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 tight junction 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. Cancer Res; 73(12); 3649–60. ©2013 AACR.

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 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′-TCCGGATGGAATTCCCATG-3′ (shRNA1), and 5′- CTGT-ACTAGGAGAGCCAAATA-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 582 patients in the PubMed GEO database as previously described (see Supplementary Methods; ref. 29).

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 582 patients with breast adenocarcinoma (29). Approximately two-thirds (426/582) 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/M 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 ROR1, tumors had significantly higher expression levels of genes associated with epithelial cells, such as CDH1 (encoding E-cadherin), TJPI (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), or VIM (encoding vimentin), than ROR1H 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). Interrogation 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 CXC4 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 CXC4 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 CXCR4).
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. 3A). EMT-related genes in primary breast cancer cells isolated from patients. C, heatmap showing the expression of EMT-related genes isolated from cells treated with ROR1-siRNA or CTRL-siRNA. D, immunoblot of protein lysates of MDA-MB-231, HS-578T, or BT549 (as indicated on the bottom) transfected with CTRL-shRNA or ROR1-shRNA, as indicated at the top. E, immunoblot of protein lysates of MCF7 transfected with a control vector or a ROR1-expressing vector, as indicated at the top.

Figure 1. High-level expression of ROR1 in breast cancer is associated with shorter metastasis-free survival and EMT gene signature. A, graph was derived from published data available through the PubMed GEO database (GSE2603, GSE5327, GSE2034, and GSE12276). Kaplan-Meier curves depict the prognostic impact of ROR1 expression on overall metastasis-free survival. For each analysis, 582 cases were segregated into tertiles with group designated ROR1H representing the one-third of the patients who had tumors with the highest levels of ROR1 mRNA and the group designated ROR1L, representing the one-third of patients who had cancers with the lowest levels of ROR1 mRNA. The one-third of patients who had tumors with intermediate expression of ROR1 mRNA was designated as ROR1M. Metastasis-free survival was determined by Kaplan-Meier analyses, and statistical differences were determined by log-rank test. The number of patients in each category, the total metastatic events, and the corresponding P values (χ2 test) are shown in the embedded tables. B, heatmap showing the expression of ROR1 (top), EMT-related genes in primary breast cancer cells isolated from patients. C, heatmap showing the expression of EMT-related genes isolated from cells treated with ROR1-siRNA or CTRL-siRNA. D, immunoblot of protein lysates of MDA-MB-231, HS-578T, or BT549 (as indicated on the bottom) transfected with CTRL-shRNA or ROR1-shRNA, as indicated at the top. E, immunoblot of protein lysates of MCF7 transfected with a control vector or a ROR1-expressing vector, as indicated at the top.

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MDA-MB-231, HS-578T, or BT549 cells typically exhibited the stellate morphology of mesenchymal cells in vitro (9). However, cells silenced for ROR1 assumed the more spherical morphology of epithelial cells (Fig. 2A). Furthermore, suppressing ROR1 induced increased expression of E-cadherin and CK-19, but reduced expression of vimentin in MDA-MG-231 (Fig. 2B). Similar results were also observed for HS-578T or BT549 cells. On the other hand, compared with untreated cells or cells transfected with a control vector, ROR1-negative MCF7 cells developed a morphologic resemblance to mesenchymal cells and had decreased expression of epithelial markers (e.g., CK19 and E-cadherin), and increased expression of mesenchymal markers, such as vimentin, when transfected to express ROR1 (Supplementary Fig. S2C and S2D). Cells silenced for ROR1 with either 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
volume of 300 mm$^3$ (dotted line, Fig. 3B). 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 RAG-/-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 Cui et al. Cancer Res; 73(12) June 15, 2013 Cancer Research

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Targeting ROR1 Inhibits Breast Cancer Metastasis

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., H5-5787 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<sup>−/−</sup>/γ<sup>−/−</sup> mice. Following injection of 5 × 10<sup>6</sup> cells, the mice were given an intravenous injection of control IgG or D10 at 50 mg/kg and then sacrificed 3 days later. The <i>ex vivo</i> 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 × 10<sup>6</sup> 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 basalt-type 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

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(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 <i>in vivo</i> lung photon flux. Right, representative H&E-stained sections of the lungs. I, graph depicts the normalized <i>in vivo</i> lung photon flux. J, representative bioluminescence images of tumor-injected mice treated with IgG (top) or D10 (bottom). K, histogram depicts the lung weight-index. L, representative H&E-stained sections of the lungs from animals treated with control IgG (IgG) or anti-ROR1 (D10), as indicated at the top of the panel. Data are shown as mean ± SEM. *<i>P</i> < 0.05; **<i>P</i> < 0.01; ***<i>P</i> < 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, SNAI2, 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 expres-
sion 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 embry-
genesis, epithelial cells undergo this transition, allowing them
to lose cell–cell contact and cell polarity, acquire gene expres-
sion signatures of mesenchymal cells, and develop the
increased motility and invasiveness required to seed develop-
ing 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
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 func-
tion 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 associ-
ation of high-level expression of Wnt5a with a more favor-
able 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 influ-
ence 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
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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 assess-
ments; Ling Zhang, Daniel Martinez, Dr. Rongrong Wu, Dr. Juan Yu, and Esther
Avery for technical help with in vivo experiments and in vitro protein analysis; and Dr. Weizhou 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.

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