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ORIGINAL ARTICLE

Palladin promotes invasion of pancreatic cancer cells by enhancing invadopodia formation in cancer-associated fibroblasts

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The stromal compartment surrounding epithelial-derived pancreatic tumors is thought to have a key role in the aggressive phenotype of this malignancy. Emerging evidence suggests that cancer-associated fibroblasts (CAFs), the most abundant cells in the stroma of pancreatic tumors, contribute to the tumor's invasion, metastasis and resistance to therapy, but the precise molecular mechanisms that regulate CAFs behavior are poorly understood. In this study, we utilized immortalized human pancreatic CAFs to investigate molecular pathways that control the matrix-remodeling and invasion-promoting activity of CAFs. We showed previously that palladin, an actin-associated protein, is expressed at high levels in CAFs of pancreatic tumors and other solid tumors, and also in an immortalized line of human CAFs. In this study, we found that short-term exposure of CAFs to phorbol esters reduced the number of stress fibers and triggered the appearance of individual invadopodia and invadopodial rosettes in CAFs. Molecular analysis of invadopodia revealed that their composition resembled that of similar structures (that is, invadopodia and podosomes) described in other cell types. Pharmacological inhibition and small interfering RNA knockdown experiments demonstrated that protein kinase C, the small GTPase Cdc42 and palladin were necessary for the efficient assembly of invadopodia by CAFs. In addition, GTPase activity assays showed that palladin contributes to the activation of Cdc42. In mouse xenograft experiments using a mixture of CAFs and tumor cells, palladin expression in CAFs promoted the rapid growth and metastasis of human pancreatic tumor cells. Overall, these results indicate that high levels of palladin expression in CAFs enhance their ability to remodel the extracellular matrix by regulating the activity of Cdc42, which in turn promotes the assembly of matrix-degrading invadopodia in CAFs and tumor cell invasion. Together, these results identify a novel molecular signaling pathway that may provide new molecular targets for the inhibition of pancreatic cancer metastasis.

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INTRODUCTION

Pancreatic cancer (pancreatic ductal adenocarcinoma) is one of the most aggressive malignancies found in humans. Although pancreatic cancer is relatively rare, it is the fourth leading cause of cancer-related deaths in the United States because of its high lethality (95–98% mortality rate within 5 years) and pattern of early metastasis.[1] It is now well established that both the extracellular matrix (ECM) and the cells within the stromal compartment surrounding epithelial-derived cancer cells contribute to the progression of pancreatic cancer and many other types of solid tumors.[2–4] The most abundant cells in the tumor stroma are cancer-associated fibroblasts (CAFs), also known as carcinoma-associated fibroblasts (CAFs) or tumor myofibroblasts. Emerging evidence suggests that CAFs contribute to tumor progression through the secretion of pro-invasive and pro-proliferative growth factors, and through the generation of growth-enhancing mechanical signals.[5–7] This hypothesis has been confirmed in mice, using mixed-cell recombination approaches in which neoplastic cells are injected either with or without accompanying CAFs. With a variety of cancer cell types, mixing the neoplastic cells with CAFs results in the formation of larger tumors and more metastases when compared with injecting neoplastic cells alone.[8–10] Thus, CAFs are likely to be critical players in the process of tumor progression and invasion, which highlights the importance of understanding the molecular mechanisms that control the invasion-promoting activities of CAFs. A recent study using an innovative cell culture system demonstrated an active role for CAFs in tumor invasion via remodeling of the ECM.[11] This study showed that CAFs can generate channels within the matrix that create passage ways for the metastasizing tumor cells. These data suggest that matrix remodeling by CAFs actively promotes the collective invasion of tumor epithelial cells.

Palladin is an actin-associated protein that is expressed at high levels in the CAFs of pancreatic tumors and other invasive tumor types.[12–14] Palladin has an essential role in the assembly and maintenance of multiple types of actin-dependent structures,

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including stress fibers, dynamic dorsal ruffles and matrix-degrading podosomes in vascular smooth muscle cells.[15,16] Palladin was first shown to have a role in protrusive motility in knockdown experiments with cultured cells, in which palladin expression was found to be required for neurite extension in cortical neurons.[17] More recently, the phenotype of the palladin null mouse (embryonic lethal, with defects in body wall closure both dorsally and ventrally) demonstrate that palladin has an essential role in normal cell motility during embryonic development.[18] Our recently published results have established that a specific variant of palladin (isoform 4) is dramatically upregulated in CAFs at an early stage in the development of pancreatic tumors.[13] In addition, we showed previously that palladin upregulation is specific to highly invasive ductal adenocarcinomas and was not observed in less invasive types of pancreas tumors.[13] A correlation between high palladin levels in the stroma and poor clinical prognosis was also demonstrated in renal cancer, suggesting a specific role for palladin in the metastasis-promoting behavior of CAFs.[14] Here, we show that, on stimulation with phorbol esters, CAFs are able to form invadopodia, a matrix-degrading actin-rich structure that is normally found in invasive cell types. The formation of invadopodia in CAFs is modulated by the small GTPase Cdc42 and by palladin expression levels. In addition, we show that palladin has a role in the regulation of Cdc42 activity. Finally, we show that palladin expression enhances the invasive behavior of CAFs *in vitro* and also tumor progression *in vivo*. Together, these results suggest that CAFs utilize palladin-dependent signaling pathways to remodel the ECM, which in turn promotes metastasis of tumor cells.

RESULTS

Invadopodia formation and matrix degradation in pancreatic CAFs

Accumulating evidence suggests that the ability of tumor cells to cross a basement membrane is facilitated by specialized subcellular structures termed invadopodia.[19,20] Both invadopodia and structurally similar organelles termed podosomes are actin-based membrane protrusions that are enriched with matrix metalloproteinases. These structures have been described in several types of tumor cells and hematopoietic cells,[21,22] but invadopodia formation in human CAFs has not been described previously. This gap in knowledge has been partially due to the absence of reliable CAF cell line models of the type used in the current study. We examined the ability of CAFs, which were isolated from collagenase-treated human pancreatic tumors, to form invadopodia in response to short-term treatment with phorbol esters. This treatment is widely used to stimulate the assembly of invadopodia and podosomes in other cell types.[23] CAFs were treated with phorbol 12-myristate 13-acetate (PMA), fixed and labeled with rhodamine–phalloidin to visualize F-actin (Figure 1a). Untreated CAFs showed typical actin stress fibers spanning the cell body. After 1 h of treatment, most cells showed increased membrane ruffling activity, and the number of stress fibers was strongly reduced. In addition, actin puncta became visible either behind the leading edge or clustered within the cell body, with about 30% of cells displaying both individual puncta and rosettes (Figure 1b).

