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Palladin Contributes to Invasive Motility in Human Breast Cancer Cells

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SUMMARY

Cancer metastasis involves multiple steps including detachment of the metastatic cells from neighboring cells, the acquisition of motility and invasion to other tissue. All of these steps require the reorganization of the actin cytoskeleton. In this study, we found that the protein palladin, a molecular scaffold with an important function in actin organization, is expressed at higher overall levels in tumors compared to benign breast tissue, and also significantly higher in four invasive breast cancer cell lines when compared to four non-invasive cell lines. In addition, we found that palladin plays a key role in the formation of podosomes. Podosomes are actin-rich structures that function in adhesion and matrix degradation and have been found in many invasive cell types. Our results show that phorbol ester treatment stimulated the formation of palladin-containing podosomes in invasive, but not in non-invasive cell lines. More importantly, palladin knockdown resulted in decreased podosome formation and a significant reduction in transwell migration and invasive motility. Palladin overexpression induced podosome formation in the non-invasive MCF7 cells, which are otherwise unable to form podosomes, suggesting that palladin plays a critical role in the assembly of podosomes. Overall, these results indicate that palladin overexpression contributes to the invasive behavior of metastatic cells.

Keywords

podosomes; migration; actin; metastasis

INTRODUCTION

Breast cancer is the second leading cause of death in women in Western countries (Dumitrescu and Cotarla, 2005). The spread of cancer cells to distant sites in the body is the

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major cause of death in breast cancer patients, rather than the primary tumors themselves. In order for cells to metastasize, they must first become detached from neighboring cells, then acquire a motile phenotype, and finally, gain the ability to invade through the extracellular matrix to permit both intravasation and extravasation. Each of these steps involves the dynamic reorganization of specific types of actin arrays. Thus, it is not surprising that many different regulators of the actin cytoskeleton have been implicated in the process of metastasis; examples include cofilin, Arp 2/3, LASP-1, Mena, AFAP-110 and profilin (Di Modugno et al., 2007; Grunewald et al., 2007a; Roy and Jacobson, 2004; Wittenmayer et al., 2004; Yamaguchi and Condeelis, 2007; Zhang et al., 2007).

Palladin is an actin-associated protein that plays an essential role in the assembly and maintenance of multiple types of actin-dependent structures, including both contractile stress fibers in fibroblasts and dynamic dorsal ruffles and podosomes in vascular smooth muscle cells (Goicoechea et al., 2006; Parast and Otey, 2000). Palladin was first shown to play a role in protrusive motility in knockdown experiments with cultured cells, in which palladin expression was found to be required for normal neurite extension in cortical neurons (Boukhelifa et al., 2001). More recently, the phenotype of the palladin null mouse (embryonic lethal, with defects in body wall closure both dorsally and ventrally) demonstrated that palladin plays an essential role in normal cell motility during embryonic development (Luo et al., 2005).

Since palladin is required for normal cell motility during embryonic development, this raises the possibility that palladin could also be involved in the pathological cell motility that is characteristic of metastatic cancer cells, and multiple lines of evidence support this idea. In an analysis of pancreatic and colorectal cancers, palladin was found within a cluster of invasion-specific genes (Ryu et al., 2001). More recently, the Condeelis lab found that the human palladin gene was strongly upregulated (~3 fold) in aggressively motile cells, as compared to the nonmotile cells in the primary tumor (Wang et al., 2004). Finally, a mutation in the human palladin gene was recently implicated in an unusual and highly penetrant form of familial pancreatic cancer, which is an extremely invasive disease (Pogue-Geile et al., 2006). Together, these results suggest that palladin could play a key role in the abnormal behavior of metastatic cancer cells. However, these results are essentially correlative in nature, and the precise mechanism by which palladin contributes to invasive cancer is not known.

The most common localization of palladin is in regularly-spaced puncta along stress fibers of well-spread cells; however, palladin have also been shown to localize to dorsal ruffles, cell-cell junctions and podosomes. Recent advances in the cell motility field have shown that many types of invasive cells form podosomes (Calle et al., 2006; Yamaguchi et al., 2006). Podosomes are highly dynamic structures involved in the adhesion of cells to solid substrates, and they also play a role in tissue invasion and matrix remodeling (Buccione et al., 2004; Gimona and Buccione, 2006; Linder, 2007). Podosomes were originally discovered in monocyte-derived cells, but they have recently been observed in other cell types that migrate across tissue boundaries as part of their physiological function (Carman et al., 2007; Gringel et al., 2006). It has been demonstrated that treatment of cells with phorbol esters such as phorbol dibutyrate (PDBu) induces the formation of podosomes and also

triggers partial disassembly of actin stress fibers. The podosomes induced by PDBu treatment possess the standard podosome features, containing an F-actin core surrounded by a ring of adhesion proteins such as vinculin and talin, and exhibiting a tubular, column-like morphology arising perpendicularly from the bottom of the cells (Gimona et al., 2003; Hai et al., 2002).

In this study, we found that palladin levels in breast cancer patients is higher in malignant tissues than in normal breast samples. In addition, highly invasive breast cancer cell lines express significantly higher levels of palladin than non-invasive cell types. Our results also show that the expression level of palladin is correlated with their ability to form podosomes and that knocking down palladin resulted in a decrease in their ability to form PDBu-induced podosomes, as well as in reduced migration and invasion. These results suggest that palladin plays an important role in promoting podosome formation, and thus high levels of palladin expression may contribute to the invasive motility of metastatic breast cancer cells by facilitating the assembly of these matrix-degrading structures. However, our results also show that overexpression of palladin alone is not sufficient to trigger enhanced invasive motility in cultured human breast cancer cells. Overall, these results support a model in which a cohort of genes is coordinately upregulated in metastatic cells, each contributing to specific aspects of invasive cell motility and cell survival.

RESULTS

Palladin protein levels correlate with metastatic potential in human breast cancer

Previous microarrays results showed that overexpression of palladin correlates with invasive motility in human breast cancer cells implanted in a mouse host (Wang et al., 2004). To further explore palladin's function in breast cancer, immunohistochemistry was performed on paraffin-embedded sections of breast tissue. Histological sections of normal breast tissues ($n = 5$), primary tumors ($n = 10$) and metastasis ($n = 5$) were stained with palladin antibody, and representative images of each group are shown Figure 1A. In normal breast, palladin was detected in ductal epithelial cells and also in occasional blood vessels (not shown). In primary tumors, palladin was detected in the neoplastic cells. Palladin staining was significantly stronger in metastatic tumors. Since metastases are derived from a highly invasive subset of cells in the original tumor, this result is consistent with the idea that palladin overexpression contributes to invasive cell motility. However, immunohistochemistry is only semi-quantitative, so we also investigated palladin levels in patient samples using immunoblot analysis. As shown in Figure 1B, palladin levels were significantly higher overall in primary tumors, as compared to normal breast tissue, although there was variation from sample to sample which may reflect the varying amount of tumor versus normal cells in each sample, or differences in the biologic behavior of these primary tumors. Palladin levels were also high in the three samples of metastatic disease, although these were also variable within this small sample size. To assure approximately equal sample loading in each lane, the blots were also analyzed for GAPDH (a standard house-keeping protein) (Figure 1B). These results support the hypothesis that deregulated palladin expression may be a common feature of invasive cancer. However, both our results obtained with patient samples and the previously published microarray results demonstrate only a

correlation and do not directly demonstrate a role for palladin in invasive cell motility. Therefore, we turned to a cultured cell model system to explore palladin's cellular function in more detail.

