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CONTRACTING ORGANIZATION: University of Alabama at Birmingham
Birmingham, AL 36294

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14. ABSTRACT

Basal-like breast cancers (BLBC) are poorly differentiated and display an aggressive clinical behavior, which has been attributed to the presence of cancer stem cells (CSC). We have previously shown TRA-8, a monoclonal antibody to death receptor 5 (DR5), is an effective preclinical treatment for BLBC. This study examines the effect of TRA-8 alone and in combination with chemotherapy (Adriamycin and Taxol) on CSC populations from BLBC cell lines and patient samples. BLBC cell lines were sorted for CSC markers CD44+, CD24- and ALDHHigh and treated with TRA-8 +/- chemotherapy and examined for cytotoxicity, inhibition of tumorsphere formation and reduction in CSC maker expression after treatment. Combination treatment of BLBC cells with TRA-8 and Adriamycin or Taxol produced additive to synergistic cytotoxicity against sorted (CD44+/CD24-/ALDHHigh) cells and significantly inhibited tumorsphere formation ($p<0.001$). ALDH activity was also reduced following combination treatment ($p<0.001$). These results show that TRA-8 anti-DR5 antibody alone or in combination with chemotherapy killed the bulk and CSC population in BLBC.

15. SUBJECT TERMS
Breast cancer stem cells, tumor initiating cells, TRA-8, Death receptor 5, Adriamycin, Taxol, Notch

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- **B. Supporting Data**
- **C. Manuscript**

Introduction

Basal-Like Breast Cancer (BLBC) accounts for 13% of all breast carcinomas (1). It is characterized by a unique mRNA profile with CK5/6 expression, inactivation of *BRCA1* and lack of estrogen receptor and HER-2 amplification (1-3). BLBC is considered one of the most aggressive, metastatic, and chemoresistant breast cancer subtypes (4). Its poor prognosis is linked to enrichment for tumor initiating cancer stem cells (CSC) (5). The *Cancer Stem Cell Model* hypothesizes that tumors, similar to normal tissue, are organized in a cellular hierarchy, with CSC, as the initiating cells with potentially limitless proliferation abilities (6). The more 'differentiated' descendants, which account for the majority of the tumor population, may also be able to proliferate, but regenerative ability is limited (6). Traditional chemotherapy agents target these differentiated cells, but unfortunately fail to kill the stem cell progenitor population (7). The chemoresistant stem cells are thought to be responsible for recurrence and metastasis of many tumor types (7). Several investigators have shown that BLBC cell lines and patient samples contain a subpopulation of breast cancer stem cells (BrCSC) (8, 9). These BrCSC are identified based on their enhanced tumorigenicity, tumorsphere forming ability, expression of $CD44^+/CD24^-$, elevated enzymatic activity of aldehyde dehydrogenase (ALDH), and dysregulation of self-renewing pathways, including Wnt, Hedgehog, and Notch signaling (10, 11). BrCSC also overexpress ABC efflux transporters, detoxification enzymes, and have slower turnover rate that make this sub-population likely to become resistant to chemotherapy (12, 13). Thus an effective BLBC therapeutic strategy must kill these propagating and chemoresistant BrCSC in addition to the proliferating non-stem cell cancer population (14). Our group has previously shown TRA-8, a monoclonal antibody to death receptor 5 (DR5), as a single agent is an effective preclinical treatment for BLBC (15, 16). This study examines the effect of TRA-8 alone and in combination with chemotherapy (Adriamycin and Taxol) on CSC populations from BLBC cell lines. Considering recent clinical findings that chemotherapy enriches for BrCSC (7), I also investigated a novel non-chemotherapy combination approach to target basal-like BrCSC. In this regard gamma secretase inhibitors (GSI) block Notch pathways known to regulate self-renewal in BrCSC, they increase surface expression of DR5 (unpublished collaborators' results), and they block anti-apoptotic pathways regulated by Notch (17-19). Therefore, I hypothesize that pre-treatment of basal-like BrCSC with GSI will further enhance anti-DR5 mediated apoptosis by TRA-8 monoclonal antibody. Thus, my **central hypothesis** is that CSC obtained from BLBC cell lines and patient samples will be sensitive to apoptosis induced by anti-DR5 monoclonal antibody (TRA-8) as a single agent or in combination with standard chemotherapy or Notch inhibitors.

