines for study, we have opted to conduct our initial characterization studies using another well characterized and versatile nonmalignant MEC line MCF10A. To begin with we have been able to prepare PTPN4 wild type and active site mutant tagged at the N terminus either by 3 tandem copies of the HA epitope or EGFP or at the C terminus with 3 tandem copies of the HA epitope. We also constructed a PTPN4 expression construct in which PTPN4 was left unmodified (ie not tagged) at any termini. HA tagged or untagged versions were also prepared either as monocistronic or bicistronic with IRES (internal ribosome entry site) EGFP expression constructs under control of a tet regulated minimal CMV promoter in a murine retrovirus. All versions were prepared with the 5 ‘ UTR (untranslated) region of PTPN4 removed and replaced by a concensus Kozak sequence for optimal translation (see Figure 2)

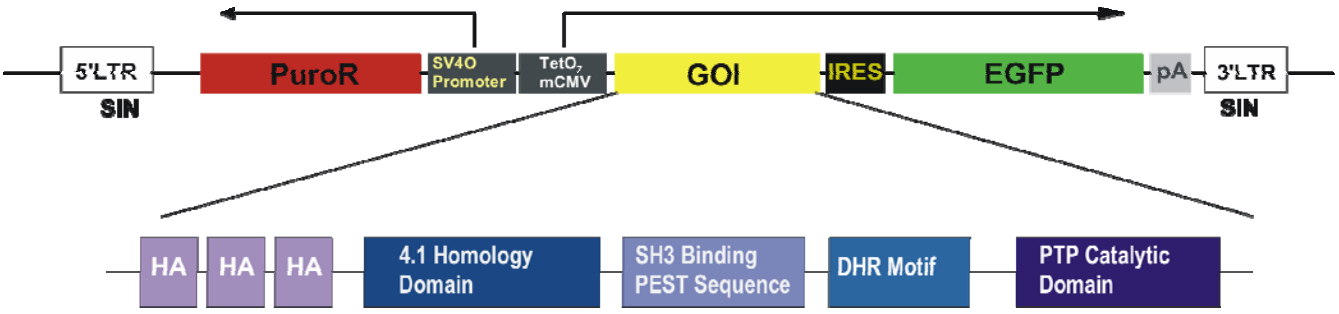
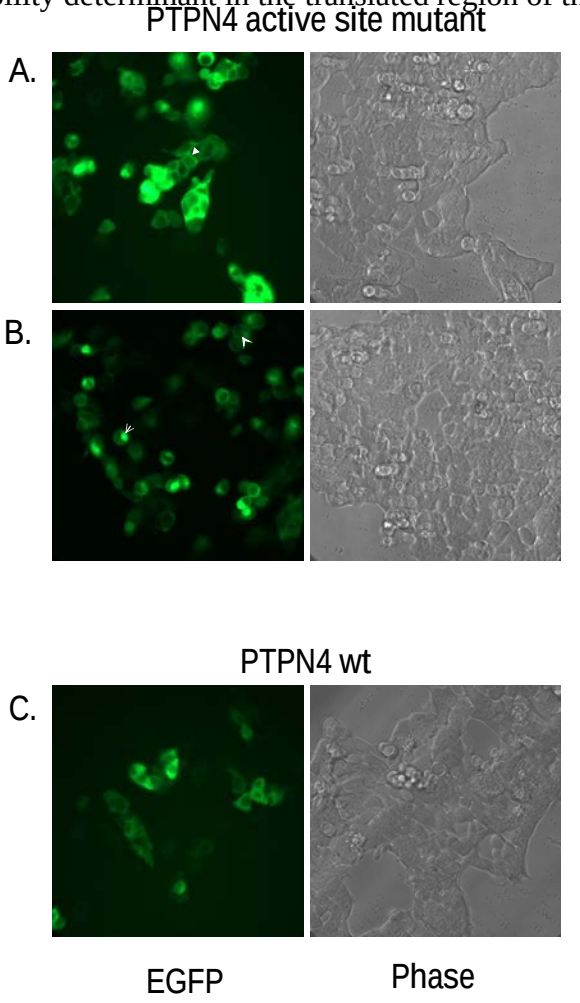


Figure 2. Inducible ectopic expression of triple HA-tagged WT-PTP-MEG1 was achieved using the above constructs. WT-PTP-MEG1 was controlled by a Tet-responsive regulatory promoter co-expressed with EGFP bicistonically.

Because BM-induced MEC morphogenesis is a well timed and coordinated sequence of events and PTP Meg 1 and D1 may function at specific points in this process (refer to previous progress reports) we have focused most of our effort towards preparing cell lines using the RetroTet ART tripartite tetracycline (Tet) inducible retroviral expression system (Rossi et al., 1998). Importantly, however after we began to work with these constructs in cell lines, we found that whereas all constructs expressed robustly in HEK 293 cells by transient transfection (measured by EGFP fluorescence and/or western blot for HA epitope; see Figures 3a-c) expression was relatively weak in MCF10A MECs transduced by these SAME retroviral constructs and a retrovirus expressing the tTA tetracycline VP16 fusion transactivator. Despite repeated attempts we consistently observed that EGFP expression was extremely weak in the fusion constructs in MCF10A MECs (either when cells were grown as conventional two dimensional (2D) monolayers or within a reconstituted basement membrane (rBM) in a three dimensional (3D) format), although otherwise identical constructs for other genes expressed EGFP robustly. We obtained similar results using the HMT-3522 S1 nonmalignant MEC line in parallel studies. Although we are also attempting PTPN4 expression studies using another nontransformed MECs obtained from Vimla Bands laboratory to rule out the possibility that the difficulties were have been encountering are cell line specific, we have reached the tentative conclusion that the PTPN4 transgene may be regulated negatively at a post transcriptional level in nonmalignant MECs but not HEK 293 cells. This conclusion is all that more likely because we have noted that upon malignant transformation of breast cells, PTPN4 (MEG1) expression become aberrantly and chronically elevated and that upon phenotypic reversion of HMT-3522 T4 tumor cells in a 3D assay the PTPN4 levels rise

