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Receptor tyrosine kinase-like orphan receptor-1 (ROR1) expression in breast cancer stem cells can be targeted for anti-cancer-stem-cell therapy

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Receptor tyrosine kinase-like orphan receptor-1 (ROR1) expression in breast cancer stem cells can be targeted for anti-cancer-stem-cell therapy

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Grace Liu

Committee in charge:

Thomas J. Kipps, Chair
Eric J. Bennett, Co-Chair
Ella Tour

2015
The Thesis of Grace Liu is approved and is acceptable in quality and form for publication on microfilm and electronically:


University of California, San Diego

2015
DEDICATION

In recognition of God who showed me what it means to trust in Him. Without you, I would have never made it to where I am today.

In recognition to my family who encouraged me to pursue a higher education to finish this program strong. To my mother and father who have been such wonderful teachers in patience and kindness. I wouldn’t have been able to do all the experiments without these valuable traits.

In recognition of Brian, thanks for supporting me mentally and emotionally and knowing when to calm my “hangry” episodes with food. Thanks for pushing me to not procrastinate too much and showing me the joys in life. You are the best! :)

In recognition of Sam, thank you for being my guide and introducing me into the lab. To those countless late hours and the crazies, I’m glad I had the chance to work with you. Best of luck in med school! You’ll be a great doctor!

In recognition of Suping, I thank you for being my mentor through these years. At first I didn’t know anything at all and ruined experiments, but you have taught me to become independent in my experiments and obtain useful data. However, I think the most valuable thing that you have taught me is the process of thought: to think about how things function, to research, and to implement an experimental design. Thank you for this!

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Lastly, in recognition of Dr. Thomas J. Kipps, I thank you for the opportunity to study and research in your lab. I have learned so many wonderful things and have made good friends here. Also, I have grown a huge interest in cancer research. There’s still so much to learn, but you have provided me a strong foundation to start my career in biological sciences. Thank you so much!
What we find changes who we become.

*Peter Morville*
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LIST OF ABBREVIATIONS

AKT        protein kinase B
ALDH1      aldehyde dehydrogenase 1
BAA        bodipy-aminoacetate
BAAA       bodipy-aminoacetaldehyde
CD         cluster of differentiation
CLL        chronic lymphocytic leukemia
CREB       cyclic-amp response element binding-factor
CSC        cancer stem cell
DEAB       diethylaminobenzaldehyde
DMEM       dulbecco’s modified eagle medium
EMT        epithelial-mesenchymal transition
EpCAM      epithelial cell adhesion molecule
ELDA       extreme limiting dilution analysis
FBS        fetal bovine serum
FDA        fluorescein diacetate
hIgG       human immunoglobulin G
MDR1       multidrug resistance protein 1
MEBM       mammary epithelial basal medium
MEGM       mammary epithelial basal medium supplemented with growth factors
NOD        non-obese diabetic
PBS        phosphate buffered saline
PDX        patient derived xenograft
PE         phycoerythrin
Pe-Cy7     phycoerythrin-Cy7
PI          propidium iodide
PI3K       phosphoinositide 3’ kinase
PKB        protein kinase B
RNA        ribonucleic acid
ROR1       receptor tyrosine kinase-like orphan receptor 1
ROR2       receptor tyrosine kinase-like orphan receptor 2
SCID       severe combined immune-deficient
SEM        standard error of the mean
shRNA      short hairpin ribonucleic acid
TGFβ       transforming growth factor
YB-1       Y-box binding protein 1
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ABSTRACT OF THESIS

Receptor tyrosine kinase-like orphan receptor-1 (ROR1) expression in breast cancer stem cells can be targeted for anti-cancer-stem-cell therapy

by

Grace Liu

Masters of Science in Biology
University of California, San Diego, 2015

Thomas J. Kipps, Chair
Eric J. Bennett, Co-Chair

Receptor tyrosine kinase-like orphan receptor 1 (ROR1) plays an important role in embryonic development and is down-regulated after birth. Although ROR1 is not expressed in the majority of normal adult tissues, ROR1 upregulation has been found in cancers. Here, we established five breast cancer patient derived xenografts in mice to investigate whether ROR1-expressing cells display cancer stem cell (CSC) features of sphere formation,
invasion, and increased tumorigenicity and whether UC-961, a humanized anti-ROR1 monoclonal antibody could inhibit those features. We found that ROR1\(^+\) cells formed 3- to 4-fold more spheres and were 2- to 3-fold more invasive than ROR1\(^-\) cells. Furthermore, ROR1\(^+\) cells had tumorigenic cell frequencies that were at least 10 times higher than ROR1\(^-\) cells. When high ROR1-expressing breast cancer cells were treated with UC-961, the cells were inhibited in their capacity to invade Matrigel, self-renew, and engraft tumors in mice by 2-fold. Because CSCs have been associated with increased chemotherapy resistance and thought to be involved in cancer recurrence, we examined the combined effect of UC-961 and Taxol on breast cancer growth and found that they synergistically inhibit breast cancer cell line growth, self-renewal, and tumor formation. In summary, the data suggests that ROR1 associates with CSC characteristics and that targeting ROR1 with monoclonal antibodies with or without Taxol can provide novel therapeutic strategies for cancer treatment.
INTRODUCTION

Within a single type of cancer, such as breast cancer, there exist tumor cells that express different morphologies and phenotypes (Magee et al., 2012). This is called tumor heterogeneity and it is what makes cancers very difficult to eliminate (Liu et al., 2014). The cancer stem cell model suggests that there is a small population of slow-dividing cells that have increased tumorigenicity and differentiating capacity compared to other cells (Magee et al., 2012). Tumorigenicity is the ability of a cell to form a tumor and differentiating capacity refers to the ability of a cell to alter its phenotype and function. Furthermore, CSCs are said to be hierarchically organized where they self-renew to maintain a small population of stem cells and differentiate into various cell types that lack stem cell properties (Dick, 2008).

