LOSS OF TUMOR SUPPRESSOR MERLIN IN ADVANCED BREAST CANCER IS DUE TO POST-TRANSLATIONAL REGULATION

K. Adam Morrow1#, Shamik Das1#, Brandon J. Metge1, Keqiang Ye4, Madhuri S. Mulekar2, J. Allan Tucker3, Rajeev S. Samant1, Lalita A. Shevde1

1Department of Oncologic Sciences, Mitchell Cancer Institute, 2Department of Mathematics and Statistics, 3Department of Pathology, University of South Alabama, Mobile, AL; 4Department of Pathology and Laboratory Medicine, Emory University, GA. #Both authors contributed equally to the work.

Running title: Merlin is lost in breast cancer

Address correspondence to: Lalita A. Shevde, Ph.D., Department of Oncologic Sciences, USA-Mitchell Cancer Institute, 1660 Springhill Ave. Mobile, AL 36604.; Tel: 251-445-9854; E-mail: lsamant@usouthal.edu

Keywords: NF2, Merlin, Osteopontin, Akt, breast cancer

Background: The role of Merlin in breast cancer is unknown.
Results: Merlin protein is degraded in advanced breast cancer due to osteopontin-initiated signaling.
Conclusion: Merlin is regulated at the post-translational level in breast tumors.
Significance: We have defined a functional role for Merlin in limiting breast tumor growth and elucidated the utility of Merlin as an important biomarker in breast cancer.

SUMMARY
Unlike malignancies of the nervous system, there were no mutations identified in Merlin in breast cancer. As such, the role of the tumor suppressor, Merlin, has not been investigated in breast cancer. We assessed Merlin expression in breast cancer tissues by immunohistochemistry and by real-time PCR. The expression of Merlin protein (assessed immunohistochemically) was significantly decreased in breast cancer tissues (even though the transcript levels were comparable) simultaneous with increased expression of the tumor promoting protein, osteopontin (OPN). We further demonstrate that the loss of Merlin in breast cancer is brought about, in part, due to OPN-initiated Akt-mediated phosphorylation of Merlin leading to its proteasomal degradation. Restoring expression of Merlin resulted in reduced malignant attributes of breast cancer, characterized by reduced invasion, migration, motility, and impeded tumor (xenograft) growth in immunocompromised mice. The possibility of developing a model using the relationship between OPN and Merlin was tested with a logistic regression model applied to immunohistochemistry data. This identified consistent loss of immunohistochemical expression of Merlin in breast tumor tissues. Thus, we demonstrate for the first time, a role for Merlin in impeding breast malignancy, identify a novel mechanism for the loss of Merlin protein in breast cancer and have developed a discriminatory model using Merlin and OPN expression in breast tumor tissues.

Merlin (Moesin-Ezrin-Radixin-Like protein), encoded by the NF2 gene, is a tumor suppressor that is frequently inactivated in tumors of the nervous system (1-7). Merlin complexes with ERM (Ezrin-Radixin-Moesin) proteins that link the cytoskeleton to glycoproteins in the cell membrane (7). Merlin is critically involved in regulating cell growth and proliferation. In vitro, Merlin mediates contact inhibition and inhibits invasiveness (8,9). Underlying the tumor suppressor function of Merlin is likely a combination of the signaling pathways that attribute its ability to suppress Ras and Rac (9-11), negatively regulate FAK, downregulate expression of cyclin D1 (12), inhibit the p21-activated kinase, Pak1 (13) and interfere with the interaction between CD44 and Hyaluronan...
The stability of Merlin protein is regulated, in part, by Akt-mediated phosphorylation at Threonine 230 and Serine 315. Phosphorylation at these amino acids leads to Merlin degradation by ubiquitination. The reduced levels of Merlin in tumors of the nervous system are predominantly brought about by mutations or loss of heterozygosity. However, Merlin’s role in breast cancer has been largely ignored due to early, sporadic studies that did not detect mutations in tumor tissues. OPN is a secreted phosphoglycoprotein that acts as an effector of tumor progression and metastasis at several levels. Elevated OPN is a marker for advanced breast cancer and multiple other cancer histotypes. OPN-initiated signaling activates NF-κB, PI-3-kinase and Akt pathways and manifests as enhanced cell proliferation and survival, migration and adhesion. We report here that while the transcript levels of Merlin are unaltered in breast cancer tissues, there is loss of Merlin expression at the protein level in breast tumors, concomitant with an increase in OPN expression. Our studies revealed that OPN-initiated signaling induced Akt-mediated phosphorylation and degradation of Merlin in breast cancer cells. Further, restoration of Merlin in breast cancer cells functionally impeded their malignant behavior. Logistic regression consistently identified decreased Merlin staining intensity in tumor tissues. It also showed that given the Merlin intensity, OPN ameliorates discrimination between normal and tumor tissue. Thus, our studies provide evidence that the availability of Merlin in breast tumors is regulated at the post-translational level. This is exciting from the perspective that Merlin was not found to be mutated or compromised at the transcript level in breast cancers. We have also defined a functional role for Merlin in limiting breast tumor growth and elucidated the utility of Merlin as an important biomarker in breast cancer.

**EXPERIMENTAL PROCEDURES**

**Cell Culture** - MCF10AT, MCF7, MDA-MB-231 and MDA-MB-435 cells were cultured as previously described. SUM159 cells were grown in DMEM/F-12 supplemented with FBS, insulin, and hydrocortisone in a humidified 5% CO₂ environment. The lineage infidelity of MDA-MB-435 cells has been discussed in several papers. We used this cell line as a model due to the fact that it naturally expresses copious OPN. Stable Merlin-expressing transfectants of MDA-MB-231 and SUM159 cells were generated by transfecting a Merlin-expressing construct. Empty-vector was transfected as control; stable transfectants were selected on G418.