The expression of palladin in breast cancer cell lines was examined by immunoblot analysis using total cell lysates Figure 2A. For these experiments, we utilized eight lines of human breast cancer cells. These cell lines are well characterized, and their invasive potentials have been established: T47D, BT474, ZR75.1 and MCF-7 cells are non-invasive, while BT549, Hs578T, MDA-MB-231 and SUM159 are invasive in nude mice and also invasively motile *in vitro* (Flanagan et al., 1999; Kuperwasser et al., 2005; Zajchowski et al., 2001). The expression levels of palladin were normalized against those of tubulin and the results are presented in arbitrary units and shown in Figure 2B. Among the breast cancer cell lines, high levels (approximately 80 arbitrary units) of palladin were detected in the highly invasive BT549, Hs578, MDA-MB-231 and SUM159 cells, whereas very low levels (approximately 5 arbitrary units) were detected in the non-invasive T47D, BT474, ZR75.1 and MCF7 cells. The correlation of palladin expression level with the metastatic potential/invasiveness of breast cancer cells suggests that palladin may be part of the mechanism governing the migratory and invasiveness of breast cancer cells.

Invasive breast cancer cells form podosomes in response to PBDu treatment

The presence of podosomes correlates with invasiveness in a variety of human cancer cell lines (Bowden et al., 1999; Kelly et al., 1998; Monsky et al., 1994; Seals et al., 2005). Podosomes are believed to facilitate migration across basement membranes through the activity of membrane-bound proteases that digest the extracellular matrix (Linder and Aepfelbacher, 2003). We examined the ability of both non-invasive and invasive breast cancer cells to form podosomes in response to phorbol ester stimulation. MCF7, MDA-MB-231 and SUM159 cells were treated with two phorbol esters, PBDu and PMA, fixed and processed for immunofluorescence. Podosomes, identified by their dot-like concentrations of F-actin, were found both individually and in clusters scattered over the ventral membrane (Figure S1). Z-series analyses of individual podosome structures (Figure S1, insets) shows that the adhesion protein vinculin, which is an intrinsic component of podosomes, closely surrounds the actin core (Figure S1). Figure 3A shows that podosome formation was detected only in the invasive cell lines MDA-MB-231 and SUM159 cells shortly after addition of either PDBu (Figure 3A) or PMA (not shown), but not in the non-invasive MCF7 cells, which have very low levels of endogenous palladin (Figure 3A).

Palladin localizes to podosomes and enhances podosome formation

Palladin has been shown previously to play an essential role in podosome formation in vascular smooth muscle cells (Goicoechea et al., 2006). We hypothesized that the high levels of palladin expression in MDA-MB-231 and SUM 159 cells could facilitate the formation of podosomes in these invasive breast cancer cells. To determine if palladin is recruited to these actin-based structures, immunostaining was used to visualize endogenous palladin in invasive MDA-MB-231 and SUM159 cells after stimulation with PDBu. Figure 3B shows that palladin was clearly enriched in the podosomes and co-localized with actin in cells double-labeled for palladin and F-actin.

To determine the role of palladin in PDBu induction of podosomes, we examined the effect of palladin knockdown on the cellular response to PDBu. siRNA oligos were used to knock down the expression of palladin. Figure 3C shows that when palladin siRNA transfected cells were treated with PDBu, a significant percentage of the siRNA-transfected cells were unable to form podosomes. When these results were quantified, we found that palladin knockdown reduces the percentage of cells that form podosomes from 36% to 19% (Fig. 3C). These results suggest that palladin plays an important role in increasing the efficiency of podosome formation induced by PDBu.

Palladin knockdown in SUM159 cells impairs cell migration and invasion

We found that palladin is overexpressed in highly invasive SUM159 cells compared with the non-invasive cell line MCF7. This finding, together with the previous demonstration that palladin is localized to dynamic structures such as dorsal ruffles and podosomes (Goicoechea et al., 2006) prompted us to investigate the role of palladin in SUM159 cell migration and invasion using serum as a chemo-attractant. SUM159 cells were transfected with siRNAs and 96 h later, were collected and placed in the upper half of the inserts, which were then incubated for 24 h. Western blot analysis shows that treatment with palladin siRNA leads to a ~90% reduction in palladin expression in SUM 159 cells (KD), as compared to cells treated with control siRNA (Figure 4A). Representative photomicrographs illustrate that SUM159 cells transfected with control siRNA migrate and invade faster compared with cells transfected with palladin siRNAs (Figure 4B-C). Following the quantification of stained cells, the percentage of migration and invasion was calculated by normalization of the number of knockdown cells, to the number of control cells that moved through the filters showing that palladin knockdown reduces both, the percentage of cells that migrate through the membrane by 60% (Figure 4B) and the percentage of cells that invade through the Matrigel by 50% (Figure 4C). In contrast, MCF-7 cells neither migrated nor invaded through the filters, either with or without palladin siRNA treatment (Figure 4B-C). These results suggest that palladin plays an important role in cell migration and invasion.

Palladin overexpression in MCF7 cells induces podosome formation

We have shown so far that palladin knockdown results in decreased podosome formation, and in a significant reduction in the efficiency of transwell migration and invasive motility of SUM159 cells which express high levels of palladin. We also have shown that in the absence of exogenous palladin, MCF7 cells (which have low endogenous levels of palladin) do not form podosomes at all. Next, we wanted to determine whether experimental overexpression palladin was sufficient to drive PDBu-induced formation of podosomes. We infected MCF7 cells with an adenovirus vector encoding either GFP (control) or myc-palladin (OE). We overexpressed palladin to levels that are comparable with those found in invasive cells such as Hs578, MDA-MB-231 and SUM159 cells (Figure 5A). Double immunostaining was used to visualize myc-palladin and actin in MCF7 cells after stimulation with PDBu (Figure 5B). When cells were infected with adenovirus vector encoding myc-palladin, podosome formation was observed in 10% of cells (Fig. 5C).