Normal stem cells are undifferentiated cells that are able to self-renew and can differentiate into various cell types to form tissue, blood, and others. Self-renewal is the process whereby stem cells divide to make more stem cells. Like normal stem cells, cancer stem cells (CSCs) are able to self-renew, propagate tumor heterogeneity and regenerate tumors (Reya et al., 2001).

In addition, studies have indicated that these CSC populations express distinct cell surface markers and can be consistently isolated. As a result of heterogeneity, not all CSC markers are expressed in all cancers nor are they expressed in similar levels in subtypes of corresponding cancers. In brain tumors, researchers found that CSCs that expressed CD133 (Clusters of Differentiation 133) were able to differentiate into cells resembling the original
tumor but not normal cells or cells that did not express CD133 (Singh et al., 2003). Furthermore, these CD133-positive cells were able to initiate and grow tumors in the brains of mice whereas CD133-negative cells were not (Singh et al., 2003; Singh et al., 2004).

In breast cancer, CD44^+CD24^- CSC populations were able to form tumors in mice whereas CD44^+CD24^+ and CD44^- populations did not form tumors (Al-Hajj et al., 2003). Similar to CD44^+CD24^- CSCs, aldehyde dehydrogenase-1 (ALDH1) activity has been characterized to be another CSC marker for various cancers including breast (Ginestier et al., 2007; Charafe-Jauffret et al., 2013). ALDH activity is measured by conversion of a fluorescent substrate, BODIPY-aminoacetaldehyde (BAAA), into BODIPY-aminoacetate (BAA) which is retained in the cells. The fluorescence can then detected by a flow cytometer. As a control, diethylamino-benzaldehyde (DEAB) is used as a specific inhibitor of ALDH to eliminate background fluorescence. With the characterization and categorization of these CSC populations in various tumors, targeted therapy against CSC populations would be able to impair tumor progression.

Currently, depending on the patient’s cancer status, patients may be given a neoadjuvant therapy drug, which is therapy given before primary therapy, to reduce the tumor size so that it can be surgically removed. Primary therapy involves surgery to reduce or remove the tumor. After the primary therapy, adjuvant therapy is given through chemotherapy, radiation, and/or others to further kill cancer cells and prevent metastasis (the spread of cancer
into other tissues). In a breast cancer study, researchers found that breast cancer patients who received adjuvant or neoadjuvant systemic therapy had improved survival, but also a high risk of recurrence 5 years after therapy (Brewster et al., 2008). CSCs have been implicated in increased chemotherapy resistance by evading apoptosis through quiescence, an upregulation of drug efflux transporters, DNA repair mechanisms and others (Vinogradov et al., 2013; Skvortsov et al., 2015). Thus current chemotherapies such as Taxol, which prevents microtubule disassembly of dividing cells, are unable to fully eradicate the CSC population. On the contrary, targeting CD44+ CSC populations in chronic lymphocytic leukemia (CLL) with a monoclonal antibody resulted in the clearance of CLL cells in vivo (Zhang et al., 2013). Accordingly, targeted therapies are needed to address the heterogeneity of cancers as well as the small population of cancer stem cells that can evade current chemotherapies.

It has been suggested that Receptor Tyrosine Kinase-like Orphan Receptor 1 (ROR1) may be a potential cancer stem cell marker as well as a therapeutic target (Zhang et al., 2012a; Zhang et al., 2014). ROR1 belongs to a family of receptor tyrosine kinase-like orphan receptors that are important during embryonic development particularly the cardiac, respiratory, and skeletal systems (Masiakowski et al., 1992; Green et al., 2008; Matsuda et al., 2001). Mice lacking ROR1 develop respiratory problems, skeletal defects leading to dwarfism, and facial abnormalities (Nomi et al., 2001). During these embryonic stages ROR1 is expressed, but is down-regulated after birth and is
not expressed in normal adult tissues (Oishi et al., 1999; Matsuda et al., 2001; Hojjat-Farsangí et al., 2014).

ROR1 has been found to be upregulated in subtypes of cancers, but not in their respective normal tissues or cells including ovarian, colon, lung, bladder, pancreatic, and B-cell chronic lymphocytic leukemia (Zhang et al., 2012b; Baskar et al., 2008). Studies have shown that ROR1 interacts and binds with Wnt5a leading to a signaling cascade that activates phosphoinositol 3' kinase (PI3K) which phosphorylates protein kinase B (PKB, also known as AKT) (Zhang et al., 2012a; Grumolato et al., 2010; Fukuda et al., 2008). Phosphorylation of AKT in turn phosphorylates the cyclic-AMP response element binding-factor (CREB), which is involved in regulating the cell cycle. In addition, ROR1 may also interact with transforming growth factor-β (TGF-β) which has been found to also activate the PI3K/AKT pathway to increase cell survival, proliferation, migration, and invasion (Vo et al., 2013; Tirino et al., 2013).