**Western Blotting Analysis** - Immunoblotting was done with anti-Merlin (Santa Cruz Biotech, Santa Cruz, CA), phospho-Ser473-Akt (Cell Signaling, Danvers, MA), total Akt (Cell Signaling), anti-mouse HA (Santa Cruz), anti-phospho-Ser315 Merlin (provided by Dr. Keqiang Ye), and anti-GAPDH (Cell Signaling). Anti-rabbit or anti-mouse HRP conjugated secondary antibody was used for detection and blots were developed with SuperSignal substrate (Pierce, Rockford, IL) and exposed using a Fuji LAS3000 imager.

**Transfection and Drug Treatment** - Cells were transfected with empty vector, Merlin (WT; wild-type) or T230A S315A Merlin mutant and treated with clasto-Lactacystin β-Lactone (Sigma, St. Louis, MO) for 2 hours. Recombinant OPN (100 ng/mL) (R&D Systems, Minneapolis, MN) was added and cells were lysed after 6 hours. Where indicated, cells were first treated with Akt inhibitor IV (Calbiochem) in serum free media for 30 min followed by 100 ng/mL human rOPN for 16 hours.

**Immunoprecipitation** - Cells were transfected with pcDNA3.1 HA-ubiquitin alone or in combination with pIRES2-myc-Merlin and incubated for 24 hrs. Cells were treated with 10μM Lactacystin, 100ng/ml OPN and 10uM AKT inhibitor IV for 12 hrs and lysed in NP-40 buffer. The lysate was immunoprecipitated with anti-Merlin antibody and the immunoprecipitate was assessed by immunoblotting.

**Real-time quantitative PCR of tissue array** - TissueScan plates (Origene, Rockville, MD) were assessed for the expression of OPN and Merlin transcripts using the manufacturer’s protocol. The reaction was carried out in a Bio-
RadiCypher iQ5 using the following program: activation step of 50°C for 2 minutes, then 42 cycles of 95°C for 5 minutes, 95°C for 15 seconds, and 60°C for 1 minute. Data was expressed as fold change (2^-ΔΔCT). Statistical analysis was conducted using JMP version 7.0.1 (SAS, Inc., Cary, NC). A 5% level of significance was used to determine significance of results. The data were summarized using mean, standard deviation, and standard error of mean. The Pearson’s correlation coefficient was used to determine correlation between numerical variables such as age. Wilcoxon test was used to compare C\textsubscript{T} levels of Merlin and OPN by group (normal or tumor), grade, and stage. p-value of < 0.05 was considered significant between groups.

**Soft agar colonization assay** - Cells were seeded in soft agar in triplicate in a 6-well plate, allowed to grow for 2-3 weeks, stained with crystal violet solution. Colonies with > 50 cells were microscopically counted.

**Foci formation assay** - Cells were transfected with empty vector or pcDNA3.1-Merlin or pcDNA3.1- T230A S315A-Merlin, detached and re-seeded in media containing selection antibiotics. Foci formed were counted after 10-14 days.

**Animal studies** - Cells (1 million) suspended in HBSS (Invitrogen) were injected into the exposed third mammary fat pad of female athymic nude mice. Orthogonal tumor measurements were recorded twice-weekly. Mean tumor diameter was calculated as the square-root of the product of orthogonal measurements. These studies were conducted under IACUC-approved protocol.

**Immunohistochemistry** - Breast tumor tissue microarrays from NCI Cooperative Breast Cancer Tissue Resource (Supplementary Data 1) were immunohistochemically stained for OPN (AKm2A1; Santa Cruz) and Merlin (A-19; Santa Cruz) using the streptavidin biotin complex method. Staining intensity was quantitated with computer-assisted image analysis in a Dako ACIS III Image Analysis System (Glostrup, Denmark).

**Statistical Analyses** - Associations between intensities of Merlin and OPN expressions and patient’s clinicopathologic data were assessed using the Wilcoxon rank test for categorical data and the Pearson’s correlation coefficient for numerical data. The percentages of normal and tumor tissues expressing Merlin or OPN were compared using a Chi-square test. The significance of percentages of samples expressing Merlin or OPN as compared to the chance occurrence was determined using the exact binomial test. The univariate and multiple logistic regression models were fit to a binary variable normal versus tumor with Merlin and OPN as possible predictors. The possibility of developing a model using the relationship between OPN and Merlin was tested with a logistic regression model on a selected cohort of the data, scoring only the positive staining events from normal tissues for Merlin and the positive staining events from tumor tissue for OPN. The selection criteria were based on the fact that Merlin is a tumor suppressor, with a strong expression in normal tissue, whereas OPN - a tumor promoting protein, is known to be overexpressed in tumor tissue. The Chi-square test was used to assess the usefulness of model for prediction of likelihood of tumor. The effect likelihood ratio test was used to assess the usefulness of predictor variables in the model. The area under the ROC curve was used to determine the predictive ability of models and in model selection. All statistical analyses were performed using software JMP v 7 (SAS Inc.). All results with p-value < 0.05 were considered statistically significant.

Statistical analyses of in vitro data: Statistical differences between groups were assessed using the Mann-Whitney test, t-test or ANOVA, using GraphPad Prism 4 software. Statistical significance was determined if the analysis reached 95% confidence. The precise p-values are listed in the corresponding figure legends.

**RESULTS**

Merlin and OPN are inversely expressed in breast cancer tissues - We probed a breast tumor tissue microarray comprising 75 invasive breast cancer cases and 9 normal breast tissues for Merlin by immunohistochemistry. The expression of Merlin did not change significantly
with respect to ethnicity, age, ER or PR status or tumor size. However, there was an overall decrease in Merlin staining in invasive breast cancer tissues grades I-III (Infiltrating Ductal Carcinoma, IDC) compared to normal tissues (Fig. 1A, images c-f (tumor) relative to a-b (normal)). The expression of Merlin was significantly lower regardless of the nodal involvement. Notably, of the 75 carcinoma tissues, 56 (75%) tissues had lost expression of Merlin (p=0.0000097) (Fig. 1, B & C). In contrast, the expression of OPN was increased in breast cancer cases compared to normal breast tissues (Fig. 1A, images i-l (tumor) relative to g-h (normal)) (p = 0.0097) (Fig. 1, D). Relative to normal tissues, a greater proportion of the tumor tissues showed OPN expression (Fig. 1, E). Overall, of all the 3 grades combined, 43 tissues out of the 56 tissues showed no staining for Merlin simultaneous with increased staining for OPN. Thus, 77% (43 out of 56 Merlin-negative tissues) of the tissues that had lost Merlin expression showed increased OPN expression (p=0.000031). Specifically, the primary tumors from 23 out of the 24 cases with distant metastasis showed no staining for Merlin (p=0.000001). Of these, 20 cases (80%) showed increased OPN staining (p=0.00077) (Fig. 1, F). Thus, our studies showed that Merlin protein expression is lost in invasive breast cancer and the loss of Merlin is accompanied by an increased expression of OPN.