Because palladin expression levels correlate with the invasiveness of breast cancer cells, we also examined whether palladin overexpression could promote cell migration and/or cell

invasion in non-invasive MCF7 cells, or enhance the invasion of SUM159 cells. Cells were infected with control and palladin-overexpressing adenovirus vectors (Figure 6A) and 24 h later, were collected and placed in the upper half of the inserts, which were then incubated for a further 24 h. Figure 6B and 6C shows that overexpression of palladin does not have any effect in the migration or invasion of SUM159 cells. Consistently with the results showed above, MCF7 cell did not migrate or invade either with or without overexpressed palladin levels. Together, these results suggest that palladin plays an important role in podosome formation but is not sufficient to promote cell migration and invasion.

DISCUSSION

Palladin's function has been explored previously in multiple types of cultured cells and also in knockout mice (Goicoechea et al., In Press). These studies have shown that palladin expression correlates closely with cell motility: palladin protein is more abundant in embryonic tissues (which are highly plastic) than in adult mammals (Parast and Otey, 2000), and in many adult organs, palladin is upregulated after injury near the wound-site, where cell migration and tissue remodeling occur (Boukhelifa et al., 2003; Jin et al., 2007; Ronty et al., 2006). This suggests that in normal cells, palladin expression is tightly controlled to allow for regulated transitions from non-motile to motile phenotypes. The present study was directed at understanding the potential role of palladin in a pathological type of cell migration, the invasive motility that characterizes malignant breast cancer cells. The role of palladin in breast cancer metastasis has not been investigated previously; however, multiple lines of evidence suggest that palladin could be critically involved in this process. First, palladin is a key regulator of the actin cytoskeleton. Palladin has been shown previously to be required for the maintenance of a normal actin cytoskeleton in a wide variety of cells (Goicoechea et al, In Press). Recently, a palladin knockout mouse was generated, and fibroblasts from this mouse displayed defects in cell motility, cell adhesion and actin organization, demonstrating that normal palladin levels are required for normal actin assembly (Luo et al., 2005). Second, palladin has been implicated in other highly invasive cancers. The human palladin gene is point-mutated in an inherited form of pancreatic cancer and also overexpressed in many cases of sporadic pancreatic cancer (Pogue-Geile et al., 2006). Pancreatic cancer is lethal in 95-99% of cases, due to the extremely aggressive invasive motility of pancreatic cancer cells, suggesting the idea that deregulated palladin may contribute to this pathological cell behavior. Third, palladin is overexpressed in the most invasive population of cancer cells, as demonstrated by the results of two independent gene expression studies (Ryu et al., 2001; Wang et al., 2004). This correlation spurred us to test directly the hypothesis that high levels of palladin expression contribute to invasive motility.

Our results showed that palladin levels are much higher in more invasive than in noninvasive breast cancer cells: the most highly invasive breast cancer cell lines express palladin at levels that are approximately eighty times of those expressed by the majority of weakly invasive breast cancer cells. As the invasiveness of cancer cells is dependent on increased migratory and invasive properties, we have tested the hypothesis that the mechanism of action of palladin overexpression in breast cancers and cell lines is to promote the migration and invasiveness of breast cancer cells. Both gain of function (by

overexpression) and loss of function (by siRNA-mediated knockdown) established a critical role of palladin in the formation of podosomes by human breast cancer cells. Overexpression of palladin promotes podosome formation but has no effect on cell migration. These results are in accordance with Wang et al, who proposed that it is the activity of a pathway as a whole and not the change in expression of any one particular gene that determines the invasive and metastatic phenotype of the tumor (Wang et al., 2005).

The question remains: how exactly does palladin contribute to these processes? We have recently shown that palladin binds to actin and that it plays a role as an actin-crosslinking protein (Dixon et al., 2008) leading us to the hypothesis that palladin may be involved in migration and invasion by organizing the actin networks at sites of protrusion, by crosslinking actin filaments into bundles. Many results obtained to date suggest that palladin also acts as a potent molecular scaffold with a critical role in organizing actin filaments into functional arrays. Palladin binds to a large number of actin-associated proteins, including ezrin, VASP/Mena/EVL, profilin, alpha-actinin, Lasp-1 and Eps8 (Boukhelifa et al., 2006; Boukhelifa et al., 2004; Goicoechea et al., 2006; Mykkanen et al., 2001; Rachlin and Otey, 2006; Ronty et al., 2004). In addition, palladin binds to signaling intermediaries such as ArgBP-2 and SPIN-90 (Ronty et al., 2007; Ronty et al., 2005), and palladin expression is required for the actin-cytoskeleton rearrangements that occur downstream of active Src (Ronty et al., 2007), suggesting that palladin regulates the organization of the actin cytoskeleton via multiple molecular pathways.

It has been proposed that genes encoding many components of the pathways that cause migration, including both stimulatory and inhibitory parts of each pathway, are coordinately upregulated in invading cells (Wang et al., 2005). It is worth noting that many of palladin's binding partners have been shown to be upregulated in invasive cancers. For example, Lasp-1 has been implicated in the motility of ovarian cancer and breast cancer cells (Grunewald et al., 2007a; Grunewald et al., 2006; Grunewald et al., 2007b; Lin et al., 2004). In addition, palladin binds to alpha-actinin, a widely expressed actin-crosslinking protein (Ronty et al., 2004) that is also overexpressed in metastatic sub-populations of cancer cells (Hatakeyama et al., 2006; Honda et al., 1998; Suehara et al., 2006). Eps8 is increased in pancreatic cancer, and its expression is required for dynamic actin-based cell protrusions and intercellular cytoskeletal organization (Welsch et al., 2007). The strong binding interaction between palladin and its binding partners, and their high degree of co-localization in cells and tissues, suggest that these proteins may have a shared function in motility and adhesion that may be deregulated in cancer cells.

In conclusion, we provide insight into the role of palladin in cancer metastasis stemming from its important involvement in actin organization and cell motility. Future studies will evaluate the relationship between palladin expression and metastatic potential in animal models and cancer progression in breast cancer patients. Further studies are required to delineate whether palladin and/or any of its regulators represent novel targets for therapeutic interventions in human cancer.

EXPERIMENTAL PROCEDURES

Cell lines

Eight breast cancer cell lines were used: T47D, BT474, ZR75.1, MCF7, BT549, Hs578, MDAMB-231 and SUM159. T-47D cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS) and 10 μ g/ml of insulin. MDA-MB-231, MCF7, BT474, and SKBR3 cells were cultured in DMEM containing 10% FBS. BT-549 cells were cultured in RPMI supplemented with 10% FBS. ZR-75-1 were obtained from ATCC and cultured in DMEM supplemented with 5% fetal bovine serum (FBS), 2 mM Lglutamine. Hs578 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 2 mM glutamine. SUM159 cells were cultured in Ham's F12 with 5% calf serum, 5 μ g/ml insulin, and 1 μ g/ml hydrocortisone. All cell lines were grown at 37°C and 10% carbon dioxide.

