Targeting ROR1 Inhibits Epithelial–Mesenchymal Transition and Metastasis

Bing Cui, Suping Zhang, Liguang Chen, Jianqiang Yu, George F. Widhopf II, Jessie-F. Fecteau, Laura Z. Rassenti, and Thomas J. Kipps

Abstract

Metastasis is responsible for 90% of cancer-related deaths. Strategies are needed that can inhibit the capacity of cancer cells to migrate across the anatomic barriers and colonize distant organs. Here, we show an association between metastasis and expression of a type I receptor tyrosine kinase–like orphan receptor, ROR1, which is expressed during embryogenesis and by various cancers, but not by normal postpartum tissues. We found that expression of ROR1 associates with the epithelial–mesenchymal transition (EMT), which occurs during embryogenesis and cancer metastasis. Breast adenocarcinomas expressing high levels of ROR1 were more likely to have gene expression signatures associated with EMT and had higher rates of relapse and metastasis than breast adenocarcinomas expressing low levels of ROR1. Suppressing expression of ROR1 in metastasis-prone breast cancer cell lines, MDA-MB-231, HS-578T, or BT549, attenuated expression of proteins associated with EMT (e.g., vimentin, SNAIL-1/2, and ZEB1), enhanced expression of E-cadherin, epithelial cytokeratins (e.g., CK-19), and reduced expression of vimentin and EMT marker proteins (e.g., ZO-1), and impaired their migration/invasion capacity in vitro and the metastatic potential of MDA-MB-231 cells in immunodeficient mice. Conversely, transfection of MCF-7 cells to express ROR1 reduced expression of E-cadherin and CK-19, but enhanced the expression of SNAIL-1/2 and vimentin. Treatment of MDA-MB-231 with a monoclonal antibody specific for ROR1 induced downmodulation of vimentin and inhibited cancer cell migration and invasion in vitro and tumor metastasis in vivo. Collectively, this study indicates that ROR1 may regulate EMT and metastasis and that antibodies targeting ROR1 can inhibit cancer progression and metastasis.

Introduction

Metastasis is responsible for 90% of cancer-related deaths (1, 2). This process includes the physical translocation of primary tumor cells across anatomic barriers to distant organs with subsequent colonization (1, 3, 4). Some poor prognostic gene signatures suggest that cells in some primary tumors are predisposed to metastasis (1–3, 5, 6). Recent attention has focused on a cell biologic program called epithelial–mesenchymal transition (EMT), which is thought to factor prominently in tumor self-renewal, progression, and metastasis (1, 4, 7, 8). EMT confers on neoplastic epithelial cells, the traits necessary in tumor self-renewal, progression, and metastasis (9). Through the use of multiple pathways involved in embryonic morphogenesis and wound healing, cancer cells also can concomitantly acquire attributes that enable invasion and metastasis (10). Furthermore, work to define cancer stem cells (CSC), which may account for relapse after therapy, has identified a variety of traits that are associated with one or more subpopulations of CSCs within various tumors (1, 4). Some studies have found that non-CSCs can become CSC-like cells upon acquiring characteristics of cells in EMT (11, 12). As such, cancer cells that undergo EMT may exhibit a CSC phenotype and acquire invasive properties that promote extravasation, angiogenesis, self-renewal, and uncontrolled growth at metastatic sites (3, 4, 13).

Receptor tyrosine kinases (RTK) play critical roles in cell differentiation, proliferation, angiogenesis, and migration (14–16). In this regard, RTK-like orphan receptor 1 (ROR1) is an evolutionarily conserved, type-I membrane protein that is expressed during embryogenesis (14, 17). The ROR1 homolog in C. elegans, Cam-1, is required for cell migration, polarity, and asymmetric cell divisions (18). In mammalian development, ROR1 is expressed by neuroprogenitor cells (NPC), which adopt properties of mesenchymal cells that migrate to the neocortex (19). Suppressed expression of ROR1 in NPCs causes their precocious differentiation into neurons, which have reduced migratory potential (20).

ROR1 is also expressed by a variety of different cancers (21), but not by normal postpartum tissues (22–25). Although ROR1 might have little if any detectable tyrosine kinase activity...
(23, 26), functional data indicate that ROR1 can contribute to noncanonical WNT signaling to promote neoplastic cell survival (22). This is noteworthy, as noncanonical WNT signaling apparently plays a role in breast cancer metastasis (16). We found that approximately a third of human breast cancer cases expressed high levels of ROR1, which was associated with activation of AKT, cAMP response element-binding protein (CREB), and enhanced tumor cell growth (21, 27). Here, we examined whether ROR1 factors in breast cancer metastasis.

