ORIGINAL ARTICLE

Palladin contributes to invasive motility in human breast cancer cells

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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 with benign breast tissue, and also expressed significantly higher in four invasive breast cancer cell lines when compared with 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.
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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 major cause of death in breast cancer patients, rather than the primary tumors themselves.

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, for example cofilin, Arp 2/3, LASP-1, Mena, AFAP-110 and profilin (Roy and Jacobson, 2004; Wittenmayer et al., 2004; Di Modugno et al., 2007; Yamaguchi and Condeelis, 2007; Zhang et al., 2007; Grunewald et al., 2007a).

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 (Parast and Otey, 2000; Goicoechea et al., 2006). 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) showed that palladin plays an essential role in normal cell motility during embryonic development (Luo et al., 2005).

As 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 ($\sim$3-fold) in aggressively motile cells, as compared with the non-motile 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,

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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 (Gringel et al., 2006; Carman et al., 2007). It has been shown that treatment of cells with phorbol esters such as phorbol-12,13-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 (Hai et al., 2002; Gimona et al., 2003).

In this study, we found that palladin levels in breast cancer patients are higher in malignant tissues than that 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 of 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

Earlier 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 (data not shown). In primary tumors, palladin was detected in the neoplastic cells. Palladin staining was significantly stronger in metastatic tumors. As 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 semiquantitative, thus 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 with normal breast tissue, although there was a 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 analysed for GADPH (a standard housekeeping 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 earlier published microassay results show only a correlation and do not directly show 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, namely T47D, BT474, ZR75.1 and MCF-7 cells are non-invasive, whereas BT549, Hs578T, MDA-MB-231 and SUM159 are invasive in nude mice and also invasively motile in vitro (Flanagan et al., 1999; Zajchowski et al., 2001; Kuperwasser et al., 2005). 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, Hs578T, 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.
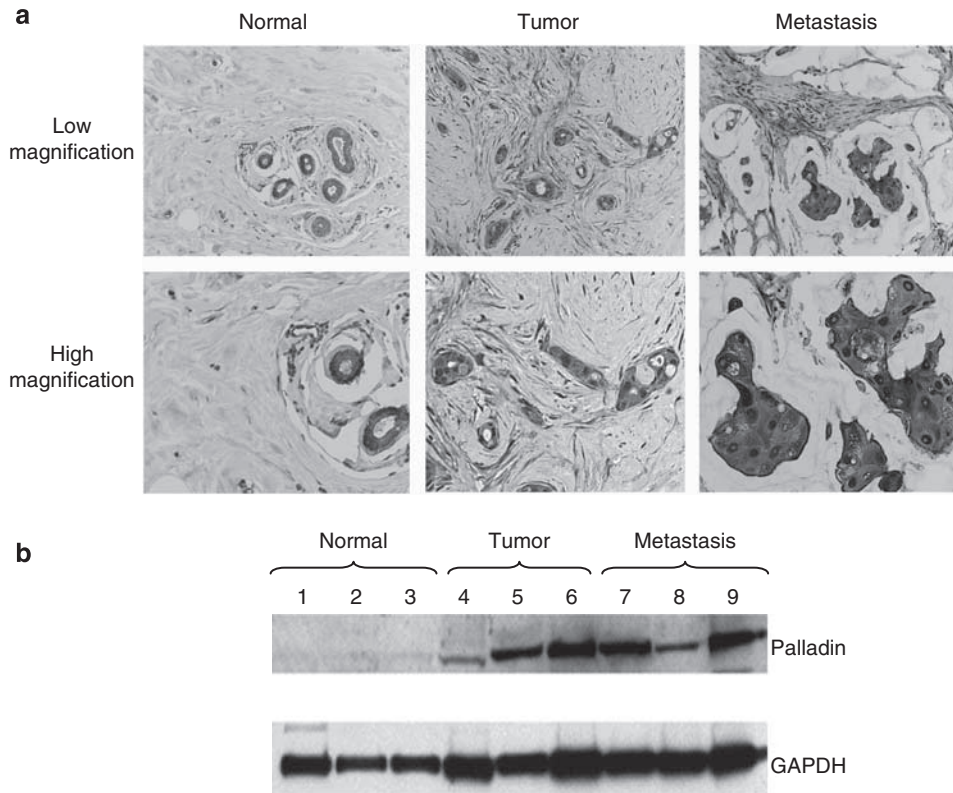
Figure 1 Palladin expression is increased in patient samples of breast cancer compared with normal breast. (**a**) Immunohistochemical analysis of normal, primary breast cancer tumors and metastatic tumors: comparison of the palladin staining in the tissue sections shows an apparent increase in staining 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. GADPH (a standard housekeeping protein) was used as a loading control.