The transcript levels of Merlin are unaltered in breast cancer tissues while those of OPN are increased - We queried the expression of Merlin in breast tumor tissues at two levels: amount of the transcript and the extent of protein expression. We assessed the transcript levels in tissues from 41 breast cancer patients and 7 normal control tissues. The transcript levels of Merlin did not show any appreciable changes (p > 0.05) between normal and breast tumor-derived tissues; there was also no change in the Merlin transcript levels across the different grades of tumors or the disease stage (Fig. 2, A-C). In contrast, the transcript levels of OPN were significantly (p < 0.01) greater in the tumor tissues relative to normal tissues. The OPN transcript levels also increased significantly in tissues derived from grades II and III tumors and with progression of the disease stage (Fig. 2, D-F).

Merlin suppresses malignant behavior of breast cancer cells - Merlin’s role as a tumor suppressor is characterized in tumors of the nervous system. In order to determine the role of Merlin in impacting malignant behavior of breast cancer cells, we restored the expression of Merlin in two human breast cancer cell lines, SUM159 (Fig. 3, A) and MDA-MB-231 (Fig. 3, B). We assessed the malignant attributes of the resultant Merlin-expressing transfectants. Expression of Merlin caused a significant reduction in the ability of breast cancer cells to form foci (Fig. 3, C & D), invade through Matrigel (Fig. 3 E & F), laterally migrate (Fig. 3, G) and grow under anchorage-independent conditions (Fig. 3, H). When injected into the mammary fat pad of female athymic nude mice, the Merlin-expressing SUM159 cells showed notable (p < 0.05) latency in the appearance of palpable tumors (Fig. 3, I). While the tumors formed by vector control cells were evident beginning at 10 days post-injection, those formed by the mixed pool and clone 6 were palpable 19 days and 54 days after injection, respectively. The Merlin-transfectant A1 and A2 clones of MDA-MB-231 also demonstrated a significantly (p < 0.05) reduced growth rate (Fig. 3, F). The Mixed Pool of Merlin transfectants of both, SUM159 and MDA-MB-231 cells showed a modest, but significant reduction on tumor growth rate. This may be likely due to a mixed population of Merlin-expressing and non-expressing cells. Cumulatively, restoration of Merlin expression in both breast cancer cell lines resulted in reduced malignant behavior.

OPN targets Merlin for Akt-mediated proteasomal degradation - We hypothesized that Akt signaling initiated downstream of OPN could regulate Merlin. Thus, to directly test the effects of OPN on the post-translational regulation of Merlin, specifically the stability of Merlin protein, we transfected SUM159 breast cancer cells with Merlin cDNA and treated with recombinant OPN. OPN causes a decrease in the protein levels of Merlin (Fig. 4, A). Treatment with the proteasome inhibitor, Lactacystin, rescued the levels of Merlin in OPN-treated cells, suggesting that OPN-initiated signaling targeted
Merlin for proteasome-mediated degradation. OPN interacts with a variety of cell surface receptors including CD44 and multiple integrins to activate signaling via the Akt pathway (31,38,39). To assess the role of Akt in OPN-initiated degradation of endogenous Merlin, we treated MCF7 cells (which express Merlin but do not express detectable levels of OPN) with recombinant OPN. Treatment with OPN caused phosphorylation of Akt concomitant with a decrease in the levels of endogenous Merlin suggesting that degradation of Merlin can be initiated by signaling downstream of OPN via Akt (Fig. 4, B, Lane 3). MCF7 cells were also treated with Akt inhibitor IV in addition to OPN (Lanes 3, 4, 5). While the levels of Akt phosphorylation predictably decreased after treatment, the levels of endogenous Merlin were restored by the inhibition of Akt phosphorylation even in the presence of OPN (Fig. 4, C, Lane 2), suggesting that inhibition of Akt activation downstream of OPN blocks the effects on degradation of Merlin. As seen in the accompanying Table, co-treatment with the Akt inhibitor blocks the effects of OPN allowing for a total recovery of endogenous Merlin (Lanes 4 & 5).

Phosphorylation of Merlin via Akt targets it for degradation by the proteasome (15,40,41). Thus, in order to determine if OPN can induce ubiquitination of endogenous Merlin leading to its proteasomal degradation, MCF10AT cells were transfected with a HA-ubiquitin expressing construct. In the presence of OPN, endogenous Merlin undergoes some ubiquitination that is evident as a smear (Fig. 4, C, Lane 2). This smear persisted in the presence of Lactacystin (Fig. 4, C, Lane 3), suggesting that Merlin was likely ubiquitinated in the cells in the presence of OPN. The beads by themselves do not non-specifically bind the HA-tagged ubiquitinated proteins in the cells (Supplementary Data 2). To specifically assess the role of activated Akt induced by OPN we co-treated with an Akt inhibitor. Ubiquitination of endogenous Merlin was abolished in the presence of Akt inhibitor, suggesting that OPN-induced Akt phosphorylation caused degradation of endogenous Merlin via the ubiquitin-proteasome pathway (Fig. 4, C, Lane 5). Similar results were observed in SUM159 cells constitutively expressing Merlin. Merlin ubiquitination was enhanced when co-treated with OPN and was abolished in the presence of Akt inhibitor re-affirming the role of Akt downstream of OPN in modulating the stability of Merlin (Supplementary Data 3, A). The converse was seen when we treated MDA-MB-435 cells with the proteasome inhibitor, Lactacystin (10 - 25μM) and the PI-3-kinase inhibitor, wortmannin (100nM). The MDA-MB-435 cells do not express detectable levels of Merlin, but express abundant OPN. Combined treatment with Lactacystin and wortmannin restored Merlin expression in the cells, suggesting that the PI-3-kinase/Akt pathway, in conjunction with the activities of the proteasome, regulates the protein levels of Merlin in the cells (Supplementary Data 3, B).

