Treatment With Paclitaxel Enriches Breast Cancer Xenografts For ROR1$^+$ Cancer Stem Cells

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Introduction

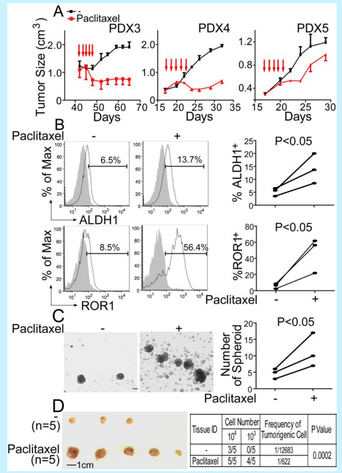
Despite recent advances, many patients with breast cancer relapse after apparently responding well to chemotherapy, often developing metastatic disease. This is thought due to the post-treatment survival of a small number of breast cancer stem cells (CSC), which are relatively resistant to chemotherapy, have self-renewal capacity, can repopulate the tumor, and spread to distant sites. ROR1 is a type I orphan-receptor tyrosine-kinase-like surface protein that is expressed during embryogenesis and by various cancer cells, but not on normal adult tissues. Its expression is associated with less-well-differentiated tumors with high metastatic potential (Zhang et al *Am J Pathol*, 2012, PMCID 3509760; Cui et al *Cancer Res*, 2013, PMCID 3832210). Conceivably, ROR1 also is expressed by CSC, as has been demonstrated recently for ovarian CSC (Zhang et al PNAS, 2014, PMCID 4260559).

In this study, we evaluated the expression of ROR1 in human primary breast cancer stem cells and breast cancer cells that received paclitaxel treatment. We also examined the activity of newly developed anti-ROR1 monoclonal antibodies (mAbs, e.g. UC-961) that was found to have activity against ROR1-expressing ovarian cancer cells and B-cell leukemia.

Materials and Methods

Tumorigenicity Assay

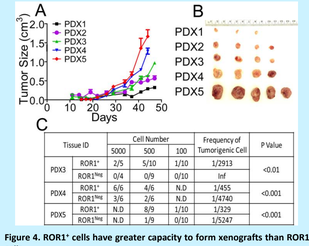
Various numbers of FACS-purified cells or cultured cell lines were suspended in Mammary-Epithelial Growth Medium (MEGM), mixed with Matrigel (BD Biosciences, San Diego, CA) at 1:1 ratio and then transplanted into mammary pad of NOD/SCID mice. We monitored the mice weekly for development of tumors. To test effect of UC-961 alone or in combination with paclitaxel on the engraftment of primary breast tumor cells, 1X10^6 single cells isolated from PDX5 were injected into the mammary pad of 4- to 6-week-old Rag2$^{-/-}\gamma_c^{-/-}$ mice. When tumor size reached 200 mm^3, 14.6 mg/Kg paclitaxel was injected intravenously for consecutive 5 days or/and 10mg/Kg of UC-961 was injected intravenously biweekly.



Tissue ID	Cell Number 10^4	10^3	Frequency of Tumorigenic Cell	P Value
-	3/5	0/5	1/12683	0.0002
Paclitaxel	5/5	4/5	1/822	

Figure 1. Evaluation of PDX after paclitaxel treatment for expression of ROR1 and ALDH1

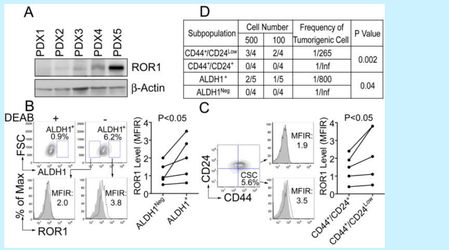
(*A*) 1X10^6 single cells from PDX tumors from 3 patients were implanted into the mammary pad of female mice (n=5 per patient). 13.4 mg/Kg paclitaxel was injected i.v., at times indicated by the red arrows. Tumor growth was monitored over time. Data shown are the mean tumor sizes for each group ± SEM (n=5). (*B*) A single cell suspension isolated from PDX4 tumors removed from untreated mice (-), or from mice treated with paclitaxel (+), were examined for expression of ALDH1 enzymatic activity (upper panel) or ROR1 (lower panel) via flow cytometry. The mean proportions of cells expressing ALDH1 or ROR1 in different PDX tumors (PDX3-5) from untreated mice or mice treated with paclitaxel are indicated in each histogram with a line connecting the values to each PDX. DEAB serves as ALDH1 inhibitor. P<0.05, as determined by Student's t-test (n=3). (*C*) Equal numbers of cells isolated from tumors of untreated mice versus mice treated with paclitaxel were assessed for their capacity to form spheroids. Representative photomicrographs of spheroids were shown in the left panel. Right panel provides the numbers of spheroids formed from three individual tumors (PDX3, PDX4, or PDX5, from mice bearing PDX that were untreated or treated with paclitaxel. Spheroids with sizes greater than 100 µm were counted via microscopy. Scale bar: 100 µm. P<0.05, as determined by Student's t-Test (n=3). (*D*) Single cells isolated from PDX4 removed from untreated mice, or from mice that received treatment with paclitaxel, were re-implanted into the mammary pad of female Rag2$^{-/-}\gamma_c^{-/-}$ mice (n= 5). Tumor incidence in animals of each group (n=5) was recorded. Right panel provided representative photographs for tumors removed from mice that were untreated (control) or treated with paclitaxel. Frequency of tumorigenic cells and probability estimates provided in the right panel were computed using Extreme Limiting Dilution Analysis (ELDA) software.