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 (Monsky et al., 1994; Kelly et al., 1998; Bowden et al., 1999; 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 (Supplementary Figure S1). Z-series analyses of individual podosome structures (Supplementary Figure S1, insets) shows that the adhesion protein vinculin, which is an intrinsic component of podosomes, closely surrounds the actin core (Supplementary Figure S1). Figure 3a shows that podosome formation was detected only in the invasive cell lines MDA-MB-231 and SUM159 shortly after addition or either PDBu (Figure 3a) or PMA (data 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 earlier 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 SUM159 cells could facilitate the formation of podosomes in these invasive breast cancer cells. To determine whether 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% (Figure 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 earlier
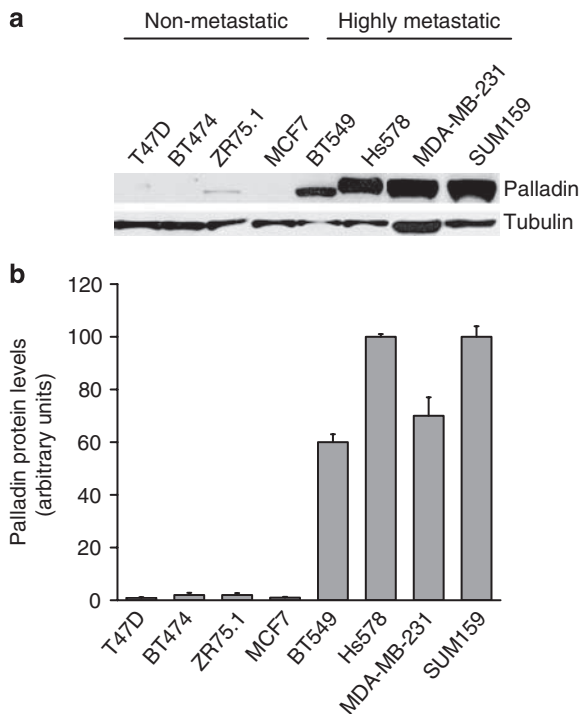

Figure 2 Palladin levels correlate with invasive potential in human breast cancer cells. (**a**) Western blot analysis was used to determine the amount of palladin protein in whole-cell lysates of eight different human breast cancer cell lines, namely T47D, BT474, ZR75.1, MCF7, BT549, Hs578T, MDA-MB-231 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.

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 chemoattractant. SUM159 cells were transfected with siRNAs, and 96 h later, they 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 SUM159 cells (KD), as compared with 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 (Figures 4b and c). After the quantification of stained cells, the percentages of migration and invasion were 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 (Figures 4b and c). These results suggest that palladin plays an important role in cell migration and invasion.

