Wnt5a induces ROR1 to recruit cortactin to promote breast-cancer migration and metastasis

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INTRODUCTION

ROR1 is a conserved oncoembryonic surface protein expressed in breast cancer. Here we report that ROR1 associates with cortactin in primary breast-cancer cells or in MCF7 transfected to express ROR1. Wnt5a also induced ROR1-dependent tyrosine phosphorylation of cortactin (Y421), which recruited ARHGEF1 to activate RhoA and promote breast-cancer-cell migration; such effects could be inhibited by cirmtuzumab, a humanized mAb specific for ROR1. Furthermore, treatment of mice bearing breast-cancer xenograft with cirmtuzumab inhibited cortactin phosphorylation in vivo and impaired metastatic development. We established that the proline at B41 of ROR1 was required for it to recruit cortactin and ARHGEF1, activate RhoA, and enhance breast-cancer-cell migration in vitro or development of metastases in vivo. Collectively, these studies demonstrate that the interaction of ROR1 with cortactin plays an important role in breast-cancer-cell migration and metastasis.

RESULTS

Wnt5a induces ROR1 to complex with cortactin in breast-cancer PDX

We examined eight primary breast-cancer patient-derived xenografts (PDXs) (Supplementary Fig. 1). Table 1 provides the diagnosis, histologic grade, patient’s age and race, and tumor-cell expression of estrogen receptors (ER), progesterone receptors (PR), HER2, ROR1, cortactin, or Wnt5a (see also Supplementary Fig. 1). Only one PDX (PDX1) was HER2+, one (PDX5) was ER+, and six (PDX3–8) expressed high-level ROR1. All PDX were histologic grade 3 and expressed cortactin and Wnt5a. None of the PDX were PR+.

Mass spectrometry (MS) analyses of anti-ROR1 mAb immune precipitates (i.p.) from lysates of ROR1-expressing breast-cancer PDX revealed cortactin in addition to ROR1 (Fig. 1a). Immunoblot analyses of anti-ROR1 or anti-cortactin immune precipitates confirmed that cortactin complexed with ROR1 in breast-cancer PDX cells (Fig. 1b–e). However, we did not detect such ROR1–cortactin complexes in lysates prepared from breast-cancer–PDX cells cultured overnight in serum-free media unless they were treated with exogenous Wnt5a (Fig. 1f, g), suggesting...
MCF7 cells were generated from breast ductal carcinoma cells and level of phosphorylated cortactin within 5 min (Fig. 2a, b). Because breast-cancer PDX cells with exogenous Wnt5a could enhance the levels of Y421-phosphorylated cortactin over time (Supplementary Fig. 1). Culture of breast-cancer cells of some patients,23,31 and that high levels of ROR1, and that such effects could also be blocked by treatment with cirmtuzumab (Fig. 2g).

Wnt5a stimulates ROR1-dependent cortactin phosphorylation and enhances the migration of breast-cancer PDX cells

Previous studies found that cortactin may be phosphorylated in breast-cancer cells of some patients,23,31 and that high levels of phosphorylated cortactin associated with enhanced cancer-cell migration, metastasis, and adverse prognosis.18,20,33 We found that treatment of breast-cancer PDX with cirmtuzumab blocked Wnt5a from inducing ROR1 to complex with cortactin (Fig. 1h, i).

Wnt5a stimulates ROR1-dependent cortactin phosphorylation and increases the migration capacity of MCF7–ROR1 cells

We found that Wnt5a stimulates ROR1-dependent cortactin phosphorylation and increases the migration capacity of MCF7–ROR1 cells. We treated the breast-cancer PDX cells with cirmtuzumab, a humanized mAb specific for a functional epitope of ROR1 that is distinct from the epitope recognized by the anti-ROR1–mAb 4A5, which we used to generate the anti-ROR1 i.p. We found that treatment of the breast-cancer PDX with cirmtuzumab blocked Wnt5a from inducing ROR1 to complex with cortactin (Fig. 1h, i).

Wnt5a stimulates ROR1/cortactin to complex with ARHGEF1 and activates RhoA

We found that treatment with Wnt5a also enhanced the migration of MCF7–ROR1 cells (Supplementary Fig. 7a, c). We selected the concentration of Wnt5a, 200 ng/ml (Fig. 2c, d), indicating that the Wnt5a-induced phosphorylation of cortactin was dependent on ROR1. We also found that cirmtuzumab could also inhibit Y421 phosphorylation of cortactin in secondary tumors generated from primary breast-cancer PDX cells in vivo (Fig. 2e, f).

Other studies demonstrated that Wnt5a could enhance migration of breast-cancer cells.15,14 In this study, we found that Wnt5a enhances the migration of serum-starved breast-cancer PDX cells that expressed ROR1, and that such effects could also be blocked by cirmtuzumab (Fig. 2g).