Materials and Methods

Cell culture
Breast cancer cell lines MDA-MB-231, HS-578T, BT549, MDA-MB-435s, MDA-MB-436, MDA-MB-157, MDA-MB-134, MCF7, BT-474, MDA-MB-453, SKBR3, MDA-MB-330, BT-483, and T47D were obtained from the American Type Culture Collection and maintained as described (28). The LM2-4175 and BoM-1833 metastatic sublines of MDA-MB-231 cells were gifts from Joan Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY).

Silencing of human ROR1
Silencing of ROR1 was achieved by targeting the sequences 5’-TCCGGATGGATCCATG-3’ (shRNA1), and 5’- CTTT-ACTAGGAGACGCCAATA-3’ (shRNA2) as described (27; see Supplementary Methods).

Analysis of metastasis
MDA-MB-231, LM2-4175, or BoM-1833 were subcutaneously injected into the second mammary fat pad, the tail-vein, or cardiac chamber of female RAG-/-/γc-/- mice. Tumor growth and metastasis were monitored via bioluminescence imaging, measured as photons per second per centimeter squared per steradian (p/s/cm²/sr; see Supplementary Methods).

Gene expression analysis from GEO datasets
We analyzed the published microarray datasets of 382 patients in the PubMed GEO database as previously described (see Supplementary Methods; ref. 29). See Supplementary Methods for detailed methods and references.

Results

ROR1 associates with metastatic cancer phenotypes
We examined for ROR1 in 14 distinct breast cancer epithelial cell lines, including 6 basal-type lines and 8 luminal-type lines. Each of the basal-type lines expressed ROR1, which generally was not expressed by luminal-type lines. Moreover, the high expression of ROR1 was associated with aggressive tumor phenotypes such as triple-negative ER-PR-HER2- and high-level migration/invasion capacity (Supplementary Table S1 and Supplementary Fig. S1; ref. 30).

ROR1 is associated with early metastatic relapse in breast adenocarcinoma
We interrogated the PubMed GEO database on cancer cells of 382 patients with breast adenocarcinoma (29). Approximately two-thirds (246/382) of these patients did not have detectable cancer in the regional lymph nodes at the time of surgery and were not administered adjuvant therapy. The remaining cases had detectable disease in regional lymph nodes and received adjuvant therapy. Among 582 cases, 46% relapsed (n = 270) and had a median metastasis-free survival time of 22.1 months. We segregated patients into 3 groups based upon their relative cancer cell expression of ROR1. Patients with tumors having the upper-third level of ROR1 mRNA expression (ROR1H) had a significantly shorter metastasis-free survival than patients with tumors that had the lower-third level (ROR1L) or intermediate-level (ROR1M) of ROR1 (P < 0.0001; Fig. 1A). Moreover, patients with ROR1H tumors had higher rates of metastasis to lung (P = 0.002), bone (P = 0.004), or brain (P = 0.04) than did patients with ROR1L or ROR1M breast cancers; (Supplementary Fig. S2). ROR1H cancers had significantly lower proportions of tumors with favorable prognostic features such as estrogen/progesterone receptors or HER2, than cancers with ROR1L or ROR1M (Supplementary Table S2). High-level expression of ROR1 was also carried out as an independent factor in predicting shorter metastasis-free survival (Supplementary Table S3). Patients with ROR1H tumors had a higher rate of metastasis, earlier relapse, and poorer survival than patients with ROR1L or ROR1M tumors, irrespective of ER, PR, or HER2 status (Supplementary Fig. S3). Furthermore, interrogation of the GSE2034, GSE2603, GSE5327, and GSE12276 array data for EMT gene signatures in breast cancer revealed that ROR1H tumors had significantly higher expression levels of genes associated with epithelial cells, such as CDH1 (encoding E-cadherin), TJP1 (encoding ZO1), and TJP3 (encoding ZO3), but lower expression levels of genes associated with mesenchymal cells, such as SNAI2 (encoding Snail-2), ZEB1 (encoding ZEB-1), CDH2 (encoding N-Cadherin), and VIM (encoding vimentin), than ROR1L tumors (Fig. 1B).