Specific Aims

Aim 1. To isolate $CD44^+/CD24^-/ALDEFLUOR^+$ cancer stem cells from established human breast cancer cell lines and patient plural effusion samples and evaluate their functional characteristics such as tumorigenicity, tumorsphere formation and chemoresistance.

Aim 2. To evaluate *in vitro* induction of apoptosis by anti-DR5 monoclonal antibody in BrCSC isolated from cell lines and patient samples, alone and in combination with chemotherapy agents (Adriamycin and Taxol), and a gamma secretase inhibitor (GSI).

Aim 3. To evaluate the DR5/DDX3/IAP apoptosis regulatory mechanism in populations of cells enriched for BrCSC isolated from basal and luminal cell lines.

Aim 4. To evaluate the *in vivo* therapeutic efficacy of anti-DR5 alone and in combination with Adriamycin, Taxol, or GSI against xenograft models established from BrCSC.

Body

Aim #1:

Task 1. To isolate $CD44^+/CD24^-/ALDEFLUOR^+$ cancer stem cells from established human breast cancer cell lines and patient plural effusion samples and evaluate their functional characteristics such as tumorigenicity, tumorsphere formation and chemoresistance.

Task 1a. Analyze sorted BrCSC population for retention of BrCSC markers (months 1-5)

- **Re-analyze sorted population 3 h, 12 h, 24 h, and 48 h after sorting**
- **Allow sorted cells to attach and analyze after every passage for four passages**
- **Implant sorted cells and analyze after 5-10 mm tumor is formed**

To address this task, I analyzed the tumorsphere forming ability of BrCSC. I sorted for $ALDH^+/CD44^+/CD24^-$ and $ALDH^-/CD44^+/CD24^-$ in 2LMP and SUM159 cell lines and evaluated tumorsphere formation at 24, 48 and 72 h time points (**Figure 1A**). Up to 48 h after plating BrCSC made more tumorspheres then the non-BrCSC. However by 72 h the $ALDH^-/CD44^+/CD24^-$ SUM159 cells started forming tumorspheres/aggregates. I hypothesized that this is due to the plating conditions enriching for BrCSC over time. To test this hypothesis, I sorted 2LMP and SUM159 for $ALDH^+$, $ALDH^-$ and total unseparated population. Cells were plated in low attachment plates in mammosphere media and analyzed by flow cytometry at 6, 12, 24, 48, and 72 h after plating (**Figures 1A, 1B**). The results indicate that marker retention is only conserved for 12 h and drops to the pre-sorted percentage by 48 h. Interestingly, the unlabeled total population also acquired CSC marker expression by 12 h and subsequently returned to the pre-sorted population marker expression levels. These results indicate that sorting is not necessary to enrich for CSC characteristics if experiments are conducted within 12 h. Also, treatments should not be carried out for more than 48 h because after this time point there is a loss of BrCSC maker expression. Initial attempts to analyze *in vivo* BrCSC markers did not prove conclusive.

Task 1b. Functional BrCSC assays (months 6-10)

- **tumorsphere formation of 2LMP, SUM159, T47D, BT-474, HCC1187, HCC1143 cell lines**
- **chemoresistance assay using tumorsphere and attached cell culture conditions**
- **implant of $ALDH^+/CD44^+/CD24^-$ sorted populations into NOD/SCID mice to verify tumorigenicity**
- **microarray analysis of sorted cells**

To address task 1b, I focused my studies on BLBC cell lines and expanded the list to include a total of 10 cell lines (SUM149, HCC38, 2LMP, SUM159, MB436, BT549, HCC1187, BT20, HCC70, HCC1143). CD44, CD24, and ALDEFLUOR marker expression was analyzed on all 10 cell lines (**Table 1**). All 10 BLBC cell lines grew under low attachment mammosphere media conditions and most produced a mixture of tumorsphere and aggregate morphologies by

48 h. Only one cell line, BT20 produced spheroids that had a smooth continuous outer membrane.