Table 1. Data on breast-cancer patient-derived xenograft (PDX), including tumor diagnosis, grade, sex, age, race, and expression information of ER (estrogen receptor), PR (progesterone receptor), HER2, ROR1, cortactin, or Wnt5a. Here, “yes” means “expressed,” and “no” means “not expressed.”
by using lysates made from ROR1\(^+\) breast-cancer PDX cells revealed that ARHGFE1 associated with cortactin (Supplementary Fig. 9a, b), and more specifically Y421-phosphorylated cortactin (Supplementary Fig. 9c). We noted that the ARHGFE1 i.p. generated from lysates of breast-cancer PDX treated with cortactin siRNA had less capacity to generate activated RhoA than the ARHGFE1 i.p. of PDX treated with control, nonspecific siRNA (Supplementary Fig. 9d). Moreover, Wnt5a induced less RhoA–GTP in MCF7–ROR1 cells treated with cortactin-specific siRNA than in MCF7–ROR1 cells treated with nonspecific control siRNA (si-Ctrl) (Supplementary Fig. 9e). Furthermore, Wnt5a-induced activation of RhoA could be blocked by cirmtuzumab in PDX cells.
Fig. 1 Association of ROR1 with cortactin in breast-cancer patient-derived xenografts (PDXs). a Cortactin peptide identified by 2D-nanoLC–MS/MS in anti-ROR1 (cirtuzumab) immune precipitates (i.p.) of lysates of PDX4 (representative of two PDXs). b Immunoblot of i.p. by using anti-ROR1 mAb or control IgG (Ctrl-IgG), as indicated on the top, by using lysates of PDX3 (representative of three PDXs). The bottom panel is an immunoblot of the whole-cell lysate (WCL) probed with anti-cortactin mAb. c Black columns indicate the mean relative interaction of cortactin with ROR1 (error bars indicate S.D.) for PDX3, PDX4, and PDX5. d Immunoblot of i.p. by using anti-ROR1 mAb or Ctrl-IgG, as indicated on the top, by using lysates of PDX4 (representative of three PDXs). The bottom panel is an immunoblot of the WCL probed with anti-cortactin mAb. e Black columns indicate the mean relative interaction of cortactin with ROR1 (±S.D.) for PDX3, PDX4, and PDX5. f Immunoblot of anti-ROR1 i.p. from lysates of serum-starved PDX5 cells (representative of three PDXs) that subsequently were treated for 30 min without (−) or with (+) Wnt5a (100 ng/ml), as indicated on the top. The bottom panel is an immunoblot of the WCL probed with anti-cortactin mAb. g Columns indicate the mean relative interaction of cortactin with ROR1 (±S.D.) for PDX3, PDX4, and PDX5 that had been treated with Ctrl-IgG or cirtuzumab, without (−) or with (+) Wnt5a, as in 1f and indicated below (P < 0.01, two-tailed Student’s t test).

( Supplementary Fig. 9f, g). We also noted that exogenous Wnt5a could induce activation of RhoA in MDA-MB-231 cells, which prior studies found expressed ROR1, but not in MDA-MB-231 cells silenced for ROR1 with ROR1-specific siRNA (Supplementary Fig. 10).

P841 is required for ROR1 to bind and activate cortactin/ARHGEF1. Cortactin contains a SH3 domain, which can bind to –P—X—P—X—P—X—X—P—X—X—P— sites that typically are found within the PRDs of other proteins, including ROR1. We previously described that PRD or proline at 841 of ROR1 was required for ROR1/cortactin association and phosphorylation of cortactin in CLL cells. Here, we examined whether the ROR1–PRD was necessary for ROR1 to complex with cortactin in breast-cancer cells. Accordingly, we transfected MCF7 cells with an expression vector driving expression of either wild-type ROR1 or a truncated ROR1 housing a deletion of the entire PRD (ΔPRD–ROR1) (Fig. 3a, b; Supplementary Fig. 4). In contrast to the anti-ROR1 i.p. from lysates of MCF7–ΔPRD–ROR1 cells, the anti-ROR1 i.p. from lysates of MCF7–ΔPRD–ROR1 cells did not have detectable cortactin (Fig. 3d).

Accordingly, we analyzed various mutants of ROR1 that each had a substitution of alanine (A) for proline (P) at position 784, 808, 826, or 841 in one of the putative SH3-binding sites within the ROR1–PRD (Fig. 3c; Supplementary Fig. 4). Comparable amounts of ROR1 were expressed by MCF7 cells transfected to express the wild-type (WT) or any one of the ROR1 mutants (Fig. 3e; Supplementary Fig. 4). Following treatment with Wnt5a, ROR1 with a P5784A substitution at 784, 808, or 826 each could complex with cortactin and recruit ARHGEF1 as effectively as the WT/ROR1 (Fig. 3e). However, the mutant with a P784A substitution at 841 of ROR1 (P841A) did not associate with cortactin (Fig. 3e).

