Exosomes Mediate Stromal Mobilization of Autocrine Wnt-PCP Signaling in Breast Cancer Cell Migration

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SUMMARY

Stroma in the tumor microenvironment plays a critical role in cancer progression, but how it promotes metastasis is poorly understood. Exosomes are small vesicles secreted by many cell types and enable a potent mode of intercellular communication. Here, we report that fibroblast-secreted exosomes promote breast cancer cell (BCC) protrusive activity and motility via Wnt-planar cell polarity (PCP) signaling. We show that exosome-stimulated BCC protrusions display mutually exclusive localization of the core PCP complexes. Fzd-Dvl and Vangl-Pk. In orthotopic mouse models of breast cancer, coinjection of BCCs with fibroblasts dramatically enhances metastasis that is dependent on PCP signaling in BCCs and the exosome component, Cd81 in fibroblasts. Moreover, we demonstrate that trafficking in BCCs promotes tethering of autocrine Wnt11 to fibroblast-derived exosomes. This work reveals an intercellular communication pathway whereby fibroblast exosomes mobilize autocrine Wnt-PCP signaling to drive BCC invasive behavior.

INTRODUCTION

The tumor-associated microenvironment, which consists of extracellular matrix (ECM), cancer-associated fibroblasts (CAFs), inflammatory immune cells, and tumor-associated vasculature, plays a critical role during tumorigenesis (Joyce and Pollard, 2009). CAFs, which are abundant in tumor-associated stroma, secrete several factors, including HGF and SDF1, to promote epithelial cell neoplastic transformation and cancer cell proliferation (Bhowmick et al., 2004; Orimo et al., 2005). In addition, CAFs interact with other stromal components to stimulate tumor-enhancing inflammation and angiogenesis (Erez et al., 2010; Orimo et al., 2005). Moreover, ECM remodeling by CAFs

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is essential for tumor cell invasion and metastasis (Gaggioli et al., 2007; Goetz et al., 2011). Although CAFs are important constituents of the tumor stroma, the paracrine signals that mediate direct crosstalk between CAFs and tumor cells in metastasis are poorly understood.

Exosomes are membrane vesicles that originate in large multivesicular bodies (MVBs) and are released in the extracellular milieu upon fusion of MVBs with the plasma membrane (Simons and Raposo, 2009; Théry et al., 2009). Exosomes have the same topology as a cell and contain a broad array of biologically active material (Simons and Raposo, 2009; Théry et al., 2009). Several cellular components of the tumor microenvironment and cancer cells secrete exosomes that function in an autocrine or paracrine manner to promote tumor-induced immune suppression, angiogenesis, and premetastatic niche formation (Peinado et al., 2012; Taylor and Gercel-Taylor, 2011). Currently, it is unknown whether CAFs secrete exosomes and whether these microvesicles support cancer cell metastasis.

The planar cell polarity (PCP) signaling pathway controls tissue polarity whereby cells orient themselves within a plane perpendicular to the apical-basal axis (Gray et al., 2011; Seifert and Mlodzik, 2007). In vertebrates, PCP signaling accounts for the orientation of hair bundles in inner ear sensory cells, the orientation of the hair follicles in the epidermis, and the convergence and extension (CE) movements of mesodermal and neuroectodermal cells (Gray et al., 2011; Seifert and Mlodzik, 2007). PCP signaling is transduced by a core module of conserved proteins that includes the seven-pass transmembrane Frizzled (Fzd) receptors, the four-pass transmembrane Van-Gogh-like (Vangl) proteins, the cytoplasmic proteins, Prickle (Pk), and the adaptor protein Dishevelled (Dvl). A hallmark of core PCP signaling is the asymmetric subcellular localization of specific protein complexes that propagates across polarized tissue (Gray et al., 2011; Seifert and Mlodzik, 2007). An outstanding question has been how the distribution of core PCP components is regulated in different tissues. In vertebrates, Wnt ligands play an important role by signaling through Fzd receptors to regulate PCP and CE movements (Gray et al., 2011; Gros et al., 2009). Wnt ligands comprise a family of secreted signaling molecules that regulate a plethora of developmental and physiological processes (Willert and Nusse, 2012). Due to acylation and glycosylation, Wnts associate tightly with the plasma membrane and the ECM (Willert and Nusse, 2012). Several mechanisms have thus been proposed to explain how Wnts might function as long-range signaling molecules. These include lateral diffusion by association with heparan sulfate proteoglycans (HSPGs), solubilization by high-density lipoproteins, carrier proteins (Coudreuse and Korswagen, 2007; Willert and Nusse, 2012), and exosomes (Gross et al., 2012). However, more investigation is required to establish the significance of these mechanisms in different Wnt-dependent processes.

There is a wealth of knowledge on the role of "canonical" Wnt/ β-catenin signaling in cancer. In contrast, much less is known about the Wnt-PCP pathway. Several core PCP pathway components, including Fzd, Vangl, and Dvl, are overexpressed in a number of tumors and are associated with tumor cell growth, migration, and metastasis (Jessen, 2009). However, it is unclear whether these components act in the context of the core PCP pathway and what molecular mechanisms underlie the potential role of Wnt-PCP signaling in cancer. Here, we report that exosomes secreted from fibroblasts, including human breast CAFs, stimulate breast cancer cell (BCC) protrusive activity, motility, and metastasis that are dependent on the exosome tetraspanin, Cd81. We show in a mouse model that exosomedriven metastasis is dependent on the core PCP pathway in BCCs and report that PCP components distribute in a mutually exclusive fashion in the protrusions of single, motile, and malignant cells. Surprisingly, exosome activity is dependent on Wnt11 produced in BCCs, and we demonstrate that fibroblastderived exosomes are internalized by BCCs and thereby loaded with Wnt11. These studies reveal a key role for fibroblast-derived exosomes in mobilizing autocrine Wnt-PCP signaling in BCCs to stimulate invasive behavior and metastasis in animal models.