Materials

Antibodies against the following proteins were used: palladin (polyclonal antibody and monoclonal 1E6 antibody previously characterized by Parast and Otey (Parast and Otey, 2000). Tubulin alpha (Lab Vision Corporation); GAPDH and cortactin (Santa Cruz) and alpha-actinin (Sigma). Protease inhibitor cocktail for mammalian tissues were from Sigma. TransIT siQuest TransIT-LT1 transfection reagent was from Mirus. Alexafluor-488 and Alexafluor-568 anti-mouse IgG and anti-rabbit IgG conjugated secondary antibodies were from Molecular Probes.

Immunofluorescence Staining

Breast cancer cells lines MCF7, MDA-MB-231 and SUM159 were grown on glass coverslips and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), then permeabilized in 0.2% Triton X-100 and incubated with the specific primary antibodies for 1 h. Primary antibodies were detected with Alexafluor-488 and Alexafluor-568 anti-mouse IgG and anti-rabbit IgG conjugates. Coverslips were examined with a Nikon TE200-U microscope with 20x and 60x objective lenses, an optional 1.5x tube lens and a Hamamatsu Orca-ER camera. Images were processed using Adobe Photoshop 7.0 (Adobe Systems Inc). Podosome formation was induced by the addition of 1 μ M phorbol-12,13-dibutyrate (PDBu; Sigma-Aldrich), as previously described (Gimona et al., 2003; Hai et al., 2002). Confocal image acquisition and analysis were performed using a Leica SP2 AOBS confocal microscope with a 63 \times , 1.4-numerical-aperture apochromatic Leica lens (Leica Microsystems). Scanning was performed with the xz axis, using three independent laser sources (364-nm-UV, 488-nm-Ar, 568-nm-Kr lasers), as required. Images were processed using Leica and Adobe Photoshop software.

siRNA Knockdown

To knock down the expression of palladin by RNA interference, two 21-base oligonucleotides were purchased from Dharmacon Research. RNA sequences were the following: sense, 5'-CUACUCCGUGUCACAUUAUU-3' and antisense, 5'-UAAUGUGACAGCGGAGUAGUU-3'. As a control we used siCONTROL Non-Targeting

siRNA #2 from Dharmacon. MCF7 and SUM159 cells were transfected using the TransIT siQuest transfection reagent following manufacture's instructions. Briefly, 1.5 ml of serum-free medium (Life Technologies) and 20 μ l of transfection reagent per plate were preincubated for 15 minutes at room temperature. After incubation, 225 μ l of 1 μ M siRNA was added to the diluted TransIT siQuest reagent and the mixture was incubated for 15 minutes at room temperature for complex formation. Then, the entire mixture was added to the cells, resulting in a final concentration of 25 nM siRNAs. Cells were assayed between 72 and 96 hours after transfection.

Palladin overexpression using adenoviral system

Mouse mycPalladin and GFP were first cloned into pENTR and then recombined into pAd/CMV/V5-DEST using Gateway recombination technology according to the manufacturer's instructions. Viruses were produced using the Virapower Adenoviral Expression System according to manufacturer's instructions (Invitrogen).

Cell Lysis and Immunoblot

MCF7, MDA-MB-231 and SUM159 cells cultured on 100 mm tissue culture dishes were briefly rinsed with phosphate-buffered saline and then scraped into a lysis buffer containing 50 mM Tris (pH 7.0), 150 mM NaCl, 1% Triton X-100, and a protease inhibitor cocktail for mammalian tissues. The supernatant was collected after centrifugation at 14,000 rpm for 15 min. The cell lysates were either analyzed by immunoblot or processed for migration and invasion assays or frozen with liquid nitrogen and stored at -80°C for future use. For the immunoblot, lysates were boiled in 2X Laemmli buffer, and 20 μ g of protein were resolved by SDS-PAGE in each lane of a 4-12% gel. The proteins were transferred to nitrocellulose and immunoblotted. For imaging, IRdye700 or 800-conjugated secondary antibodies were used with an Odyssey infrared imaging system (Licor). In some cases, immunocomplexes were visualized using the Western Lights Chemiluminescence Detection kit from Perkin-Elmer.

Immunohistochemistry

Paraffin sections of human specimens of normal breast, primary breast cancer, and metastatic breast cancer were obtained from the University of North Carolina Tissue Procurement Facility. The primary antibody for palladin was a commercial polyclonal obtained from Proteintech Group at 1:200 dilution. Briefly, the slides were incubated at 60°C for 15 minutes and then deparaffinized in xylene. Endogenous peroxidase activity was then quenched by incubation for 30 minutes in 0.3% H_2O_2 in methanol. Next, the slides were steamed for 25 minutes in citrate buffer. After adequate cooling, the specimens were placed in diluted normal blocking serum (based on secondary antibody). The slides were then incubated overnight with the primary antibody in a humidity box at 4°C and then placed in diluted biotinylated secondary antibody for 30 minutes at room temperature. The slides were incubated in VECTASTAIN Elite ABC Reagent (Vector Laboratories) for 30 minutes at room temperature followed by peroxidase substrate solution until the desired stain intensity was obtained. Finally, the slides were counterstained in Hemotoxilyn and dehydrated before mounting with a coverslip.

Migration assay

MCF7 and SUM159 siRNA transfected cells and their parental control were serum starved for 2 h, harvested, and resuspended in serum free medium. Cells (5×10^4) were added to the top chambers of 24-well Transwell plates and complete medium was added to the bottom chambers. After 24 hours of incubation at 37°C and 5% CO₂, the nonmigrating cells present on the upper surface of the filter were removed using a sterile cotton swab. The cells that were able to migrate through the chamber onto the lower surface of the filter were fixed and stained with Diff-Quick (American Scientific Products). The number of migrating cells was counted with entire fields and the results were calculated as migration rate in relation to parental control cells. Each experimental condition was done in triplicates and repeated at least 3 times.