We examined whether Wnt5a could induce phosphorylation of RhoA in MCF7 cells transfected with W/T ROR1 or any one of the ROR1 mutants. Each of the various transfected MCF7 cell lines expressed levels of cortactin that were comparable to that of the MCF7 parental cell line (Fig. 3f). We observed that Wnt5a induced phosphorylation of cortactin and activation of RhoA, and enhanced the motility of MCF7 cells expressing ROR1 with a P784A substitution at 784, 808, or 826 as effectively as MCF7–ROR1 cells expressing W/T ROR1 (Fig. 3f, g, h). However, Wnt5a did not induce such effects with MCF7–ΔPRD–ROR1 cells, MCF7–ROR1(P841A) cells, or MCF7 cells, which lacked ROR1 altogether (Fig. 3f, g, h).

P841 is required for ROR1 to enhance MCF7-metastatic development, which can be inhibited by cirtuzumab. Previous studies demonstrated that ROR1+ breast-cancer cells had a greater capacity to metastasize than breast-cancer cells silenced for ROR1. Consistent with these findings, we found that the numbers of metastatic foci detected in the lungs of mice 1 (Fig. 4a, b) or 3 weeks (Fig. 4c, d) following intravenous injection of MCF7–ROR1 cells were significantly greater than those detected in the lungs of mice injected with equal numbers of MCF7 cells. On the other hand, the numbers of metastatic foci detected in mice injected with MCF7–ROR1(P841A) cells were significantly less than those found in mice injected with MCF7–ROR1 cells (Fig. 4a–d), but comparable to the numbers of metastatic foci detected in mice injected with MCF7 cells, or in mice injected with MCF7–ROR1 cells treated at days-0 and -14 with cirtuzumab (10 mg/kg) (Fig. 4e–h).

DISCUSSION

In the present study, we found that Wnt5a induces ROR1 to associate with cortactin, which undergoes tyrosine phosphorylation in breast-cancer PDX cells or MCF7 cells transfected to express ROR1. Moreover, in response to Wnt5a, ROR1/cortactin complexed and stimulated ARHGEF1, which induced activation of RhoA and enhanced cell migration. Silencing expression of cortactin with cortactin-directed siRNA inhibited Wnt5a-enhanced cell migration. Collectively, these studies demonstrate that cortactin plays a critical role in ROR1-dependent, non-canonical Wnt5a signaling, leading to increased tumor-cell migration and metastasis.

The ROR1–PRD was essential for ROR1 to complex with cortactin and increase breast-cancer-cell migration in response to Wnt5a. Cortactin houses a SH3 domain, permitting it to associate with other proteins that have suitable PRD or proline motifs, which typically reside with the PRD. We found that the proline residue at position 841 of ROR1 was indispensable for it to recruit cortactin. Moreover, in contrast to W/T ROR1 or ROR1 with proline-to-alanine substitutions at other sites, the mutant ROR1 with an alanine instead of a proline at position 841 (ROR1(P841A)) was unable to complex with cortactin, enhance cortactin phosphorylation, recruit ARHGEF1, activate RhoA, or enhance cell migration/metastasis of MCF7 cells. Thus, this residue plays an important role for ROR1 to bind and phosphorylate cortactin, which appears necessary to enhance migration and metastasis of MCF7–ROR1 cells. This contrasts with our findings to CLL cells, which also express another cytoskeletal protein named HS1. HS1 also can complex with ROR1 at proline 841 to enhance chemokine-directed migration of CLL cells in response to Wnt5a. HS1 is not expressed in cancers derived from non-hematopoietic cells, such as breast cancer. Instead, we find that the ROR1–cortactin interaction is critical for enhanced migration and metastasis of breast-cancer cells independent of expression of HS1.

Primary breast cancers that express relatively high levels of phosphorylated cortactin have a greater capacity for migration/metastasis and are associated with a less favorable prognosis than breast cancers with low-to-negligible levels of phosphorylated
Factors other than Wnt5a, such as activation of Src-family kinases, may also induce cortactin phosphorylation in breast-cancer cells. However, our findings indicate that phosphorylation of cortactin may also be in response to Wnt5a, which is generally present at high levels in breast carcinomas relative to that noted in normal breast tissues. Consistent with this notion, we found that the level of phosphorylated cortactin rapidly attenuated in breast-cancer PDX cultured in serum-free conditions. This suggests that Wnt5a-mediated cortactin phosphorylation may play a role in breast cancer cell migration.