even further. The data suggest that regulatory pathways may exist in nontransformed MECs that restrict PTPN4 expression and that these pathways/mechanisms either do not exist OR alternately have become dysfunctional following malignant transformation in MECs. In addition, these regulatory pathways do not appear to be functioning as effectively in HEK 293 cells (see Figure 3). Indeed, the evidence suggests that these regulatory mechanisms likely reside at the level of mRNA stability as poor expression was transferred to EGFP in *cis* in the bicistronic constructs. Human PTPN4 has a relatively long 5' UTR (untranslated) region that is both GC rich and contains three short open reading frames upstream of the coding ATG. These structural features are consistent with a complex post transcriptional regulatory mechanism for PTPN4 but as indicated removing the 5' UTR and addition of a Kozak concensus sequence resulted in a poorly expressing construct in MCF10A cells. It is possible that the 5'UTR acts positively to increase expression of the normal gene in MECs and that its absence unmasked an mRNA instability determinant in the translated region of the gene as has been seen for c-fos.



PTPN4 wild type (C.) or active site mutant (phosphatase dead) (A. and B.) tagged at the N terminus transiently transfected and expressed constitutively in HEK 293 cells. EGFP fluorescence was observed predominately in the cytoplasm but in selected cells could be seen concentrated at cell-cell junctions (A. solid arrow head), and in rounded cells cortically outlining the plasma membrane (B., solid barbed arrow). Occasional cells had dense cytoplasmic accumulations of EGFP fluorescence to one side of the nucleus which suggest submembranous compartments such as the golgi or ER (B., open barbed arrow). These localizations are consistent with an actin binding FERM domain containing protein and are probably dynamic and were observed for both wild type and mutant PTPN4 24 hours after transfection but were less prominent for the wild type than the mutant after 48 hours consistent with a stabilization/slow turnover of substrate interactions in the mutant. Whereas expression was robust in 293 cells the same constructs did not express well (extremely weak fluorescence) in normal immortalized MEC MCF10A cells (although a control EGFP only construct under the same promoter did) suggesting that in MECs PTPN4 may be regulated at the post transcriptional level.

Figure 3a-c: Ectopic expression of EGFP-tagged wild type or mutant PTPN4

Curiously the triple HA N terminally tagged PTPN4 but not the C terminally tagged bicistronic expression construct with EGFP gave a higher level of EGFP expression than the corresponding untagged PTPN4 construct suggesting that the triple HA tag at the 5' end of the mRNA may have some influence on the potency of the putative instability determinant consistent with a complex interaction of the 5'UTR with this determinant in the endogenous mRNA. Interestingly it also appeared that EGFP expression was higher for the bicistronic expression construct with the N terminally triple HA tagged PTPN4 active site mutant than the wild type in MCF10A cells. The same was observed in Sf9 insect cells infected with a six histidine N terminally tagged PTPN4 wild type and active site mutant. These constructs differ from each other only with respect to a change in the codons that substitute serine for the active site cysteine, suggesting the intriguing possibility that PTPN4 may be under some form of negative feedback regulation that is dependent on its own phosphatase activity. If true based upon our current data given that mammary tumor cells express elevated levels of PTPN4 (see previous progress reports) it is possible that this feedback regulation is missing or altered following malignant transformation. As such these observations hint at possible complex levels of regulation of gene expression in normal MECs that merit further study. Towards this goal we are currently preparing mammary tumor lines that express various PTPN4 constructs for further study. From the point of view of studying the effects of PTPN4 over-expression of wild type and active site mutant (dominant negative) PTPN4 in MEC the poor expression of the present constructs poses some unforeseen technical difficulties in using these to study their function in normal and malignant MECs. We are at present in the process of re-preparing constructs replete with the 5'UTR of the endogenous gene to examine the possibility that this will improve expression of the transgene. If so this should point to the existence of the putative interaction with the 5'UTR and an mRNA instability determinant in the translated region as well as improve ectopic expression of the transgene for analysis of protein function.

Characterization of the effect of ectopically, chronically elevating PTPN4 (MEG1) expression in nontransformed MECs

Given the difficulty that we encountered achieving good ectopic expression of PTPN4 in nontransformed MECs we decided to prepare stable populations of retrovirally-infected, tet regulatable populations of triple HA-tagged PTPN4 wild type and to obtain nonmalignant MEC lines that expressed levels of ectopic PTPN4, similar to what we could detect in the transformed mammary tumor lines (we are currently preparing similar populations of MECs expressing a mutant PTP dead phosphatase and an activated PTP; see Figure 2). We were able to achieve this goal by transiently inducing expression of PTPN4 in MCF10A MECs using the bicistronic EGFP constructs and then rapidly sorting the cell population that expressed detectable, stable EGFP using FACS sorting. To this end we obtained several uniform populations of MECs with elevated ectopic expression (to levels we detected in tumors; refer to previous reports) of tet regulatable PTPN4 expression. We then immediately inhibited the PTPN4 expression by adding tetracycline to the media to avoid aberrant effects of chronic PTPN4 expression on MEC behavior and cell selection and expanded the cell population. We then re expressed the PTPN4 by removing the tetracycline, confirmed its uniform expression through observation of robust EGFP (bicistronic) expression in the expanded cell population (see Figure 4 right immunofluoresence images), as well as immunostaining for the HA-tagged PTPN4 protein and immunoblotting for HA-tagged PTPN4 in cell lysates (see Figure 4; left immunoblot). From these studies we could also determine that PTPMEG1 expression is localized to the plasma membrane in regions associated with β1 integrin and focal adhesion complexes (see Figure 4 farthest right hand image).