We analyzed the presence of known invadopodia markers to determine whether the actin-rich puncta formed in CAFs in response to phorbol esters were invadopodia. Adhesion-associated and actin-binding proteins (such as vinculin and cortactin) were found in the invadopodia-like structures of CAFs (Supplementary Figure S1). As protein kinase C (PKC) mediates phorbol ester-stimulated podosome and invadopodia formation in several cell lines,[23–25] we determined whether PKC also regulates assembly of invadopodia in CAFs. Cells were pre-treated with the PKC inhibitor Gö6976 for 30 min, and then activated by PMA (60 min in the presence of the inhibitor). The PKC inhibitor reduced the number and percentage of cells that formed invadopodia by threefold (Figure 1c; $P < 0.05$), indicating that PKC has a role in PMA-induced invadopodia formation in CAFs.

A defining trait of the invadopodia described in tumor cells is their ability to degrade the ECM.[20,26,27] To determine if the invadopodia detected in CAFs possess this property, we performed a well-established *in vitro* matrix degradation assay.[28] CAFs were seeded onto glass coverslips pre-coated with fluorescently labeled gelatin and treated for 1 h with PMA. The black dots in the fluorescent gelatin represent areas of focal degradation of the matrix (Figure 1d). These dots colocalized with actin-rich invadopodia in CAFs, indicating that in these cells, PKC stimulation results in the assembly of actin-rich, matrix-degrading structures that closely resemble the invadopodia described in invasive epithelial cancer cells. Taken together, these data show that PKC-dependent, matrix-degrading invadopodia are not unique to neoplastic and hematopoietic cells but can also form in CAFs.

CAFs are known to express α-smooth muscle actin, and thus are considered to be a type of myofibroblast, and phenotypically distinct from normal fibroblasts. To ask if normal fibroblasts share with CAFs the ability to assemble invadopodia, we treated normal primary human fibroblasts with phorbol esters, then fixed and stained the cells with phalloidin. Neither individual invadopodia nor invadopodial rosettes were detected in normal fibroblasts (Figure 2a). To extend our observations to activated myofibroblasts from other sources, we utilized immortalized cell lines (immortalized mouse pancreatic stellate cells clone 2 (imPSC-C2) and imPSC-C3) from activated stellate cells isolated from mouse pancreas.[29,30] Previous studies have established that activated stellate cells are a major source myofibroblasts in the fibrotic pancreas, and of CAFs in pancreas tumors. We tested the ability of these mouse pancreatic myofibroblasts to form invadopodia in response to phorbol ester stimulation. Both imPSC-C2 and imPSC-C3 were treated with two phorbol esters, PMA and phorbol-12,13-dibutyrate (PDBu), fixed and labeled with rhodamine–phalloidin to visualize F-actin. Invadopodia were found both individually and in rosettes in both clones of imPSC shortly after addition of either PMA (Figure 2b) or PBDu (Supplementary Figure S2). As a final confirmation that CAFs can assemble invadopodia, we assayed the ability of primary CAFs to respond to phorbol ester treatment, using both mouse CAFs obtained from a xenografted human tumor, and human CAFs cultured from an explanted patient sample. Invadopodia were detected in both types of primary CAFs (Supplementary Figure S3). We showed previously that primary and immortalized human CAFs have high levels of palladin when compared with normal fibroblasts.[13] To investigate palladin levels in imPSC-C2 and imPSC-C3, we performed western blot analysis using human normal gingival fibroblasts as a control. As expected, the two mouse PSC clones show that palladin is upregulated when compared with normal fibroblasts (Figure 2c), and similar to the levels detected in human CAFs. The expression levels of palladin were normalized against those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the results are presented in Figure 2d. Approximately a fivefold increase in palladin levels were detected in the activated myofibroblasts compared with normal fibroblasts. These results suggest that a high level of palladin expression is a relevant molecular feature underlying the mechanism that governs invadopodia formation in activated myofibroblasts.

Palladin localizes to PMA-induced invadopodia and enhances invadopodia formation after phorbol ester stimulation

To determine whether palladin is recruited to invadopodia of CAFs, immunostaining was used to visualize endogenous palladin after stimulation with PMA. Palladin was strongly enriched in the invadopodia and colocalized with actin in cells that were double-stained with anti-palladin antibodies and phalloidin (Figure 3a).