ROR1 expression in cancer has been correlated with high expression of phosphorylated AKT (p-AKT) and phosphorylated CREB (p-CREB) which leads to enhanced cancer cell growth, proliferation, and survival (Cicenas et al., 2008; Zhang et al., 2012a). Furthermore, cancers expressing ROR1 have been correlated with more aggressive disease and epithelial-mesenchymal transition (EMT) (Cui et al., 2013; Kong et al., 2011). EMT is a process where epithelial cells become mesenchymal stem cells by reorganizing their cell
structure/phenotype and increasing their properties of cell motility and ability to invade the extracellular matrix.

Studies have shown that silencing ROR1 with short hairpin RNA (shRNA) in ovarian cancer cell lines significantly decreased p-AKT, p-CREB, and EMT markers which correlated with a decrease in cell viability, invasion, and metastasis (Zhang et al., 2012a; Cui et al., 2013; Zhang et al., 2014). Furthermore, transplantation of cells with ROR1-shRNA inhibited tumor engraftment in mice (Zhang et al., 2014). In a study performed on ovarian cancer, transplantation of ROR1-positive (ROR1+) cancer cells in mice led to increased tumorigenicity compared to ROR1-negative (ROR1-) populations which showed reduced tumorigenicity (Zhang et al., 2014).

Recently, a novel fully humanized monoclonal antibody called UC-961 has been shown to inhibit ovarian cancer stem cell spheres from forming as well as decrease tumorigenesis (Zhang et al., 2014). Humanized antibodies are created from non-human species that have been modified to be similar to antibodies produced naturally in humans. UC-961 also known as cirmtuzumab recognizes the extracellular domain of ROR1 and has high specificity towards ROR1+ cells with low cytotoxicity for ROR1- cells. Since ROR1 has restricted expression on some human cancers, UC-961 may be a promising source of therapy either alone or in combination with current therapies such as Taxol (Cui et al., 2013).

In this study, we examined the significance of ROR1 biologically and clinically in breast cancer. More specifically, we focused on whether ROR1-
expressing cells exhibit CSC characteristics of sphere formation, cell invasion, and tumorigenicity. Furthermore, we studied whether targeting ROR1 with a humanized monoclonal antibody against ROR1, named UC-961, could inhibit those CSC characteristics. Lastly, we investigated the combined effect of UC-961 therapy with the chemotherapy agent Taxol on breast cancer growth in vitro and in vivo.
METHODS

Animal Models

Rag/γc-/- and NOD/SCID mice 6- to 12- week old mice were used in the study under the laboratory animal guidelines of the National Institutes of Health (NIH). Mice were housed in specific pathogen-free conditions and fed ad libitum.

Cell Culture

Breast cancer cell lines HS578T and NIH3T3 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS, Invitrogen), 50μg of penicillin G, and 50μg of streptomycin sulfate.

Establishing Breast Cancer PDX Models

The five breast PDX tissues (BR1936, BR1367, BR0851L, BR1126, and BR0869) were maintained and grown in Rag/γc-/- and NOD/SCID mice by retransplanting isolated single cells or frozen tissue from parent tumor in a mixture of mammary epithelial basal medium supplemented with growth factors and Matrigel (BD Biosciences).

Single-cell Isolation from Breast PDX Tissues

Primary breast tumor tissues from PDX models were minced then enzymatically and mechanically dissociated using human Tumor Dissociation Kit and GentleMACS Dissociator (Miltenyi Biotec) following the manufacturer’s protocol. Percoll Plus (GE Healthcare Life Sciences) was used to remove
dead cells and red blood cells from the sample through density gradient centrifugation following the manufacturer’s protocol. Cell viability was verified through trypan blue staining and a hemacytometer.

For transplantation of ROR1 sorted cells, NOD/SCID mice were injected in their mammary fat pads with different cell numbers of FACS-purified cells. Cells were prepared in a 1:1 ratio of mammary epithelial basal medium (MEBM, LONZA) and Matrigel growth factor reduced (BD Biosciences). Tumor formation was monitored twice weekly.

To test the effect of UC-961 and Taxol in breast tumor cell engraftment, BR1936 primary cells were injected orthotopically into the second mammary fat pad of Rag/yc-/- mice. When tumor volumes reached 0.2cm^3, the mice were treated with 10mg/kg UC-961 (once weekly) and/or 13.4mg/kg taxol (once a day for 5 consecutive days), intravenously. Tumor volumes were monitored three times a week.

**Sphere-Formation and Self-Renewal Assay**

For sphere-formation assays, 100-5000 viable primary cells isolated from breast cancer PDX models were plated on 6-well, Ultra-low Attachment plates (Corning) in MEBM growth medium supplemented with various growth factors (MEGM) (Lonza, Walkersville, MD) for 2-3 weeks. Spheres were then counted under an inverted light microscope (Nikon).

For self-renewal assays, primary breast cancer cells were first grown for 2-3 weeks on an Ultra-Low Attachment Surface Flask (Corning, Lowell, MA) to obtain 1\textsuperscript{st} passage spheres. Resulting spheres were then enzymatically
and mechanically digested into single cells with Cell Dissociation Buffer (GIBCO, Carlsbad, CA) supplemented with 0.125% trypsin-EDTA (GIBCO) and 20- to 25-gauge needles. Resulting viable cells were washed, centrifuged, and resuspended in MEGM. Then, 1 to 1000 cells were plated in 96-well ultra-low attachment plates (Corning) and were treated with either hIgG, Taxol (3nM; Hospira), UC-961 (50ug/ml) or in combination. Cells were retreated with UC-961 after 72 hours in the respective wells. The number of wells containing spheres (>50um) were counted using an inverted microscope (Nikon) 1 week later.