Silencing the expression of OPN reduced the overall levels of ubiquitinated Merlin; in combination with Akt inhibitor and Lactacystin, abrogating OPN expression caused a notable decrease in the ubiquitinated Merlin (Supplementary Data 3, C).

OPN initiated signaling causes phosphorylation of Merlin at Serine 315 - The loss of Merlin in the presence of OPN is caused by the phosphorylation of Merlin at the Ser315 position (Fig. 5, A). Specifically, phosphorylation of Merlin at this residue has been reported to target it for proteasome-mediated degradation (15,40). This form of Merlin was detectable upon inhibition of proteasomal degradation with Lactacystin in presence of OPN. We further determined that while OPN is able to induce degradation of Merlin, the Merlin mutant T230A S315A (that cannot be phosphorylated by Akt) is resistant to the effects of OPN (Fig. 5, B). Thus, cumulatively, our results suggest that OPN activates Akt-mediated signaling that causes phosphorylation of Merlin at Ser315. This event targets Merlin for ubiquitin-mediated degradation in breast cancer cells.

Degradation-resistant Merlin functionally restricts malignant behavior - We assessed the ability of the Merlin mutant T230A S315A for its ability to impact the properties of breast cancer cells in the perspective of OPN signaling. Both, the wild-type Merlin and the T230A S315A Merlin mutant significantly (p < 0.05) reduced...
the numbers of foci formed by the SUM159 cells (Fig. 5, C). In order to test the effectiveness of T230A S315A Merlin mutant under conditions of elevated OPN expression, we tested the ability of Merlin to impact the foci formation capability of SUM159-OPN (stably expressing OPN) cells. While wild-type Merlin cannot impact the foci formation capability of the SUM159-OPN cells, the T230A S315A Merlin mutant brings about a significant (p < 0.05) reduction in the numbers of foci formed (Fig. 5, D). Similar results were obtained in the assessment of anchorage-independent growth in a soft-agar colonization assay (Fig. 5, E), suggesting that the degradation-resistant T230A S315A Merlin mutant retains its ability to effectively blunt malignant attributes in presence of OPN.

**OPN enhances tissue identification and discriminatory power of Merlin** - In order to assess the discriminatory power of Merlin and OPN, we applied a logistic regression model to a binary variable of normal & tumor tissue to our data. The Chi-square test for appropriateness of model (p = 0.0448; ROC (Receiver Operating Characteristic) curve area = 0.7220) indicates that Merlin has a discriminatory power for distinguishing between normal and tumor tissues (Fig. 6, A). The logistic regression also showed that OPN by itself is not a good discriminator between normal and tumor tissues (p = 0.2878; ROC area = 0.6040) (Fig. 6, B). Further, multiple logistic regression showed that OPN does not increase the discriminatory power of Merlin (p = 0.162; ROC area = 0.723) (Fig. 6, C). Towards the possibility of developing a model that uses the unique inverse relationship between OPN and Merlin, we applied a logistic regression model to a selected cohort of the data, scoring only the positive staining events from normal tissues for Merlin and the positive staining events from tumor tissue for OPN. As seen in Figure 6, D, it is apparent that the logistic model for Merlin alone, using this data set is very good at discriminating between normal and breast tumor tissues (p < 0.0001; R² = 0.43; ROC area = 0.93). Furthermore, given the Merlin intensity, OPN expression ameliorates tissue identification with increased discriminative power of the model (n=46; p < 0.0001; R² = 0.81; ROC area = 0.9917) (Fig. 6, E). We then applied model developed from this training set to our selected data and we found that out of the 46 samples queried, only 2 samples were misclassified (Fig. 6, F) resulting in 96% probability of correct classification.

**DISCUSSION**

While Merlin has been extensively explored in tumors arising from the nervous system, its role in breast cancer is understudied. Early studies reported that mutations in Merlin were not detected in breast cancer (19). In a separate study, Yaegashi et al reported infrequent involvement of mutations in the NF2 gene (encoding for Merlin) in an independent cohort of 60 breast cancer patients (20). Dai et al reported that the estrogen-response gene and tumor suppressor, NHREF, likely acts in conjunction with Merlin to transduce a growth suppressive signal (42). Thus, while there are sporadic references regarding Merlin in breast cancer, the functional and biological roles of Merlin in breast cancer have largely been ignored due to the absence of detectable mutations and the lack of reports of change at the transcript level.

In this study, we have seen that the level of Merlin transcript does not appreciably change in breast tumor tissues. Thus, it was intriguing to note a significant decrease in the immunohistochemical staining for Merlin, suggestive of the fact that Merlin protein expression is lost in breast cancer. In contrast, the oncoprotein, OPN showed an increase in expression at the transcript levels as well as at the protein level. OPN binding to cell surface receptors, such as the integrins, cause several signal transduction pathways to turn on culminating in enhanced proliferation, migration and survival (22). Our studies demonstrate that OPN induces Akt-mediated phosphorylation of Merlin that targets Merlin for ubiquitin-mediated degradation in breast cancer cells resulting in decreased overall cellular pools of endogenous Merlin.