PTP-MEG1 (N4) is expressed robustly and localizes to the plasma membrane in nonmalignant MECs

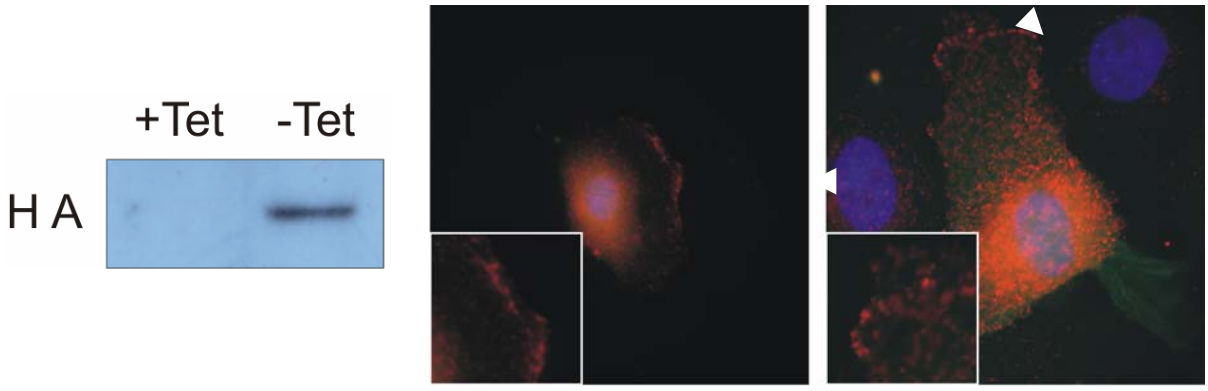


Figure 4. Immunoblot and immunofluoresence of HA-tagged ectopically expressed PTP-MEG1 in MCF10As plated as monolayers on matrix-coated glass coverslips showing that ectopic PTP-MEG1 is robustly expressed and localizes to the plasma membrane in the cells and is associated with structures reminiscent of lamellipodia.

Despite these encouraging results, because visualization of the HA-tagged protein necessitated the fixation of the breast cells we are unable at present to comment upon the dynamic state of PTPMEG1 in MECs during morphogenesis or its localization and behavior in response to exogenous stimuli such as growth factors or changes in extracellular matrix (ECM) organization, composition or stiffness (matrix force; refer to earlier progress report for details). Therefore, to address this issue we now are in the process of constructing YFP-tagged PTPMEG1 expression constructs and preparing lentiviral stable expressing MEC lines to be used in conjunction with spinning disco confocal microscopy time lapse imaging to visualize PTPMEG1 behavior during three dimensional reconstituted basement membrane-induced morphogenesis.

However, despite these difficulties, after we confirmed uniform and elevated, sustained expression of PTPN4 in our MEC population we were also able to conduct studies on MEC behavior in conventional 2D monolayer cultures and in rBM 3D cultures to assess the consequence of increasing PTPN4 expression towards that observed in the tumors cells on their functional behavior. Thus far we could show that elevating the expression of PTPMEG1 in the nonmalignant MECs greatly and significantly enhances integrin-dependent adhesion as illustrated by classic integrin adhesion assays. This increased matrix adhesion behavior was determined to be similar for adhesion to collagen I, laminin, basement membrane and fibronectin. This suggests that because we could not demonstrate specific enhancement of PTPMEG1 expressing MEC adhesion to a specific ECM substrate, our preliminary conclusion is that PTPMEG1 likely exerts a general enhancement of integrin-dependent adhesion in breast cells (see Figure 5). These data suggest that PTPMEG1 is functioning at an intracellular level that generally modulates integrin-dependent adhesion. We also noted that ectopic expression of PTPMEG1 greatly elevated MEC growth in 2D monolayers (data not shown).