Matrigel Invasion Assay

The invasive potential of the parental MCF7 and SUM159 cells and the palladin knockdown cells was assessed using the Matrigel invasion chambers from BD Biosciences. Cells were cultured for 48 hours in complete media, harvested by trypsinization, and counted, and 5×10^4 cells were placed onto the top insert. One chamber consists of a cell insert and a well. The bottom of the cell insert is covered with a filter containing multiple 8- μ m pores and is coated with a basement membrane matrix (Matrigel). Cells, in 500 μ l of serum-free DMEM media, were seeded in the cell insert and placed in the well, which was filled with 750 μ l of DMEM and supplemented with 10% fetal bovine serum. After 24 hours of incubation cells were treated as described above for the migration assay.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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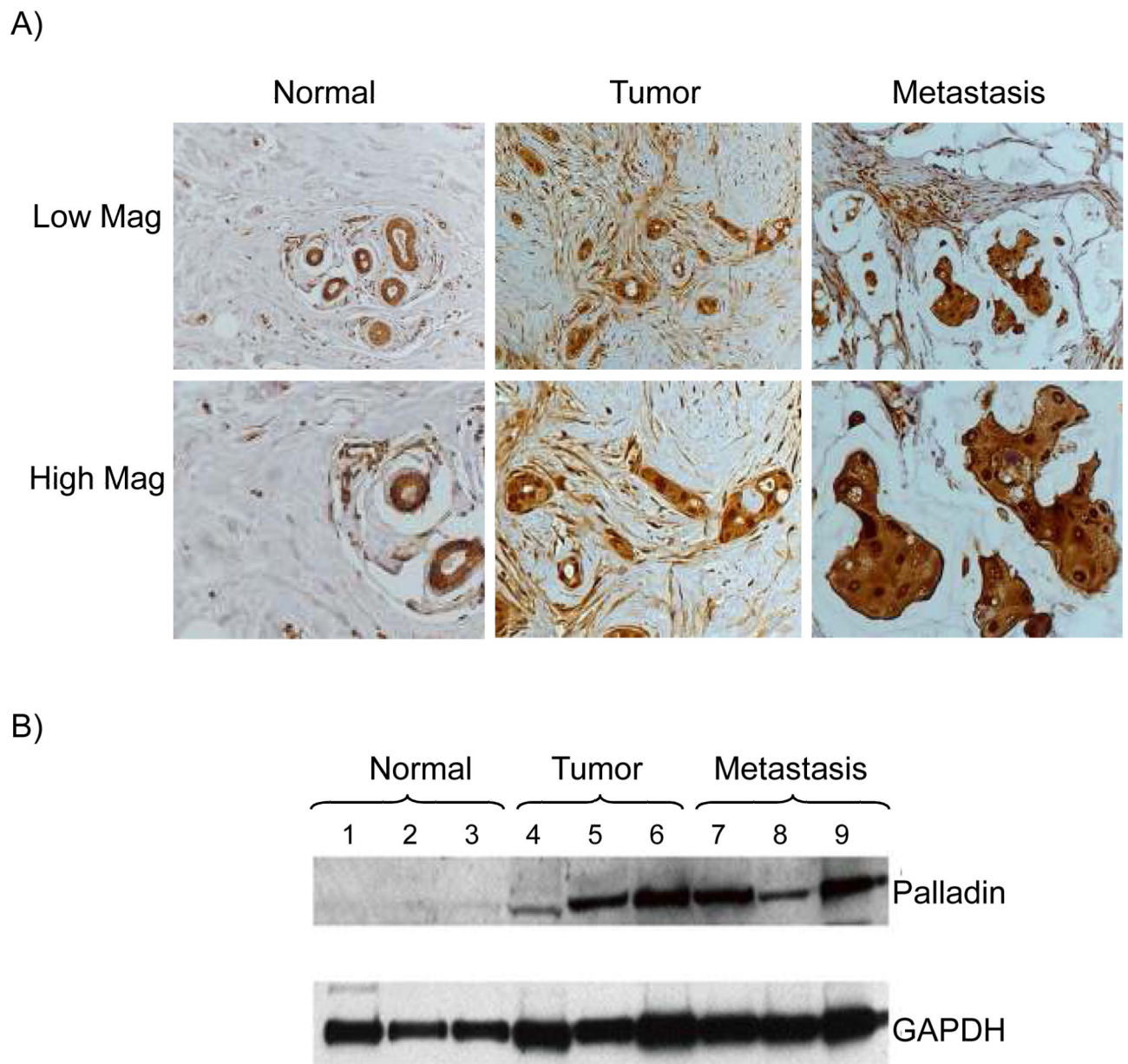


Figure 1. Palladin expression is increased in patient samples of breast cancer compared with normal breast

A. Immunohistochemical analysis of normal, primary breast cancer tumors and metastatic tumors: comparison of the palladin staining in the tissue sections demonstrates an apparent increase in staining in metastatic tumors. **B.** Palladin levels are increased in primary tumors and metastatic tumors: western blots were used to determine the levels of palladin in normal, primary breast cancer tumors and metastatic tumors. GAPDH (a standard house-keeping protein) was used as a loading control.

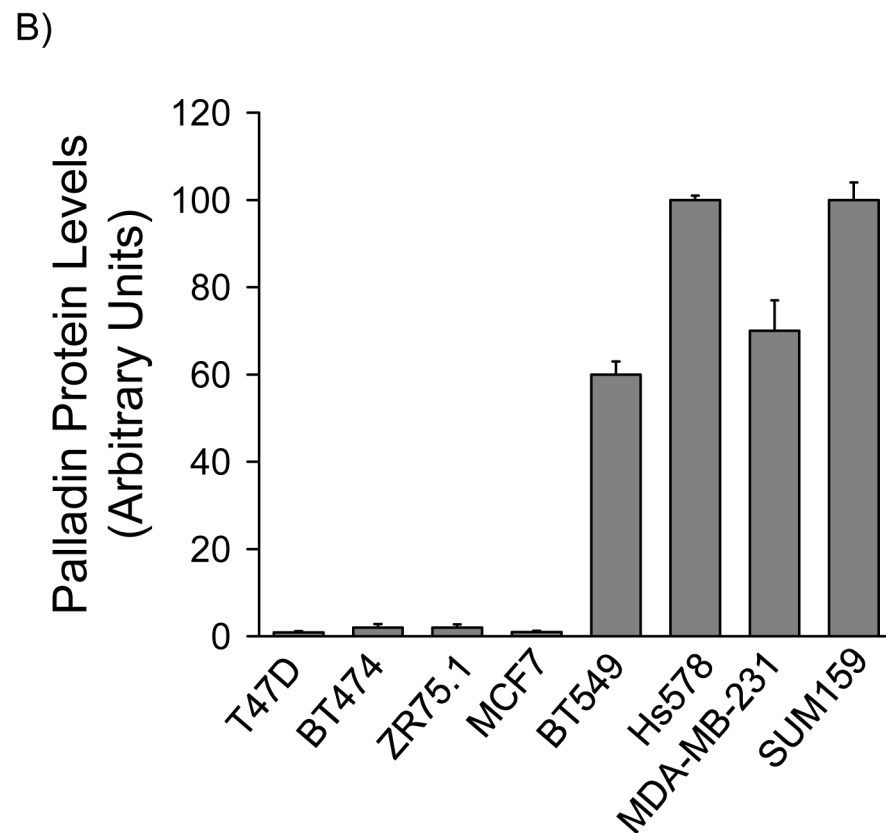
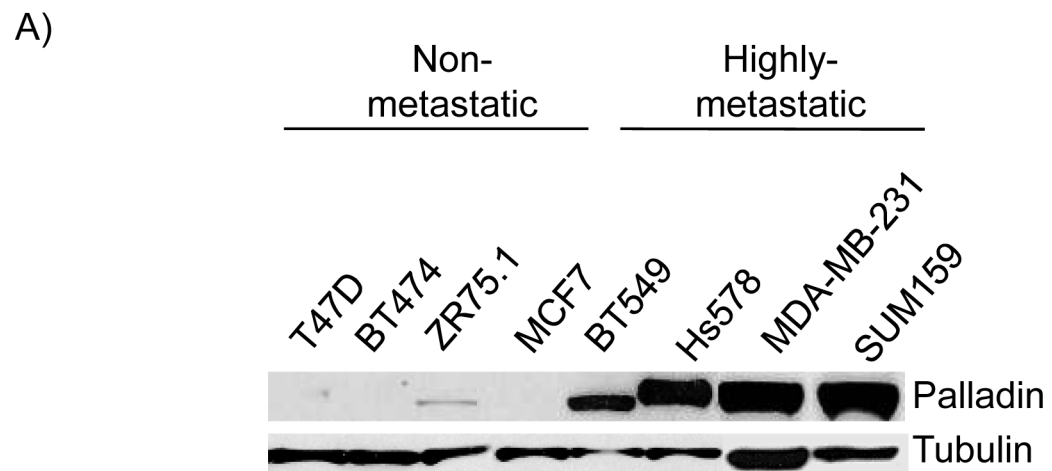