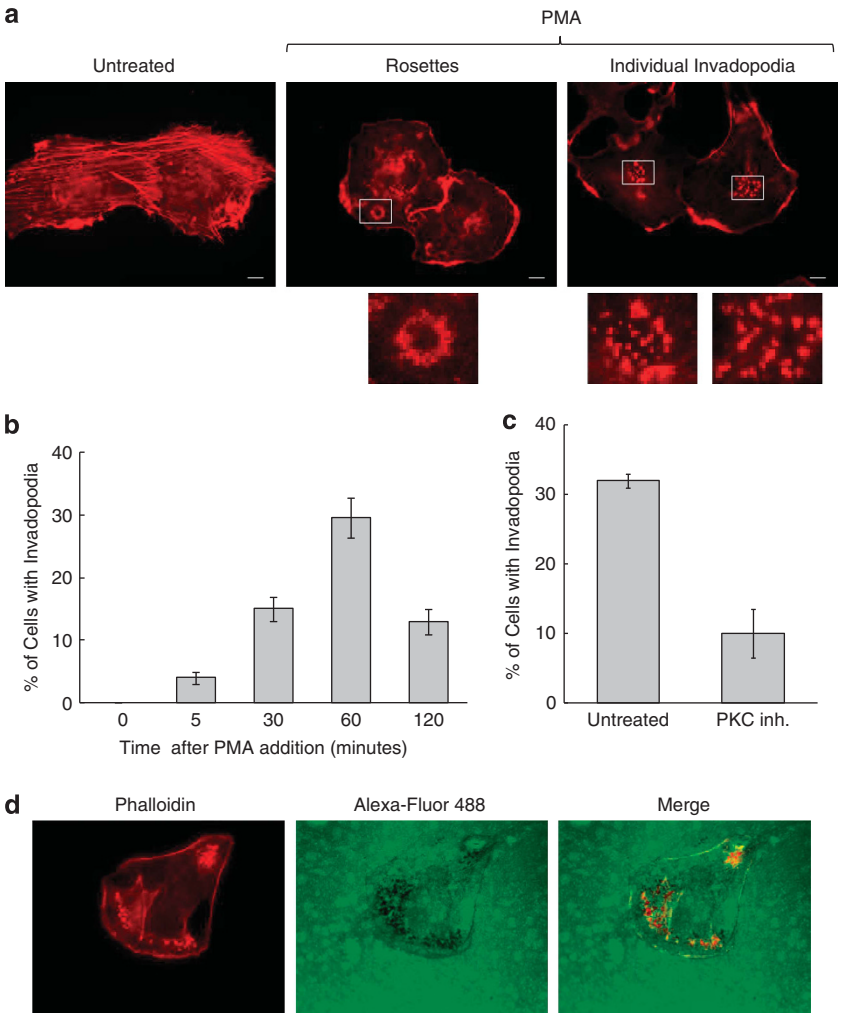

Figure 1. PMA treatment induces invadopodia formation and matrix degradation in human CAFs. Human CAFs were treated for 1 h with PMA, fixed and stained with rhodamine–phalloidin to visualize F-actin. Boxes indicate individual invadopodia and rosettes. (**a**) Representative pictures of individual invadopodia and rosettes. (**b**) Time course of invadopodia formation. (**c**) Effect of 0.1 μM PKC inhibitor Gö6976 on invadopodia formation. Invadopodia formation was significantly decreased after treatment of CAFs with PKC inhibitor (PKC inh, 10%) as compared with control cells (untreated, 32%). (**d**) CAFs were cultured on Alexa-488-labeled gelatin-coated coverslips overnight, and then treated with PMA for 1 h before fixation. Cells were then fixed and stained with rhodamine–phalloidin to visualize actin-rich invadopodia. Arrowheads denote invadopodia-degrading matrix and the degradation sites. Scale bar = 10 μm.

To determine the functional role of palladin in the assembly of invadopodia, we examined the effect of inactivating this gene on the cellular response of CAFs to PMA. Small interfering RNA (siRNA) oligos were used to knockdown the expression of palladin. Western blot analysis showed a ~90% reduction of palladin expression in CAFs (siRNA) compared with cells treated with control siRNA (CTRL; Figure 3b). The ability of CAFs to form invadopodia was strongly impaired when palladin expression was silenced (Figure 3c). Palladin knockdown reduced the percentage of cells that form invadopodia by more than twofold ($P<0.05$; Figure 3c). These results reveal that palladin has an important role in invadopodia formation in CAFs.

Cdc42 is involved in PMA-induced invadopodia assembly

Cdc42 is considered a master regulator of the core actin polymerization machinery found in invadopodia in vascular smooth muscle cells, metastatic rat carcinoma mammary cells and human melanoma cells.[31–33] Although Cdc42 has been reported to have a role in podosome and invadopodia formation in multiple cell lines, the cellular function of Cdc42 in CAFs is largely unexplored. To determine the role of Cdc42 in invadopodia formation in CAFs, we examined the effect of Cdc42 knockdown on the cellular response to PMA. Western blot analysis showed that treatment with Cdc42 siRNA reduced Cdc42 expression by 90% in CAFs (siRNA) compared with cells treated with control siRNA (CTRL; Figure 4a). When Cdc42-knockdown cells were treated with PMA, a large percentage of the siRNA-transfected cells were unable to form invadopodia. Indeed, Cdc42 knockdown reduced the percentage of cells that form invadopodia by fourfold (Figure 4b; $P<0.05$), demonstrating that Cdc42 has an important role in regulating the assembly of invadopodia in CAFs.

Palladin regulates the activity of Cdc42

We have previously shown that palladin has a role in the regulation of Rac1 activity in vascular smooth muscle cells.[16] Rac1 and Cdc42 pathways can share the same components, and in many cases they bind to the same effectors and can be activated by the same guanine nucleotide exchange factors (GEFs).[34] This led us to speculate that palladin may also be modulating Cdc42 activity. Like other GTPases, Cdc42 cycles between an active,

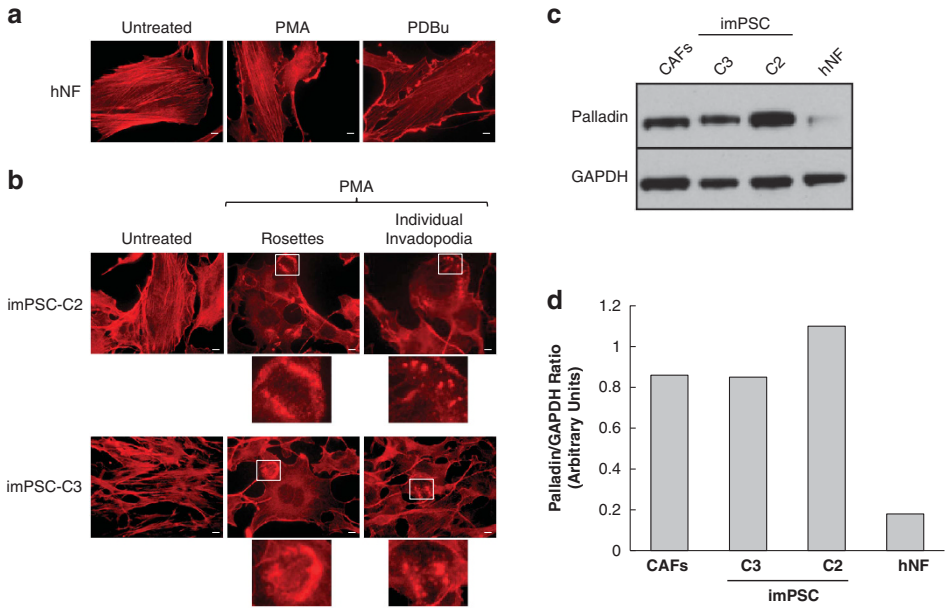

Figure 2. Mouse PSCs form invadopodia after phorbol ester treatment and contain high levels of palladin. (**a**) Human normal fibroblasts (hNFs) were treated with phorbol esters (50 ng/ml PMA and 1 μM phorbol-12,13-dibutyrate (PDBu)), fixed and stained with rhodamine–phalloidin to visualize F-actin. (**b**) Immortalized mouse PSC clone C2 (imPSC-C2) and immortalized mouse PSC clone 3 (imPSC-C3) were treated with PMA, fixed and stained with rhodamine–phalloidin to visualize F-actin. Boxes indicate individual invadopodia and rosettes. Scale bar = 10 μm. (**c**) Whole-cell lysates of human CAFs, mPSC-C2 and mPSC-C3 were analyzed by western blot using the palladin monoclonal antibody 1E6. Blots were also stained for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control for equal loading. (**d**) Quantification of palladin protein levels in hPSC, imPSC-C3, imPSC-C2 and human normal gingival fibroblasts (hNF). The ratio of palladin to total cellular GAPDH is shown on the left axis.