In vitro chemoresistance will be further described in aim 2 as part of the TRA-8 and chemotherapy combination studies. A more rigorous test of CSC chemoresistance was performed using an *in vivo* model. NOD/SCID mice were implanted ini the mammary fat pad with $2x10^6$ cells and treated IP with 2 mg/kg of Adriamycin every 7 days for a total of three treatments. The tumors were harvested 24 h after the last treatment. *In vivo* treatment with chemotherapy enriched for BrCSC (68% compared to 27% control) (**Figure 2**). Such results have been reported in patients clinically treated with chemotherapy, however this is the first report of an *in vivo* Adriamycin treated NOD/SCID mouse model to show that Adriamycin treated tumors were enriched for BrCSC.

Sorted $ALDH^+$ cells have been reported to be the most tumorigenic subpopulation in BLBC cell lines. $ALDH^{+/-}$ 2LMP cells were sorted and NOD/SCID mice were immediately implanted with 20,000, 5,000, 1,000 cells per mouse (4 mice per dilution, for a total 24 mice) and their tumor growth followed for 65 days (**Figure 3**). The results show that sorted $ALDH^+$ cells are enriched for tumorigenic cells that produce more rapidly growing and larger tumors than obtained with $ALDH^-$ cells. Microarray analysis of sorted cells was not conducted.

Aim #2:

(Task 2). To evaluate *in vitro* induction of apoptosis by anti-DR5 monoclonal antibody in BrCSC isolated from cell lines and patient samples, alone and in combination with chemotherapy agents (Adriamycin and Taxol), and GSI.

Task 2a. Chemotherapy + TRA-8 combination therapy (Taxol and Adriamycin) (month 11-13) For all combination treatments, I will sort $ALDH^+/CD44^+/CD24^-$ from 2LMP, SUM159, T47D, BT-474, HCC1187, HCC1143; treat in attached and tumorsphere assays. I will also plate parental unsorted populations and treat in attached conditions, then analyze surviving cells for BrCSC markers. Patient samples will be cut into 5 or 8 mm cores and slices. Pleural effusions will be treated in non-attached conditions similar to tumorsphere assay. The majority of patient samples will be treated with TRA-8 in combination with GSI.

It was important to evaluate whether normal breast cells and stem cells are affected by treatment with TRA-8. To this end, I tested TRA-8 sensitivity against the MCF10A normal breast cell line (**Figure 4**). There was no cytotoxic effect to the total population with TRA-8 alone or in combination with Taxol and Adriamycin, and the $ALDH^+$ cells were also unaffected by treatment (maintained 5%). *In vitro* evaluation of BrCSC anti-DR5 apoptosis was completed and fully described in the manuscript attached in Appendix C. Please refer to figures in the manuscript when reading through abbreviated results. DR5 expression was examined on $CD44^+/CD24^-/ALDH^+$ cells of the HCC1143, 2LMP, and SUM159 cell lines (**Manuscript Figure 1**). Sorted BrCSC exhibited comparable DR5 expression to unsorted parental population. I then investigated whether TRA-8 was triggering caspase 8 and 3 activation in BrCSC. **Manuscript Figure 2** shows a representative analysis of the 2LMP cell line with rapid induction of caspase 3 and 8 cleavage. A similar result was obtained with the SUM159 cell line. A comprehensive cytotoxicity analysis identified IC_{50} values for sorted and unsorted populations

(**Manuscript Table 1**). In 5/8 cell lines, the BrCSC enriched cells were significantly more sensitive than the parental cells. This was an unexpected finding.