RESULTS

L Cell Fibroblasts Stimulate BCC Protrusive Activity, Motility, and Invasion

In the course of investigating the core PCP pathway in BCC motility, we discovered that conditioned media from mouse fibroblast L cells, hereafter referred to as active-conditioned medium (ACM), potently stimulated the protrusive activity and motility of human breast adenocarcinoma MDA-MB-231 cells (Figures 1A-1C; Movie S1 available online). Upon ACM treatment, MDA-MB-231 cells formed long and dynamic protrusions that extended in different directions throughout the treatment (Figure 1B; Movie S1). Furthermore, ACM-treated cells moved significantly faster with more directional persistence than control cells (Figure 1C; Figures S1A and S1B; Movie S1). We also examined three-dimensional (3D) Matrigel cultures, which revealed that ACM stimulated formation of MDA-MB-231 cell protrusive structures (Figure 1D). ACM also induced protrusive structures and motility in other mammary cell lines of epithelial origin, including human carcinoma SUM-159PT, MDA-MB-468, and T-47D cells, mouse carcinoma EMT-6 cells, tumorigenic EpRas cells, and immortalized EpH4 cells (Figures S1C and S1D). This indicates that the L-cell-secreted factor(s) has a potent and prevalent effect in inducing mammary cell protrusive activity and migration.

Enhanced cell protrusive activity, motility, and protrusive growth in a 3D environment are typically correlated with increased metastatic potential. MDA-MB-231 cells are weakly metastatic (Minn et al., 2005) and thus provide a good model to study Lcell-induced BCC metastasis. Therefore, we injected MDA-MB-231 cells either alone or together with L cells in the mammary fat pad of SCID mice (Figure 1E). Four weeks postinjection, the mice were sacrificed, and their primary tumors and lungs were collected for analysis. In fat pads coinjected with L cells and MDA-MB-231 cells, tumor growth was robust (Figure S1E) when compared to fat pads injected with comparable numbers of either MDA-MB-231 cells alone, or L cells alone, which possess tumorigenic activity (Sanford et al., 1956). Next, we examined the lungs of tumor-bearing mice for MDA-MB-231 metastatic lesions using an antibody specific for human vimentin (Figure 1F). This revealed that MDA-MB-231 cells alone formed few lung lesions after 4 weeks of primary tumor growth (Figure 1F). In contrast, when coinjected with L cells, both the number and the size of MDA-MB-231 metastatic lesions were significantly enhanced (Figures 1F-1H). To confirm that this was not unique to MDA-MB-231 cells, we developed a second mouse model using SUM-159PT cells, whose motility is also stimulated by ACM (Figure S1D). Orthotopic injection of SUM-159PT cells alone produced almost no metastases at 4 weeks postimplantation, consistent with previous reports (Ma et al., 2007). However, when coinjected with L cells, nests of SUM-159PT cells were readily apparent in the lung parenchyma (Figure S6D), similar to MDA-MB-231 cells. Of note, our subsequent work showed that enhanced metastasis in either model was not due to accelerated growth of the primary tumor in the coinjected group (Figures S1E, 2D–2F, 6B–6D, and S6D). Altogether, these results demonstrate that fibroblast-secreted factors promote BCC protrusive activity, motility, and metastasis in mouse models.

The Core PCP Pathway Is Required for Fibroblast-Induced BCC Motility and Metastasis

Smurf1 and Smurf2 are ubiquitin ligases that are critical regulators of cell polarity, protrusive activity, and BCC motility (Ozdamar et al., 2005; Sahai et al., 2007; Viloria-Petit et al., 2009; Wang et al., 2003). Accordingly, siRNA knockdown of Smurf1 and Smurf2 in MDA-MB-231 cells inhibited ACM activity (Figure S2A). Smurf1 and Smurf2 are critical regulators of PCP and CE movements in mice (Narimatsu et al., 2009), which prompted us to explore whether ACM-induced protrusions and motility might be mediated through the core PCP pathway. For this, we used siRNA to downregulate expression of the core PCP pathway components, including Fzd6, Dvl1, Pk1, and Vangl1 (Figures 2A, 2B, S2B, and S2C). Strikingly, interference with DvI1, Fzd6, and VangI1 inhibited ACM-induced cell protrusions and motility (Figures 2A, 2B, and S2D), whereas Pk1 knockdown led to almost complete cessation of movement (Figures 2B and S2D; Movie S2). Knockdown of Fzd3, Fzd7, and Dvl2, which are also associated with PCP signaling, inhibited motility, albeit more modestly, suggesting functional redundancy (Figures S2B and S2C). In ACM-stimulated SUM-159PT cells, interference



Figure 1. L-Cell-Secreted Factors Promote BCC Protrusive Activity, Motility, and Metastasis

(A) Experimental schematic. ACM produced by conditioning DMEM with L cells for 3 days was added to MDA-MB-231 cells as described in Experimental Procedures, and the protrusive activity and motility of individual cells were imaged by time-lapse microscopy for 18 hr.

(B) Representative images of MDA-MB-231 cells treated with control media (DMEM) or ACM are shown. Highlighted are short membrane protrusions in control (white hollow arrow) versus extended protrusions in ACM-treated cells (white arrows).