**Matrigel Cell Invasion Assay**

Primary breast cancer cells were plated in invasion chambers (8 µm pore inserts, BD Biosciences) at a concentration of 1x10^5 cells/ml in MEBM. The lower chambers were filled with serum-free conditional medium. Conditional medium was created by starving 70-80% confluent NIH3T3 cells in DMEM for 48 hours. The medium was collected then frozen at -80°C then thawed for use. Cells on the upper side of the inserts were scraped off, then fixed and stained with Diff-Quick staining kit (IMEB Inc, San Marcos, CA). Images were taken using a Nikon inverted microscope.

**Immunoblot Analyses**

Cells from primary tumor tissues or spheres were lysed in phosphate buffered saline (PBS) containing 1% NP40, 0.1% SDS, 0.5% sodium deoxyylate, and protease inhibitors (Pierce). Size-separated proteins were transferred to nitrocellulose membranes, and then incubated with primary
antibodies specific for ROR1, Bmi-1, Snail-1, and β-actin (Cell Signaling Technology, Danvers, MA). After washing the membranes, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase. The blots were prepared for enhanced chemiluminescence and autoradiography. The protein concentration was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL).

**Flow-Cytometry Analysis and Sorting**

Primary cells were obtained from primary tumors and blocked with Fc-blocking reagent (Miltenyi Biotec, BD Pharmingen). For ROR1 staining and sorting, blocked cells were stained with Alexa-647-conjugated 4A5, phycoerythrin (PE)-conjugated anti-epithelial cell adhesion molecule (EpCAM), and biotin-labeled anti-H2Kd (BD Biosciences) for 20 minutes on ice without light. Cells were washed twice using FACS Buffer (PBS containing 3% FBS). Secondary phycoerythrin-Cy7-conjugated streptavidin (PE-Cy7, BD Pharmingen) was used to detect bound anti-H2Kd. Cells were washed twice more with FACS Buffer. Cells were stained with Fluorescein Diacetate (FDA, Life Technologies) to detect live cells for 10 minutes on ice and then washed twice.

For CD44+/CD24- analysis, blocked primary cells were stained with Fluorescein-conjugated anti-CD44, PE-conjugated anti-CD24 (BD Pharmingen), Alexa-647-conjugated 4A5, and biotin-labeled anti-H2Kd with PE-Cy7-conjugated streptavidin.
To detect ALDH1 activity, blocked primary cells were incubated with ALDEFLUOR assay buffer containing ALDH substrate (BAAA, StemCell Technologies). In parallel, half the cells were treated with the ALDH1 inhibitor, diethylaminobenzaldehyde (DEAB, 50mmol/L) all cells were incubated for 15 minutes at 37°C with shaker. Cells were washed twice and then stained with Alexa-647-conjugated 4A5, and EpCAM-PE for 20 minutes on ice. Afterwards, cells were washed twice. Cells treated with DEAB were used in the gating strategy to define cells with ALDH1 activity.

Propidium Iodide (PI, Biovision, Mountain View, CA) was added right before analysis and sorting using a FACs-Calibur or FACS-Aria flow cytometer (Becton Dickinson). Data were analyzed using the FlowJo Software (Tree Star). Viable cells were determined by calculating the percentage of FDA+/PI- populations. Mouse cells were excluded with H2Kd staining and human breast epithelial cells were included with EpCAM staining.

**Statistical Analysis**

To obtain P values, data were analyzed with GraphPad software using Student’s T-test or ELDA software.

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RESULTS

ROR1 expression in breast PDX models

We first established five breast cancer patient-derived xenograft (PDX) models in mice and examined their relative ROR1 expression by flow cytometry and western blot (Figure 1). Flow cytometry analysis for ROR1 expression revealed that BR1936 had the highest mean fluorescence intensity ratio (MFIR) of 6.6 which was about 2-fold higher than BR1367 (MFIR: 3) and almost 3-fold higher than BR0851L (MFIR: 2.4) (Figure 1A). Furthermore, BR1126 and BR0869 had MFIR of 1.3 and 1.1, respectively (Figure 1A). The relative ROR1 expression was also confirmed by western blot (Figure 1B).

Previous studies have identified breast CSCs by cell surface expression of CD44+/CD24^{low/-} and by aldehyde dehydrogenase-1 (ALDH1) activity (Al-Hajj et al., 2003; Ginestier et al., 2007). We wanted to examine the association of ROR1 with these established markers. The total proportions of CD44^{+}/CD24^{low/-}, total CD44^{+}, and ALDH1^{+} cells were analyzed for each PDX model (Table 1A). ROR1 expression was found to be higher in CD44^{+}/CD24^{low/-} compared to non-CD44^{+}/CD24^{low/-} populations and in ALDH1^{+} compared to ALDH1^{-} populations (Table 1B). Furthermore, an increased proportion of CD44^{+}/CD24^{-} expression and ALDH1 activity were found in ROR1^{+} populations compared to ROR1^{-} populations (Table 1C).