Fig. 2 Wnt5a induces ROR1-dependent phosphorylation of cortactin and enhances migration of breast-cancer PDX cells. a) Immunoblot analysis of lysates prepared from serum-starved PDX5 (representative of three PDXs) that subsequently were treated with Wnt5a (100 ng/ml) for the times indicated above (in minutes). b) Columns indicate the mean relative tyrosine phosphorylation of cortactin at Y421 (pCortactin) (±S.D.) for PDX4, PDX5, and PDX6 treated for 0, 1, or 5 min with Wnt5a (P < 0.05, two-tailed Student’s t test). c) Immunoblot analysis of lysates prepared from serum-starved PDX5 (representative of three PDXs) that subsequently were treated with Ctrl-IgG or cirmtuzumab (10 μg/ml), without (−) or with (+) Wnt5a, as indicated above. d) Columns indicate the mean relative pCortactin (±S.D.) for PDX4, PDX5, and PDX6 cells treated with Ctrl-IgG or cirmtuzumab for 2 h, and subsequently treated for 5 min without (−) or with (+) Wnt5a, as indicated below (P < 0.01, Student’s t test). e) Immunoblot analysis of lysates of PDX4 harvested from mice treated with Ctrl-IgG or cirmtuzumab (10 mg/kg), as indicated on top, and probed for pCortactin or Cortactin, as indicated on the left. f) Columns indicate the mean relative pCortactin (±S.D.) for PDX3, PDX4, and PDX5 (P < 0.01, two-tailed Student’s t test). g) Columns indicate the mean cell migration at 10 h (±S.D.) in the absence (−) or presence (+) of exogenous Wnt5a (200 ng/ml) for serum-starved PDX4, PDX5, and PDX6 that were treated with Ctrl-IgG or cirmtuzumab (10 μg/ml). Data are from three independent experiments (P < 0.05; P < 0.01, Student’s t test).
media unless we added exogenous Wnt5a. We find that Wnt5a induces phosphorylation of cortactin, which may be dependent on the activity of Src.37,38 In any case, our results reveal that the association of cortactin phosphorylation with metastasis may reflect differences in Wnt5a-induced ROR1-dependent signaling. Consistent with this notion are studies demonstrating that high-level expression of ROR1 in breast cancer is associated with increased rates of metastases and poorer survival.8,9

We previously found that Wnt5a could induce ROR1 to recruit and activate ARHGEF1, leading to activation of RhoA in leukemia cells.43 However, the mechanism for this was unclear, as ARHGEF1 does not contain an SH3-binding domain. Here we demonstrate that association of ARHGEF1 to ROR1 appears mediated by cortactin. In this light, cortactin may be considered an adaptor protein for ROR1, functioning to enhance cell migration and/or metastasis by recruiting and activating ARHGEF1 to ROR1 to provide localized activation of RhoA.44 Consistent with this model
is the observation that breast-cancer cells with reduced levels of cortactin following treatment with cortactin-specific siRNA had reduced chemotaxis in response to Wnt5a, implicating that cortactin plays a critical role in breast-cancer-cell migration/metastasis.

One study found that Wnt5a could also enhance macrophage-induced invasiveness of MCF7 cells in vitro. Furthermore, another study found that forced expression of Wnt5a enhanced activation of RhoA in 21PT and 21NT breast-cancer-cell lines, but only increased cellular motility in 21NT cells. As noted in the present study, MCF7 cells lack expression of ROR1, suggesting that some of the effects of Wnt5a on cell invasiveness may be independent of ROR1. In any case, our study indicates that Wnt5a

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Fig. 4 Proline at 841 of ROR1 is critical for enhancing development of metastatic foci of MCF7–ROR1. a HE staining of lung tissue from a representative tumor-bearing mouse engrafted with MCF7 or MCF7-expressing ROR1 (MCF7–ROR1) or the mutant form of ROR1, ROR1P841A (MCF7–P841A) at 1 week after intravenous (i.v.) tail-vein injections of equal numbers of viable cells. Green arrows indicate metastatic foci (objective: 40×). b Each symbol represents the number of metastatic foci that were found in the lungs of each animal in the groups indicated below (mean ± S.D., n = 5). P < 0.01; P < 0.05, two-tailed Student’s t test. c HE staining of lung tissue of mice injected as in (a), but 3 weeks after i.v. injection of equal numbers of viable cells, as indicated on top. Green arrows indicate metastatic foci (objective: 40×). d Each symbol represents the number of metastatic foci found in the lungs of each mouse per group (mean ± S.D., n = 5) (P < 0.05, two-tailed Student’s t test). e HE staining of lung tissue from a representative tumor-bearing mouse injected i.v. with MCF7–ROR1 cells 1 week earlier and treated with nonspecific human IgG (IgG) or cirmtuzumab (10 mg/kg), as indicated on top. Green arrows indicate metastatic foci (objective: 40×). f Each symbol represents the number of metastatic foci that were found in the lungs of each animal in each group (mean ± S.D., n = 5) (P < 0.01, two-tailed Student’s t test). g HE staining of lung tissue from a representative tumor-bearing mouse injected i.v. with MCF7–ROR1 cells 3 weeks earlier and treated with nonspecific human IgG (IgG) or cirmtuzumab (10 mg/kg), as indicated on top. Green arrows indicate metastatic foci (objective: 40×). h Each symbol represents the number of metastatic foci that were found in the lungs of each animal in each group (mean ± S.D., n = 5) (P < 0.01, two-tailed Student’s t test).
induces ROR1-dependent cortactin phosphorylation, which prior studies found could associate with the Arp2/3 complex to facilitate cytoskeletal reorganization, stabilized branched actin networks, and lamellipodia formation to enhance cellular motility.\textsuperscript{25,47,48}