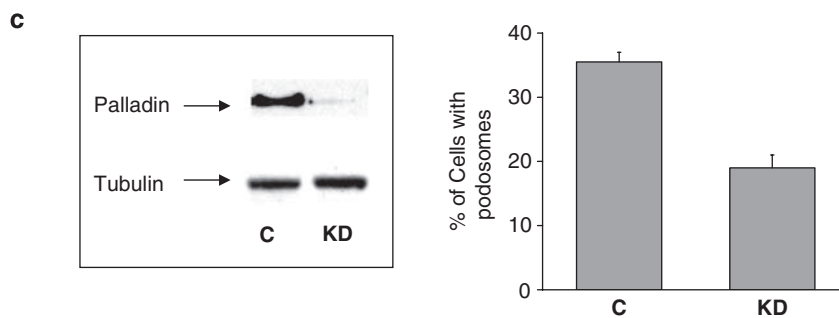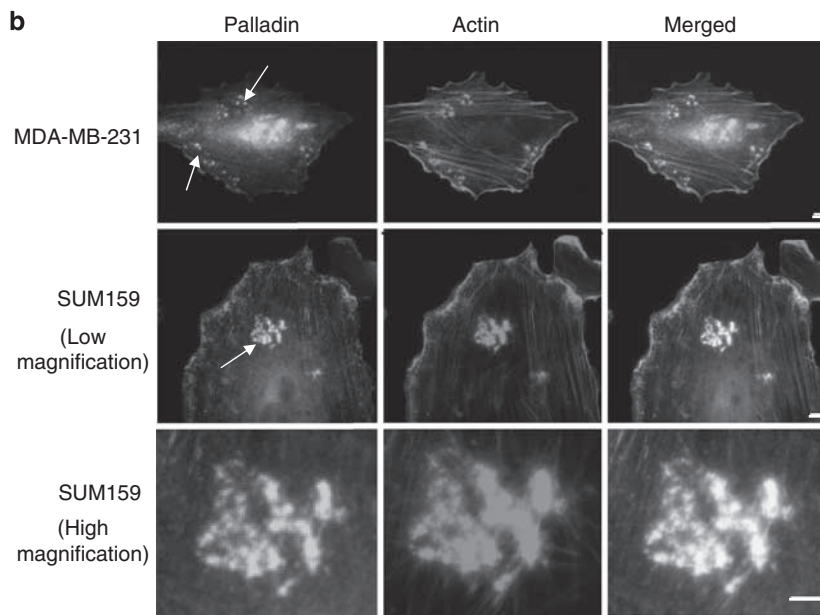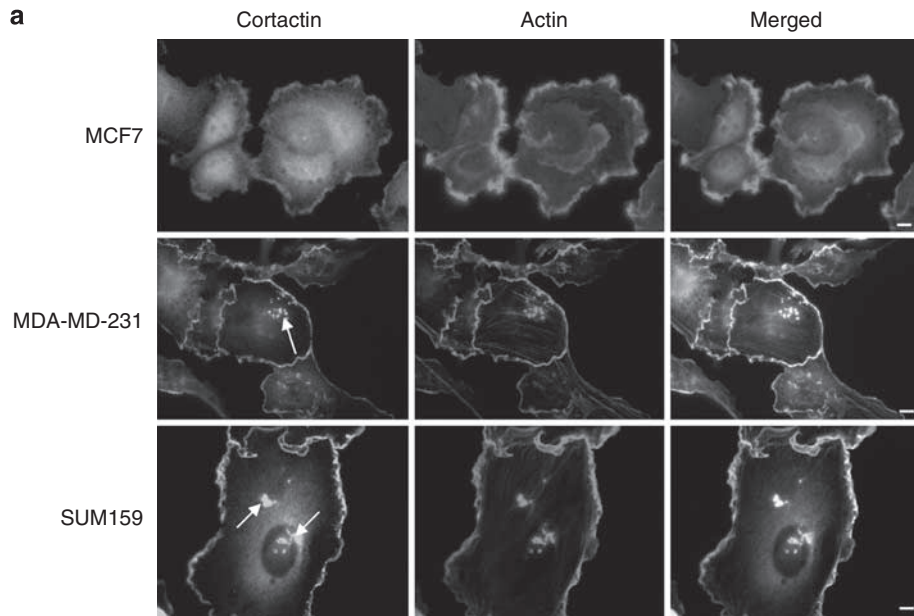*Palladin overexpression in MCF7 cells induces podosome formation*

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 of palladin was sufficient to drive the PDBμ-induced formation of podosomes. We infected MCF7 cells with an adenovirus vector encoding either green fluorescent protein (GFP) (control) or myc-palladin (OE). We overexpressed palladin to levels that are comparable with those found in invasive cells such as Hs578T, MDA-MB-231 and SUM159 cells

Figure 3 Palladin localizes to PDBu-induced podosomes and enhances podosome formation. (**a**) MCF7, MDA-MB-231 and SUM159 cells were plated on fetal bovine serum (FBS)-coated coverslips and treated with the phorbol ester PDBu for 20 min. Co-labeling with rhodamine phalloidin and a 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 min. 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 panel: 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 min. 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 μm.