Ubiquitin-mediated degradation of tumor suppressors such as p53, PML, PTEN and VHL has also been documented to be responsible for the decreased availability of the respective proteins in tumor cells (43,44). We showed that degradation of endogenous Merlin is one of the
ways by which OPN-initiated signaling removes the check of this tumor suppressor. OPN is a secreted protein. Hence it is available to the tumor cells in their microenvironment. Given this fact, the implications of our findings can have important considerations for understanding and appreciating the effects that OPN can have on tumor cells. OPN levels increase during pathogenesis of breast cancer. OPN is also available to the tumor cells from the surrounding stromal and inflammatory cells that infiltrate the tumor. OPN-initiated signaling via Akt results in phosphorylation of Merlin and its subsequent degradation. Being a secreted protein that utilizes a variety of receptors, OPN can influence signaling in surrounding tumor cells causing a reduction in Merlin protein levels as a ‘bystander effect’ resulting in a widespread degradation-induced loss of Merlin. As such, while OPN has been reported to induce ubiquitin-mediated degradation of Stat1 (45), ours is the first study to report that OPN causes degradation of a tumor suppressor protein. Although in breast cancer Merlin may not be a prototypic tumor suppressor gene that conforms to the classic definition of Knudsen’s two-hit hypothesis, our study clearly demonstrates that Merlin has a tumor suppressor activity in breast cancer. Restoration of Merlin in breast tumor cells (less than 2-fold upregulated relative to normal tissues; Supplementary Data 4) functionally blunted their malignant properties. As such, the inverse relationship between Merlin and OPN that we observed in clinical specimens is far from just coincidental. Logistic regression identified Merlin intensity as a good predictor for immunohistochemical identification of tumor tissue. It also showed that enhanced staining intensity of OPN ameliorates tissue identification, when combined with the staining intensity of Merlin in breast tumor tissues. The significance of Merlin expression and its function in breast cancer had been ignored thus far. As such, this is the first study reporting a functional role for Merlin in breast cancer and is also the first report of OPN in causing the degradation of a tumor suppressor protein. Thus, our studies elucidate the utility of Merlin and OPN as important biomarkers in breast cancer and also identify a novel mechanism for the loss of Merlin expression in breast cancer.

ACKNOWLEDGMENTS
This work is supported by NIH grants CA138850 to L.A.S. & CA140472 to R.S.S., Mayer Mitchell Award to L.A.S., and the USA-Mitchell Cancer Institute.
References