ROR1 breast cancer cell lines
We suppressed expression of ROR1 in basal-type breast cancer cell lines (e.g., MDA-MB-231) using short-hairpin RNAs (shRNA), which targeted either of 2 different sequences in ROR1. Expression of ROR1 protein was inhibited in cells transfected with either ROR1-shRNA1 or ROR1-shRNA2, in contrast to cells transfected with a control shRNA (CTRL-shRNA; Supplementary Fig. S4A). Interrigation of the array data for gene expression differences between MDA-MB-231 transfected with CTRL-shRNA or ROR1-shRNA (GEO accession: GSE31631) revealed that cells silenced for ROR1 had higher expression levels of KRT19 (encoding CK19), and lower expression levels of CXCR4 and VIM than parental or control-treated MDA-MB-231 cells (27). These findings were confirmed by qRT-PCR (Supplementary Fig. S4B and S5A). Flow cytometry analyses have also shown that cell surface expression of CXCR4 was lower in cells silenced for ROR1 (Supplementary Fig. S5B).

We examined for EMT-associated markers in cells transfected with CTRL-shRNA or ROR1-shRNA. Suppressing ROR1 with either ROR1-shRNA or ROR1-shRNA1/2 in either MDA-MB-231, HS-578T, or BT549 attenuated expression of mRNA and proteins associated with EMT (e.g., vimentin, SNAIL-1/2,
and ZEB1). Conversely, suppressing ROR1 increased expression of epithelial cytokeratins (e.g., CK-19). Although we did not observe significant changes in the TJP1 mRNA encoding ZO-1 in any of the 3 cell lines examined, cells silenced for ROR1 expressed higher levels of this tight junction protein, suggesting that ZO-1 might be under posttranscriptional control (Fig. 1C). Immunoblotting of protein lysates of MDA-MB-231, HS-578T, or BT549 cells transfected with ROR1-shRNA1 or ROR1-shRNA2 had a reduced capacity for migration/invasion, and for chemotaxis toward CXCL12, than cells treated with CTRL-shRNA (Fig. 2E and F, and Supplementary Fig. S5C).
Silencing ROR1 inhibits orthotopic lung metastasis

We compared the metastatic potential of CTRL-shRNA–transfected versus ROR1-shRNA–transfected MDA-MB-231 cells that were stably transfected using a luciferase/GFP-expression vector (Fig. 3A). Injection of 2.5 to 10 × 10^5 cells into the subcutaneous mammary fat pad of immunodeficient RAG–/–γc−/− mice generated primary tumors at the site of injection. We did not observe significant differences in the progressive increases in bioluminescence of tumors resulting from injection of CTRL-shRNA–transfected or ROR1-shRNA–
transfected cells until 3 or more weeks after the injection of at least $1 \times 10^6$ cells, as noted previously (Supplementary Fig. S7; ref. 27).

To examine for differences in rates of "spontaneous" cancer metastasis, the primary tumors resulting from injection of $1 \times 10^6$ cells were surgically removed when they reached a...
Because of different growth rates, the median number of days from cell injection to surgical removal of the primary tumors was significantly greater for mice injected with cells silenced for ROR1 (40 ± 2.5 days) than for mice that received equal numbers of CTRL-shRNA-transfected cells (31 ± 0.5 days; Fig. 3B). The extirpated primary tumors had similar volume, weight, and ex vivo bioluminescence (Fig. 3C–E). Following removal of the primary tumor, we monitored for metastatic disease via bioluminescence. Animals injected with CTRL-shRNA-transfected cells had significantly greater bioluminescence in the lung or liver at the time of primary tumor excision than did the mice engrafted with cells silenced for ROR1 at the later time when they had their primary tumors excised (Fig. 3E and F). Animals injected with cells silenced for ROR1 had less detectable increase in lung bioluminescence relative to that of mice injected with CTRL-shRNA-transfected cells (Fig. 3G). The animals were sacrificed 21 days after their primary tumors were excised. At that time, the extirpated lungs (Fig. 3H–J) and livers (Fig. 3K and L) of mice injected with CTRL-shRNA-transfected cells had significantly greater bioluminescence and weight compared with mice injected with ROR1-silenced cells. Moreover, the lungs and livers of mice injected with CTRL-shRNA–transfected cells universally had extensive
metastatic disease, which was not the case for mice injected with ROR1-silenced cells (Fig. 3J–L).