(C) MDA-MB-231 cells treated as in (B) were traced, and the average speed was determined. Results are plotted as a boxplot where the box spans the 25th and 75th percentile, the line marks the median speed, and the whiskers mark the minimum and maximum data points (n = 30 cells per group). ***p < 0.0001. (D) MDA-MB-231 cells grown in Matrigel for 3 days were treated as indicated and imaged by phase-contrast microscopy. Higher-power view of boxed area is

shown in lower panels. Scale bars, 90 μ m.

with Dvl3, Pk1, or Fzd7 expression analogously inhibited motility (Figure S2E). We next examined if the core PCP pathway is important for the enhanced metastasis of MDA-MB-231 cells upon coimplantation with L cells. For this, we focused on Pk1, the only Pk isoform expressed in MDA-MB-231 cells (data not shown), thus allowing efficient interference with the pathway. Using three different shRNAs, we stably knocked down Pk1 by 53%-86% in MDA-MB-231 cells and, as in transient knockdowns, observed reduced motility in response to ACM (Figure S2F). Cells harboring Pk1 or control shRNA were then coinjected with L cells in the mammary fat pad of mice (Figure 2C). Strikingly, Pk1 downregulation inhibited the appearance of Lcell-induced MDA-MB-231 micrometastatic foci (Figure 2D) to basal levels (Figures 2E and S2G). Importantly, Pk1 knockdown in MDA-MB-231 cells did not affect the rate of primary tumor growth (Figures 2F and S2H), suggesting that PCP signaling specifically promotes metastasis. Collectively, these results demonstrate that the core PCP pathway in tumor cells is necessary for fibroblast-induced BCC motility and in vivo metastasis.

Core PCP Components Distribute Asymmetrically in ACM-Induced Cell Protrusions

A unique characteristic of PCP components is their asymmetric subcellular distribution orthogonal to the apical-basal axis of polarity (Gray et al., 2011; Seifert and Mlodzik, 2007). In the Drosophila wing epithelium, the proximal subset of proteins, consisting of Pk and Van Gogh, and the distal subset, consisting of Fzd, Dvl, and Diego, localize at the plasma membrane on opposite sides of cell-cell contacts (Seifert and Mlodzik, 2007). Similarly, in vertebrates, these protein complexes localize asymmetrically (Ciruna et al., 2006; Gray et al., 2011; Narimatsu et al., 2009), raising the question of how PCP promotes motility of individual BCCs. Therefore, we examined the localization of core PCP pathway components in MDA-MB-231 cells. In ACMstimulated cells, Fzd6 was enriched and colocalized with Dvl1 at the leading front of protrusions in association with filamentous actin (F-actin) (Figures 3A, S3A, and S3B). In contrast, unstimulated cells displayed little colocalization of Fzd6 and Dvl1 (Figure 3A). Next, we examined Pk1 using a Pk1-specific Fab antibody fragment (Figure 3B). This revealed that Pk1 colocalized extensively with cortical F-actin, as previously found by Veeman et al. (2003), and was aligned along the nonprotrusive cell membrane (Figure 3B). Moreover, in ACM-stimulated cells, we observed Pk1 staining at the base of cell protrusions that was mutually exclusive to Fzd6 staining (Figure 3B). Similarly, ectopically expressed Flag-Vangl1 was also enriched along the nonprotrusive cell cortex in both unstimulated and ACM-stimulated cells. Furthermore, in ACM-induced cell protrusions, Vangl1 localization was mutually exclusive to Fzd6 (Figure 3C). We also examined ACM-stimulated SUM-159PT cells and observed Fzd7 enriched at the tip of cell protrusions, marked by F-actin networks, where it colocalized with Dvl3 (Figures S3C and S3D). These results demonstrate that in ACM-stimulated BCCs, Fzd-Dvl and Vangl-Pk are asymmetrically distributed with respect to cellular protrusions, in a manner analogous to planar-polarized epithelial cells. To the best of our knowledge, such asymmetric distribution of PCP components with respect to cellular protrusions has not been previously reported in single, motile, and malignant cells.

Autocrine Wnt11 Mediates Fibroblast-Induced BCC Protrusive Activity and Motility

The distribution and function of core PCP components in ACMstimulated motility and metastasis suggested that L cells secrete a factor(s) that regulates PCP signaling. How extracellular cues control PCP signaling in vertebrates is poorly understood, but genetic studies in developmental models show that Wnt ligands, including Wnt5a and Wnt11, are key regulators of the pathway (Gray et al., 2011; Gros et al., 2009). Therefore, we examined whether L cells express Wnt ligands and found, to our surprise, that Wnt5a and Wnt11 were not expressed in L cells (data not shown). Furthermore, siRNA-mediated knockdown in L cells of Porcupine, which is an acyltransferase essential for Wnt lipidation and secretion (Willert and Nusse, 2012), did not affect ACM activity (Figures S4A and S4B). Therefore, we considered whether ACM controls autocrine Wnt signaling in MDA-MB-231 cells. First, we knocked down Porcupine expression in MDA-MB-231 cells (Figure S4C) and observed potent inhibition of ACM-induced protrusive activity and motility (Figure 4A). Furthermore, knockdown of Wnt11, but not Wnt5a, also interfered with ACM activity (Figures 4B and S4C; Movie S3). Thus, autocrine Wnt11 in MDA-MB-231 cells is necessary for ACMinduced motility. Next, we tested the association between Wnt11 and its putative receptor, Fzd6, by immunofluorescence. In control, unstimulated cells, endogenous Wnt11 displayed a punctate distribution (Figure 4C) that was abolished upon Wnt11 knockdown (Figure S4D), thus confirming the specificity of the signal. Furthermore, in unstimulated cells, Wnt11 was concentrated inside the cells, consistent with Wnt vesicular trafficking (Coudreuse and Korswagen, 2007), and displayed little colocalization with Fzd6 (Figure 4C). In contrast, upon ACM treatment, endogenous Wnt11 was observed in puncta at the tip of cell protrusions where it colocalized with Fzd6 (Figures 4C and S4E). Altogether, these results indicate that ACM stimulates autocrine Wnt11 association with Fzd6 and activation of PCP signaling during BCC protrusive activity and motility.