ROR1 expression is associated with increased sphere formation, cell invasion, and tumorigenicity
The ability to form spheres is a characteristic of cancer stem cells (Lobo et al., 2007; Iglesias, et al., 2013). To determine if ROR1 expression is associated with increased sphere formation, we performed sphere formation assays with the five breast PDX models. We found that PDX tumors with higher ROR1 expression produced more spheres than those with lower or non-detectable ROR1. BR1936 formed 2.5- to 3-times more spheres than BR1367 and BR0851L, respectively (Figure 2A). Moreover, BR0851L formed 3-times more spheres than BR1126 and no spheres were detected for BR0869 (Figure 2A). By immunoblot analyses, we found that sphere cultures enriched for ROR1-expressing cells in comparison to the initial tumor-cell population (Figure 2B). Next we isolated ROR1+ and ROR1- breast cancer cells from BR1936, BR1367, and BR0851L by staining single-cell suspensions with an anti-ROR1 monoclonal antibody and performing FACS sorting using the gates shown in the bottom contour plot (Figure 2C). ROR1+ cells cultured as spheres formed 3-times more spheres than ROR1- cells did for BR1936, BR1367, and BR0851L (Figure 2D).

CSCs are more invasive than their non-CSC counterparts (Al-Hajj et al., 2013). To evaluate the invasive capacity of the five breast PDX models, we performed Matrigel cell invasion assays. BR1936 and BR1367 had about 2.5-fold increased invasion compared to BR0869 (Figure 3A). BR0851L was not significantly more invasive in comparison to BR0869 whereas BR1126 was almost 2-fold more invasive than BR0869 (Figure 3A). We performed FACS sorting on BR1936, BR1367, and BR0851L to isolate ROR1+ and ROR1- cells
using the similar gating strategy as before (Figure 2C). Sorted ROR1$^+$ and ROR1$^-$ breast cancer cells were analyzed for their invasive capacity by cell invasion assays. ROR1$^+$ (filled bars) cells invaded Matrigel 2- to 3-fold times more than ROR1$^-$ (open bars) cells (Figure 3B).

To determine the tumorigenic capacity of the five breast PDX models, we injected female rag/γc-/- mice with breast cancer cells isolated from the PDX models and monitored their tumor sizes for 7 weeks. BR1936 and BR1367 grew most rapidly out of the 5 breast cancer types whereas BR0851L, BR1126, and BR0869 grew more slowly and formed smaller tumors in comparison to BR1936 and BR1367 (Figure 4A,B).

Since BR1936 and BR1367 had the highest ROR1 expression of the 5 PDX models (Figure 1A,B), we wanted to see if ROR1 expression was associated with tumorigenicity, which is another CSC characteristic (Al-Hajj et al., 2003; Ginestier et al., 2007). By FACS sorting for ROR1$^+$ and ROR1$^-$ cells, we isolated viable breast cancer cells for 4 of the 5 PDX models (Figure 4C). Different cell numbers were then injected into the mammary pad of female rag/γc-/- mice and monitored for tumor growth for 8 weeks. Mice injected with ROR1$^+$ cells had increased tumor incidence compared to mice injected with ROR1$^-$ cells in each of the examined PDX models (Figure 4D). Taken together, these results demonstrate an association between ROR1 expression and CSC characteristics which supports our characterization of ROR1 as cancer stem cell marker in breast cancer.
UC-961 can inhibit tumor cell invasion, stem cell self-renewal, and tumor engraftment

In a previous study, our lab had shown that ROR1 can be targeted with a humanized antibody, UC-961, in ovarian cancer (Zhang et al., 2014). Here, we wanted to examine the effect of UC-961 on breast cancer cell invasion, self-renewal, and tumor engraftment. Using breast cancer cells isolated from two of the highest ROR1-expressing breast cancer PDX models (BR1936 and BR1367), we performed Matrigel invasion assays by treating cells with UC-961 and PBS (control). UC-961 treated BR1936 and BR1367 cells had about 60% reduction in cell invasion compared to control treated cells (Figure 5A). After culturing BR1936 cells in sphere culture, we dissociated the spheres back into single cells and examined their capacity to form spheres again, which is the ability to self-renew. Using the Extreme Limiting Dilution Analysis (ELDA) software (Hu et al., 2009), we determined that 1 of every 97 control treated BR1936 cells had the ability to self-renew whereas 1 of every 203 UC-961 treated cells had the ability to self-renew (Figure 5B). To determine the effect of UC-961 on tumor engraftment, we injected the mammary pads of female rag/yc-/- mice with BR1936 cells and then treated the mice, intravenously, with UC-961. We found that UC-961 treated mice formed significantly smaller tumors compared to control (PBS) mice where the tumor weights were half of the control mice (Figure 6).

UC-961 in combination with Taxol synergistically inhibits breast cancer cell line growth
Current treatments for breast cancer involve chemotherapy drugs such as Taxol. While Taxol is capable of eliminating the bulk of the tumor, a small population of CSCs still remains and can repopulate the tumor. In the present study, ROR1-expression seems to associate with CSC characteristics such as increased tumorigenicity among others. Thus we wanted to determine what effect UC-961, which targets ROR1-expressing cancer cells, would have in combination with Taxol on breast cancer cells. We performed BrdU ELISA assays on the breast cancer cell line HS578T to measure cell growth rates after treatment. A combination of various drug doses was used for UC-961 (0 µg, 15 µg, 30µg, and 60µg) and Taxol (0 nM, 30 nM, 100 nM, and 300 nM). The percent inhibition for the various drug combinations were then plotted (Figure 7). Using the Bliss independence model, we analyzed the interaction between UC-961 and Taxol (Zhao et al., 2014). Synergistic interactions were found between all doses of UC-961 and 300 nM Taxol at the 5% significance level.