(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 (Figure 5c).

Because palladin expression levels correlate with the invasiveness of breast cancer cells, we also examined
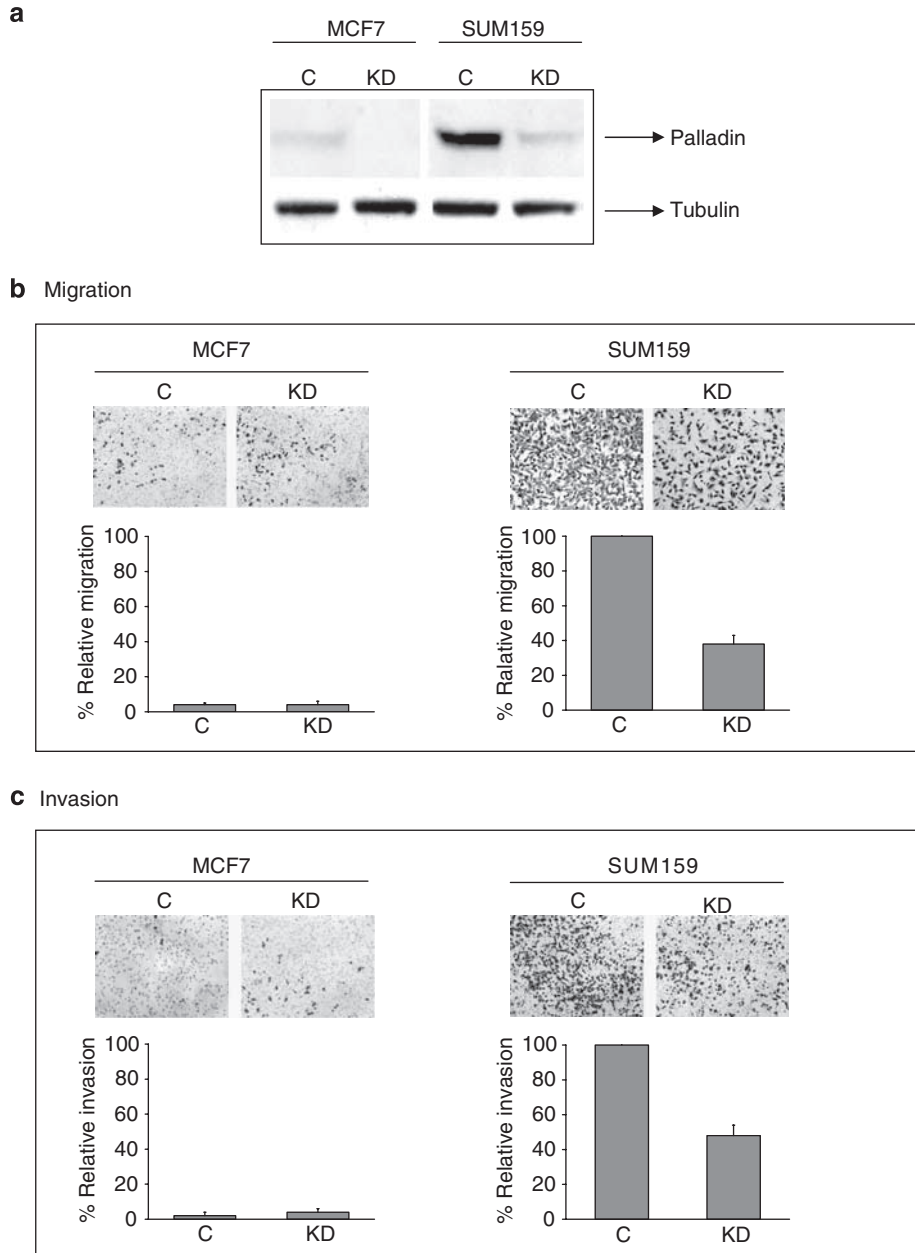
a