Identification and Functional Characterization of L-Cell-Secreted Exosomes

Our results indicated that L cells secrete factor(s) that promote autocrine Wnt11-PCP signaling in BCCs. To identify the factor(s), we fractionated ACM by size exclusion chromatography, which

⁽E–H) MDA-MB-231 cells were injected alone or together with L cells in the mammary fat pad (mfp) of mice (E). Lungs from mice harboring the indicated tumors were stained for vimentin, and representative micrometastases corresponding to the median size of the population are shown (F; higher-power view of boxed area is shown in lower panels. Scale bars, 35μ m). The number of metastatic colonies in lungs harboring MDA-MB-231 only (n = 4) or MDA-MB-231 plus L cells (n = 7) was quantified (G; mean colony number ± SEM; p = 0.03, using two-tailed unpaired t test with Welch's correction) and the area determined (H; bars indicate the mean ± SEM; p = 0.0004, using two-tailed unpaired t test with Welch's correction). See also Figure S1 and Movie S1.





(A and B) MDA-MB-231 cells transfected with the indicated siRNA to Dvl1, Fzd6, Pk1, Vangl1, or a scramble sequence (Scr) were treated and presented as in Figure 1B. Cell speed was quantified (B) and plotted as box and whisker plots as in Figure 1C (n = 33 cells per group). ***p < 0.0001.

(C–E) Schematic of the experimental design (C). Lung lesions from mice harboring the indicated tumors were detected as in Figure 1F, and representative images are shown (D; scale bars, 35μ m). Metastatic colonies in mice injected with MDA-MB-231 (MDA) cells in the presence (n = 10 mice per group) or absence (n = 6 mice per group) of L cells were quantified (E; mean colony number ± SEM; p = 0.048, using two-tailed unpaired t test with Welch's correction). ns, not significant. (F) Tumor growth, in mice described in (C)–(E), was measured at the indicated time points and plotted as the mean volume ± SEM. See also Figure S2 and Movie S2.

revealed the activity in a single high molecular weight fraction (S6) (Figures S5A–S5C). Further purification by ion-exchange chromatography yielded a final active fraction (Q3) (Figures S5D–S5F) that was subjected to mass spectrometry (MS) (Table S1). Analysis using David bioinformatics tools followed by manual annotation revealed that the active fraction was highly enriched in proteins associated with vesicles (Table S2), in particular exosome components such as the tetraspanin Cd81 and its partners Igsf8 and Ptgfrn (Zöller, 2009) (Table S1).

Exosomes have specific structural and molecular characteristics that are distinct from other cell-derived microvesicles (Théry et al., 2009). Therefore, to verify the presence of exosomes in ACM, we analyzed total ACM and the S6/Q3 active fractions by electron microscopy (EM) (Figure 5A). This revealed cup-shaped, 30–100 nm diameter structures that are typical of exosomes (Théry et al., 2009). Moreover, we confirmed the identity of these vesicles by immunostaining for exosome markers, namely Cd81, Igsf8, and Ptgfrn (Figure 5B). Exosomes float at 1.13–1.19 g/ml density in sucrose gradients (Théry et al., 2009). We found that L-cell-secreted vesicles also floated at 1.13–1.15 g/ml density (data not shown), and proteomic analysis revealed that this fraction was highly enriched in known exosome components, further confirming their presence in ACM (Figure 5C).

To determine whether exosomes produced by L cells are sufficient to induce BCC protrusive activity and motility, we isolated exosomes from ACM by differential ultracentrifugation (Théry et al., 2006). This revealed that the 100,000 \times g pellet, which contains the exosomes, possessed ACM stimulatory activity, whereas activity in the supernatant was depleted (Figure 5D). Next, we interfered with exosomes by depleting ACM of cholesterol, which is enriched in exosome membranes and plays a role in exosome secretion (Simons and Raposo, 2009). Depletion of cholesterol using the cholesterol chelator, methyl β -cyclodextrin, interfered with exosome integrity, as determined by loss of Cd81-positive exosomes and inhibited ACM-induced MDA-MB-231 cell motility (Figure S5G). Furthermore, immunodepletion of exosomes using antibodies against Cd81, Igsf8, or Ptgfrn inhibited ACM activity (Figure 5E). Analysis of Cd81, Igsf8, or Ptgfrn immunoprecipitates by MS also revealed numerous exosome components (data not shown), thus verifying successful immunocapture. Altogether, these results demonstrate that L-cell-secreted exosomes modulate BCC protrusive activity and motility.

Cd81-Positive Exosomes Promote BCC Motility and Metastasis

Exosomes are rich in proteins, RNAs, and lipids that bestow upon them diverse functions in different biological contexts (Simons and Raposo, 2009; Théry et al., 2009). Therefore, to identify component(s) important for the Wnt-PCP pathway, we subjected ACM to heat denaturation, which interfered with its activity (Figure S6A), suggesting that protein components are important. Our proteomic analysis (Figure 5C) revealed that tetraspanins Cd63, Cd81, and Cd82, as well as their interacting partners, Ptgfrn and Igsf8, were highly abundant in L cell exosomes. Therefore, we knocked down Cd63, Cd81, Cd82, Igsf8, and Ptgfrn using siRNA in L cells and found that downregulation of Cd81 expression inhibited ACM-induced MDA-MB-231 cell motility (Figure S6B) close to that of unstimulated cells. In contrast, interference with Cd63, Cd82, and Ptgfrn expression in L cells had no effect, whereas interference with Igsf8 expression slightly enhanced activity (Figure S6B). We conclude that Cd81 functions in the context of exosomes to regulate fibroblast-induced BCC motility.