In conclusion, the present study demonstrates a previously unrecognized ROR1/cortactin/ARHGEF1-dependent pathway leading to activation of RhoA in response to Wnt5a in breast-cancer cells. The reported findings highlight a pathway for potential drug development targeting ROR1-dependent Wnt5a-induced signaling. In this regard, we found that cirtuzumab could block the capacity of Wnt5a to induce ROR1 to complex and phosphorylate cortactin, recruit ARHGEF1, and activate RhoA, thereby suppressing breast-cancer-cell migration/metastasis. It should be noted that in these studies, cirtuzumab was injected on the same day on which mice were challenged with cancer cells injected intravenously to study the effect of blocking ROR1 signaling on circulating breast-cancer cells. Other studies have demonstrated that treatment with cirtuzumab may mitigate the risk for relapse and metastasis of breast-cancer PDX treated with paclitaxel.\textsuperscript{49} Clinical studies are underway to examine the safety and activity of cirtuzumab used in combination with paclitaxel for treatment of patients with advanced breast cancer (https://clinicaltrials.gov/ct2/show/ NCT02776917). Such studies will be required to determine whether cirtuzumab may have activity in patients with established metastases. In any case, this study provides added rationale for the clinical evaluation of this humanized anti-ROR1 mAb in the treatment of patients with breast cancer or other ROR1-expressing cancers.\textsuperscript{26,51}

\textbf{METHODS}

\textbf{Cell culture}

MCF7, MDA-MB-231 cells (purchased from ATCC), or MCF7 cells transfected with different ROR1 constructs, were cultured in DMEM medium with 10% FBS, 1% penicillin/streptomycin, maintained at 37°C in a humidified atmosphere of 5% CO\textsubscript{2} and tested negative for mycoplasma contamination. Media and supplements were purchased from Life Technologies (Carlsbad, CA, USA).

For serum starvation of breast-cancer PDX cells, freshly isolated PDX cells were cultured in mammary-epithelial basal medium without growth factors, purchased from Lonza.

\textbf{Immunoprecipitation analysis}

Immunoprecipitation analysis was performed as described.\textsuperscript{52} Cells were lysed in a buffer containing 1% Nonidet P-40, 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM EDTA with protease inhibitors (Roche). The lysates were cleared by centrifugation at 16,000 x g for 15 min. Immune precipitates were isolated using protein A agarose beads, followed by immunoblot or MS analysis. Antibodies for immune precipitation were as follows: ROR1 (Cell Signaling Technology, Danvers, MA, USA; dilution 1:1000, catalog#2109), or ROR1 (Cell Signaling; dilution 1:1000, catalog#4102), which were detected with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling). The integrated optical density (IOD) of bands was evaluated by densitometry and analyzed by using Gel-Pro Analyzer software (Media Cybernetics). All the samples for the same experiment were prepared at the same time, and blots were processed in parallel.

\textbf{Nucleofection of plasmids and siRNA}

Cell line Nucleofector Kit for siRNA or plasmid transfection was purchased from Lonza. Cells (2 x 10\textsuperscript{5}) were suspended in 100 μl of Nucleofector Solution with plasmids (pcDNA3.1 vector expressing human ROR1) or siRNA (GE Dharmacon, Lafayette, CO) and transfected with the Nucleofector II device (program P-020). The transfected cells were cultured in six-well plates in complete medium for 48 (plasmids) or 72 h (siRNA) and then subjected to immunoblot analysis and assays. Endofree Plasmid Maxi Kits (QIAGEN) were used to purify plasmids for transfection. G418 (800 μg/ ml) was used for selection of stable MCF7 transfectants, which were then examined via flow cytometry or western blot.

\textbf{MS analysis}

MS analysis was performed as described previously.\textsuperscript{52} Briefly, bound proteins were digested by trypsin (Roche) directly on beads for the MS analysis. Digested peptides were separated by online 2D-nanoLC and detected by LTQ linear ion-trap mass spectrometers. Each sample took 22.5 h to analyze, and about 200,000 MS/MS spectra were collected for each run. Raw data were extracted and searched by using Spectrum Mill (Agilent, v3.03) database search software against the NCBI ref seq database limited to human taxonomy (version 44).

\textbf{RhoGEF nucleotide exchange activity assay}

RhoGEF exchange assay kit was purchased from Cytoseekten and used as per the manufacturer’s instructions. For in vitro guanine nucleotide exchange activity on RhoA, ARHGEF1 was immunoprecipitated from MCF7-ROR1 cells that previously transfected with si-Ctrl or si-Cortactin. Reactions were measured in a Teco Spectrofluor plus fluorimeter (λ\textsubscript{ex} = 360 nm, λ\textsubscript{em} = 460 nm). Pull-down ARHGEF1 was added after 120 s. Readings were taken at 30°C every 1 min for a total reaction time of 44 min. The exchange curve can be achieved by exporting raw data to Microsoft Excel and analyzing the data by using GraphPad Prism 6.0.