**Silencing ROR1 inhibits experimental lung and bone metastasis**

We administered the ROR1-shRNA- or CTRL-shRNA–transfected MDA-MB-231 to 6-week-old RAG2−/−γc−/− mice via intravenous (5 × 10⁵ cells) or intracardiac (1 × 10⁵ cells) injection to evaluate for differences in metastatic potential of cells injected into either the venous or arterial blood. All animals that received CTRL-shRNA–transfected cells via the lateral tail vein died within 32 days due to lung metastasis, whereas mice that received ROR1-silenced cells survived significantly longer (Fig. 4A). Animals injected with CTRL-shRNA–transfected cells had 19-fold or 60-fold greater bioluminescence in the lungs at day 21 or day 28, respectively, than mice injected with cells silenced for ROR1 (Fig. 4B). We also sacrificed animals in another experiment at various times to examine the lungs for metastatic disease. Although nascent metastatic foci were readily detected at 3 days after injection of CTRL-shRNA–transfected cells, few, if any, metastatic foci could be detected in the lungs of animals injected with ROR1-silenced cells, even at later time points (Fig. 4C–E). Moreover, lungs extirpated from mice injected with CTRL-shRNA–transfected cells had significantly greater ex vivo bioluminescence and median weight (3-fold and 6-fold on days 21 and 28, respectively) than lungs of mice injected with ROR1-silenced cells (Fig. 4F and G, data not shown). The metastatic foci that developed in animals injected with CTRL-shRNA–transfected cells also expressed higher levels of phospho-AKT and phospho-CREB and had higher proportions of proliferating cells than the few metastatic foci that we detected in mice injected with ROR1-silenced cells, which instead expressed higher levels of CK-19 and lower levels of vimentin (Supplementary Fig. S8).

We also examined for metastatic disease following injection of 1 × 10⁵ cells into the left cardiac ventricle. All mice that received CTRL-shRNA–transfected cells died within 30 days of this injection, whereas animals injected with ROR1-silenced cells survived significantly longer (Fig. 4H). Mice injected with CTRL-shRNA–transfected cells developed substantial femoral/pelvic area bioluminescence, which was not detected in mice injected with tumor cells silenced for ROR1 (Fig. 4I and J). We sacrificed animals on day 21 and found that the isolated femoral/pelvic bones of mice injected with CTRL-shRNA–transfected cells had high bioluminescence (Fig. 4K) due to extensive marrow metastasis (Fig. 4L), which was not apparent in mice injected with cells silenced for ROR1.

BoM1833 and LM2-4175 were selected from MDA-MB-231 to have different tissue tropism, BoM-1833 metastasizing...
Figure 5. An anti-ROR1 mAb D10 inhibits cancer-cell migration and metastasis. A, D10 mAb causes internalization of ROR1. MDA-MB-231 cells were stained with control-IgG-Alexa647 (red) or D10-Alexa647 for 30 minutes on ice and then either kept on ice (blue) or transferred to 37°C for 1 hour (orange) before flow cytometry. B, confocal microscopy of D10-stained (green) MDA-MB-231 cells before and after 1-hour incubation at 37°C. C, MDA-MB-231 cells were treated with or without control IgG (IgG) or D10 for 24 hours before staining with a fluorochrome-labeled, non-cross-blocking anti-ROR1 mAb, without loss in viability. Mean fluorescence intensity (MFI) of treated cells is shown (***: P < 0.001 by one-way ANOVA). D, representative immunoblots probed for vimentin (top) or β-actin (bottom) of lysates prepared from MDA-MB-231 before (0 hour) or after 1, 4, or 24 hours treatment with D10 or control IgG. The ratios of vimentin to β-actin band-intensity are provided below. E, immunoprecipitates of MDA-MB-231 cell-lysate using control IgG (IgG) or anti-ROR1.
preferentially to the bone and LM2-4175 to the lung (3). We found that each of these cell lines still expressed ROR1 (Supplementary Fig. S9A). We suppressed ROR1 in each with ROR1-shRNA (Supplementary Fig. S9B and S9C), allowing us to examine the ROR1 dependence of organ-specific metastasis following intravenous injection of $2 \times 10^7$ LM2-4175 or intracardiac injection of $1 \times 10^6$ BoM-1833 into 6-week-old RAG$^{-/-}$γc$^{-/-}$ mice. Mice injected with LM2-4175 silenced for ROR1 had a significantly lower median increase in lung bioluminescence and significantly longer median survival than did mice injected with CTRL-shRNA–transfected LM2-4175 (Supplementary Fig. S9D and S9E). Also, the lungs of mice that isolated 21 days after the injection of ROR1-silenced LM2-4175 had significantly lower median weight, ex vivo bioluminescence, and fewer and smaller metastatic foci than mice injected with CTRL-shRNA–transfected LM2-4175 (Supplementary Fig. S9F–S9H). Similarly, mice injected with BoM-1833 silenced for ROR1 had significantly lower increases in skeletal bioluminescence than did mice injected with equal numbers of CTRL-shRNA–transfected BoM-1833 (Supplementary Fig. S9I and S9J). Moreover, necropsy of animals sacrificed 21 days after intracardiac injection revealed few, if any, detectable metastatic foci in the bone or liver. This was in marked contrast to the extensive metastatic disease detected at each of these sites in animals injected with CTRL-shRNA–transfected BoM-1833 (Supplementary Fig. S9J and S9K).