Figure 2. Palladin levels correlate with invasive potential in human breast cancer cells

A- Western blot analysis was used to determine the amount of palladin protein in whole-cell lysates of eight different human breast cancer cell lines: T47D, BT474, ZR75.1, MCF7, BT549, Hs578T, MDA-MB-231 and SUM159. **B-** Quantification of palladin protein levels in breast cancer cells. The ratio of palladin to total cellular tubulin is shown on the left axis, and this amount was found to correlate closely with the invasive potential of the eight cell lines.

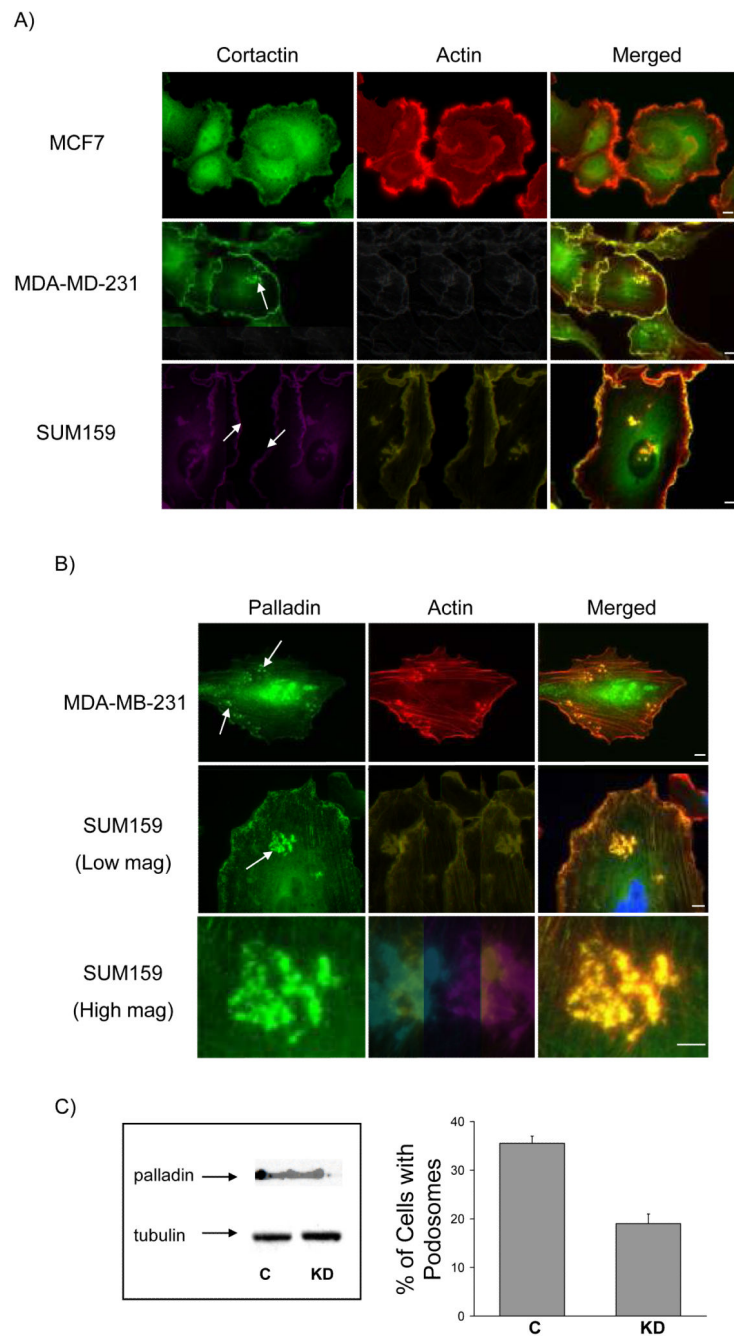


Figure 3. Palladin localizes to PDBu-induced podosomes and enhances podosome formation
A- MCF7, MDA-MB-231 and SUM159 cells were plated on FBS-coated coverslips and treated with the phorbol ester PDBu for 20 minutes. Co-labeling with rhodamine-phalloidin and polyclonal anti-cortactin antibody was used to detect podosomes. Note that only the invasive MDA-MB-231 and SUM159 breast cancer cell lines form podosomes after PDBu treatment and not the non-invasive MCF7 cell line. **B-** MDA-MB-231 and SUM159 cells were plated on FBS-coated coverslips and treated with the phorbol ester PDBu for 20 minutes. After fixation, endogenous palladin was detected by immunofluorescence. Co-

labeling with phalloidin and anti-palladin antibody reveals that palladin was consistently detected in podosomes of the both invasive cell lines. Top two panels: Low magnification images of MDA-MB-231 and SUM159 breast cancer cell lines. Bottom: High magnification image of SUM159 cells to show detail. C- SUM159 cells transfected with control siRNA oligos and siRNA oligos targeting palladin were plated on FBS-coated coverslips overnight and then treated with PDBu for 20 minutes. Left panel- Western blot analysis was used to detect palladin levels in cells treated with control oligos (C) or palladin- specific siRNA oligos (KD). RNA interference reduced expression by ~90%. Right panel- Podosome formation was significantly reduced in palladin knockdown cell (KD, $19 \pm 5 \%$), as compared with control cells (C, $36 \pm 3 \%$). Results are representative of three independent experiments in which at least 300 transfected cells were counted. Scale Bar = $10\mu\text{m}$