\textbf{RhoA activation assay}

RhoA activation assay reagents were purchased from Cytoskeleton and used as per the manufacturer’s instructions. Briefly, GTP-bound active RhoA was pulled down with Rhotekin-RBD beads for 1 h at 4°C, and then subjected to immunoblot analysis. Immunoblots of whole-cell lysates were used to assess for total RhoA. The IOD of bands was evaluated by densitometry and analyzed by using Gel-Pro Analyzer software (Media Cybernetics).

\textbf{Flow cytometry analysis}

Flow cytometry analysis was performed as described.\textsuperscript{53} Anti-ROR1 mAb (4A5) conjugated with Alexa Fluor 647 (4A5–Alexa Fluor 647) generated in our laboratory was used to stain cells at 4°C for 20 min. The stained cells were washed twice with FACS buffer (PBS, pH 7.4, 3% FBS) and examined by 4-color, multiparameter flow cytometry by using a dual-laser FACSCalibur (BD Biosciences). Data were analyzed by using FlowJo software (TreeStar).

\textbf{Site-specific mutation}

Site-specific mutations were performed as described previously.\textsuperscript{53} In brief, mutation constructs were generated with QuikChange Site-Directed Mutagenesis System (Invitrogen) on the basis of the parental construct (wild-type ROR1), according to the manufacturer’s instructions. The mutations for each construct were verified by DNA sequencing. The following primer sets were used:

P(784A), 5′-CAGTGAATTCGTAACGCGGCGATCT-3′ (sense) and 5′-C TCTGTAATTTGGCATATGCCTGCAATGC-3′ (antisense); P(808A), 5′-GATTGGTCTTTTGTGATGCCTGCAATGC-3′ (sense) and 5′-G TCTGGATATTCGCGGGCGGCGCAATGATTG-3′ (antisense); P(826A), 5′-CAATTGGATACACCATCCTGCTAGATCGACG-3′ (antisense) and 5′-GAGGACGCTGATATCACCGAGATTG-3′ (antisense);

Published in partnership with the Breast Cancer Research Foundation
Cell migration assay

The cell migration assay was performed as described. Briefly, cells were collected by treatment with trypsin/EDTA solution, washed twice with serum-free medium, centrifuged, resuspended in medium containing 0.1% bovine serum albumin, and then placed in media at a concentration of 2.5 × 10³/mL. Each cell suspension was placed onto separate top chambers of a transwell culture polycarbonate insert with 6.5-mm diameter and 8 μm of pore size (Corning). We added Wnt5a at 200 ng/ml in the lower compartment of the chamber. After incubation at 37°C for 10 h, wells were washed with PBS and fixed with 4% paraformaldehyde. The cells on the apical side of each insert were removed by scraping. Cells that migrated through the pores to the lower chamber were stained with Diff-Quick staining kits (IMB Inc., San Marcos, CA). Stained cells were analyzed by counting under the Nikon inverted microscope.

Animal and PDX models

In total, 4- to 6-week-old female Rag2−/−,γc−/− mice were used in this study, following the care and use of laboratory animal guidelines of the National Institutes of Health (NIH). The mice were housed in laminar-flow cabinets under specific pathogen-free conditions and fed ad libitum. The PDX models were established by using mechanically minced fresh breast-cancer specimens. Early passages of primary-tumor tissues from these PDX models were mechanically minced, and enzymatically and mechanically dissociated by using GentleMACS Dissociator (Miltenyi Biotec) in accordance with the manufacturer’s protocol. Dead cells and erythrocytes were removed through density-gradient centrifugation by using Percoll Plus (CC-17-5442-01; GE Healthcare Life Sciences) following fixation in formalin for pathology review, as described in the protocol.

To test the effects of cirmtuzumab on the tyrosine phosphorylation of cortactin (Y421) in the engraftment of primary breast tumors, 1 × 10⁴ single cells isolated from PDX tumors were suspended in mammary growth medium, mixed with Matrigel (BD Biosciences, San Diego, CA) at 1:1 ratio, and injected into the mammary pad of 4- to 6-week-old Rag2−/−,γc−/− mice. When tumor size reached 200 mm³, 10 mg/kg of cirmtuzumab or Ctrl-IgG was injected intravenously biweekly for 1 month. PDX tumors were considered significant. GraphPad Prism 6.0 (GraphPad Software Inc.) was used to perform analysis for significance.

Study approval

Primary breast-tumor specimens were collected from patients, who provided written informed consent on a protocol approved by the Institutional Review Board of the University of California, San Diego (approval number 090401), in accordance with the Declaration of Helsinki. The animal study protocol was approved by the University of California San Diego Institutional Animal Care and Use Committee (approval number S03037).