Tissue ID		Cell Number 5000	500	100	Frequency of Tumorigenic Cell	P Value
PDX3	ROR1$^+$	2/5	5/10	1/10	1/2913	<0.01
	ROR1^{Neg}	0/4	0/9	0/10	Inf	
PDX4	ROR1$^+$	6/6	4/6	N.D	1/455	<0.001
	ROR1^{Neg}	3/6	2/6	N.D	1/4740	
PDX5	ROR1$^+$	N.D	8/9	1/10	1/329	<0.001
	ROR1^{Neg}	N.D	1/9	0/10	1/5247	

Figure 4. ROR1$^+$ cells have greater capacity to form xenografts than ROR1^{Neg} cells

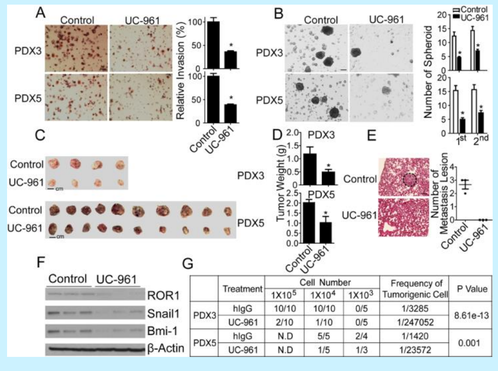
(*A*) 1X10^6 cells from each PDX sample in 50 µl were mixed with equal volumes of Matrigel and then injected into the mammary pad of female Rag2$^{-/-}\gamma_c^{-/-}$ mice. Tumor growth was monitored over time for 48 days. (*B*), Representative photographs of each PDX removed at 48 days, as described in (*A*). (*C*) Tumor incidence in animals implanted with ROR1$^+$ or ROR1^{Neg} cells isolated from each of the various breast PDX. Frequency of tumorigenic cells and probability estimates were computed using ELDA software. N.D indicates not done.



Subpopulation	Cell Number 500	100	Frequency of Tumorigenic Cell	P Value
CD44$^+$/CD24^{Low}	3/4	2/4	1/265	0.002
CD44$^+$/CD24$^+$	0/4	0/4	1/Inf	
ALDH1$^+$	2/5	1/5	1/800	0.04
ALDH1^{Neg}	0/4	0/4	1/Inf	

Figure 2. ROR1 expression is associated with breast CSC markers

(*A*) Immunoblot analysis for ROR1 in cell lysates of each of five PDX tumors using anti-rabbit anti-ROR1 antibody. β-Actin served as loading control. (*B-C*), Flow cytometric analysis of breast PDX samples. (*B*) Cells from primary tumors were stained with 4A5 or control mAb, and with ALDOFLUOR without (-) or with (+) the ALDH1-inhibitor, DEAB, as indicated at the top. The open boxes in the each contour plot indicate the gates used for identifying cells with ALDH1 activity, the proportions of which are indicated. The open boxes in the left of the contour plots depict the gates used to identify cells that are certain to lack ALDH1 activity. In the bottom row are histograms depicting the fluorescence of cells within these boxes that were negative (left) or positive (right) for ALDH1 activity. Right panel provides the staining intensity for ROR1 in ALDH1$^+$ versus ALDH1^{Neg} cells from each of five different PDX tumors. (*C*) Cells were stained with CD44, CD24, 4A5, or control mAb. CD44$^+$/CD24^{Low} or CD44$^+$/CD24$^+$ cells were analyzed further for ROR1. The shaded histograms depict the fluorescence of cells stained with an isotype-control mAb, whereas the open histograms depict the fluorescence of cells stained with 4A5. Right panel provides ROR1 staining intensity of CD44$^+$CD24^{Low} versus CD44$^+$CD24$^+$ cells from each of five different PDX. The number in each plot provides the mean fluorescence intensity ratio (MFIR) for ROR1, which is derived from the mean fluorescence intensity (MFI) of cells labeled with the anti-ROR1 mAb divided by MFI of cells labeled with an antibody of irrelevant specificity. (*D*), CD44$^+$/CD24^{Low} versus CD44$^+$/CD24$^+$ cells or ALDH1$^+$ versus ALDH1^{Neg} cells isolated from PDX4 or PDX5 were implanted into mammary pad of immune-deficient mice (n=4-5). The numbers of mice with tumors 2 months after engraftment over the number of mice injected in each group are shown in the table. Frequency of tumorigenic cells and probability estimates were computed using ELDA software.