b Migration

c Invasion

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 SUM159 cells (KD), as compared with 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 SUM159 cells, whereas 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 SUM159 cells, whereas 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 h after plating. (Representative microphotographs of migrating and invading cells are included.)

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-over-expressing adenovirus vectors (Figure 6a), and after 24 h, they were collected and placed in the upper half of the inserts, which were then incubated for a further 24 h. Figures 6b and c show 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.
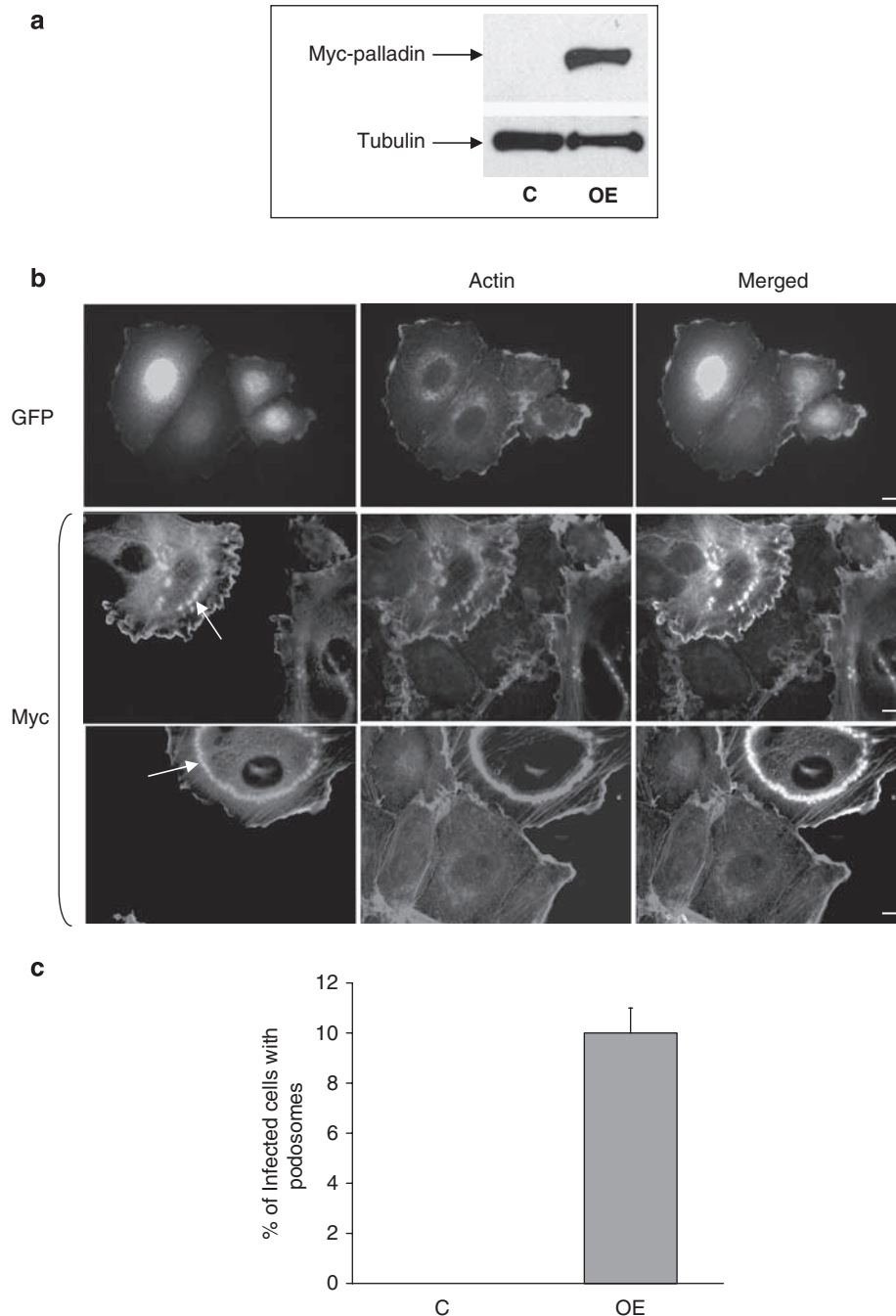
a