**UC-961 in combination with Taxol inhibits breast cancer self-renewal and tumor formation more so than either agent alone**

We found that the self-renewal capacity of BR1936 was further inhibited when UC-961 was combined with Taxol than with either agent alone in vitro (Table 2). Using the ELDA software, 1 of every 31 control (hlgG) treated BR1936 cells had the ability to self-renew (Table 2). On the other hand, 1 of every 84 UC-961 and 1 of every 97 Taxol treated cells had the ability to self-renew (Table 2). More importantly, we found that UC-961 in combination with
Taxol was capable of inhibiting self-renewal by a factor of 4 compared to either agent alone and by a factor of 13 compared to control treated cells where 1 of every 404 cells had the capacity to self-renew (Table 2).

Next we sought to determine the effect of UC-961 and Taxol in vivo. BR1936 cells were injected into 6-12 week old female rag/γc-/- mice. When tumor sizes reached 0.2cm³, mice were intravenously treated with UC-961 alone (given once weekly), Taxol alone (given once a day for 5 consecutive days), or in combination. Control tumors formed larger tumors most rapidly whereas UC-961 treated tumors were slightly delayed in their growth (Figure 8A). Taxol treated tumors did not start grow in size again until 10 days after treatment stopped (Figure 8A). The combined treatment of UC-961 and Taxol, however, was capable of inhibiting tumor formation in mice for more than 20 days after treatment (Figure 8A). Analysis of these treated tumors by western blot show decreased ROR1 expression when tumors are treated with UC-961 alone (Figure 8B). In addition, ROR1 expression was greatly increased when tumors were treated with Taxol alone whereas ROR1 expression for UC-961 in combination with Taxol treated tumors did not increase (Figure 8B).

Cells isolated from the treated tumors were retransplanted into mice and the tumors were allowed to form without additional treatment. After 45 days, mice were sacrificed and tumors were extracted. In the control group, 5 out of 5 mice formed tumors (Figure 8C). UC-961 or Taxol treated mice formed tumors in 2 out of 5 and 3 out of 5 mice, respectively (Figure 8C).
Moreover, UC-961 in combination with Taxol prevented tumor formation in all 5 mice (Figure 8C).

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DISCUSSION

Our study in breast cancer characterizes ROR1 as a cancer stem cell (CSC) marker in promoting sphere growth, cell invasion, cancer stem cell self-renewal, and tumorigenesis. Breast stem cell markers such as CD44+/CD24- and ALDH1 activity have been documented in several reports (Al-Hajj et al., 2003; Stingl 2008, Ginestier et al., 2007). However, these markers are expressed normal stem cells in addition to CSCs (Ghebeh et al., 2013). As a result, therapies against these markers could also harm normal cell function leading to inefficient elimination of cancer cells and unwanted toxicities. On the other hand, studies have shown that ROR1 is differentially expressed on cancer cells but not on their respective normal cells (Baskar et al., 2008; Borcherding et al., 2014).

Analysis of previously established CSC markers, CD44+/CD24- and ALDH1 activity, on breast cancer PDX models revealed that ROR1 is co-expressed with the CSC markers giving evidence that ROR1 is expressed on cancer stem cells. Interestingly, we could not detect CD44+/CD24- population in BR1936 even though it expressed the highest ROR1 levels. These findings highlight the complexities of tumor heterogeneity in that the phenotype of each tumor is uniquely shaped by the tumor’s microenvironment (Mannello 2013; Marusyk et al., 2010; Chen et al., 2015). In addition, the findings suggest that ROR1 could be used as another marker to isolate CSCs together with markers such as CD44+/CD24- and ALDH1 activity.
Previous studies found that CSC populations have increased sphere formation, cell-invasion, and tumorigenic capacity (Liu et al., 2012; Sheridan et al., 2006; Scheel et al., 2012; Charafe-Jauffret et al., 2013). Sphere formation has been established as a characteristic of cancer stem cells (CSCs) in various cancers such as brain and breast (Reynolds et al., 1996; Dontu et al., 2003). Spheres have been shown to contain stem cell-like features such as self-renewal and differentiation, which makes sphere formation a valuable tool to assess CSC properties (Liu et al., 2011; Weiss et al., 1996). Similarly, when breast cancer cells were cultured as spheres, we found that the expression of ROR1 increased and that ROR1^+ breast cancer cells had greater sphere forming capacity compared to ROR1^- cells indicating a potential role of ROR1 in maintaining the CSC population. CSCs have also been thought to be responsible for cancer metastasis. Several studies have indicated that CSCs can be created through epithelial-mesenchymal transition (EMT) and that CSCs have increased capacity to invade the extracellular matrix, migrate to another location, and repopulate the tumor (Morel et al., 2008; Mani et al., 2008; Kong et al., 2011). Our data confirms a study on ovarian cancer stem cells where ROR1^+ cells had increased capacity to form spheres, invade Matrigel, and form tumors compared to ROR1^- cells (Zhang et al., 2014).