Different tetraspanins have tumor-promoting (Tetraspanin 8 and Cd151) or -suppressing functions (Cd9 and Cd82) (Zöller, 2009). To investigate the importance of Cd81 in metastasis, we generated L cells in which Cd81 was constitutively knocked down by two different shRNAs (Figure 6A). This interfered with ACM activity but, importantly, did not affect the secretion of exosomes because the levels of other exosome markers, including Igsf8, Ptgfrn, and Flotillin1, were unchanged (Figure S6C). Next, we coinjected Cd81-knocked-down L cells together with MDA-MB-231 cells, using our orthotopic mouse model (Figure 6B). Metastasis of MDA-MB-231 cells was significantly enhanced upon coinjection with control shRNA-expressing L cells, as above (Figures 6B and 6C). In sharp contrast, there was a severe suppression in the number of metastatic nodules when Cd81-deficient L cells were coinjected (Figure 6C). Similarly, L-cell-stimulated metastatic SUM-159PT cells in the lung parenchyma were also strongly suppressed by downregulation of Cd81 (Figure S6D). Importantly, loss of Cd81 expression in L cells did not significantly affect the growth rate or size of the primary tumor (Figures 6D and S6D), confirming that the enhanced metastatic potential of BCCs was not secondary to increased tumor growth. Altogether, these studies suggest that L-cell-secreted Cd81-positive exosomes enhance the metastatic potential of BCCs by regulating their motility.

To assess whether stromal Cd81 expression might be associated with human breast cancer, we analyzed a publicly available gene expression data set (GSE4823) of stroma isolated from invasive ductal carcinoma and normal breast reduction tissue (Finak et al., 2006). This revealed that, whereas CD81 expression was significantly upregulated in human breast tumor-associated stroma (Figure S6E), the expression of IGSF8, PTGFRN, CD63, and CD82 was unchanged (data not shown), consistent with the notion that Cd81 functions in the tumor-associated stroma to promote tumorigenesis. Next, we investigated whether primary human breast CAFs, like L cells, secrete exosomes that promote BCC motility. For this, we prepared conditioned media from CAFs isolated from two patients (CAF1 and CAF2) (Hosein et al., 2010). Both CAF populations secreted exosomes positive for Cd81, Igsf8, and Flotillin1 (Figure 6E) that also stimulated MDA-MB-231 cell motility (Figure 6F) in a Pk1-dependent manner (Figure 6G). These CAF exosomes also stimulated MDA-MB-231 cell protrusions that displayed asymmetric accumulation of Fzd6 and Dvl1, similar to L-cell-derived exosomes (Figures S6F and S6G). Taken together, our studies indicate that secretion of Cd81-positive exosomes is an important feature of human CAFs that can stimulate BCC motility via autocrine Wnt-PCP signaling.

L Cell Exosomes Mobilize MDA-MB-231-Produced Wnt11

To explore the molecular mechanisms whereby Cd81-positive exosomes activate autocrine Wnt-PCP signaling, we first



assessed exosome secretion by similar numbers of L cells or MDA-MB-231 cells (Figure 7A). Analysis of Cd81, Igsf8, Ptgfrn, and Flotillin 1 levels revealed that MDA-MB-231 cells produced significantly less exosomes compared to L cells (Figure 7A). Interestingly, MDA-MB-231 total cell lysates showed significant expression of Cd81, suggesting that these cells are deficient in exosome biogenesis (Figure S7A). These results are consistent with our finding that exosome production and Cd81 expression are important for PCP signaling. Next, we sought to determine how L cell exosomes might activate autocrine Wnt11-PCP signaling by examining their internalization by MDA-MB-231 cells. For this, we generated L cells stably expressing Cd81-EYFP and confirmed that the fusion protein is secreted in exosomes (Figure S7B). We then treated MDA-MB-231 cells with conditioned media from either control or Cd81-EYFP-expressing L cells. Immunoprecipitation of Cd81-EYFP from lysates of recipient MDA-MB-231 cells showed association with these cells (Figure 7B), whereas immunofluorescence revealed that Cd81-EYFP accumulated in vesicular structures (Figure 7C). Exosomes have previously been reported to be internalized by endocytosis (Simons and Raposo, 2009; Théry et al., 2009), and Wnt ligands are trafficked via endosomes from the plasma membrane of Wnt-producing cells, a process that is thought to be necessary for their maturation and long-range signaling (Coudreuse and Korswagen, 2007). Thus, we examined endogenous Wnt11 in MDA-MB-231 cells and observed its colocalization with Cd81-EYFP in vesicular structures in the perinuclear region of the cell, consistent with previously reported Wg subcellular localization (Coudreuse and Korswagen, 2007) (Figure 7C). We conclude that Wnt11 ligands gain access to L cell exosomes within the endocytic pathway.

Once taken up by endocytosis, exosomes can be recycled back to the extracellular milieu by the recipient cells (Théry et al., 2009). Therefore, we wondered whether Wnt11 could be loaded onto recycling L cell exosomes. To test this hypothesis, we generated MDA-MB-231 cells stably expressing Wnt11 tagged at the C terminus with HA to facilitate detection of the ligand (Figure S7C). Wnt11-HA-expressing MDA-MB-231 cells were then treated with either control media (DMEM) or ACM for 20 hr to allow exosome trafficking. These MDA-MB-231-conditioned media were next subjected to differential ultracentrifugation and immunoblotting for Wnt11-HA and Cd81 (Figure 7D). Although the Wnt11-HA signal was too weak to be detected in total conditioned media, the ligand was readily detected in exosomes isolated from ACM conditioned by Wnt11-HA-expressing cells. In contrast, Wnt11-HA was not present in the exosome pellet from MDA-MB-231-conditioned control media (Figure 7D). Furthermore, immunogold labeling and EM revealed Wnt11-HA in exosomes isolated from MDA-MB-231-conditioned ACM (Figure 7E). In contrast, analysis of either ACM or MDA-MB-231-conditioned control media revealed no Wnt11-positive exosomes (data not shown).