Secondary tumorsphere formation was also examined to test if a CSC enriched population was also sensitive to TRA-8. Indeed, similar results were obtained with the sorted cells, where secondary tumorsphere formation was significantly inhibited by treatment with TRA-8 ($p<0.001$) (**Manuscript Figure 3**). *In vitro* treatment with TRA-8 decreased the percentage of ALDH+ cells in 2LMP and SUM159 BLBC cell lines. In contrast, treatment with chemotherapy (Adriamycin or Taxol) enriched for ALDH expression (**Manuscript Figure 4**). TRA-8 inhibited tumorigenicity of BrCSC in 2LMP orthotopic mouse models (**Manuscript Figure 5**). Only 2 of 5 tumors developed in the *ex vivo* TRA-8 (20 nM) treatment group and 5 of 5 tumors grew in the Adriamycin (500 nM) treatment group ($p<0001$). Similar results were obtained with the SUM159 cell line (data not shown).

Combination treatment of 2LMP cells with TRA-8 and Adriamycin or Taxol produced additive to synergistic cytotoxicity against sorted cells ($CD44^+/CD24^-/ALDH^+$) (**Figure 5A**). *In vitro* treatment with TRA-8 in combination with Taxol or Adriamycin produced a decrease in the percentage of BrCSC (**Figure 5B**). Patient pleural samples were characterized by immunohistochemistry for Adenocarcinoma (Moc31 positive staining) and non-mesothelial (negative Calretinin staining). Dissociated clusters from one patient were analyzed for TRA-8 sensitivity, and the IC_{50} value was ~100 ng/mL. Un-dissociated clusters were also pre-treated with Adriamycin for 24 h followed by 24 h incubation with TRA-8 showed minor combination effect (**Figure 6**).

Task 2b. Notch inhibition+TRA-8+GSI (months 14-16)

Not yet performed

Task 2c. TRA-8, chemotherapy plus GSI combination (months 17-19)

Not yet performed

Aim #3:

(Task 3) To evaluate the DR5/DDX3/IAP apoptosis regulatory mechanism in populations of cells enriched for BCSC isolated from basal and luminal cell lines. (months 19-25) **Complex evaluation will be done using Immunoprecipitation (IP) of DR5 followed by Western Blot analysis of DDX3 and cIAP to look at total levels of the protein. I will also use AlphaLISA, which is a very sensitive method to detect levels of protein expression, based on the magnitude of the fluorescence.**

Not yet performed

Aim #4:

(Task 4) To evaluate the *in vivo* therapeutic efficacy of anti-DR5 alone and in combination with Adriamycin, Taxol or GSI against xenograft models established from BSCS. (months 11-30)

a. Sort cells injected into MFP, allow tumors to grow, then treat with TRA-8 in combination with chemotherapy and GSI (months 11-25)

b. Parental cells injected into the MFP, allow tumors to grow, enrich for BrCSC with chemotherapy then treat with TRA-8 or GSI/chemotherapy combination (months 19-25)

c. Develop luciferase expressing cells from 2LMP, SUM159, T47D, BT-474, HCC1187, HCC1143 to test metastatic model of treatment (months 25-30)

Not yet performed

Key research accomplishments:

- BrCSC marker expression was shown to be maintained for ~24 h after sorting, and cells grown in mammosphere media/low attachment plates acquire elevated levels of ALDH activity by 12 h.
- Elevated ALDH activity can identify cells that are better able to form tumorspheres and are more tumorigenic.
- Established an *in vivo* chemotherapy treatment model in NOD/SCIDs that enrich for BrCSC markers.
- TRA-8 does not affect normal breast cells or normal breast stem cells.
- BrCSC enriched populations have DR5 expression similar to parental cells.
- TRA-8 treatment of basal-like BrCSC populations activates caspase 8 and 3.
- TRA-8 treatment of BrCSC populations dramatically inhibits tumorsphere formation.
- BrCSC from 5/8 cell lines were significantly more sensitive to TRA-8 then parental cells.
- Tumorigenicity of BrCSC was inhibited by treatment with TRA-8.
- Combination treatment with TRA-8 and Taxol produced synergistic cytotoxicity of parental and BrCSC tumorspheres.
- Combination treatment reduced BrCSC ALDH expression.
- A breast cancer patient pleural effusion sample was sensitive to TRA-8 and combination treatment.