Collectively, these data suggest that breast cancer stem cells express ROR1. Further, we showed that breast cancer cells expressing ROR1 can be targeted with a monoclonal antibody, UC-961, to inhibit cell-invasion, CSC self-renewal, and tumor engraftment. Although UC-961 was capable of
inhibiting these CSC properties by about 50%, some tumor cells could still form tumors with sizes comparable to control treated mice. While UC-961 may be able to target the small population of CSCs, it may be unable to target the majority of cells that are already rapidly proliferating and do not express ROR1. The result would be a delay in tumor growth rather than the elimination of growth. Our research supports studies that have targeted ROR1 with antibodies to inhibit ROR1⁺ leukemia cell engraftment as well as ovarian cancer sphere formation and engraftment (Widhopf et al., 2014; Zhang et al., 2014).

Chemotherapy drugs such as Taxol can eliminate the majority of the tumor, but a small population of CSCs is not eradicated. Since CSCs have the capacity to differentiate into various cell types, they are capable of repopulating the tumor, thus leading to cancer recurrence and drug resistance (Yardley, 2013; Marquette et al., 2012). Studies have shown that Taxol can induce the phosphorylation of downstream pathways which activates Y-box binding protein 1 (YB-1). YB-1 acts as a transcription factor to regulate the transcription of genes including CD44 and others involved with drug resistance such as the multidrug resistance protein 1 (MDR1) (To et al., 2010; Bourguignon et al., 2008). Moreover, Taxol induction of CD44 resulted in enhanced CSC features such as self-renewal and sphere growth (To et al., 2010).

Consistent with studies that have shown that Taxol can enhance the CSC population, we found that Taxol elevated ROR1 levels whereas Taxol
combined with UC-961 brought ROR1 levels back down in vivo (Larzabal et al., 2013). It is possible that because Taxol eliminates the rapidly proliferating cells and enhances ROR1 levels, UC-961 treatment becomes more effective at targeting ROR1-expressing cells. Thus resulting in the synergy observed in vitro where cell growth and self-renewal were further inhibited by UC-961 and Taxol. It is not clear, however, whether the enhanced ROR1 levels are due to the induction of ROR1 gene transcription and translation or the enrichment for ROR1 expressing cells that are more drug resistant thereby surviving Taxol treatment whereas non-ROR1 expressing cells are eliminated. In addition, when cells from the various treated tumors were re-engrafted into new mice, we found that the cells from combined treatment were unable to form tumors. Due to the small number of mice, however, future studies would be needed using a larger number of mice to validate these findings.

In summary, the present study suggests that in breast cancer, ROR1 expresses cancer stem cell characteristics which can be targeted using a monoclonal antibody, UC-961. Furthermore, combining a chemotherapy agent, Taxol, with UC-961 can inhibit tumor cell growth and prevent tumor engraftment. Understanding the mechanisms behind how Taxol enhances ROR1 and how UC-961 can decrease ROR1 in cancer cells will provide novel strategies for cancer treatment.
Acknowledgements

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Figure 1: Relative ROR1 expression on breast PDX models.

Five breast cancer patient-derived xenografts (PDX) were grown in mice until tumors formed. Single viable cells isolated from the tumors were analyzed by (A) flow cytometry or (B) western blotting for ROR1 expression. B-actin is used as a loading control.
Figure 2: ROR1 expression is associated with increased sphere formation.

(A) Isolated single cells from BR1936, BR1367, BR0851L, BR1126, and BR0869 were cultured and the average number of spheres formed were counted (right). Representative images of spheres that formed are shown (left). Statistical significance is indicated by asterisks (*). (*P<0.05, **P<0.01, ***P<0.001, using Student’s t-test, n=4 for each cell type). Error bars indicate SEM. (B) Immunoblot analyses of cell lysates prepared from primary tumors (BR1936, BR1367, and BR0851L), and primary tumor cells cultured as spheres. Cell lines Jeko-1 (ROR1+) and Ramos (ROR1-) were used as positive and negative controls, respectively. Blots were probed for ROR1 and β-actin. (C) Sorting strategy to isolate ROR1+ versus ROR1- cells. Isotype control (upper contour plot) and sorted sample (lower contour plot) are shown. Open boxes are the gates used to obtain ROR1- (left) or ROR1+ (right) cells. (D) Sorted ROR1+ and ROR1- cells isolated from BR1936, BR1367, or BR0851L were cultured as spheres. Representative images of spheres formed are shown (left). Bar graph (right) indicates average numbers of spheres formed in three separate culture wells. Filled bars indicate ROR1+ cells. Open bars indicate ROR1- cells. Statistical significance is indicated by asterisks (*). (*P<0.05, **P<0.01, using Student’s t-test, n=3 for each cell type). Error bars indicate SEM.
Figure 3: ROR1 expression is associated with increased invasiveness.

(A) Matrigel cell invasion assay was performed on isolated single cells from BR1936 (black bar), BR1367 (grey), BR0851L (white), BR1126 (checkered), and BR0869 (striped). Representative cell invasion images (left) and relative invasion % bar graph (right) are shown. Statistical significance is indicated by asterisks (*) in comparison to BR0869. (*P<0.05, **P<0.01, ***P<0.001, using Student’s t-test, n=4 for BR1936, BR1367, BR0851L; n=2 for BR1126, BR0869). (B) Matrigel cell invasion assay was performed on ROR1+ and ROR1- sorted cells for BR1936, BR1367, and BR0851L. Representative images (left) and relative invasion % bar graph (right) are shown. Statistical significance is indicated by asterisks (*). (*P<0.05, **P<0.01, using Student’s t-test, n=2 for each cell type)
Figure 4: ROR1 expression is associated with tumorigenicity.