b

c

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**) Green fluorescent protein (GFP) and palladin-overexpressing MCF7 cells were plated on fetal bovine serum (FBS)-coated coverslips and treated with the phorbol ester PDBu for 20 min. 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 $= 10 \, \mu m$.

Discussion

Palladin's function has been explored earlier 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, that is 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; Ronty
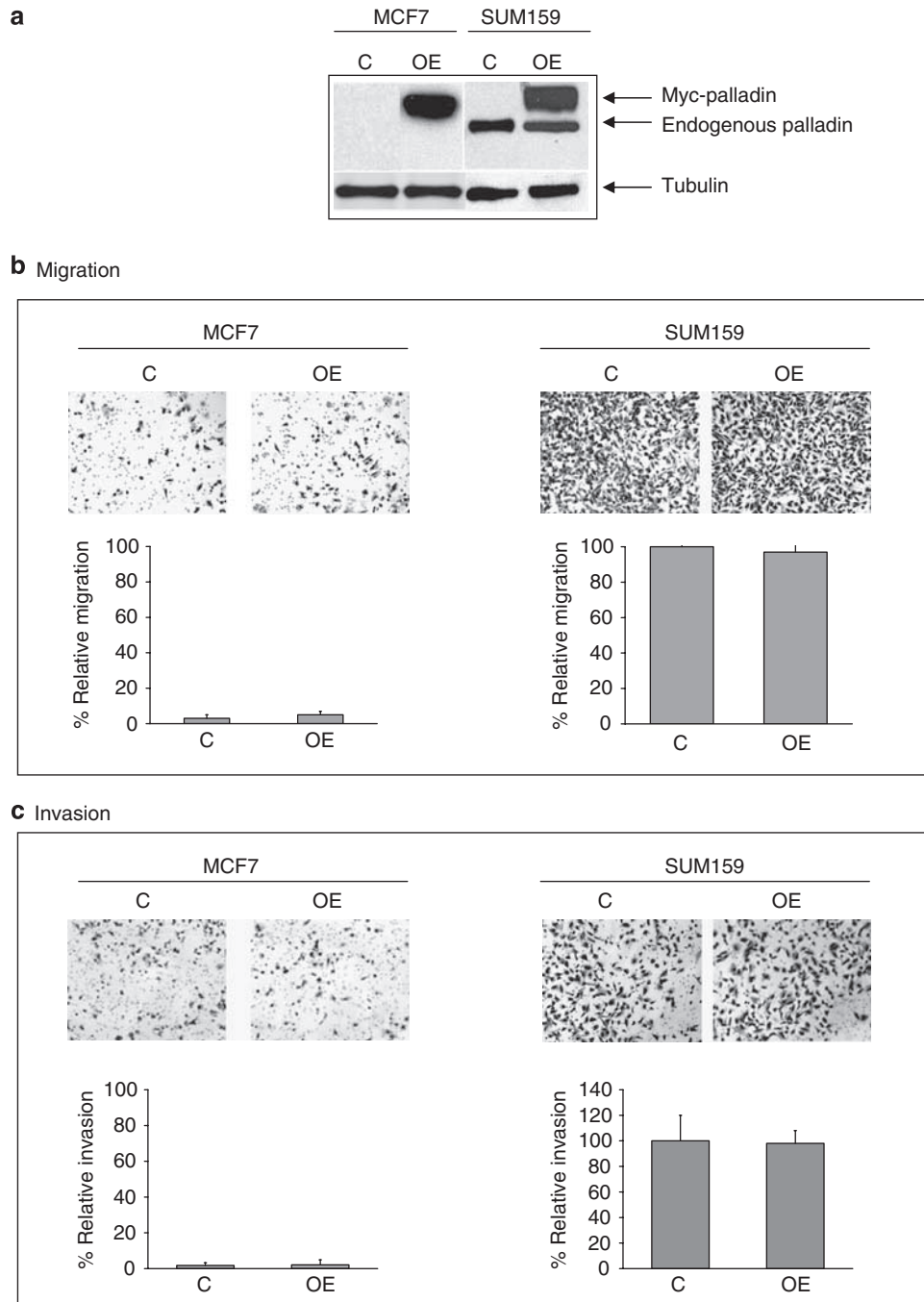
Figure 6 Palladin overexpression does not affect cell migration of either invasive or non-invasive breast cancer cells. (**a**) The levels of palladin in MCF7 and SUM159 cells infected with adenovirus expressing either green fluorescent protein (GFP) (C, control) or myc-palladin (OE, overexpression) were assessed by western blot. (**b**) Transwell migration assay of MCF7 (left) and SUM159 (right) cells expressing either GFP (C) or myc-palladin (OE). (**c**) Matrigel invasion assay of control MCF7 (left) and SUM159 (right) cells expressing either GFP or myc-palladin (OE). For both assays, cells on the lower surface of the filter were fixed and counted 20 h after plating. (Representative microphotographs of migrating and invading cells from three different experiments are included.)