We next conditioned either control media or ACM with Wnt11-HA-expressing MDA-MB-231 cells and analyzed Wnt11-HA internalization by parental MDA-MB-231 cells not expressing Wnt11-HA (Figure S7D). This revealed that a similar amount of Wnt11-HA was internalized from either conditioned control media or conditioned ACM, suggesting that Wnt11-HA secretion and internalization are not dependent on exosomes. We then tested if in the absence of MDA-MB-231 cells, incubation of Wnt11-HA-conditioned control media with naive ACM might support Wnt11-HA loading on exosomes (Figure 7F). However, when Wnt11-HA-conditioned control media were simply mixed with ACM, little or no Wnt11-HA associated with exosomes, despite their efficient purification under these conditions (Figures 7F and 7G), contrasting the efficient loading of Wnt11-HA onto ACM exosomes in the presence of MDA-MB-231 cells (Figures 7F and 7G). Collectively, our data support a model in which autocrine Wnt-PCP signaling is mobilized by fibroblast-derived exosomes that are loaded with Wnt11 upon trafficking in BCCs to drive metastasis (Figure S7E).

DISCUSSION

Communication between cancer cells and the associated stroma plays a key role in driving tumor progression, but how stromal cues might act to specifically promote cancer metastasis is less clear. Here, we reveal that fibroblast-secreted exosomes play a key role in promoting BCC motility and metastasis by mobilizing autocrine Wnt-PCP signaling in tumor cells (Figure S7E). In particular, we found that L-cell-secreted exosomes have a potent stimulatory effect on the protrusive activity, motility, and metastasis of BCCs and that human CAFs also secrete stimulatory exosomes. Furthermore, we found that Cd81 is important for L-cell-induced BCC metastasis because interference with Cd81 strongly suppressed lung metastases but had no effect on the growth of the primary tumor. This suggests that Cd81-positive exosomes play a key role in a metastasis-specific pathway that is separable from stromal signals that promote growth of the primary tumor. Indeed, Cd81 is significantly overexpressed in stroma associated with human invasive ductal carcinoma (Figure S6E). These results indicate that fibroblast exosomes and, in particular, CAF exosomes have a cell migration-promoting role during metastasis, and it will be of interest to define the impact of this pathway on the progression of human breast cancer.

Several PCP pathway components regulate cancer cell motility and invasion (Jessen, 2009). However, it is unclear

Figure 3. Core PCP Complexes Localize Asymmetrically in ACM-Stimulated BCCs

(A–C) Confocal images of MDA-MB-231 cells cultured in DMEM or ACM, as described in Figure 1A, are shown. Endogenous Fzd6 (green [first column] and white [second column]) was immunostained in parallel with endogenous Dvl1 (A), endogenous Pk1 (B), or ectopically expressed Vangl1 (C) (red [first column] and white [third column]). F-actin-rich, cell protrusive structures were detected by phalloidin staining (blue [first column] and white [fourth column]). The localization of Fzd6 at the leading edge of ACM-induced cell protrusions is indicated (A–C, green arrows [second column]). The colocalization of Dvl1 (A, red arrows) with Fzd6 (green arrows) at ACM-induced cell protrusions is indicated (yellow arrows). Pk1 (B) and Vangl1 (C) staining along the nonprotrusive cell membrane flanking ACM-induced cell protrusions are indicated (red line). Note that Pk1 (B) and Vangl1 (C) are depleted from the leading front of ACM-induced protrusions that is marked by rich F-actin and Fzd6 staining (green arrows). The insets show a higher magnification of the indicated white, boxed areas. Scale bars, 10 µm. See also Figure S3.



Figure 4. Autocrine Wnt11 Is Required for ACM-Stimulated BCC Protrusive Activity and Motility

(A and B) MDA-MB-231 cells transfected with siRNA targeting Porcupine (Porcn) (A), Wnt5a (B), Wnt11 (B), a scramble sequence (Scr), or under mock conditions were treated and presented as in Figure 1B. Cell speed was quantified (bottom) and plotted as box and whisker plots (n = 27 cells per group in A, and n = 30 cells per group in B). ***p < 0.0001.

(C) MDA-MB-231 cells treated as indicated were immunostained for endogenous Fzd6 (green [first column] and white [second column]) and Wnt11 (red [first column] and white [third column]). F-actin-rich, cell protrusive structures were detected by phalloidin staining (blue [first column] and white [fourth column]). Colocalization of Fzd6 (green arrows) and Wnt11 (red arrows) at the leading edge of ACM-induced cell protrusions is indicated (yellow arrows). The insets are a higher magnification of the boxed areas. Scale bars, 10 µm. See also Figure S4 and Movie S3.

Figure 5. L-Cell-Derived Exosomes Stimulate BCC Motility

(A and B) Exosomes isolated as described in Experimental Procedures from total ACM and the active fraction from size exclusion chromatography (S6) or ionexchange chromatography (Q3) were imaged by EM. Cup-shaped microvesicles with sizes between 40 nm (arrowhead) and 100 nm (arrow) are indicated and enlarged in the bottom of (A). ACM exosomes immunolabeled for different exosome markers are shown in (B).

(legend continued on next page)

whether PCP signaling per se is directly involved in cancer. Our results provide compelling evidence that PCP signaling promotes breast cancer metastasis because all the core PCP components we tested were critical for ACM-induced protrusive activity and motility, and disruption of Pk1 expression potently inhibited L-cell-induced BCC metastasis without affecting primary tumor growth. In developmental contexts, PCP complexes are asymmetrically organized in individual cells across the plane of the tissue (Gray et al., 2011; Seifert and Mlodzik, 2007), whereas here, we show asymmetry with respect to the protrusions of ACM-stimulated, single, and motile cells, with Fzd and DvI at the leading edge and VangI-Pk in the flanking cortical regions. Importantly, in dynamically rearranging tissue, such as the zebrafish neural keel and mouse neuroectoderm, Pk1 is also asymmetrically distributed but does not show organization across the tissue (Ciruna et al., 2006; Narimatsu et al., 2009). Thus, we propose that core PCP signaling regulates cancer cell protrusive activity and motility in an analogous fashion to its role in regulating cytoskeletal dynamics and CE movements during development.