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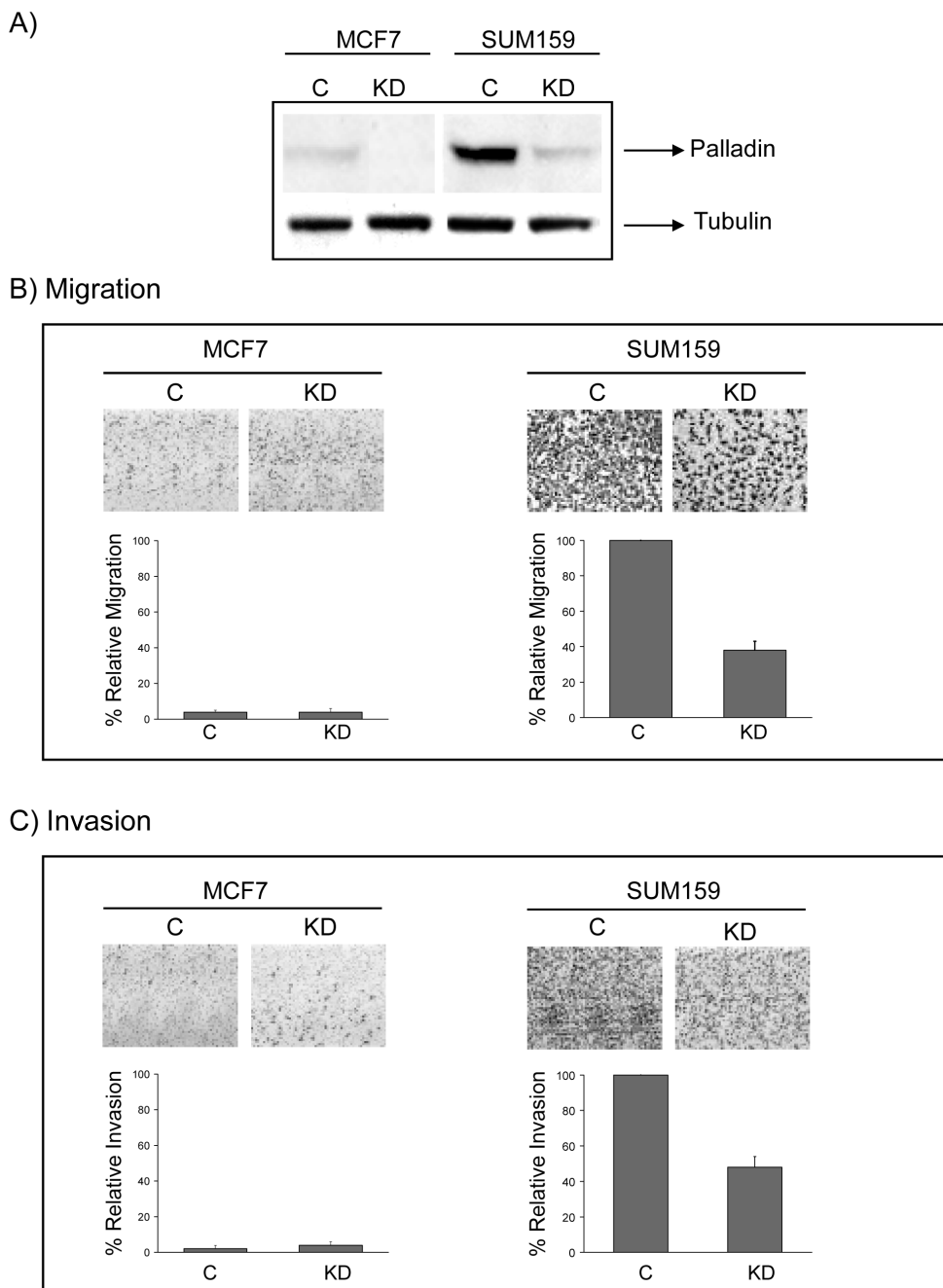


Figure 4. Palladin knockdown reduces migration and invasion of invasive breast cancer cells
A- Western blot analysis shows that treatment with palladin siRNA leads to a ~90% reduction in palladin expression in SUM 159 cells (KD), as compared to cells treated with control siRNA (C). **B-** Transwell migration assay of control siRNA (C) and palladin siRNA (KD) transfected MCF7 and SUM159 cells. Palladin knockdown reduces the number of cells migrating through a transwell filter by ~60% in SUM-159 cells, while the non-invasive MCF-7 cells are non-migratory either with or without palladin siRNA treatment. **C-** Matrigel invasion assay of control siRNA (C) and palladin siRNA (KD) transfected MCF7

and SUM159 cells. Palladin knockdown reduces the number of cells invading through a Matrigel-coated transwell filter by ~50% in SUM-159 cells, while the non-invasive MCF-7 cells are non-invasive either with or without palladin siRNA treatment. For both assays, cells on the lower surface of the filter were fixed and counted 20 hours after plating (Representative microphotographs of migrating and invading cells are included).

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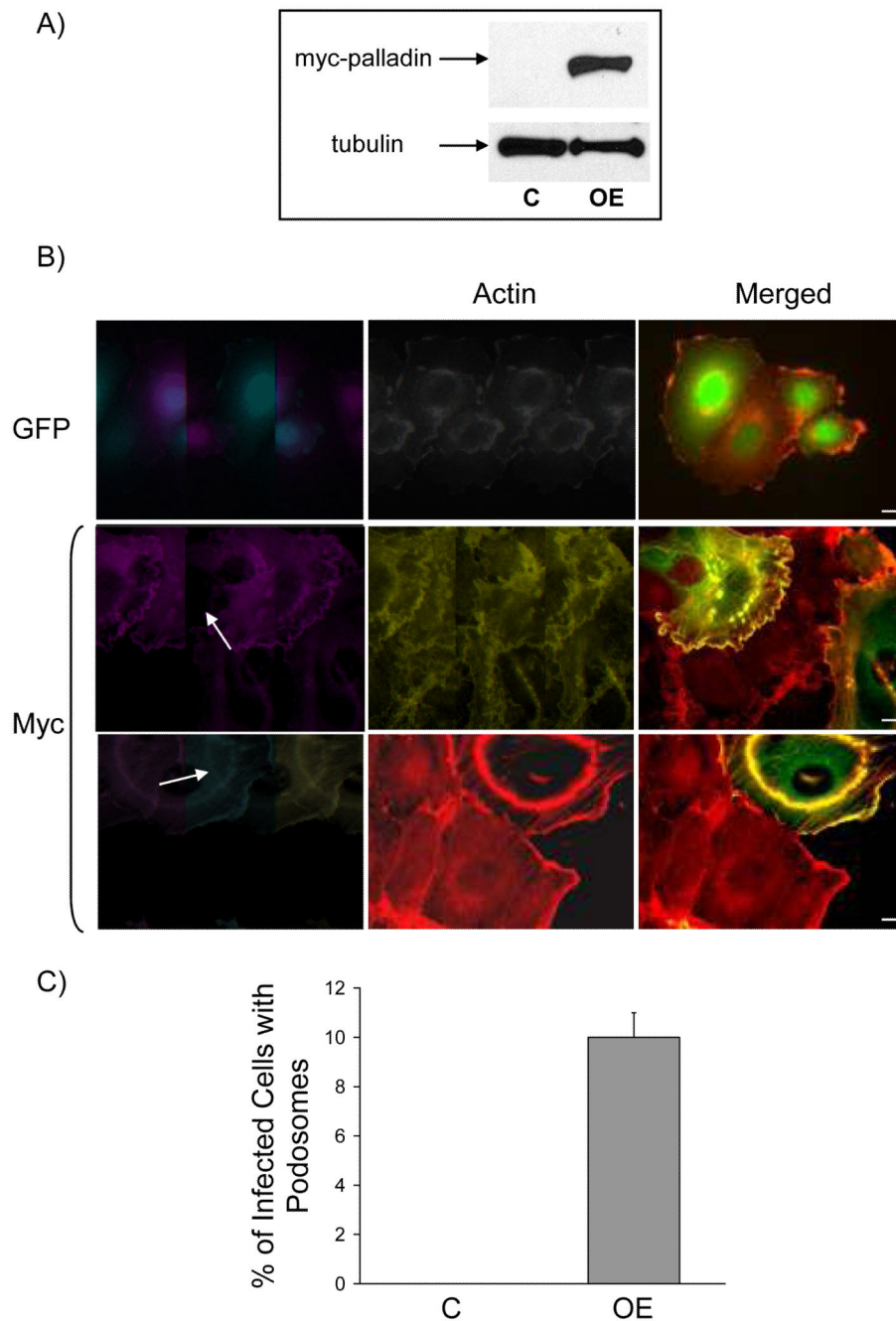