**An anti-ROR1 antibody inhibits cancer metastasis**

We generated monoclonal antibodies (mAb) specific for the extracellular domain of ROR1 and selected one (D10), which could induce rapid downmodulation of surface ROR1 at 37°C (Fig. 5A). Treatment of MDA-MB-231 with D10 caused ROR1 internalization, as assessed via confocal microscopy (Fig. 5B). This resulted in significant reduction of ROR1, as assessed via flow cytometry using a different mAb specific for a distinct, non–cross-blocking epitope of ROR1 (Fig. 5C). Treatment of MDA-MB-231 with D10 also reduced expression of cytoplasmic vimentin (Fig. 5D), which was bound to ROR1 in communoprecipitation studies (Fig. 5E). Treatment with D10 also significantly inhibited the migration and invasion capacity of MDA-MB-231 in vitro (Fig. 5F and G). D10 also could inhibit the migration/invasion capacity of other ROR1+ cancer cell-lines (e.g., H5-578T and BT549; Supplementary Fig. S10).

We assessed whether D10 could inhibit invasion and metastasis of MDA-MB-231 injected into the tail vein of RAG$^{-/-}$γc$^{-/-}$ mice. Following injection of $5 \times 10^6$ cells, the mice were given an intravenous injection of control IgG or D10 at 50 mg/kg and then sacrificed 3 days later. The ex vivo bioluminescence of the lungs from animals given D10 was significantly lower than that of animals treated with control IgG (Fig. 5H). Moreover, the lungs of animals that received control IgG had multiple metastatic foci, which were not detectable in mice treated with D10. In another experiment, each mouse received an intravenous injection of $5 \times 10^3$ MDA-MB-231 and then given 3 weekly intravenous injections of control IgG or D10 at 5 mg/kg. Mice treated with D10 developed significantly less pulmonary bioluminescence than mice given control IgG (Fig. 5I and J). When sacrificed at day 35, the lungs of D10-treated mice had significantly lower median weight (Fig. 5K) and fewer metastatic foci (Fig. 5L) than lungs of control-treated animals. Collectively, these data indicate that D10 can inhibit metastasis in immunodeficient mice.

**Discussion**

Here, we show that ROR1 associates with EMT and cancer metastasis. Patients with breast cancers that had high-level expression of ROR1 had shorter metastasis-free survival than patients with cancers that had intermediate-to-negligible ROR1. Moreover, expression of ROR1 was peculiar to basaltype cell lines that had high metastatic potential. Silencing ROR1 reduced the expression of proteins implicated in cancer metastasis, such as SNAIL-1/2, ZEB1, CXCR4, and vimentin, but increased the expression of epithelial and tight junction proteins, such as CK-19 and ZO-1, indicating that ROR1 was necessary for maintaining the mesenchymal phenotype. Silencing ROR1 markedly impaired the capacity of cancer cells to migrate into tissues and establish tumor foci at distant sites, underscoring the importance of ROR1 in cancer metastasis. Importantly, perturbing the cell surface expression of ROR1 with an anti-ROR1 mAb could disrupt the capacity of such cells to migrate or form metastatic foci, indicating that targeting ROR1 may mitigate the risk for cancer metastasis.