1. Lau, Y. K., Murray, L. B., Houshmandi, S. S., Xu, Y., Gutmann, D. H., and Yu, Q. (2008) *Cancer Res* **68**, 5733-5742
2. Rushing, E. J., Cooper, P. B., Quezado, M., Begnami, M., Crespo, A., Smirniotopoulos, J. G., Ecklund, J., Olsen, C., and Santi, M. (2007) *J Neurooncol* **85**, 297-305
3. Muranen, T., Gronholm, M., Lampin, A., Lallemand, D., Zhao, F., Giovannini, M., and Carpen, O. (2007) *Hum Mol Genet* **16**, 1742-1751
4. Begnami, M. D., Palau, M., Rushing, E. J., Santi, M., and Quezado, M. (2007) *Hum Pathol* **38**, 1345-1350
5. Fouladi, M., Helton, K., Dalton, J., Gilger, E., Gajjar, A., Merchant, T., Kun, L., Newsham, I., Burger, P., and Fuller, C. (2003) *Cancer* **98**, 2232-2244
6. James, M. F., Han, S., Polizzato, C., Plotkin, S. R., Manning, B. D., Stemmer-Rachamimov, A. O., Gusella, J. F., and Ramesh, V. (2009) *Mol Cell Biol* **29**, 4250-4261
7. Sainio, M., Zhao, F., Heiska, L., Turunen, O., den Bakker, M., Zwarthoff, E., Luchman, M., Rouleau, G. A., Jaaskelainen, J., Vaheeri, A., and Carpen, O. (1997) *J Cell Sci* **110 (Pt 18)**, 2249-2260
8. Poulikakos, P. I., Xiao, G. H., Gallagher, R., Jablonski, S., Jhanwar, S. C., and Testa, J. R. (2006) *Oncogene* **25**, 5960-5968
9. Kim, H., Lim, J. Y., Kim, Y. H., Park, S. H., Lee, K. H., Han, H., Jeun, S. S., Lee, J. H., and Rha, H. K. (2002) *Mol Cells* **14**, 108-114
10. Morrison, H., Sherman, L. S., Legg, J., Banine, F., Isacke, C., Haipek, C. A., Gutmann, D. H., Ponta, H., and Herrlich, P. (2001) *Genes Dev* **15**, 968-980
11. Morrison, H., Sperka, T., Manent, J., Giovannini, M., Ponta, H., and Herrlich, P. (2007) *Cancer Res* **67**, 520-527
12. Xiao, G. H., Gallagher, R., Shetler, J., Skele, K., Altmare, D. A., Pestell, R. G., Jhanwar, S., and Testa, J. R. (2005) *Mol Cell Biol* **25**, 2384-2394
13. Kissil, J. L., Johnson, K. C., Eckman, M. S., and Jacks, T. (2002) *J Biol Chem* **277**, 10394-10399
14. Bai, Y., Liu, Y. J., Wang, H., Xu, Y., Stamenkovic, I., and Yu, Q. (2007) *Oncogene* **26**, 836-850
15. Tang, X., Jang, S. W., Wang, X., Liu, Z., Bahr, S. M., Sun, S. Y., Brat, D., Gutmann, D. H., and Ye, K. (2007) *Nat Cell Biol* **9**, 1199-1207
16. Nunes, F., Shen, Y., Niida, Y., Beauchamp, R., Stemmer-Rachamimov, A. O., Ramesh, V., Gusella, J., and MacCollin, M. (2005) *Cancer Genet Cytogenet* **162**, 135-139
17. Wallace, A. J., Watson, C. J., Oward, E., Evans, D. G., and Elles, R. G. (2004) *Genet Test* **8**, 368-380
18. Lasota, J., Wasag, B., Dansonka-Mieszkowska, A., Karcz, D., Millward, C. L., Rys, J., Stachura, J., Sobin, L. H., and Miettinen, M. (2003) *Lab Invest* **83**, 1361-1371
19. Arakawa, H., Hayashi, N., Nagase, H., Ogawa, M., and Nakamura, Y. (1994) *Hum Mol Genet* **3**, 565-568
20. Yaegashi, S., Sachse, R., Ohuchi, N., Mori, S., and Sekiya, T. (1995) *Jpn J Cancer Res* **86**, 929-933
21. Craig, A. M., Nemir, M., Mukherjee, B. B., Chambers, A. F., and Denhardt, D. T. (1988) *Biochem Biophys Res Commun* **157**, 166-173
22. Shevde, L. A., Das, S., Clark, D. W., and Samant, R. S. (2009) *Curr Mol Med*
23. Shevde, L. A., Samant, R. S., Paik, J. C., Metge, B. J., Chambers, A. F., Casey, G., Frost, A. R., and Welch, D. R. (2006) Clin Exp Metastasis 23, 123-133
24. Kim, Y. W., Park, Y. K., Lee, J., Ko, S. W., and Yang, M. H. (1998) J Korean Med Sci 13, 652-657
25. Tuck, A. B., and Chambers, A. F. (2001) J Mammary Gland Biol Neoplasia 6, 419-429
26. Tuck, A. B., O'Malley, F. P., Singhal, H., Harris, J. F., Tonkin, K. S., Keriovlet, N., Saad, Z., Doig, G. S., and Chambers, A. F. (1998) Int J Cancer 79, 502-508
27. Rudland, P. S., Platt-Higgins, A., El-Tanani, M., De Silva Rudland, S., Baraclough, R., Winstanley, J. H., Howitt, R., and West, C. R. (2002) Cancer Res 62, 3417-3427
28. Furger, K. A., Menon, R. K., Tuck, A. B., Bramwell, V. H., and Chambers, A. F. (2001) Curr Mol Med 1, 621-632
29. Singhal, H., Bautista, D. S., Tonkin, K. S., O'Malley, F. P., Tuck, A. B., Chambers, A. F., and Harris, J. F. (1997) Clin Cancer Res 3, 605-611
30. Rodrigues, L. R., Teixeira, J. A., Schmitt, F. L., Paulsson, M., and Lindmark-Mansson, H. (2007) Cancer Epidemiol Biomarkers Prev 16, 1087-1097
31. Das, R., Mahabeleshwar, G. H., and Kundu, G. C. (2003) J Biol Chem 278, 28593-28606
32. Philip, S., Bulbule, A., and Kundu, G. C. (2004) Glycoconj J 21, 429-441
33. Rangaswami, H., Bulbule, A., and Kundu, G. C. (2006) Glycoconj J 23, 221-232
34. Metge, B. J., Frost, A. R., King, J. A., Dyess, D. L., Welch, D. R., Samant, R. S., and Shevde, L. A. (2008) Clin Exp Metastasis 25, 753-763
35. Lacroix, M. (2009) Cancer Chemother Pharmacol 63, 567
36. Rae, J. M., Creighton, C. J., Meck, J. M., Haddad, B. R., and Johnson, M. D. (2007) Breast Cancer Res Treat 104, 13-19
37. Chambers, A. F. (2009) Cancer Res 69, 5292-5293
38. Robertson, B. W., and Chellaiah, M. A. Exp Cell Res 316, 1-11
39. Zhang, G., He, B., and Weber, G. F. (2003) Mol Cell Biol 23, 6507-6519
40. Okada, M., Wang, Y., Jang, S. W., Tang, X., Neri, L. M., and Ye, K. (2009) Cancer Res 69, 4043-4051
41. Ye, K. (2007) Cell Adh Migr 1, 196-198
42. Dai, J. L., Wang, L., Sahin, A. A., Broemeling, L. D., Schutte, M., and Pan, Y. (2004) Oncogene 23, 8681-8687
43. Yang, Y., Kitagaki, J., Wang, H., Hou, D. X., and Perantoni, A. O. (2009) Cancer Sci 100, 24-28
44. Trotman, L. C., Wang, X., Alimonti, A., Chen, Z., Teruya-Feldstein, J., Yang, H., Pavletich, N. P., Carver, B. S., Cordon-Cardo, C., Erdjument-Bromage, H., Tempst, P., Chi, S. G., Kim, H. J., Misteli, T., Jiang, X., and Pandolfi, P. P. (2007) Cell 128, 141-156
45. Gao, C., Guo, H., Mi, Z., Grusby, M. J., and Kuo, P. C. (2007) J Immunol 178, 1870-1881
FIGURE LEGENDS:

FIGURE 1. Merlin and OPN are inversely expressed in breast cancer tissues. A, Merlin is expressed in normal breast tissue (a, b); but is lost in invasive breast cancer (c-f). Conversely, OPN is expressed at very low levels in normal breast (g, h); but is upregulated in invasive breast cancer (i-l). Immunohistochemical staining was performed for Merlin and OPN on serial sections from 75 cases of invasive breast cancer and 9 normal breast tissues. We recorded loss of Merlin in 75% (56 cases) of invasive breast cancer cases. Of these 56 tissues, 43 (77%) showed concomitant increased OPN expression. Shown are representative photomicrographs of the results. a & g; b & h; c & i; d & j; e & k; f & l represent serial tissue sections. B, The staining intensity of Merlin is reduced in breast cancer tissues (Grades I, II & III) and in tumors showing distant metastasis (DM). The box and whiskers plot shows the range of staining intensities for the tissues. † indicates statistically significant difference relative to normal breast tissues. Relative to normal breast tissue, Merlin expression is significantly lower in Grade I (p = 0.0026), Grade II (p = 0.0005), Grade III (p = 0.0017) tumors and in tumors with distant metastasis (p < 0.0001). C, A greater proportion of normal breast tissues express Merlin in contrast to breast cancer tissues (node-negative and node-positive) as well as those showing distant metastasis (DM) (p = 0.0005). Relative to normal breast tissue, the levels of Merlin are significantly lower in node-negative (p = 0.0171) and node-positive (p = 0.0457) tumors and in tumors with distant metastasis (p < 0.0001). The levels of Merlin in DCIS tissues are not significantly different from normal tissue (p = 0.2026). D, The staining intensity of OPN is significantly increased († p < 0.0001) in breast cancer tissues (Grades I, II & III) and in tumors showing distant metastasis (DM) relative to normal breast tissue. E, A greater proportion of breast cancer tissues (node-negative and node-positive as well as those showing distant metastasis (DM)) express Merlin in contrast to normal breast tissues. F, 77% of patients (across all grades and with distant metastasis) show loss of Merlin expression with concomitant increase in OPN expression. Merlin is expressed in normal breast tissue; its expression is decreased in breast cancer and is negligible in cases with distant metastases. Conversely, OPN expression increases in breast cancer patients.