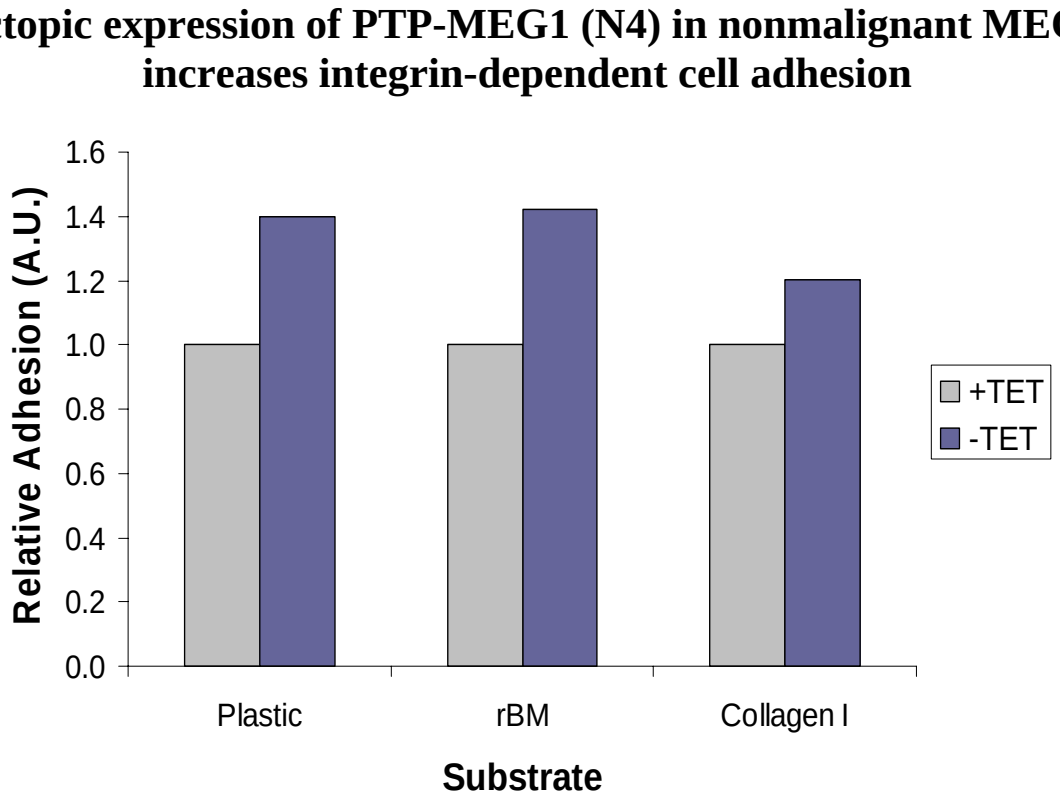


Figure 5. Non-malignant MEC adhesion to collagen and a laminin-rich basement membrane is increased following ectopic elevation of PTP-MEG1 expression.

More interestingly however, we found that PTPMEG1 enhanced the size of MEC colonies formed in response to rBM (see Figure 6 – left hand graph of colony size) and also compromised the morphology of these 3D structures (see Figure 6; see right hand photo micrograph images of three dimensional rBM-induced colonies). Upon careful examination we could show that PTPMEG1 expression disrupted mammary tissue polarity as evidenced by disorganized localization of $\alpha 6\beta 4$ integrin (see Figure 7), loss of an endogenous basement membrane (data not shown) and destabilization of cell-cell adherens junctions demonstrated by triton extractable beta catenin (see Figure 8). Studies are now in progress to explore the role of PTPMEG1 on MEC invasion and motility. Given these profound effects we are also keen on identifying PTPMEG1 substrates in nonmalignant MECs and to study the effect of PTPMEG1 on tumor behavior.

Ectopic and sustained expression of PTPMEG1 in nonmalignant MECs increases cell growth and colony size in rBM and alters tissue morphology

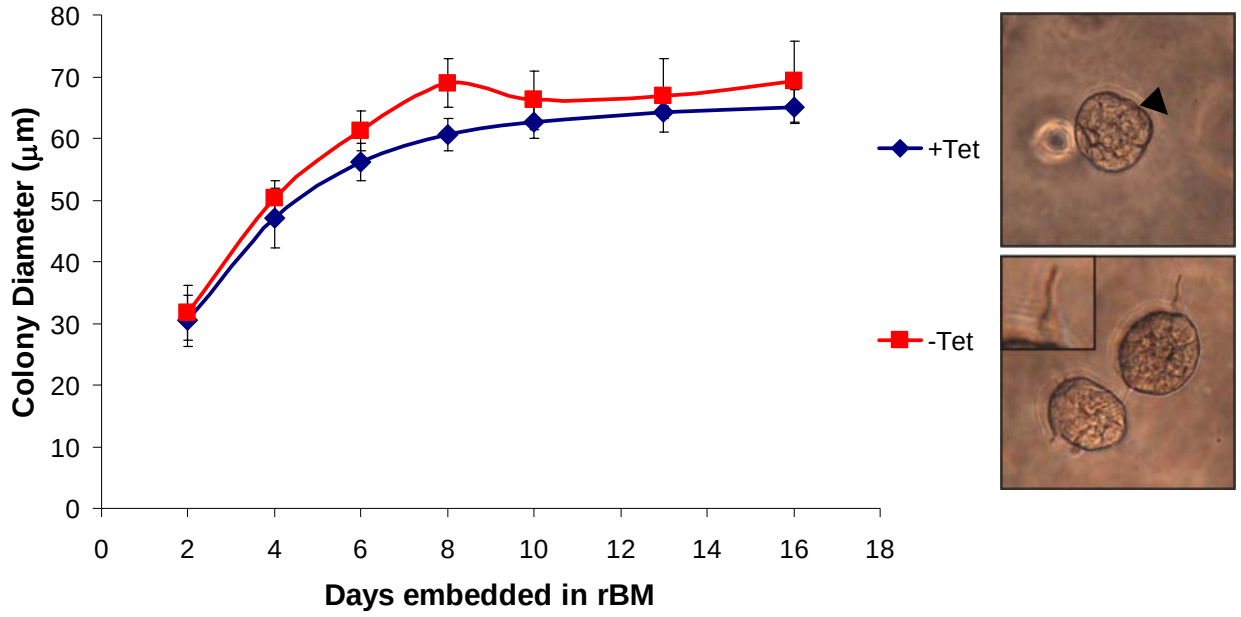


Figure 6. There is a consistent increase in growth when PTP-MEG1 is ectopically expressed in MCF10As embedded in rBM for the indicated number of days. Phase images illustrate the morphology at day 16, showing a slight increase in size and an increase in cellular protrusions with ectopic expression of PTP-MEG1.