	Treatment	Cell Number 1X10^5	1X10^4	1X10^3	Frequency of Tumorigenic Cell	P Value
PDX3	hIgG	10/10	10/10	0/5	1/3285	8.61e-13
	UC-961	2/10	1/10	0/5	1/247052	
PDX5	hIgG	N.D	5/5	2/4	1/1420	0.001
	UC-961	N.D	1/5	1/3	1/23572	

Figure 5. ROR1 mAb is able to inhibit tumor cell invasion, cancer stem cell self-renewal, tumor growth, and metastasis

(*A*) Representative photomicrographs of cells invading Matrigel; the cells were either treated with control hIgG (Control) or UC-961 at 50 µg/ml. To the right of the photomicrographs are bar graphs depicting the mean relative invasion of cells into Matrigel (±SEM) of each of the cell preparations in three independent experiments, normalized to control treated cells. Scale bar: 10 µm. (*B*) Spheroid assay for cells isolated from PDX3 or PDX5 and then treated with control hIgG or UC-961 at 50 µg/ml. The ability of the spheres treated with either control antibody or UC-961 to form secondary spheroids also is shown (2^{ns}). Spheroids (>100 mm) were counted under a stereomicroscope. Scale bar: 100µm. (*C-E*), 1 x 10^6 single cells from either PDX3 or PDX5 were implanted into mammary pads of Rag2$^{-/-}\gamma_c^{-/-}$ mice. 10 mg/Kg UC-961 or control hIgG were injected i.v biweekly, starting at two weeks post-transplantation. Photographs of tumors from mice treated with either UC-961 or control antibody were shown in (*C*). (*D*) The bar graph provides average weight of tumors from each group ± SEM (n=4-10). (*E*) HE staining of lung tissue from a representative tumor-bearing mouse that had been engrafted with cells of PDX5 via injection into the mammary pad. The scatter plot shows average numbers of metastatic foci that were found in the lungs of each of animal in each group (±SEM, n = 3). Scale bar: 100 µm. (*F*) Lysates of cells isolated from PDX5 in mice that received either control hIgG or UC-961 were examined by immunoblot for proteins as indicated on the right margin. (*G*) Single tumor cells isolated from tumors of mice that were treated with either control hIgG or UC-961 were re-implanted into the mammary pad of Rag2$^{-/-}\gamma_c^{-/-}$ mice. The numbers of mice that had tumors at two-months after implantation over the numbers of mice injected in each group are shown in the table. The frequency of tumorigenic cells and p-values were computed using ELDA software. *indicates P<0.05, as determined by Student's t-Test.

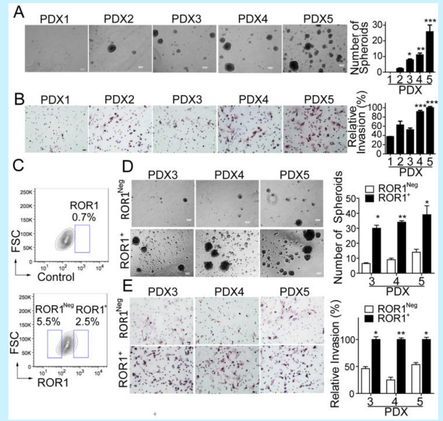
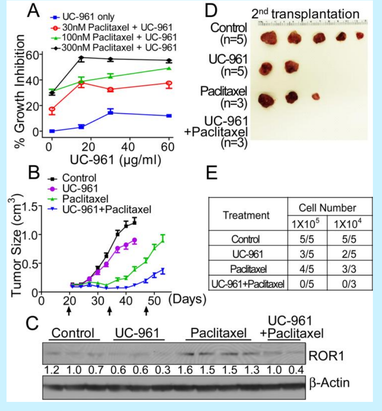