FIGURE 2. Breast tumor tissues have increased OPN transcript levels, but comparable Merlin transcript levels relative to normal breast tissues. A, The overall levels of Merlin are not significantly reduced (p=0.82) in tumor tissues relative to normal breast tissues. B, C, There is no appreciable change in the levels of Merlin across the grade (p=0.6) of the breast tumor tissues and the disease stage (p=0.15). D, The transcript levels of OPN are significantly increased (^ p=0.0028) in breast tumor tissues relative to normal breast tissues. E, F, The transcript levels of OPN are notably increased with the advance in the grade of the breast tumor (^ p=0.04) and disease stage (^ p=0.01).

FIGURE 3. Merlin can functionally suppress the malignant behavior of breast cancer cells. A, B, Stable Merlin expressing transfectants were derived from SUM159 & MDA-MB-231 cells. C, D, Restoration of Merlin significantly reduces the foci formation ability (^ p=0.005 for SUM159 and ^ p=0.003 for MDA-MB-231), E, F, invasive properties of SUM159 (^ p<0.0001) and MDA-MB-231 cells (^ p<0.0001). G, Restoration of Merlin in SUM159 cells significantly (p=0.014) reduces their ability to laterally migrate (in a wound healing assay) and H, grow under anchorage-independent conditions (^ p=0.02). I, Expression of Merlin in SUM159 cells results in increased latency and reduced tumor growth of the xenografts. The tumor size is represented as mean tumor diameter (^ p<0.0001 relative to vector controls; 4 mice were assessed per group). J, MDA-MB-231 cells restored for Merlin expression show significantly slower growth of the cells as xenografts. (^ p<0.016 relative to vector controls; 4 mice were assessed per group).

FIGURE 4. OPN targets Merlin for Akt-mediated proteasomal degradation. A, OPN (100ng/ml) causes a decrease in the levels of Merlin in SUM159 cells. Treatment with Lactacytisin (10μM) rescues Merlin in presence of OPN. B, In MCF7 cells, that express endogenous Merlin, OPN treatment activates Akt (increased levels of phospho-Akt (Ser 473), Lane 2). The levels of total Akt remain unaltered. Akt
inhibitor IV reduces levels of phospho-Akt and restores endogenous Merlin levels (Lanes 3, 4, 5). The levels of phospho-Akt were assessed 8 hours after treatment with rhOPN and Akt inhibitor, while Merlin levels were scored 16 hours after the treatment. C, OPN increases the levels of ubiquitinated endogenous Merlin (Lane 2). The smear represents ubiquitinated Merlin. Lactacystin arrests the ubiquitinated Merlin resulting in increased intensity of the signal (Lane 4). Inhibition of Akt reduces the intensity of ubiquitinated Merlin (Lane 5). MCF10AT cells were transfected with HA-ubiquitin and treated with OPN, Lactacystin and Akt inhibitor IV. Cell lysate (2mg) harvested in NP40 buffer was immunoprecipitated overnight for endogenous Merlin. The immunoprecipitate was immunoblotted with anti-HA antibody and anti-Merlin antibody. Merlin and GAPDH levels from the cell lysates are represented as inputs for the experiment. The band intensity of Merlin in all the lanes was quantitated relative to the respective GAPDH bands and is depicted in the accompanying Table and is represented as the ratio of Integrated Density Values (IDV) and also as a relative percent IDV ratio (relative to Lane 1, untreated cells). Densitometry was done using the AlphaEase program.

FIGURE 5. OPN initiated signaling causes phosphorylation of Merlin at Serine 315; the degradation resistant Merlin can reduce malignant attributes of breast cancer cells. A, Merlin is phosphorylated at Ser315 in response to OPN. Lysate from SUM159 cells transfected with Merlin and treated with OPN was probed for total Merlin and phosphorylated Merlin (Serine 315). GAPDH was used as a loading control. B, Phosphorylation of Serine 315 and Threonine 230 makes Merlin refractory to OPN. SUM159 cells were transfected with Merlin (WT) or T230A S315A Merlin mutant and treated with OPN and Lactacystin. Cell lysates were probed for total Merlin. GAPDH was used as a loading control. Mutant Merlin (T230A S315A) is not degraded in response to OPN, whereas wild-type Merlin is degraded by OPN. C, Wild-type Merlin and T230A S315A Merlin mutant can significantly (^ p < 0.05) reduce foci formation ability of SUM159 cells. Plasmids corresponding to empty-vector, wild-type Merlin and Merlin mutant were transfected into SUM159 cells. Cells were detached and re-seeded in media containing selection antibiotics. Foci were counted after 10-14 days. D, Only the degradation-resistant T230A S315A Merlin mutant can reduce foci formation in presence of elevated OPN signaling in SUM159 cells (^ p=0.003, relative to vector control). E, Wild-type Merlin and T230A S315A Merlin mutant can significantly (^ p < 0.05) reduce colony formation in soft agar by SUM159 cells. Only the degradation-resistant T230A S315A Merlin mutant can reduce the ability to grow under anchorage-independent condition in soft agar in presence of elevated OPN signaling in SUM159 cells (^ p < 0.05, relative to vector control).