We previously showed that Smurf E3 ubiquitin ligases regulate PCP and CE movements in mice by binding phosphorylated Dvl that is associated with Par6 to target Pk for degradation (Narimatsu et al., 2009). Moreover, Smurfs are critical for cancer cell motility and the metastatic phenotype (Sahai et al., 2007; Viloria-Petit et al., 2009; Wang et al., 2003). Here, we demonstrate that Smurfs, as well as core PCP components, are essential for ACM-induced BCC protrusive activity and motility. Importantly, DvI proteins are critical for directed cell motility that also depends on the Cdc42/Par6/aPKC polarity complex (Schlessinger et al., 2007). Furthermore, Smurfs in complex with Par6 control degradation of RhoA at the tips of cellular protrusions (Wang et al., 2003) that is also important for dissolution of tight junctions during loss of apical-basal polarity associated with TGF-β-dependent epithelial-to-mesenchymal transition (Ozdamar et al., 2005). Therefore, we propose that the Par-Smurf complex also engages core PCP signaling in cancer to direct cell motility and metastasis.

Our studies also revealed the unexpected finding that fibroblast-secreted exosomes are internalized into BCCs and associate with BCC-produced Wnt11 to activate PCP signaling. Wnts are subject to posttranslational modifications that drive their tight association with the plasma membrane and are required for Wnt function in vivo (Coudreuse and Korswagen, 2007; Willert and Nusse, 2012). In particular, Wnts are palmitoylated in a manner that is dependent on Porcupine, a multitransmembrane protein that resides in the ER and is essential for Wnt ligand secretion and function (Coudreuse and Korswagen, 2007; Willert and Nusse, 2012). Consistent with this, we found that

downregulation of Porcupine expression in MDA-MB-231 cells completely abrogated ACM-induced cell protrusive activity and motility. Moreover, Wnts localize to MVBs and recycling endosomes within Wnt-producing cells, and this is thought to be necessary for Wnt maturation and signaling (Coudreuse and Korswagen, 2007). In agreement with this, we found that BCCproduced Wnt11 colocalized with fibroblast-secreted Cd81 in BCC vesicular structures and demonstrated that Wnt11 tethers to Cd81-positive exosome upon trafficking in BCCs. Thus, autocrine Wnt11 is tethered to stroma-secreted exosomes within endocytic vesicles of the recipient tumor cells to activate PCP signaling (Figure S7E). Interestingly, it was recently reported that exosome-associated Wnts function during Drosophila development (Gross et al., 2012). We therefore propose that association of Wnts with exosomes is an essential and conserved mechanism that is important during development and homeostasis that is co-opted in cancer. In the future, it will thus be critical to elucidate how exosomes and their constituent components, such as Cd81, regulate Wnt signaling in other biological contexts.

EXPERIMENTAL PROCEDURES

Conditioned Media Preparation

Confluent cells were incubated with DMEM supplemented with penicillin/ streptomycin, amphotericin B, and either 0% FBS (for L cells) or 0.2% FBS (for CAFs and MDA-MB-231 cells). Three days later, the conditioned media were collected, filtered, and stored at 4°C for up to 2 months.

Single-Cell Motility Assay and Immunofluorescence

Cells were seeded at very low density in assay media prepared from mixing equal volumes of 5% FBS/RPMI with DMEM or conditioned media. Phase-contrast time-lapse movies were collected on a Leica microscope (DMIRE2) using a 10× N-Plan objective lens, an Orca-ER camera (Hamamatsu), and a MS-2000 xyz automated stage (ASI). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂ (Pathology Devices). Cell speed was determined by manually tracing the cell periphery every hour for 18 hr using Volocity software (PerkinElmer). For immunofluorescence imaging, DMEM or ACM-treated cells were grown and fixed on coverslips (Thermo Scientific) prior to immunostaining and imaging as described in the Extended Experimental Procedures.

Orthotopic Mouse Model of Breast Cancer Metastasis

Research involving animals was performed in accordance with protocols approved by the animal facility at Toronto Centre for Phenogenomics (Toronto). A total of 1×10^4 L cells and 3×10^6 MDA-MB-231 or SUM-159PT cells were implanted into the right fourth mammary fat pad of 10-week-old female C.B-17 SCID mice (Charles River, Canada). Four weeks postinjection, the mice were euthanized, and tumors and lungs were harvested for analysis. MDA-MB-231 and SUM-159PT lung metastases were detected using a human-specific vimentin antibody (Dako) as described in Extended Experimental Procedures.

⁽C) ACM exosomes were purified on a sucrose gradient, and associated proteins identified by MS were annotated using GeneCards. Proteins are grouped by function, and exosome proteins previously identified in ExoCarta are highlighted in bold.

⁽D) ACM was subjected to sequential centrifugation at 2,000 × g for 10 min, 10,000 × g for 40 min, and 100,000 × g for 14 hr, and motility induction by the corresponding pellets (P) or the final supernatant (Sup. or S) was quantified as in Figure 1C (top; n = 29 cells per group; **p < 0.001). Aliquots were also immunoblotted (IB) for exosome marker proteins as indicated (bottom).