Figure 3. ROR1$^+$ cells have greater capacity to form spheroids or to invade Matrigel

(*A*) Photomicrographs of spheroids that developed from cultured cells isolated from each of the various PDX primary tumors (Scale bar: 100 µm). The bar graph to the right depicts the average numbers of spheroids formed by cells from each PDX in triplicate wells ± SEM. Asterisks (*) indicate the statistical significance of differences in the number of spheroids of cells from different PDX samples compared with PDX1 (* for <0.05, ** for <0.01, *** for <0.001 using Dunnett's multiple comparison test). (*B*) Representative photomicrographs of invaded cells from isolated tumor cells of different PDX. To the right of the photomicrographs are bar graphs depicting the mean relative invasion of cells into Matrigel (±SEM) of each of the cell preparations in three independent experiments, normalized to that of the cells from PDX5. Scale bar: 10 µm. (*C*) Strategy for sorting ROR1$^+$ versus ROR1^{Neg} cells. The open boxes indicate the gates used to select ROR1^{Neg} (left) or ROR1$^+$ (right) cells. (*D*) Photomicrographs of spheroids formed from ROR1$^+$ or ROR1^{Neg} cells isolated from each of the PDX, as indicated on the top. Scale bar: 100 µm. The bar graph to the right depicts the average numbers of spheroids formed ± SEM by each of the cell preparations in three separate cultures, as indicated at the bottom of the histogram. (*E*) Photomicrographs of Matrigel-invading cells from ROR1$^+$ or ROR1^{Neg} cells isolated from different PDX, as indicated on the top. Scale bar: 10 µm. The bar graph to the right depicts the mean relative invasion of cells into Matrigel (±SEM) for each of the cell preparations in three independent experiments, normalized to that of the cells from PDX5. * for P<0.05, ** for P<0.01, using Student's t-Test.



Treatment	Cell Number 1X10^5	1X10^4
Control	5/5	5/5
UC-961	3/5	2/5
Paclitaxel	4/5	3/3
UC-961+Paclitaxel	0/5	0/3

Figure 6. The effect of ROR1 mAb in combination with paclitaxel on breast cancer growth

(*A*) ROR1 mAb in combination with paclitaxel had significantly greater effect on tumor growth than either treatment with paclitaxel or UC-961 alone. ROR1$^+$ HS578T cells were treated with UC-961, paclitaxel, or the combination of UC-961 and paclitaxel for 72 hours. Brdu incorporation assay was performed on treated or untreated cells. Data are shown as the mean (±SEM) percentage of cell growth inhibition of quadruplets samples from each of the treatments. (*B*) 1 x 10^6 single cells from PDX5 were implanted into mammary pads of female mice. 10 mg/kg UC-961 or 13.4 mg/Kg paclitaxel were injected intravenously. Tumor growth was monitored over time. (*C*) Immunoblot analysis using lysates from tumor cells extracted from mice that received treatment as indicated by the legend on the top. The band densities were measured with Image J, normalized to β-actin, are indicated in each lane. (*D*) Single tumor cells isolated from PDX5 of mice that received either no treatment or UC-961, paclitaxel, or both UC-961 and paclitaxel were re-engrafted into the mammary pads of Rag2$^{-/-}\gamma_c^{-/-}$ mice. The numbers of mice that had tumors after two-months were divided by the numbers of mice injected in each group are shown in the table.

Results

- Treatment with paclitaxel enriches for cells that express ROR1 and could form spheroids or re-engraft immune-deficient mice
- Expression of ROR1 in primary breast cancer cells is associated with expression of markers of CSC (e.g. ALDH1)
- ROR1+ breast cancer cells have features of CSC
- Breast cancer cells that express ROR1 have enhanced capacity to engraft immune-deficient mice
- UC-961 specific for ROR1 could inhibit the capacity of breast cancer cells to form spheroids or engraft immune-deficient mice
- Treatment of breast cancer cells with a humanzied anti-ROR1 mAb, UC-961, suppressed expression of the polycomb-ring-finger oncogene, BMI1, and proteins implicated in the epithelial-mesenchymal transition
- Treatment with paclitaxel and UC-961 is more effective against breast cancer xenografts than treatment with either agent alone

Conclusion

Combined treatment with UC-961 and paclitaxel was more effective than either treatment alone in eradicating breast cancer PDX, suggesting that therapy directed at both CSC and non-CSC may improve the treatment outcome of patients with breast cancer

Acknowledgements

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