Ectopic and sustained expression of PTPMEG1 (N4) disrupts tissue polarity in nonmalignant MECs in rBM

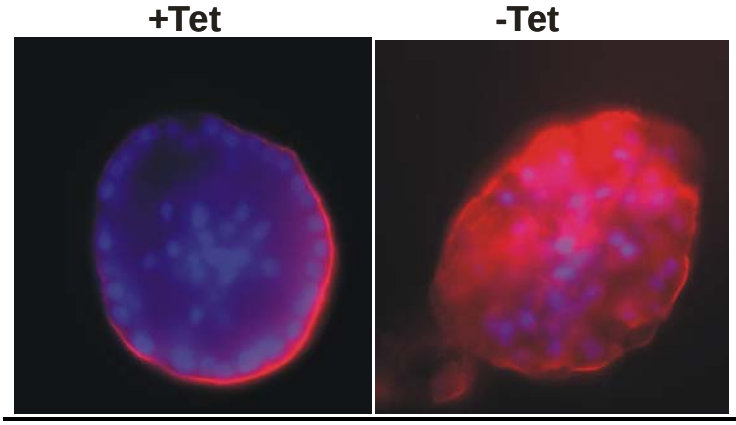


Figure 7. Immunostaining of MCF10As grown in 3D rBM for 16 days showing perturbed basal localization of β4 integrin with (-Tet) and without (+Tet) ectopic expression of WT-PTP-MEG1.

Ectopic and sustain expression of PTPMEG1 (N4) in nonmalignant MECs destabilizes cell-cell adherens junctions and disrupts mammary morphogenesis

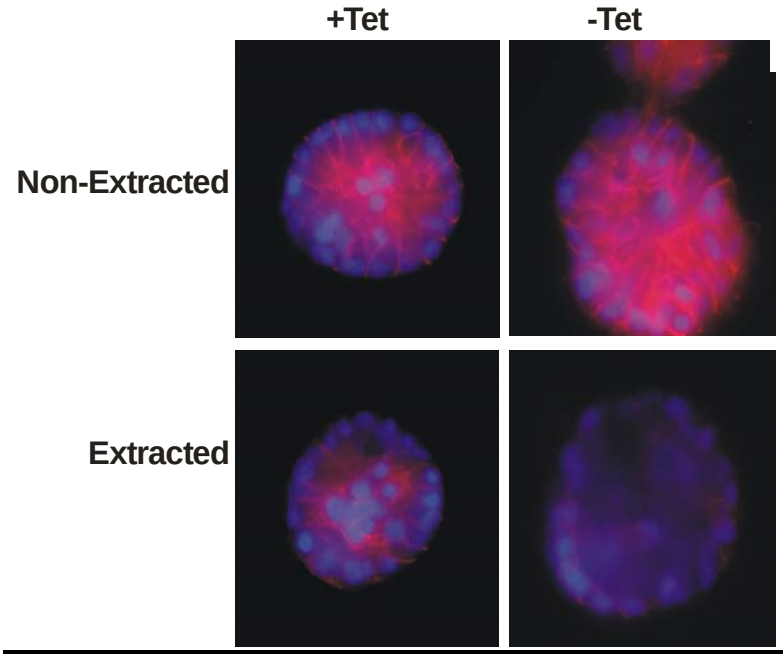


Figure 8. Immunostaining of MCF10As grown in 3D rBM for 16 days showing β-catenin localization at adherens junctions with (-Tet) and without (+Tet) ectopic expression of WT-PTP-MEG1. Note the loss of β-catenin with cytoskeleton extraction prior to fixation in cells overexpressing PTP-MEG1, indicating that adherens junctions are less stable under these conditions.

Generation of additional molecular tools for manipulating PTP Meg 1 and D1:

To augment our ongoing studies we have additionally generated various siRNA retroviral and lentiviral expression constructs for PTP Meg 1 which will be used to study the effect of knocking down Meg 1 levels on MEC morphogenesis and tumorigenesis and phenotypic reversion. This technique has proved especially successful for the assessment of another adhesion regulated PTP - PTP-PEST (see previous progress reports) as well as for the examination of the effect of the breast cancer tumor suppressor BRCA1 and a homeobox gene we identified as a key modulator of BRCA1 expression (Gilbert et al., In revision for publication in Cell). We also have available constitutive and tet inducible adenoviral expression systems and a lentiviral expression system in the laboratory that should permit rapid over-expression of wild type, dominant-negative and constitutively active MEG1 and D1 PTPs in MECs at various stages of morphogenesis. More importantly we have recently established an inducible GST-tagged-V12 Rac system that together with a proteomics approach is permitting us the ability to study the effector specificity/restriction induced for activated Rac following tissue morphogenesis. We should be able to use a similar strategy to identify tissue specific restricted PTP MEG1 access at various stages of mammary morphogenesis such as before and after adherens junction assembly and basal polarity. Lastly, as per our original outline we have also successfully constructed GST-tagged PTP Meg 1 (PTPN4) expression constructs for standard substrate trapping studies. Although we appreciate that we are behind schedule in achieving our original work tasks – given the difficulties that we have encountered with the expression constructs and also the overly ambitious goals outlined in our original proposal (refer to reviewers critiques) – we now hope that in this next and last year we will be able to identify differential PTPMEG1 substrates in nonmalignant and transformed MECs. In addition, we are in the process of addressing reviewers concerns for one manuscript and are currently preparing a second manuscript for publication that we anticipate we will submit shortly.