Data availability

The data generated and analyzed during this study are described in the following data record: https://doi.org/10.6084/m9.flgshe.9874683. Datasets supporting the figures and tables in this published article are not publicly available to protect patient privacy, but can be accessed from the corresponding author on request, upon the completion of a Data Usage Agreement, as described in the data record above. Raw breast-cancer PDX-derived mass spectrometry data are publicly available in the Japan Proteome Standard Repository (iPost repository) under the accession ID: JPTS000678. All the uncropped western blots generated during this study are available in Supplementary Fig. 11.

Received: 28 January 2019; Accepted: 20 September 2019; Published online: 25 October 2019

REFERENCES

1. Maslakowski, P. & Carroll, R. D. A novel family of cell surface receptors with tyrosine kinase-like domain. J. Biol. Chem. 267, 26181–26190 (1992).
2. Wilson, C., Goberdhan, D. C. & Steller, H. Drosophila cortactin is a potential receptor tyrosine kinase. Proc. Natl Acad. Sci. USA 90, 7109–7113 (1993).
3. Forrestor, W. C., Dell, M., Perens, E. & Garriga, G. A. C. elegans Ror receptor tyrosine kinase regulates cell motility and asymmetric cell division. Nature 400, 881–885 (1999).
4. Rodriguez-Niedenfuhr, M., Proks, F. & Christ, B. Expression and regulation of ROR-1 during early avian limb development. Anat. Embryol. 207, 495–502 (2004).
5. Broome, H. E., Rassenti, L. Z., Wang, H. Y., Meyer, L. M. & Kipps, T. J. ROR1 is expressed on hematogones (non-neoplastic human B-lymphocyte precursors) and a minority of precursor-B acute lymphoblastic leukemia. Leuk. Res. 35, 1390–1394 (2011).

6. Fukuda, T. et al. Antisera induced by infusions of autologous Ad-CD154-leukemia B cells identify ROR1 as an oncofetal antigen and receptor for Wnt5a. Proc. Natl Acad. Sci. USA 105, 3047–3052 (2008).
7. Zhang, S. et al. The onco-embryonic antigen ROR1 is expressed by a variety of human cancers. Am. J. Pathol. 181, 1903–1910 (2012).
8. Zhang, S. et al. ROR1 is expressed in human breast cancer and associated with enhanced tumor-cell growth. PLoS ONE 7, e31127 (2012).
9. Cui, B. et al. Targeting ROR1 inhibits epithelial-mesenchymal transition and metastasis. Cancer Res. 73, 3649–3660 (2013).
10. Zhang, H. et al. ROR1 expression correlated with poor clinical outcome in human ovarian cancer. Sci. Rep. 4, 5811 (2014).
11. Cui, B. et al. High-level ROR1 associates with accelerated disease progression in chronic lymphocytic leukemia. Blood 128, 2931–2940 (2016).
12. Zheng, Y. Z. et al. ROR1 is a novel prognostic biomarker in patients with lung adenocarcinoma. Sci. Rep. 6, 34447 (2016).
13. Zhu, Y. et al. Rab35 is required for Wnt5a/Dvl2-induced Rac1 activation and cell migration in MCF-7 breast cancer cells. Cell Signal 25, 1075–1085 (2013).
14. Zhu, Y. et al. Dvl2-dependent activation of Dama1 and Rhoa regulates Wnt5a-induced breast cancer cell migration. PLoS ONE 7, e37823 (2012).
15. Hasan, M. K., Rasenti, L., Wadhop, G. F., 2nd, Yu J. & Kipps, T. J. Wnt5a causes ROR1 to complex and activate cortactin to enhance migration of chronic lymphocytic leukemia cells. Leukemia. https://doi.org/10.1038/jn1375-018-0306-7 (2018).
16. Hasan, M. K. et al. Wnt5a induces ROR1 to complex with H5I to enhance migration of chronic lymphocytic leukemia cells. Leukemia 31, 2615–2622 (2017).
17. Kitamura, D., Kaneko, H., Miyagoe, Y., Ariyasu, T. & Watanabe, T. Isolation and characterization of a novel human gene expressed specifically in the cells of hematopoietic lineage. Nuc. Acid. Res. 17, 9367–9379 (1989).
18. MacGrath, S. M. & Koleske, A. J. Cortactin in cell migration and cancer at a glance. J. Cell Sci. 125, 1621–1626 (2012).
19. Patel, A. S., Schechter, G. L., Wasilenko, W. J. & Somers, K. D. Overexpression of EMS1/cortactin in NIH3T3 fibroblasts causes increased cell motility and invasion in vitro. Oncogene 16, 3227–3232 (1998).
40. Zhang, S. et al. Inhibition of chemotherapy resistant breast cancer stem cells by a ROR1 specific antibody. Proc. Natl Acad. Sci. USA 116, 1370–1377 (2019).
41. Choi, M. Y. et al. Pre-clinical specificity and safety of UC-961, a first-in-class monoclonal antibody targeting ROR1. Clin. Lymphoma Myeloma Leuk. 15(Suppl), S167–S169 (2015).
42. Choi, M. Y. et al. Phase I Trial: cirtuzumab inhibits ror1 signaling and stemness signatures in patients with chronic lymphocytic leukemia. Cell Stem Cell 22, 951–959 (2018), e9503.
43. Widhopf, G. F. 2nd et al. ROR1 can interact with TCL1 and enhance leukemogenesis in Emu-TCL1 transgenic mice. Proc. Natl Acad. Sci. USA 111, 793–798 (2014).
44. Hasman, M. K. et al. ALK is a MYCN target gene and regulates cell migration and invasion in neuroblastoma. Sci. Rep. 3, 3450 (2013).
45. Azmair, N. et al. Daple is a novel non-receptor GEF required for trimeric G protein activation in Wnt signaling. Elife 4, e07091 (2015).
46. Marangoni, E. & Poupon, M. F. Patient-derived tumour xenografts as models for breast cancer drug development. Curr. Opin. Oncol. 26, 556–561 (2014).
47. Whittle, J. R., Lewis, M. T., Lindeman, G. J. & Visvader, J. E. Patient-derived xenograft models of breast cancer and their predictive power. Breast Cancer Res. 17, 1–17 (2015).
48. Dobrolecki, L. E. et al. Patient-derived xenograft (PDx) models in basic and translational breast cancer research. Cancer Metastasis Rev. 35, 547–573 (2016).
49. Shafie, S. M. & Brist, L. A. Formation of metastasis by human breast carcinoma cells (MCF-7) in nude mice. Cancer Lett. 11, 81–87 (1980).
50. Hasman, M. K. W. I. et al. Meta-data supporting data files of the related article: Wnt5a induces ROR1 to recruit cortactin to promote breast cancer migration and metastasis. Figshare. https://doi.org/10.6084/m9.figshare.9874493 (2019).