⁽E) ACM incubated with biotinylated antibodies against Cd81, Igsf8, Ptgfrn, or control (Ctrl) IgG was immunodepleted using Streptavidin beads, and induction of motility was measured as in Figure 1C (top; n = 31 cells per group; **p < 0.001 and ***p < 0.0001). Media were also IB for Cd81, Igsf8, and Ptgfrn (bottom). See also Figure S5 and Tables S1 and S2.

Figure 6. Cd81-Positive Exosomes Stimulate BCC Motility and Metastasis

(A) Conditioned media from L cells transfected with shRNAs targeting Cd81 (shCd81#1 or shCd81#2) or a scramble sequence (shScr) were tested for induction of MDA-MB-231 cell motility quantified as in Figure 1C (top; n = 32 cells per group; **p < 0.001 and ***p < 0.0001). Total cell lysate was subjected to immunoblotting (IB) for Cd81 and actin (bottom).

(B and C) The lungs of mice harboring the indicated tumors (B; top schematic) were stained as in Figure 1F, and representative images are shown (B; bottom. Scale bars, 35μ m). Metastatic colonies were quantified (C; mean colony number \pm SEM, n = 4–7 mice per group; p = 0.046, using two-tailed Mann Whitney test). (D) The tumor volume in mice described in (B) and (C) was measured at the indicated time points and plotted as the mean volume \pm SEM.

(E and F) Conditioned media from CAFs isolated from two different patients (CAF1 and CAF2) were subjected to ultracentrifugation to isolate exosomes (100,000 \times g pellet in E; CAF1Pellet and CAF2Pellet in F), IB for Cd81, Igsf8, and Flotillin 1 (E), and tested for activity (F) in comparison to 100,000 \times g pellet from control media (F; DMEMPellet). Cell speed was quantified and is plotted as box and whisker plots (n = 33 cells per group). ***p < 0.0001.

(G) MDA-MB-231 cells transfected with Pk1 or a scramble sequence (Scr) were treated with exosomes (p, 100,000 \times g pellet) as in (F). Cell speed was quantified and plotted as box and whisker plots (n = 40 cells per group). ***p < 0.0001.

See also Figure S6.

Figure 7. Fibroblast-Secreted Exosomes Mobilize Wnt11 Expressed in BCCs

(A) Conditioned media from MDA-MB-231 (MCM) or L cells (ACM) cultured in DMEM for 1–3 days were subjected to differential ultracentrifugation to isolate exosomes (top) followed by IB for the indicated exosome markers (bottom; asterisk (*) indicates lane contained molecular weight markers).

(B) MDA-MB-231 cells were treated with CM from L cells stably expressing Cd81-EYFP (Cd81-EYFP_CM) or empty vector (pLVX_CM) for 20 hr (top). Total CM from L cells (bottom left) or anti-GFP immunoprecipitates from treated MDA-MB-231 cells (bottom right) were IB with an anti-GFP antibody as indicated.

(C) Confocal images of MDA-MB-231 cells treated for 16 hr with CM produced from L cells described in (B) are shown. Cd81-EYFP protein was detected with an anti-GFP antibody (green), and endogenous Wnt11 (red) was detected as in Figure 3C. Scale bars, 10 μ m.

(D) MDA-MB-231 cells stably expressing an empty vector or Wnt11-HA were incubated with DMEM (D) or ACM (A) for 20 hr to produce BCC-conditioned media (top, indicated in yellow), which was subjected to differential ultracentrifugation and IB for HA and Cd81 (bottom).

Exosome Isolation, Imaging, and Immunodepletion

Exosomes were isolated as previously described by Théry et al. (2006) by sequential ultracentrifugation at 2,000 × *g* for 30 min, 10,000 × *g* for 40 min, and 100,000 × *g* for 2–14 hr, washed once with PBS, and purified by centrifugation at 100,000 × *g* for 2 hr. Exosomes were imaged by whole-mount EM as detailed in the Extended Experimental Procedures. For immunodepletion, ACM was incubated with biotinylated Cd81, Igsf8, or Ptgfrn antibodies, or isotype IgG control (described in the Extended Experimental Procedures) for 20 hr at 4°C, immune complexes captured by Streptavidin T1 beads (Invitrogen), and the resultant media supernatant was filter sterilized and tested in single-cell motility assays.

Immunoprecipitation and Immunoblotting

Protein was extracted with TNTE lysis buffer. Total protein lysates were separated by SDS-PAGE, transferred onto nitrocellulose membrane, probed with primary antibodies and HRP-linked secondary antibodies as detailed in the Extended Experimental Procedures, and detected with SuperSignal reagent (Thermo Scientific).

Statistical Analysis

Statistical significance was calculated using Prism (GraphPad Software). The cell motility data were analyzed using two-tailed unpaired t test. The incidence and size of metastatic foci were analyzed using two-tailed unpaired t test with Welch's corrections or two-tailed Mann Whitney test as indicated. Values of p < 0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, four tables, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.11.024.

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(E) EM images of exosomes isolated from ACM conditioned by Wnt11-HA-expressing MDA-MB-231 cells (as described in D) and immunolabeled using a Wnt11 antibody followed by a gold-conjugated secondary antibody.

(F) Empty vector (pCAGIP)- or Wnt11-HA-expressing MDA-MB-231 cells were incubated with DMEM or ACM as described in (D) to produce Wnt11-HA_DMEM1 or Wnt11-HA_ACM1-conditioned media (yellow in schematic). These media were then mixed with an equal volume of original ACM (ACM2) or DMEM (DMEM2) to produce Wnt11-HA_DMEM1+ACM2 and Wnt11-HA_ACM1+DMEM2, respectively (orange in schematic). Exosomes were then purified by ultracentrifugation and IB as in (D) (bottom).

(G) Cd81-positive exosomes from conditioned media prepared as in (F) (orange in schematic) were immunoprecipitated using an anti-Cd81 antibody and IB as in (D) (bottom).

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