Figure 5. Palladin overexpression induces podosomes formation

A- Western blot analysis shows that treatment with adenovirus vector containing myc-palladin in MCF7 cells leads to palladin levels comparable with those found in invasive cell lines such as MDA-MB-231 and SUM159. **B-** GFP and palladin-overexpressing MCF7 cells were plated on FBS-coated coverslips and treated with the phorbol ester PDBu for 20 minutes. After fixation, palladin was detected by immunofluorescence. Co-labeling with phalloidin and anti-myc antibody reveals that myc-palladin was consistently detected in podosomes of infected cell lines. **C-** Podosome formation was induced in palladin

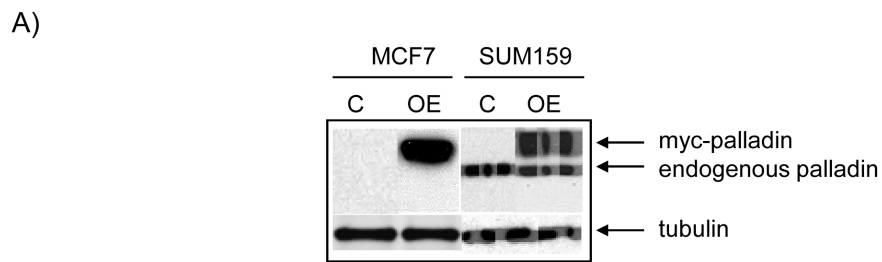
overexpressing cells (OE, $10 \pm 2\%$) as compared with control cells (C, 0 %). Results are representative of three independent experiments in which at least 100 transfected cells were counted. Scale Bar = 10 μ m

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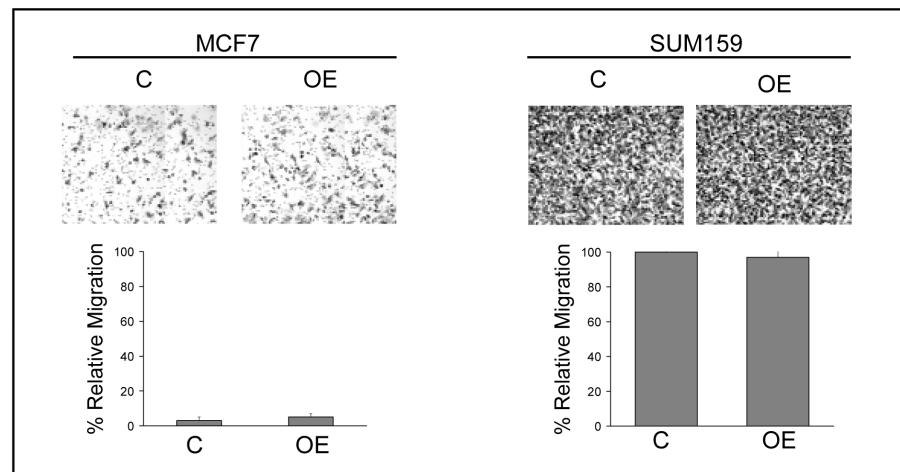
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B) Migration



C) Invasion

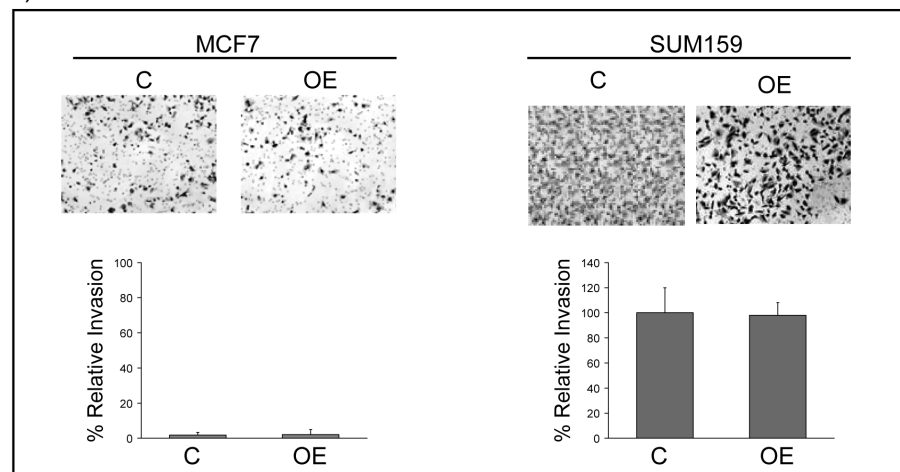


Figure 6. Palladin overexpression does not affect cell migration of either invasive or noninvasive breast cancer cells

A- The levels of palladin in MCF7 and SUM159 cells infected with adenovirus expressing either GFP (C, control) or myc-palladin (OE, overexpression) were assessed by Western blot. **B-** Transwell migration assay of MCF7 (left) and SUM159 (right) expressing either GFP (C) or myc-palladin (OE). **C-** Matrigel invasion assay of control MCF7 (left) and SUM159 (right) expressing either GFP © or myc-palladin (OE). For both assays, cells on the lower surface of the filter were fixed and counted 20 hours after plating (Representative

microphotographs of migrating and invading cells from three different experiments are included).

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