In prior studies, we observed that silencing ROR1 in MDA-MB-231 lowered the levels of p-AKT and p-CREB, which was associated with decreased rates of cell proliferation (21). However, our studies presented here indicate that this could not fully account for the reduced metastatic potential of ROR1-silenced MDA-MB-231 cells to undergo metastasis. First, MDA-MB-231 cells silenced for ROR1 had a reduced capacity to invade the lung within 24 to 72 hours (Fig. 4B and C and Supplementary Figs. S7A and S9D) after intravenous administration, when differences in the numbers of viable cells between ROR1-silenced versus control-treated cells were not apparent. Second, in the orthotopic model, we allowed for the primary tumor to grow to the same size in mice injected with either control-treated or ROR1-silenced cells before examining for metastatic foci (Fig. 3B). As such, the mice challenged with ROR1-silenced MDA-MB-231 cells had a significantly longer latency period following primary tumor engraftment before examining for metastases. Third, we compared the growth of primary tumors in orthotopic mammary fat pads versus that of

---

**Notes:**

(ROR1) were used for immunoblot analyses probed with antibodies specific for vimentin (top) or ROR1 (bottom). F, histograms provide the number of migrated MDA-MB-231 cells that were pretreated for 1 hour with D10 or control IgG. G, representative photomicrographs of migration (left) or invasion (right) of MDA-MB-231 following treatment with control IgG or D10. H, left, histograms depicting the in vivo lung photon flux. Right, representative H&E-stained sections of the lungs. I, graph depicts the normalized in vivo lung photon flux. J, representative H&E-stained sections of the lungs from animals given control IgG (IgG) or anti-ROR1 (D10), as indicated at the top of the panel. Data are shown as mean ± SEM: *, P < 0.05; **, P < 0.01; ***, P < 0.001, compared with IgG group.
metastatic disease to the lung using different inoculation cell doses and noted significant differences between mice injected with control versus ROR1-silenced MDA-MB-231 in the growth of metastatic disease in the lung at 21 days, when there was not a significant difference between the growth of the primary tumors that formed at the site of injection (Supplementary Fig. S7). Finally, treatment with D10 significantly reduced the numbers of metastatic lesions observed in mice injected with MDA-MB-231 cells, even though this antibody had little or no effect on the rate of cell proliferation in vitro. Collectively, these data argue that ROR1 influences the rate of tumor cell metastasis.

Prior studies have shown that silencing ROR1 in MDA-MB-231 resulted in decreased levels of phosphorylation at Ser-473 (phospho-AKT; 27). Consistent with this, we noted that the metastatic foci formed by MDA-MB-231 expressed high levels of phospho-AKT, which was not found in the few metastatic foci of mice injected with MDA-MB-231 cells silenced for ROR1. Activation of AKT may induce expression of Snail, Slit2, and ZEB1, which in turn can promote expression of proteins that characterize EMT (31–35). Here, we provide evidence that expression of these key EMT regulators in breast cancer cell lines is dependent upon expression of ROR1, which we and others found could promote activation of p-AKT in primary cancers and cancer cell lines (15, 21, 27).

The reduced metastatic potential of cells silenced for ROR1 may also be attributed in part to their reduced expression of chemokine receptors such as CXCR4. Expression of CXCR4 allows cancer cells to seed distant organ that have relatively high levels of CXCL12, the ligand for CXCR4 (36, 37). Moreover, inhibition of the CXCL12–CXCR4 axis can partially inhibit the in vivo metastasis of MDA-MB-231 to the lung or the marrow (36), sites with relatively high-level expression of CXCL12 (6). We observed that silencing ROR1 resulted in reduced expression of CXCR4 and inhibited capacity of these cells to migrate toward CXCL12 in vitro. This, together with the other changes noted in expression of EMT-related proteins, might account for the reduced potential for metastatic spread of MDA-MB-231 cells silenced for ROR1.

The role played by ROR1 in cancer metastases might be conscripted from its physiologic role in embryogenesis. EMT is required for normal development (7, 10); during early embryogenesis, epithelial cells undergo this transition, allowing them to lose cell–cell contact and cell polarity, acquire gene expression signatures of mesenchymal cells, and develop the increased motility and invasiveness required to seed developing organs (38). At such stages of embryogenesis, ROR1 is expressed by NPCs, which are migrating neural crest cells with mesenchymal properties in the neocortex (19). Recent studies found silencing ROR1 in NPCs decreased production of neurons in long-term culture as ROR1 signaling apparently was required to prevent premature differentiation of NPCs (20). Similarly, ROR1 might function in neoplasia to regulate the proliferative, invasive, and mesenchymal properties of CSCs.