GTP-bound state and an inactive GDP-bound state, and the active form can be isolated using an affinity precipitation approach.[35,36] We used this standard pull-down assay to investigate the activity of Cdc42 in CAFs. Overall levels of Cdc42 expression do not differ between control and palladin KD cells, but the pool of active Cdc42 is significantly reduced when palladin expression is either transiently knocked down using siRNA or stably knocked down using lentiviral delivery of short hairpin RNA (shRNA1, shRNA2), as shown in Figure 5a. Cdc42 activity in the palladin knockdown cells decreased on average >60% (Figure 5b; $P<0.05$), demonstrating that the activity of this GTPase is decreased in the absence of palladin expression.

Palladin enhances *in vitro* invasion of pancreatic cancer cells

CAFs have been shown previously to promote the invasion of tumor cells by creating channels in the ECM.[11] Our finding that palladin may be required for invadopodia formation motivated us to determine whether palladin expression is critical for the invasion-promoting activity of CAFs. We used a mixed-cell *in vitro* invasion assay that uses Matrigel-coated invasion chambers. Human pancreatic tumor cells (AsPC-1) were fluorescently labeled with CellTracker red and placed in the upper chamber together with control CAFs (AsPC-1 + CTRL CAFs) or with palladin knockdown CAFs (AsPC-1 + shRNA1 CAFs). AsPC-1 cells with no CAFs were used as a control (AsPC-1 alone). The cells were allowed to invade for 24 h and then the non-invading cells were removed from the upper well. The invading cells were fixed and the red AsPC-1 cells were counted. These results show that there is a twofold ($P<0.05$) increase in the number of AsPC-1 cells that invade through the Matrigel-coated membranes in the presence of control CAFs compared with AsPC-1 cells alone (Figure 6). In contrast, in the presence of palladin knockdown CAFs, no significant increase ($P=0.29$) in the number of invading AsPC-1 cells was observed. Importantly, the effect on palladin depletion in CAFs on AsPC-1 migration was rescued by re-expressing an shRNA-resistant palladin construct (Figure 6).

The cancer stroma is usually composed of fibrillary collagen, predominantly type I. To assess the invasion-promoting activity of CAFs on this type of matrix, we performed a mixed-cell *in vitro* invasion assay using collagen I-coated invasion chambers. Results show that there is a twofold ($P<0.05$) increase in the number of AsPC-1 cells that invade through the collagen-coated membranes in the presence of control CAFs compared with AsPC-1 cells alone (Figure 7). In contrast, no significant increase in the number of invading AsPC-1 cells was observed in the presence of palladin knockdown CAFs (shRNA1, $P=0.58$; shRNA2, $P=0.62$). These results indicate that palladin-expressing stromal cells promote *in vitro* invasion of pancreatic tumor epithelial cells through either Matrigel or collagen.

Palladin enhances *in vivo* invasion of pancreatic cancer cells

Along with others, we have shown previously that the high levels of palladin observed in samples of bulk pancreas tumors are due to palladin overexpression in stromal fibroblasts and not in neoplastic cells.[12,13,37] We also showed that palladin levels in the stroma correlate closely with tumor invasiveness in pancreas tumors, suggesting that palladin overexpression may have a critical role in CAF behavior.[13] Thus, to evaluate if palladin expression in CAFs promotes tumor growth *in vivo*, we used a mixed-cell xenograft study. The stable knockdown CAFs were mixed with AsPC-1 cells before implantation into the pancreata of immunodeficient mice, following a well-established protocol.[38,39] In parallel, mice were injected with AsPC-1 cells mixed with control CAFs, or AsPC-1 cells alone (no CAFs). The proportion of CAFs in the co-injection relative to tumor cells was 1:3 tumor-to-CAF ratio, which reflects the relative abundance of stromal cells typically observed in human pancreatic adenocarcinomas.

To accurately monitor *in vivo* tumor growth, AsPC-1 cells were stably transfected with luciferase, and bioluminescence imaging

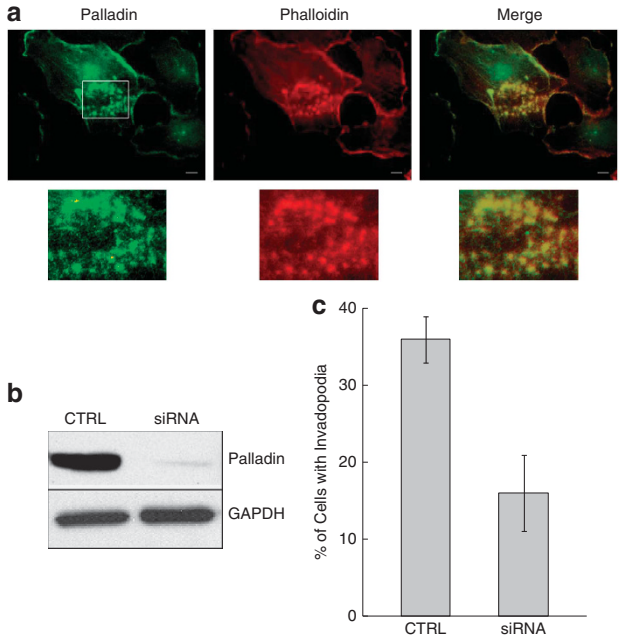

Figure 3. Palladin localizes to invadopodia and enhances invadopodia formation. (**a**) CAFs were treated for 1 h with PMA. After fixation, endogenous palladin was detected by immunofluorescence. Co-labeling with tetramethyl rhodamine isothiocyanate (TRITC)-phalloidin and polyclonal anti-palladin antibody reveals that palladin colocalizes with actin. Scale bar = 10 μm. (**b**) CAFs transfected with control siRNA oligos (CTRL) and siRNA oligos targeting palladin (siRNA) were treated with PMA for 1 h. Invadopodia formation was significantly reduced in palladin knockdown cells (siRNA, 16% of total cells displaying invadopodia), as compared with control cells (CTRL, 36% of total cells displaying invadopodia). Results are representative of three independent experiments in which at least 300 transfected cells were counted. $P < 0.05$, Student's *t*-test. (**c**) Western blot analysis was used to detect palladin levels in control (CTRL) and palladin knockdown (siRNA) cells.