KEY RESEARCH ACCOMPLISHMENTS:

- Completed the successful generation of HEK 293 cell lines expression various wild type, tagged and untagged and constitutively active, substrate trapping and PTP dead mutant PTPMEG1
- Completed the successful generation of bicistronic triple HA-tagged MEG1 expression tet regulatable nonmalignant MEC populations for morphological studies
- Completed the successful demonstration of efficient and robust expression of triple HA-tagged PTPMEG1 (N4) expression in nonmalignant MECs
- Completed the assessment of effect of ectopic and sustained expression of PTPMEG1 on MEC morphogenesis including demonstration and verification that PTPMEG1 influences tissue polarity and cell-cell adherens junctions
- Completed an assessment of the effect of ectopic and sustained expression of PTPMEG1 on nonmalignant MEC growth
- Completed the successful demonstration that PTPMEG1 enhances integrin-dependent cell adhesion in nonmalignant MECs suggesting it might function by increasing substrate adhesion to destabilize cell-cell adhesions thereby compromising MEC morphogenesis

- Established stable shRNA lentiviral and inducible adenoviral protocols for manipulating PTPMEG1 and D1 expression at various stages of mammary morphogenesis in either 2D or 3D culture formats.

- Generated recombinant expressed PTP Meg1 protein for generation of PTP Meg1 antibodies and for pull down experiments aimed at identifying candidate substrates

- Demonstrated proficiency to produce sufficient quantities of intact PTPMEG1 protein and initiated generation of producing polyclonal and monoclonal antibodies for PTPEG1

- Prepared affinity columns of full length 6-His tagged substrate trapping mutant PTPMEG1 expressed from sf9 insect cells in progress

Reportable Outcomes:

A. Manuscripts

1. Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, Reinhart-King CA, Margulies SS, Dembo M, Boettiger D, Hammer DA, **Weaver VM.** Tensional homeostasis and the malignant phenotype. Cancer Cell 2005; 8(3): 241-254.

2. Johnson KR, Leight JL, **Weaver VM.** "Demystifying three-dimensional force and tissue morphogenesis." Methods in Cell Biology: Cell Mechanics. Academic Press, San Diego, In Press.

3. Lakins, J.N., Gbegnon, M., Chrenek, M., Kang, B., Wong, P., and **Weaver, V.M.** Adhesion-linked protein tyrosine phosphatases, mammary morphogenesis and malignancy. Am J Physiol. In Revision.

4. Boettiger, D., Zahir, N., and **Weaver, V.M.** Tensional Homeostasis in Normal and Abnormal Physiology and Therapeutic Prospects. Trends in Molecular Medicine. In Press, 2006.

5. Butcher, D., Zahir, N., and **Weaver, V.M.** A Tense Situation and Tumorigenesis, Nature Reviews Cancer. In Press.

B. Abstracts

1. **Weaver VM,** Johnson KR, Lakins JN. Adhesion-linked protein tyrosine phosphatases, morphogenesis, and breast cancer progression. Poster Presentation. Era of Hope Meeting for the Department of Defense (DOD) Breast Cancer Research Program (BCRP), Philadelphia, PA, June 2005.

2. Johnson KR, Lakins JN, Friedland JC, **Weaver, VM.** Dynamic and reciprocal link between forces and PTPs in normal and malignant mammary behavior. Poster Presentation. BMES Annual Fall Meeting, Baltimore, MD, October 2005.

3. Johnson KR, Lakins JN, **Weaver VM.** Protein Tyrosine Phosphatases, Adhesion, and MEC Morphogenesis and Malignancy. Poster Presentation. 45th American Society of Cell Biology Annual Meeting, San Francisco, December 2005.

C. Oral Meetings Presentations:

1. **Weaver, V.M.** Spatial-Mechanical regulation of mammary morphogenesis and malignancy, ASCB-ECI Conference on Engineering Cell Biology, Washington, Seattle, July 16, 2005.

2. **Weaver, V.M.** A biophysical perspective of epithelial morphogenesis, malignancy and treatment responsiveness AACR Special Conference on Cancer, Proteases, and the Microenvironment, Bonita Springs, FL, December 2, 2005.

3. **Weaver, V.M.** Form, Force and Fate, Annual Basic Science Colloquium on Cell Polarity in Morphogenesis and Cancer, Northwestern University, Evanston, IL, March 24, 2006.

4. **Weaver, V.M.** The role of force and matrix organization in malignant transformation, Major Symposium on "Tumor Microenvironment," at the 97th American Association for Cancer Research Washington, DC, April 4, 2006.

5. Gilbert, P., and **Weaver, V.M.** Three dimensional control of life and death, International In Vitro Biology Society Conference, Cincinnati, OH, June 4, 2006.

6. Zahir, N., Johnson, K., Paszek, M., Leight, J., Friedland, J., Lakins, J.N. and **Weaver, V.M.** Spatial-mechanical regulation of mammary morphogenesis and malignancy, EMBO conference on Breast Cancer, Dublin, Ireland, June 6, 2006.