FIGURE 6. OPN ameliorates tissue identification and discriminatory power of Merlin. The reciprocal relationship between Merlin and OPN was assessed by logistic regression and ROC curve analyses. A, Logistic plot using Merlin as a predictor variable to distinguish between normal and tumor tissues. The effect likelihood test (p = 0.0448; ROC (Receiver Operating Characteristic) curve area = 0.722) indicates that Merlin has a discriminatory power for distinguishing between normal and tumor tissues. B, Logistic plot of OPN as a predictor variable shows that OPN by itself, is not reliably able to discriminate between normal and tumor tissues (p = 0.2872; ROC area = 0.6040). C, ROC curve for logistic model with Merlin and OPN as predictor variables to distinguish between normal and tumor tissues indicates that OPN does not augment the discriminatory power of Merlin (whole model test p = 0.0517; ROC area = 0.7234). The effect likelihood tests show that while Merlin contributes significantly (p = 0.0286) to the prediction of tumor tissue, OPN does not (p = 0.1682). D, Logistic plot using data from only the normal tissues that stained for Merlin and the entire dataset of tumor tissue staining for Merlin shows that Merlin has a very high discriminatory power for distinguishing between normal and tumor tissues (p < 0.0001; ROC area = 0.93). E, Logistic plot using data from only the tumor tissues that stained for OPN and the entire dataset of normal tissue staining for OPN shows that OPN has discriminatory power for distinguishing between normal and tumor tissues (p < 0.0007; ROC area = 0.7023). F, ROC curve for logistic model utilizing non-zero Merlin values for normal tissues and non-zero OPN values for tumor tissues as predictor variables to distinguish between normal and tumor tissues indicates that OPN augments the discriminatory power of Merlin (whole model test p < 0.0001; R^2 = 0.81; ROC area = 0.9917). The effect likelihood tests
show that both, Merlin (p < 0.0001) and OPN (p = 0.0001) contribute significantly to the prediction of tumor tissue.
FIGURE 1

A. Images of tissue sections showing different staining intensities of Merlin and OPN.

B. Bar graph showing the mean staining intensity of Merlin across different tissue characteristics. * indicates statistical significance.

C. Bar graph showing the percentage of cases with Merlin expression across different tissue characteristics.

D. Graph showing the staining intensity of OPN across different tissue characteristics. S.E.M. indicates standard error of the mean.

E. Bar graph showing the percentage of cases with OPN expression across different tissue characteristics.

F. Line graph showing the percent expression of OPN and Merlin in tissue specimens. 

Tissue characteristics and mean staining intensity of Merlin:

| Tissue Characteristic | n  | Mean Staining Intensity of Merlin | S.E.M. |
|-----------------------|----|----------------------------------|--------|
| Normal                | 9  | 74.2                             | 19.5   |
| Grade I               | 13 | 37.3                             | 13.6   |
| Grade II              | 25 | 27.8                             | 9.1    |
| Grade III             | 11 | 59.2                             | 14.2   |
| Distant Metastasis    | 24 | 3.9                              | 3.9    |

Tissue characteristics and mean staining intensity of OPN:

| Tissue Characteristic | n  | Mean Staining Intensity of OPN | S.E.M. |
|-----------------------|----|-------------------------------|--------|
| Normal                | 9  | 74.2                          | 19.5   |
| DCIS                  | 9  | 54.0                          | 17.2   |
| Node -ve              | 25 | 33.7                          | 9.2    |
| Node +ve              | 26 | 38.0                          | 9.6    |
| Distant Metastasis    | 24 | 3.9                           | 3.9    |
FIGURE 2

A

B

C

D

E

F

Relative transcript levels, $2^{-\Delta\Delta CT}$ (Mean ± S.E.M.)

Normal (n=7) Grade I (n=1) Grade II (n=13) Grade III (n=27)

Normal (n=7) Stage 1 (n=10) Stage 2 (n=20) Stage 3 (n=11)

Normal (n=7) Stage 1 (n=10) Stage 2 (n=20) Stage 3 (n=11)

Relative transcript levels, $2^{-\Delta\Delta CT}$ levels

Normal (n=7) Tum or (n=41)

Normal (n=7) Tum or (n=41)
FIGURE 4

A

| - | + | + | OPN (100ng/ml) |
| - | - | + | Lactacystin (10μM) |

Merlin

GAPDH

B

| 1 | 2 | 3 | 4 | 5 |
| - | - | 1μM | 2μM | 5μM |
| - | + | + | + | Akt inhibitor |

Merlin

P-AktSer473

Akt

GAPDH

C

IP: Merlin

IB: HA

Ubiquitinated Merlin

Merlin (IP)

Merlin (input)

GAPDH (input)

MCF10AT

Densitometric quantitation of Merlin relative to GAPDH

| 1 | 2 | 3 | 4 | 5 |
| 0.793 | 0.44 | 0.606 | 0.85 | 0.783 |

IDV Merlin/IDV GAPDH

100 55 76 107

Relative Percent IDV Ratio 98.7

TABLE

1 | 2 | 3 | 4 | 5 |
- | - | + | - | + | Akt inhibitor |
- | - | - | + | + | Lactacystin |
- | + | + | + | + | OPN |

Lactacystin (10μM)

OPN (100ng/ml)

Akt inhibitor
FIGURE 5

Table A:

| Treatment                  | Vec | Merlin (WT) | T230A S315A Merlin |
|----------------------------|-----|-------------|--------------------|
| OPN (100ng/ml)             | -   | +           | +                  |
| Lactacystin (10μM)         | -   | -           | +                  |

Figure B:

Graph C:

Percent of colonies relative to vector control (Mean ± S.E.M.)

Graph D:

Percent foci formed (Mean ± S.E.M.)
FIGURE 6

A

B

C

D

E

F

Intensity of Merlin

Intensity of OPN

Likelihood of Tumor

Likelihood of Tumor

1-Specificity

False Positive

Intensity of Merlin

Intensity of OPN

Likelihood of Tumor

Likelihood of Tumor

1-Specificity

False Positive

by guest on March 24, 2020
http://www.jbc.org/
Downloaded from

Loss of tumor suppressor Merlin in advanced breast cancer is due to post-translational regulation
K. Adam Morrow, Shamik Das, Brandon J. Metge, Keqiang Ye, Madhuri S. Mulekar, J. Allan Tucker, Rajeev S. Samant and Lalita A. Shevde

J. Biol. Chem. published online September 30, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M111.250035

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2011/09/30/M111.250035.DC1