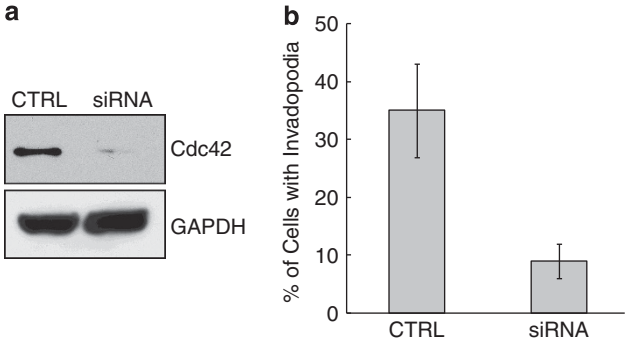

Figure 4. Cdc42 regulates invadopodia assembly in CAFs. Human CAFs transfected with control siRNA oligos and siRNA oligos targeting Cdc42 were treated with PMA for 1 h. (**a**) Western blot analysis was used to detect Cdc42 levels in cells treated with control oligos (C) or Cdc42-specific siRNA oligos (KD). (**b**) Invadopodia formation was significantly reduced ($P < 0.05$) in Cdc42 knockdown cells (KD, 9%), as compared with control cells (C, 35%). Results are representative of three independent experiments in which at least 300 transfected cells were counted.

of the mice was performed weekly as previously described.[40] Similar to the pattern that is typical of pancreas cancer in human patients, the AsPC-1 cells formed primary tumors at the site of injection and also metastasized locally within the abdomen (Figure 8a). To measure the whole-body tumor load for each animal, total photon flux was used (Figure 8b). Our results show that in animals where neoplastic cells were mixed with control CAFs, tumor growth was dramatically enhanced as compared with mice injected with tumor cells alone, in agreement with published results,[10] validating the expected behavior of this system. In contrast, palladin knockdown CAFs were significantly less efficient in promoting tumor progression than control, palladin-expressing CAFs (Figure 8b; $P < 0.05$). The possibility of CAF-induced tumorigenesis in the absence of carcinoma cells was ruled out previously by the absence of tumor development in a control group of animals inoculated with CAFs only.[10] These results, using well-controlled *in vivo* models, are congruent with our *in vitro* experiments, and demonstrate that palladin expression critically contributes to the acquisition of the pro-proliferative, pro-invasive phenotype of pancreatic CAFs. Thus, we conclude that palladin is a novel regulator of pancreatic cancer growth and invasion, at least in part via its important effects on the behavior of CAFs. Together, these results extend our understanding on how palladin regulates pancreatic cancer pathobiology through the modulation of the tumor microenvironment.

DISCUSSION

A growing body of literature supports the idea that tumor–stromal interactions have a key role in the initiation and progression of solid tumors, especially in cancers with a robust desmoplastic component such as pancreatic ductal adenocarcinoma. We have shown previously that the actin-associated protein palladin (isoform 4) is dramatically upregulated in both primary and immortalized CAFs cultured from human pancreatic ductal adenocarcinoma explants.[13] Here, we examined the role of palladin in the invasion-promoting activity of pancreatic CAFs in culture and in a mouse model of tumor progression. We used two different *in vitro* systems to assess the impact of palladin in matrix remodeling. First, we monitored invadopodia formation on the matrix protein gelatin; second, we assayed cell invasion through two different substrates: Matrigel (which is a complex mixture of matrix molecules including laminin, collagen type IV and entactin) and collagen (85% type I and 15% type III, which are main components of the desmoplastic tumor stroma). Our results show that pancreatic CAFs are able to form functional, matrix-degrading invadopodia, and also demonstrate that palladin expression in CAFs promotes invasion through tumor stroma. In addition, we show that the small GTPase Cdc42 has a critical role in the formation of CAFs' invadopodia, and that palladin levels correlate with Cdc42 activity, suggesting that palladin contributes to the regulation of Cdc42 during invadopodia formation. In mouse xenograft experiments using a mixture of CAFs and tumor cells, palladin expression in CAFs promoted the rapid growth and metastasis of human pancreatic tumor cells that were co-injected with CAFs. These data indicate that in intact animals, palladin is directly involved in facilitating tumor cell migration through the ECM, which is enriched with collagens type I and III. Taken together, these results indicate that CAFs utilize palladin-dependent pathways to activate Cdc42, assemble invadopodia and remodel important and abundant matrix molecules, which in turn may promote the ability of tumor cells to invade across a basement membrane.

The mode of invasion that is induced by invadopodia requires fast assembly of cell-matrix adhesions, efficient proteolysis of the matrix and a dynamic actin cytoskeleton. Tissue invasion is most efficient when these cellular processes are coordinated. Many actin–cytoskeleton-associated proteins act as major players in the Src-mediated organization of adhesive interactions such as podosomes and invadopodia. Moreover, there is growing evidence that actin-binding proteins have multiple roles in

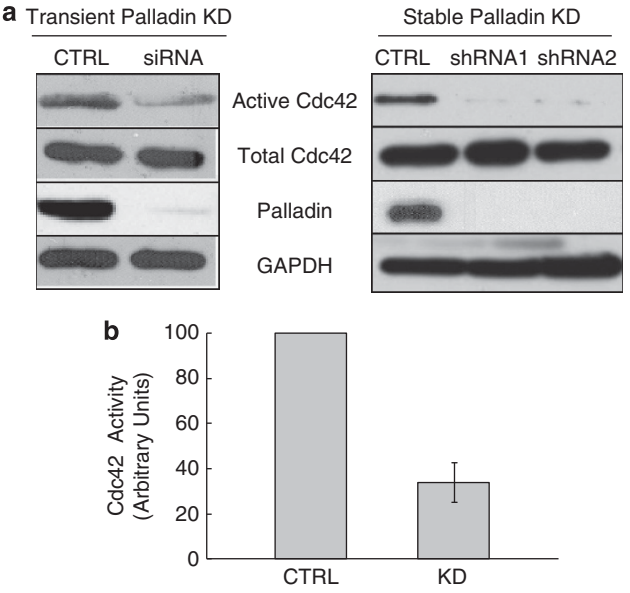

Figure 5. Palladin regulates the activity of Cdc42. (**a**) Western blot analysis of whole-cell lysates from palladin transient knockdown CAFs using control siRNA (CTRL) or siRNA targeting palladin (siRNA; left panel), and palladin stable knockdown CAFs using lentivirus (shRNA1 and shRNA2; right panel). Active Cdc42 was specifically pulled down from cell lysates containing equal amounts of protein with immobilized recombinant PAK-CRIB and analyzed by western blotting using anti-Cdc42 antibody. A representative experiment is shown. (**b**) The level of active Cdc42 was quantified by densitometric analysis of western blots and the amount of active Cdc42 was normalized to the amount of total Cdc42. Cdc42 activation was quantified from five independent assays ($P<0.05$).