7. **Weaver, V.M.** Force and the third dimension, Invited Symposium Speaker Gordon Conference on Signaling by Adhesion Receptors, Mount Holyoke College, MA, June 28, 2006.

D. Invited Institutional Presentations:

1. **Weaver, V.M.** Spatial-mechanical signaling and tumorigenesis, Science Lecture series, Jefferson Institute of Molecular Medicine, Philadelphia, PA, October 3, 2005.

2. **Weaver, V.M.** A Spatial-Mechanical Perspective of Mammary Morphogenesis, Malignancy and Treatment, Dept Surgery, USCF, San Francisco, CA, October 25, 2005.

3. **Weaver, V.M.** The tension mounts: mechanics meets morphogenesis and malignancy, MD Anderson, Houston, TX, November 2, 2005.

4. **Weaver, V.M.** A Biophysical Perspective of Mammary Gland Development and Tumorigenesis, The Cancer Institute of New Jersey, New Brunswick, NJ, February 22, 2006

5. **Weaver, V.M.** Form, Force and Fate, NIH Optical Imaging Special Interest Group (SIG), Bethesda, MA, March 15, 2006.

6. **Weaver, V.M.** Spatial-mechanical Regulation of Mammary morphogenesis, malignancy and tumor therapy, Divisions of Cancer Biology and Molecular Oncology, Department of Medicine, Evanston Northwestern Research Institute, Northwestern University, Evanston, IL, March 23, 2006

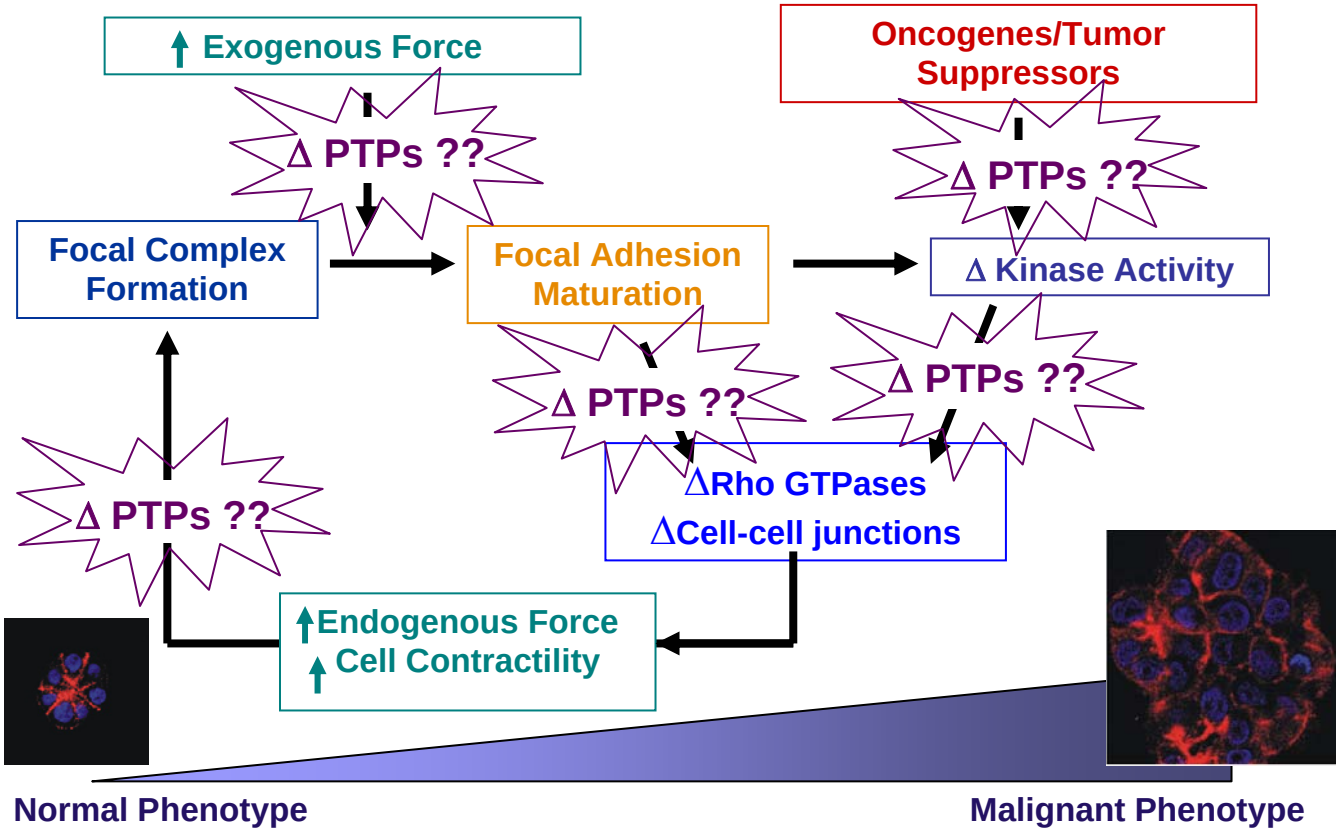
7. **Weaver, V.M.** Forcing Cell Fate, Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, D.C., March 31, 2006

Conclusions:

Since our last progress report we have generated various directly tagged PTPMEG1 expression constructs – including an EGFP direct tagged PTPMEG1 and a triple HA-tagged construct. For various reasons the EGFP-tagged expression constructs do not appear to be well-tolerated in MECs, hence we have reported here only our results obtained using the HA-tagged construct in nonmalignant MECs – however this method requires the fixation of cells and hence cannot be used to visualize PTPMEG1 in live cells undergoing morphogenesis. Therefore, as an alternative we are in the process of generating YPF-tagged PTPMEG1 expression constructs using lentiviral inducible promoters which we believe will enable us to image live cells embedded within rBM. To this end my laboratory is in the process of moving to S.F. where we have ready access to live cell imaging facilities for these types of studies including free use of a spinning disc confocal microscope and dedicated imaging facilities including live cell time lapse microscopy apparatus. We have also decided to generate polyclonal and monoclonal antibodies that we hope to use to pull down/substrate identification studies as well as for immunostaining purposes. In this regard we intend in the future to assess the levels and localization of PTPMEG1 in human breast specimens from normal and transformed breast tissue. However, to assure the DOD granting agency – we do not intend to conduct these human specimen analysis studies during the funding period for this grant. Instead, these experiments will be done to help prepare preliminary data required for the submission of an NIH grant on band 4.1 PTPs in breast cancer. As such we do NOT require an IRB as of yet and will be submitting this approval in due time. We have also successfully expressed wild type PTPMEG1 in nonmalignant MECs and completed studies on its effect on MEC morphogenesis. Our data clearly show that sustained expression of MEG1 in nontransformed MECs to a level that is expressed in tumors disrupts cell-cell adherens junctions and compromises tissue polarity. Consequently MECs expressing the MEG1 protein have compromised morphogenesis and instead assemble large, invading disorganized structures reminiscent of premalignant mammary lesions (DCIS). These results are consistent with our data showing that MEG1 is only transiently expressed in nonmalignant MECs during mammary morphogenesis and that its expression is rapidly downregulated following assembly of cell-cell adhesions. Our previous studies suggested that PTPMEG1 was somehow involved in regulating adherens junction integrity – and our data indicating PTPMEG1 localizes to sites of cell-cell junction AND alters adherens junctions is consistent with this conclusion. Our data also accord with our observations that PTPMEG1 levels remain elevated in tumor colonies that lack adherens junctions and have compromised tissue polarity. We are therefore quite anxious now in this last year to determine what the substrate interactions of PTPMEG1 are in the nonmalignant MECs. Towards this goal we will use co-IPs with our new antibody, substrate trapping mutants and our new in situ GST pull down modified method. Our most recent data suggest that PTPMEG1 may be required to maintain cell-cell adhesions – but also that mechanisms that exist in nontransformed MECs to regulate PTPMEG1 expression may not exist in tumors. Again these observations indicate that we will need to identify putative PTPMEG1 specific substrates to understand PTPMEG1 effects on tissue behavior. In addition to these achievements – we have a manuscript which has been reviewed and requires additional experiments to address reviewers concerns. We also have a second manuscript that is currently being prepared for submission for publication. In the next year we intend to publish both of these manuscripts and in addition prepare another manuscript that will focus on the identification and characterization of PTPMEG1 substrates in normal and transformed MECs. Finally, one of the goals of the DOD IDEA awards is to permit investigators to complete critical studies that will permit them to apply for and successfully compete for extramural funding through the NIH. Given our interesting observations we are now in the process of preparing an NIH RO1 application and intend to have this submitted for the June 1st grant deadline. Based upon data that we have generated during the course of this grants funding we

have assembled a working model for how we believe PTPs and specifically those of the band 4.1 family of PTPs likely operate in MEC tissues to maintain tissue homeostasis and how their function becomes aberrant and may contribute to malignant transformation and ultimately tumor metastasis.

Figure 9 . Proposed Model linking force to PTPs, tissue architecture and tissue morphogenesis in normal and transformed mammary tissue



Future Objectives to be completed within this last year of funding:

- Complete the generation of polyclonal antibodies
- Characterize newly generated polyclonal antibodies
- Begin to generate monoclonal antibodies that can be used for immunostaining
- Generate YFP-tagged PTPs for tracking PTPMEG1 location in live cells
- Set up time lapse microscopy imaging to track PTPMEG1 localization during normal mammary morphogenesis in 3D rBM cultures and in tumor colonies in rBM.
- Complete immunoblot characterization of PTPMEG1 from 2D versus 3D cultures of nonmalignant MECs and in tumor and reverted tumor lysates
- Conduct immunostaining assays to determine if we can detect endogenous protein using our generated antibodies
- Use tagged expression constructs as well as generated antibodies to conduct PTPMEG1 activity assays in MECs
- Prepare T4-2 MECs expressing wild type and mutant inactive PTPMEG1 (N4)

- Assess effect of PTPMEG1 activity on T4-2 MEC morphology in rBM and in response to a phenotypic reversion cue focusing on effects on cell-cell junctions and tissue polarity
- Conduct IP studies to identify putative substrates for PTPMEG1 in nonmalignant and transformed MECs in 2D culture
- Conduct IP studies and/or use the substrate trapping mutant PTPMEG1 expression construct to identify putative PTPMEG1 substrates in nonmalignant and malignant MECs in rBM
- Conduct preliminary biochemical characterization of PTPMEG1 substrates in nonmalignant and tumorigenic MECs
- Begin affinity column studies to identify PTPMEG1 substrates in lysates from nonmalignant and transformed MECs
- Publication of article currently in revision
- Submission and publication of article current in preparation
- Preparation, submission and hopefully publication of article on substrate identification for PTPMEG1
- Preparation and submission of NIH RO1 grant on PTPs in breast cancer focusing on PTPMEG1

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