In any case, the results of this study suggest that ROR1 is a critical regulator of the invasive properties of cancer cells that are required for metastasis. Loss of ROR1 seems to reverse EMT, allowing the cancer cells to acquire expression of epithelial cytokeratins and tight junction proteins that may tether the cell to a local microenvironment. Such changes are associated with a diminished capacity for cell migration and invasiveness, which also is observed for cancer cells treated with the anti-ROR1 mAb D10. D10 can cause cell surface downmodulation and internalization of ROR1, which seems to be associated with the cytoplasmic cytoskeletal protein vimentin. In this regard, D10 may function like a ligand for ROR1, such as Wnt5a, which we found in prior studies, could bind ROR1 to effect noncanonical Wnt signaling (22). Surface expression of Wnt5a and/or other ligands by cells within the microenvironment might serve to downmodulate ROR1 to effect reversal of EMT. High-level expression of ROR1 ligands thus might mitigate the risk of metastasis, possibly accounting for the paradoxical association of high-level expression of Wnt5a with a more favorable prognosis for some cancers (39–45). However, recent studies have found that recombinant Wnt5a actually increases MDA-MB-231 cell motility (46). This is in direct contrast to what we observe following treatment of such cells with the anti-ROR1 mAb D10. Conceivably, D10 might cause perturbations and/or turnover in ROR1, which seems to be required to maintain the functions associated with EMT. This may be due to the distinct way D10 binds to ROR1 and/or the ability of this bivalent antibody to effect ROR1 cross-linking, which may not be induced by Wnt5a unless it is expressed on the plasma membrane of cells within the tumor microenvironment. Furthermore, Wnt5a might engage receptors other than ROR1 that could influence the outcome of Wnt5a–tumor interactions (47–49); this is in contrast to D10, which is specific for ROR1.

In summary, we found that expression of ROR1 is required to maintain EMT and that targeting ROR1 can inhibit cancer metastasis. Because normal postpartum tissues generally do not express this oncoembryonic antigen, therapeutic targeting of ROR1 may offer a highly selective approach to mitigating the risk of metastatic disease.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: B. Cui, S. Zhang, L. Chen, T.J. Kipps
Development of methodology: B. Cui, L. Chen, J. Yu, J.-F. Farah-Fecteau, T.J. Kipps
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Cui, S. Zhang, L. Chen, L. Rassenti, T.J. Kipps
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Cui, S. Zhang, G.F. Widhopf II, T.J. Kipps
Writing, review, and/or revision of the manuscript: B. Cui, S. Zhang, L. Chen, G.F. Widhopf II, J.-F. Farah-Fecteau, T.J. Kipps
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Cui, L. Rassenti, T.J. Kipps
Study supervision: T.J. Kipps

Acknowledgments
The authors thank Dr. Joan Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY) for providing the LM2-4175 and BoM-1833 breast cancer cell lines; UCSD Histology Core lab for technical help with processing tissue specimens; Dr. Nissi Varki for histology and immunohistochemistry assessments; Ling Zhang, Daniel Martinez, Dr. Rongrong Wu, Dr. Juan Yu, and Esther Ling Zhang, Daniel Martinez, Dr. Rongrong Wu, Dr. Jian Yu, and Esther

Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 2013 American Association for Cancer Research.
Targeting ROR1 Inhibits Breast Cancer Metastasis

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 5, 2012; revised March 15, 2013; accepted April 5, 2013; published online June 14, 2013.

Avery for technical help with in vivo experiments and in vitro protein analysis; and Dr. Weinhou Zhang for helpful discussions and technical suggestions.

Grant Support
This study was supported by the NIH (PO1-CA081534), California Institute for Regenerative Medicine (CIRM), and the Blood Cancer Research Fund, UCSD Foundation.

References


Targeting ROR1 Inhibits Epithelial–Mesenchymal Transition and Metastasis

Bing Cui, Suping Zhang, Liguang Chen, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/73/12/3649

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2013/06/25/73.12.3649.DC1

Cited articles
This article cites 49 articles, 15 of which you can access for free at:
http://cancerres.aacrjournals.org/content/73/12/3649.full.html#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
/content/73/12/3649.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.