Female rag/yc-/- mice were injected in the mammary pad with $1 \times 10^6$ cells from BR1936, BR1367, BR0851L, BR1126, and BR0869. Tumor sizes were measured twice weekly and (A) a graph of tumor sizes for each breast PDX model is shown. (B) Images of tumors extracted from each breast PDX model (n=5). Single cells isolated from BR1936, BR1367, BR0851L, and BR1126 were sorted for ROR1$^+$ and ROR1$^-$ cells by FACS. (C) Gating strategy for ROR1$^+$ and ROR1$^-$ cells is shown. Dead cells were excluded by PI negative staining and live cells were included by FDA positive staining. Mouse cells were excluded from sorting by EpCAM positive staining (indicating human epithelial cells). (D) Different cell numbers of viable ROR1$^+$ and ROR1$^-$ sorted cells were implanted in the mammary pad of female rag/yc-/- mice and monitored for tumor growth for 8 weeks. Tumor sizes sizes $\geq 0.2 \text{cm}^3$ were considered as positive for tumor growth. The table indicates the number of mice which had tumor growth out of the total number of mice implanted with either ROR1$^+$ or ROR1$^-$ cells. The frequency of tumorigenic cell and P-value was calculated using ELDA software. N.D., not done.
Figure 5: UC-961 can inhibit tumor cell invasion and self-renewal.

Single viable cells isolated from BR1936 and BR1367 tumors were treated with PBS (control) or 50ug/ml UC-961 for 15 min on ice. (A) Matrigel cell invasion assays were then performed in duplicate for each tumor type. Invaded cells were counted 15 hours later and stained with Diff-Quick staining kit. Representative images of invasion are shown for each tumor type; with upper image (control) and middle image (UC-961 treated). The bar graphs indicate the percent relative invasion comparing UC-961 treated cells to control treated cells. Bars were normalized to control treated cells set as 100%. Statistical significance is indicated by asterisks (*). (*P<0.05, using Student’s t-test, n=2 for each treatment). (B) 1st passage BR1936 spheres dissociated into single cells were treated with PBS (control) or 50ug/ml UC-961 for 15 min on ice. Treated cells were plated at different cell numbers (1 to 1000) in MEGM for 1.5 weeks. The number of wells that contained spheres that were greater than 50um was counted. Sphere-initiating cell frequency and P-value was calculated using the ELDA software.
Figure 6: UC-961 can inhibit breast cancer tumor engraftment

Female rag/yc-/- mice were injected into the mammary pad with 5×10^4 BR1936 cells. Once tumor sizes reached 0.2 cm³, mice were treated with PBS or 10 mg/kg of UC-961 once a week, intravenously. Mice were sacrificed one month later. Tumors were extracted, weighed (lower panel), and imaged (upper panel). Statistical significance is indicated by asterisks (*). (*P<0.05, using Student’s t-test, n=10 for each treatment).
Figure 7: UC-961 in combination with Taxol synergistically inhibits breast cancer cell line growth

HS578T cells, which expresses ROR1, were treated with UC-961 alone, Taxol alone, or in combination for 72 hours at different concentrations. BrdU incorporation assays were performed.
Figure 8: UC-961 in combination with Taxol inhibits tumor formation.

(A) BR1936 (5x10⁴) cells were injected into the 2nd mammary pad of 6-12 week old female rag/γc-/- mice. Once tumor sizes reached 0.2cm³, treatment with 10 mg/kg UC-961 (once weekly), 13.4mg/kg taxol (once a day for 5 consecutive days), or in combination was given intravenously (n=10 mice per group). Tumor sizes were measured three times a week. Arrow indicates the start of treatment. (B) Control (PBS), UC-961, and taxol treated tumors (n=3 per group) were analyzed by western blot for ROR1 and β-actin. (C) Isolated breast cancer cells (1x10⁵) from UC-961 and taxol treated tumors were retransplanted into rag/γc-/- mice. Tumors were allowed to form with no treatments given to the mice for 45 days. Image of formed tumors are shown (n=5 for control and UC-961; n=3 for Taxol and UC-961+Taxol groups).
Acknowledgements

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Table 1: ROR1 expression associates with breast cancer stem cell markers.

Single cells isolated from 5 breast PDX tumors were analyzed by flow cytometry. (A) The percentage of ALDH^+^, CD44^+/CD24^−^, and total CD44^+^ expression. (B) ROR1 expression (MFIR) on ALDH^+, ALDH^−^, CD44^+/CD24^−^, and non-CD44^+/CD24^−^ populations. (C) The percentage of ALDH1 or CD44^+/CD24^−^ expression on ROR1^+^ and ROR1^−^ populations.

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<td>26.6</td>
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</table>
Table 2: UC-961 combined with Taxol inhibits breast cancer self-renewal.

1\textsuperscript{st} passage BR1936 cells were dissociated and treated with 50ug/ml hlgG, 50ug/ml UC-961, and or 3nM Taxol for 15min on ice. Treated cells were plated at different cell numbers in MEGM for 1.5 weeks. Cells were retreated with 50ug/ml hlgG and UC-961 or MEGM on the 3\textsuperscript{rd} day. The number of wells that contained spheres that were greater than 50um was counted. Sphere-initiating cell frequency and P-value was calculated using the ELDA software.

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<th>P-Value in stem cell frequencies between any of the groups</th>
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Acknowledgements

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