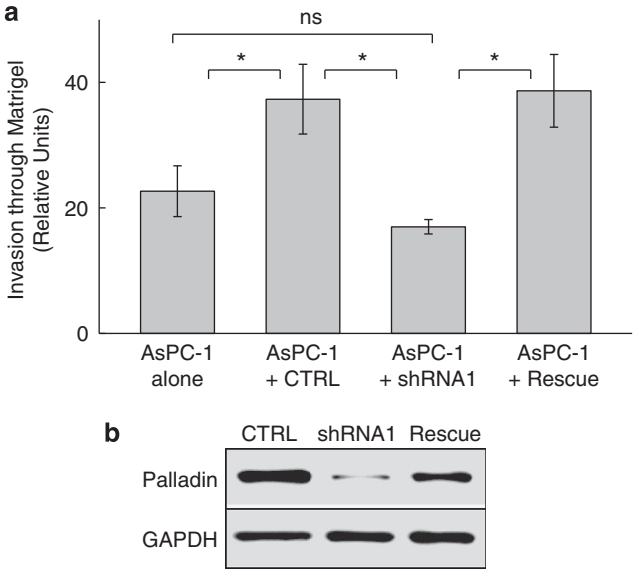

Figure 6. Palladin is required by CAFs to promote invasion of pancreatic cancer cells through Matrigel. (**a**) Fluorescently labeled AsPC-1 tumor cells were plated together with control CAFs (AsPC-1 + CTRL) or palladin knockdown CAFs (AsPC-1 + shRNA1) or rescued CAFs (transiently transfected with knockdown resistant mouse palladin). AsPC-1 cells invaded across the Matrigel-coated filter toward fetal bovine serum (FBS) containing medium in the lower chamber. Invading AsPC-1 cells were quantified as indicated in the Materials and methods section (NS = not significant, $^*P<0.05$). (**b**) Western blot analysis was used to detect palladin levels in cells treated with control shRNA (CTRL), palladin-specific shRNA (shRNA1) and shRNA resistant palladin construct expressed in adenovirus (Rescue).

tumorigenic and metastatic processes of various human tumors. Indeed, the balance between actin regulators might determine the type of adhesive structure that is formed and could account for the differences in shape and dynamics of invadopodia and podosomes.[41] Thus, it is not surprising that the actin-binding protein palladin has an important role in the assembly of invadopodia. In addition to its role as an actin-binding and -bundling protein, palladin functions as a cytoskeletal scaffolding molecule, as it interacts with a large number of actin-binding proteins with important roles in organizing actin filament arrays. Palladin's proline-rich domain binds directly to the actin-regulating proteins VASP (and its relatives Mena and EVL), profilin (actin polymerization), Eps8 (actin capping and bundling), SPIN90 (actin polymerization) and ArgBP2.[16,42–45] In addition, palladin binds to α-actinin, a widely expressed actin-crosslinking protein, and to Src itself, a kinase that is known to promote the assembly of invadopodia and podosomes. Studies from Luo *et al.*[18] have shown that fibroblasts from a palladin knockout mouse display defects in cell motility, cell adhesion and actin organization, demonstrating that normal palladin levels are required for normal actin assembly. In addition, we have previously shown that palladin influences the activity of Rac1, which is a member of Rho family of GTPases.[16] As Rho family members are key regulators of actin organization, the current evidence suggests that palladin influences actin organization through at least three distinct mechanisms: via direct crosslinking of F-actin, binding to multiple actin-regulatory proteins and altering the activity of Rho family members. All three of these molecular activities may contribute to the assembly of invadopodia in CAFs.

A growing body of evidence connects the Rho family of GTPases (RhoA, Rac1 and Cdc42) to cancer cell migration, invasion

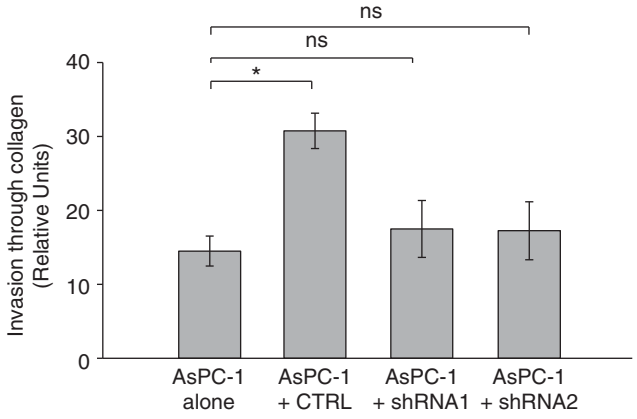

Figure 7. Palladin is required by CAFs to promote invasion of pancreatic cancer cells through collagen. Fluorescently labeled AsPC-1 tumor cells were plated together with control CAFs (AsPC-1 + CTRL) or palladin knockdown CAFs (AsPC-1 + shRNA1 and AsPC-1 + shRNA2). AsPC-1 cells invaded across the collagen-coated filter toward fetal bovine serum (FBS) containing medium in the lower chamber. Invading AsPC-1 cells were quantified as indicated in the Materials and methods section (NS = not significant, $^*P<0.05$).

and metastasis.[46,47] The deleterious role of Rho GTPases in human cancer stems from their overexpression or hyperactivation rather than the presence of activating point mutations.[48] This suggests that their regulatory proteins, guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), have a critical role in dysregulated signaling during human cancer initiation and progression. Either inhibition of a GAP or