ACKNOWLEDGEMENTS

We thank Victoria Tripple, Christine Gray, Han Zhang, and Jian Yu for assistance with the analysis. We also thank the Breast Cancer Research Foundation (Grant no. BCRF-17-120) and the Science and Technology Foundation of Shenzhen, China (Shenzhen Peacock Innovation Team Project, Grant no. KQTD2014063010068078) to support the study, and California Institute for Regenerative Medicine (CIRM) (Grant no. DR-0360924) for supporting us in generating anti-ROR1 mAbs and cirtuzumab.

AUTHOR CONTRIBUTIONS

M.K.H. and T.J.K. designed the research and conceived the project. M.K.H., G.F.W., S.Z., and T.J.K. analyzed the data, and wrote the paper. M.K.H. and S.M.L. performed cellular and mice experiments. Z.S. and S.P.B. performed mass spectrometry analysis. B.A.P. contributed to primary breast-cancer samples. M.K.H. and T.J.K. analyzed the data, and wrote the paper.

COMPETING INTERESTS

B.A.P.: Research Funding: Pfizer, Novartis, Glaxo Smith Kline, Genentech/Roche, Breast Cancer Research Foundation, Safeway Foundation to the Athena Breast Health Network, University of California Office of the President to the Athena Breast Health Network, and Patient Center Outcomes Institute (PCORI)—National Institutes of Health. Travel/honoraria past 3 years: NCCN Board Meetings/Annual Meeting, Alliance Clinical Trials Group semiannual meetings, and Athena Breast Health Network semiannual meetings—University of California consortium. Spouse: Bioalta Inc. consulting, EMG Seraona consulting and teaching, and Salk Institute licensing fees for technology involving protein-interaction technology, Merck (incorporated stock): T.J.K. Cirtuzumab was developed by T.J.K. and licensed by the University of California to Oncertal Therapeutics, Inc., which provided stock/options to the university and T.J.K. Research Funding to T.J.K.: Pharmacology/BiabVie, Breast Cancer Research Foundation, Oncertal Therapeutics, Inc., California Institute for Regenerative Medicine (CIRM), R01-CA236361 from the National Cancer Institute/NIH, and Research Agreement—VelosBio, Inc., Celgene. Travel/Honoraria 2017–2019 (T.J.K.): Pharmacology/BiabVie, Genentech/Roche, Janssen, Gilead, National Cancer Institute/NIH, Celgene, Indi Heme Review, University of Nebraska Medical Center/Research to Practice, Society of Hematologic Oncology, Shenzhen Cancer Center, European Research Initiative on CLL (ERIC), Dava Oncology, Patient Power (LLC), Breast Cancer Research Foundation German CLL Study Group (GCLLSG), iW NFL, NCCN CLL/SLL Hairy Cell Leukemia Panel Meeting, TG Therapeutics, Verastem, Bionest Partners, and OncLive. The remaining authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information is available for this paper at https://doi.org/10.1038/s41523-019-0131-9.