et al., 2006; Jin *et al.*, 2007). This suggests that in normal cells, palladin expression is tightly controlled to allow for regulated transitions from non-motile to motile phenotypes. This 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 earlier; 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 earlier 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, showing that normal palladin levels are required for a 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, because of 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 over-expressed in the most invasive population of cancer cells, as shown 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 hypoth-esis that high levels of palladin expression contribute to invasive motility.

Our results showed that palladin levels are much higher in more invasive than in non-invasive breast cancer cells, that is the most highly invasive breast cancer cell lines express palladin at levels that are approximately 80 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) estab-lished a critical role of palladin in the formation of podosomes by human breast cancer cells. Overexpres-sion of palladin promotes podosome formation but has no effect on cell migration. These results are in accordance with Wang *et al.* (2005), 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.

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 (Mykkanen *et al.*, 2001; Boukhelifa *et al.*, 2004, 2006; Ronty *et al.*, 2004; Goicoechea *et al.*, 2006; Rachlin and Otey, 2006). In addition, palladin binds to signaling intermediaries such as ArgBP-2 and SPIN-90 (Ronty *et al.*, 2005,

2007), 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 through 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 (Lin *et al.*, 2004; Grunewald *et al.*, 2006, 2007a, b). In addition, palladin binds to alpha-actinin, a widely expressed actin-cross-linking protein (Ronty *et al.*, 2004) that is also over-expressed in metastatic sub-populations of cancer cells (Honda *et al.*, 1998; Hatakeyama *et al.*, 2006; 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 im-portant 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.

Materials and methods

Cell lines
A total of eight breast cancer cell lines were used, namely T47D, BT474, ZR75.1, MCF7, BT549, Hs578T, MDA-MB-231 and SUM159. T47D cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 10 μg/ml of insulin. MDA-MB-231, MCF7, BT474, and SKBR3 cells were cultured in DMEM containing 10% FBS. BT549 cells were cultured in RPMI supplemented with 10% FBS. ZR75.1 cells were obtained from ATCC (Manassas, VA, USA) and cultured in DMEM supplemented with 5% FBS, 2 mM L-glutamine. Hs578T cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 2 mM L-glutamine. SUM159 cells were cultured in Ham's F12 with 5% calf serum, 5 μg/ml insulin and 1 μg/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 earlier characterized by Parast and Otey, 2000). Tubulin alpha (Lab Vision Corporation, Fremont, CA, USA); GAPDH (glycer-

aldehyde 3-phosphate dehydrogenase) and cortactin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and alpha-actinin (Sigma, St Louis, MO, USA). A protease inhibitor cocktail for mammalian tissues was from Sigma. A TransIT siQuest TransIT-LT1 transfection reagent was from Mirus (Madison, WI, USA). Alexafluor-488 and Alexafluor-568 anti-mouse IgG- and anti-rabbit IgG-conjugated secondary antibodies were from Molecular Probes (Carlsbad, CA, USA).