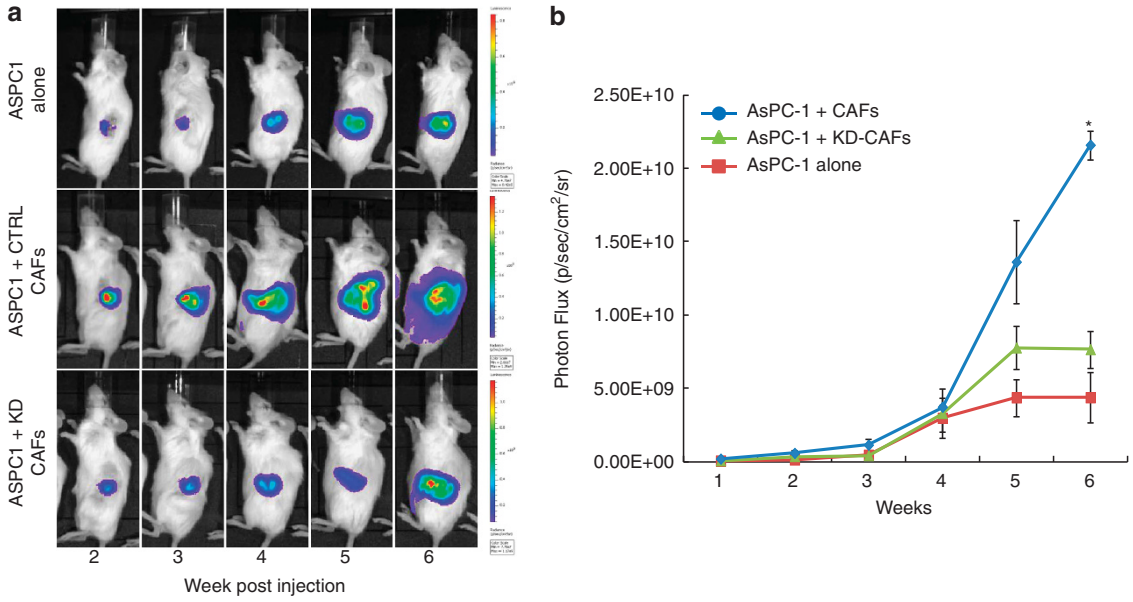

Figure 8. Palladin expression in CAFs enhances *in vivo* invasion of pancreatic cancer cells. Luciferase-expressing AsPC-1 cells were mixed with no CAFs, control CAFs, or palladin knockdown CAFs, and injected into the pancreas. Serial bioluminescence imaging of tumors reflects differential rates and efficiencies of tumor progression for the different mixed-cell types. (**a**) Representative bioluminescence images at the indicated times (weeks) post-injection with luciferase-expressing AsPC-1 cells, mixed with either control CAFs, palladin knockdown CAFs or no CAFs (AsPC1 alone). Each set of images was taken from the same mouse. (**b**) Tumor growth rates ($n = 10$ mice per group) injected with the specified mixed-cell type, as reflected by total photon flux (photons per second) per animal over the indicated time periods. Mice were injected with AsPC-1 cells with control CAFs (blue line), or palladin knockdown CAFs (green line), or no CAFs (red line). *$P < 0.05$.

upregulation of a GEF could result in increased activation of GTPases and thus promote deregulation of Rho family signaling cascades in cancer.[48] Two GEFs have been implicated directly in invadopodia/podosome formation with a role in cancer. The GEF Arhgef5 has been shown to have crucial roles in Src-induced podosome formation.[49] Faciogenital dysplasia protein Fgd1 regulates invadopodia biogenesis and ECM degradation and is upregulated in prostate and breast cancer.[50] Our results showed that Cdc42 activity is significantly reduced in palladin KD cells. This could result from reduced activity of one or more GEFs or increased activity of one or more GAPs. To date, little is known about the role of GEFs and GAPs in invadopodia biogenesis and function. Our future studies will focus on the identification of the regulatory proteins that are responsible for the activation of Cdc42 downstream of palladin.

Our current results provide mechanistic insights that expand on previously published studies that were largely correlative in nature. Along with others, we have shown that palladin is highly expressed in an isoform-specific pattern in the stroma of pancreas tumors, and that this upregulation is specific to invasive pancreatic ductal adenocarcinoma and not detected in less invasive tumor types.[12,13,37] In addition, elevated expression of stromal palladin has been shown to predict poor clinical outcome in renal cell carcinoma.[14] A recent study has shown that exogenous overexpression of palladin in normal fibroblasts imparts myofibroblast properties to these cells, promoting the invasive potential of cancer cells.[37] In the current work, we used knockdown approaches to determine the role of stromal palladin in cancer cell invasion and extended these studies into a mouse model. The results of both the transwell co-culture experiments and the mouse xenograft study suggest that palladin expression has an essential role in the pro-invasive behavior of CAFs. Previously published co-culture experiments have shown that CAFs possess the ability to generate channels within the ECM that promote the migration of skin carcinoma cells, and that CAFs enhance the metastatic behavior of cancer cells in xenograft models.[11] Our data suggest that in addition to Rho-dependent mechanisms that create tunnels through the matrix, CAFs also use invadopodia to degrade the matrix, and thus our results suggest that CAFs promote the invasion of cancer cells through multiple modes of matrix remodeling.

In conclusion, we provide novel insights into the role of palladin and Cdc42 in CAF-mediated invasion of pancreas cancer cells. These results suggest that the palladin/Cdc42 pathway will likely provide new targets for molecular therapies aimed at controlling the aggressive metastasis of pancreas cancer, one of the most dismal pathobiological behaviors of this tumor.

MATERIALS AND METHODS

Ethics statement

All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the Institutional Animal Care and Use Committee.

Cell lines

Immortalized human PSCs were characterized previously.[10] imPSC-C2 and clone 3 (imPSC-C3) were characterized previously.[30] Immortalized CAF, imPSC-C2, imPSC-C3 and primary CAFs were cultured in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and antibiotics. Pancreatic cancer cell line AsPC-1 was obtained from ATCC (ATCC # CRL-1682, Manassas, VA, USA). AsPC-1 cells were cultured in RPMI supplemented with 10% fetal bovine serum and antibiotics. All cell lines were grown at 37 °C and 10% carbon dioxide.

Materials

The following antibodies were used: palladin polyclonal (ProteinTech Group, Chicago, IL, USA); palladin monoclonal 1E6 previously characterized by Parast and Otey[15]; tubulin alpha (Lab Vision Corporation, Fremont, CA, USA); glyceraldehyde 3-phosphate dehydrogenase and cortactin (Santa Cruz, Dallas, TX, USA); vinculin (Sigma, St Louis, MO, USA); Cdc42 monoclonal (BD Biosciences, Franklin Lakes, NJ, USA). Protease inhibitor cocktail for mammalian tissues were from Sigma. TransIT siQuest and

TransIT-LT1 transfection reagent were from Mirus (Mirus, Madison, WI, USA). Alexafluor-488 and Alexafluor-568 anti-mouse immunoglobulin G and anti-rabbit immunoglobulin G-conjugated secondary antibodies were from Molecular Probes (Molecular Probes, Eugene, OR, USA). Horseradish peroxidase-conjugated secondary andibodies were from Jackson Immunoresearch (Jackson Immunoresearch, West Grove, PA, USA).

Immunofluorescence staining

Cells 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 immunoglobulin G and anti-rabbit immunoglobulin G conjugates. Coverslips were examined with a Nikon TE200-U microscope (Nikon, Melville, NY, USA) with 20 × and 60 × objective lenses, an optional 1.5 × tube lens and a Hamamatsu Orca-ER camera. Images were processed using Adobe Photoshop 7.0 (Adobe Systems Inc., New York, NY, USA). Invadopodia formation was induced by the addition of 1 μM PDBu (Sigma-Aldrich, St Louis, MO, USA), as previously described[23,51] and PMA as previously described.[52]

siRNA knockdown

The following siRNA were used in this study (sense strand): palladin, 5′-CUACUCCGCUGUCACAUUAUU-3′; Cdc42, 5′-GAUAACUCACCACUGUCC ATT-3′. As a control we used siCONTROL non-targeting siRNA #2 from Dharmacon (Dharmacon, Lafayette, CO, USA). Cells were transfected using the TransIT siQuest transfection reagent following the manufacturer's instructions. Briefly, 1.5 ml of serum-free medium (Life Technologies, Dharmacon) and 20 μl of transfection reagent per plate were preincubated for 15 min at room temperature. After incubation, 225 μl of 1 μM 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.

Cell lysis and immunoblot

Cells cultured on 100 mm tissue culture dishes were briefly rinsed with PBS 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 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 μg of protein were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in each lane of a 4–12% gel. The proteins were transferred to nitrocellulose and immunoblotted. Immunocomplexes were visualized using the Western Lights Chemiluminescence Detection kit from Perkin-Elmer (Perkin Elmer, Waltham, MA, USA).

In vitro ECM degradation assay

Cells were seeded onto coverslips that were coated with fluorescein isothiocyanate-conjugated gelatin for an *in vitro* ECM degradation assay, as described previously.[28] Briefly, glass coverslips were coated overnight with 100 lg/ml fluorescein isothiocyanate-labeled gelatin in PBS and fixed with 0.5% glutaraldehyde, washed six times with PBS, incubated with 50 lg/ml fibronectin at room temperature for 1 h, then washed once with 70% ethanol/PBS and once with medium. Cells were plated onto the coverslips and incubated at 37 °C in an incubator with 5% CO_2 for up to 24 h. Cells were fixed, permeabilized and incubated with 1% bovine serum albumin to block nonspecific staining and the F-actin was stained with rhodamine-conjugated phalloidin. The coverslips were examined using a Nikon Eclipse TE2000-U fluorescent microscope. The images were captured and analyzed, and the percentage of total area that was degraded was calculated using Nikon NIS Elements imaging software (Nikon).

Co-culture invasion assay

The invasiveness of pancreatic cancer cells was assessed based on the number of cells invading through either Matrigel-coated transwell chambers (BD Biosciences) or collagen-coated transwell chambers (Millipore, Billerica, MA, USA). AsPC-1 cells were fluorescently labeled with CellTracker red for quantification, then placed in the upper chamber together with control CAFs (AsPC-1 + CTRL) or with CAFs in which palladin was stably knocked down using shRNA delivered by lentivirus. The stable knockdown line is designated shRNA1. To restore palladin expression in the stable knockdown cells, knockdown resistant full-length mouse palladin was transiently expressed using an adenoviral vector, as previously described.[17] AsPC-1 with no CAFs were used as a control (AsPC-1 alone). The cells were allowed to invade for 24 h; then the non-invading cells were removed from the upper well. The invading cells were fixed and the red AsPC-1 cells were counted. The assay was performed at least two times in triplicate for each group.

Lentiviral constructs and transduction

GIPZ lentiviral shRNAmir (control non-silencing and human palladin shRNA) vectors (clone ID. V3LHS_310865; shRNA1 and V3LHS_310862; shRNA2 and gene access no. NM_016081) were obtained from Open Biosystem, Huntsville, AL, USA. Lentiviral packaging was done in TLA-HEK293T cells in accordance with the Trans-Lentiviral packaging system (Open Biosystems). After 12 h of transfection, the packaging cocktail was aspirated and replaced with complete medium. After 72 h, lentiviral containing supernatants were collected, centrifuged at 3000 r.p.m. for 20 min and stored at − 80 °C for further use. PSC cells were transduced with lentiviral stock (shRNA1 and shRNA2) overnight. The following day, the infection media was removed and replaced with complete medium containing puromycin (2 μg/ml) to select for shRNA1, shRNA2 or control (CTRL) shRNA-expressing cells and total cell lysates were subjected to western blot analysis for palladin expression as described.

Cdc42 activity assay

For the Cdc42 activation assay, cells were lysed in 600 μl of 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 1% Triton X-100, and protease inhibitors. Lysates were cleared at 16 000 *g* for 5 min. Supernatants were rotated with 50 μg GST-PBD (GST fusion protein containing the Rac/Cdc42 binding domain of PAK1) prebound to glutathione-Sepharose beads (GE, Fairfield, CT, USA). Beads were washed, resuspended in sodium dodecyl sulfate sample buffer and bound material was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose and immunoblotted for Cdc42.

Orthotopic model of pancreatic cancer

All animal experiments were reviewed by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. An orthotopic NSG mouse model of pancreatic cancer utilizing AsPC-1 pancreatic tumor cells labeled with firefly luciferase was used. Mice were divided into groups receiving intrapancreatic injections of (a) AsPC-1 cells alone, 0.5×10^6 per mouse; (b) AsPC-1 with control CAFs (tumor cell to CAF ratio 1:3); or (c) AsPC-1 with palladin stable knockdown CAFs (shRNA1), in a ratio of 1:3 (tumor cell to CAF). All cell suspensions, including the mixtures of AsPC-1 and CAFs, were injected in a 21 μl volume of Dulbecco's modified Eagle's medium. Bioluminescent imaging was performed weekly to quantify and localize the luciferase signal from AsPC-1 cells.

Statistical analysis

Data were analyzed using a two-tailed unpaired Student's *t*-test. A *P*-value of <0.05 was considered significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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