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, 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 $\times 20$ and $\times 60$ objective lenses, an optional $\times 1.5$ tube lens and a Hamamatsu Orca-ER camera. Images were processed using Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA, USA). Podosome formation was induced by the addition of $1\,\mu M$ PDBu (Sigma-Aldrich, St Louis, MO, USA), as described earlier (Hai et al., 2002; Gimona et al., 2003). Confocal image acquisition and analysis were performed using a Leica SP2 AOBS confocal microscope with a $\times 63$, 1.4-numerical-aperture apochromatic Leica lens (Leica Microsystems, Bannockburn, IL, USA). Scanning was performed with the xz axis, using three independent laser sources (364-nm-UV, 488-nm-Ar and 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 (Lafayette, CO, USA). RNA sequences were the following: sense, 5′-CUACUCCGCUGU CACAUUAUU-3′ and antisense, 5′-UAAUGUGACAGCG GAGUAGUU-3′. As a control we used siCONTROL Non-Targeting siRNA no. 2 from Dharmacon Research. MCF7 and SUM159 cells were transfected using the TransIT siQuest transfection reagent following the manufacturer's instructions. Briefly, 1.5 ml of serum-free medium (Life Technologies, Carlsbad, CA, USA) and $20\,\mu l$ of transfection reagent per plate were preincubated for 15 min at room temperature. After incubation, $225\,\mu l$ of $1\,\mu M$ siRNA was added to the diluted TransIT siQuest reagent and the mixture was incubated for 15 min 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 h after transfection.

Palladin overexpression using adenoviral system
Mouse myc-palladin 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 the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

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 r.p.m. for 15 min. The cell lysates were either analysed by immunoblot or processed for migration and invasion assays or frozen with liquid nitrogen and stored at $-80\,^{\circ}C$ for future use. For the immunoblot, lysates were boiled in $2\times$ Laemmli buffer, and $20\,\mu g$ of protein was 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, Lincoln, NE, USA). In some cases, immunocomplexes were visualized using the Western Lights Chemiluminescence Detection kit (Perkin-Elmer, Wellesley, MA, USA).

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 at 1:200 dilution (Proteintech Group, Chicago, IL, USA). Briefly, the slides were incubated at $60\,^{\circ}C$ for 15 min and then deparaffinized in xylene. Endogenous peroxidase activity was then quenched by incubation for 30 min in 0.3% H_2O_2 in methanol. Next, the slides were steamed for 25 min in citrate buffer. After adequate cooling, the specimens were placed in diluted normal blocking serum (based on a secondary antibody). The slides were then incubated overnight with the primary antibody in a humidity box at $4\,^{\circ}C$ and then placed in a diluted biotinylated secondary antibody for 30 min at room temperature. The slides were incubated in the VECTASTAIN Elite ABC Reagent (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature followed by peroxidase substrate solution until the desired stain intensity was obtained. Finally, the slides were counterstained in hematoxylin 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 h of incubation at $37\,^{\circ}C$ and 5% CO_2, 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, McGraw Park, IL, USA). 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 carried out in triplicates and repeated at least three 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 (BD Biosciences). Cells were cultured for 48 h 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-μm pores and is coated with a basement membrane matrix (Matrigel). Cells, in $500\,\mu l$ of serum-free DMEM media, were seeded in the cell insert and placed in the well, which was filled with $750\,\mu l$ of DMEM and supplemented with 10% FBS. After 24 h of incubation, cells were treated as described above for the migration assay.

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