Reportable outcomes:

Publications related to aims:

1. **Londoño Joshi AI**, Oliver PG, Li Y, Lee CH, Forero-Torres A, LoBuglio AF, Buchsbaum DJ: Basal-like breast cancer stem cells are sensitive to anti-DR5 mediated cytotoxicity. *Breast Cancer Res Treat*, Epub ahead of print, 2011.

Publications not related to aims:

1. Whitworth JM, **Londoño Joshi AI**, Sellers JC, Oliver PG, Muccio DD, Atigadda VR, Straughn JM, Jr, Buchsbaum DJ: The impact of novel retinoids in combination with platinum chemotherapy on ovarian cancer stem cells. *Gynecol Oncol* In press, 2012.

2. Bevis KS, McNally LR, Sellers JC, Della Manna D, **Londoño Joshi AI**, Amm H, Straughn JM, Jr, Buchsbaum DJ: Anti-tumor activity of an anti-DR5 monoclonal antibody, TRA-8, in combination with taxane/platinum-based chemotherapy in an ovarian cancer model. *Gynecol Oncol*, 121:193-199, 2011.

Awards:

1. UAB Comprehensive Cancer Center - John R. Durant Award for Excellence in Cancer Research- Graduate Student Category, 2011
2. UAB Department of Pathology - Betty Spencer Pritchett Award for Outstanding Cancer Research 2011
3. Susan G. Komen for the Cure Travel Scholarship - AACR annual conference Washington, DC. 2010
4. Howard Hughes Medical Institute - Science Education Alliance Teaching Assistant Award 2011. Janelia Farm Genomic Analysis Training travel award 2010

Other:
1. Attended annual meeting of American Association for Cancer Research, Orlando, 2011.
2. Successfully passed my doctoral candidacy examination
3. Please see additional reportable outcomes in *Curriculum Vitae* attached as Appendix A.

Conclusion:

The results show that TRA-8 anti-DR5 antibody alone or in combination with chemotherapy killed the bulk and CSC population in basal-like breast cancer cell lines and a patient sample. A multicenter phase II study of anti-DR5 antibody in combination with albumin-bound-Taxol (Abraxane) in patients with triple negative breast cancer has completed accrual. This preclinical study would predict that patients undergoing combination treatment might have an extended time to progression and increased survival compared to treatment with chemotherapy alone.

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14. Chuthapisith S, Eremin J, El-Sheemey M, Eremin O. Breast cancer chemoresistance: emerging importance of cancer stem cells. Surg Oncol 2010;19:27-32.
15. Oliver PG, Lobuglio AF, Zhou T, Forero A, Kim H, Zinn KR, Zhai G, Li Y, Lee, CH, Buchsbaum DJ. Effect of anti-DR5 and chemotherapy on basal-like breast cancer.Breast Cancer Res Treat. 2011 Sep 7. [Epub ahead of print]
16. Buchsbaum DJ, Zhou T, Grizzle WE, et al. Antitumor efficacy of TRA-8 anti-DR5 monoclonal antibody alone or in combination with chemotherapy and/or radiation therapy in a human breast cancer model. Clin Cancer Res 2003;9:3731-41.
17. Kruyt FA, Schuringa JJ. Apoptosis and cancer stem cells: Implications for apoptosis targeted therapy. Biochem Pharmacol 2010;80:423-30.
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Appendix

A. ***Curriculum Vitae***

B. **Supporting Data**

C. **Manuscript**

Appendix A

Angelina I. Londoño-Joshi

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EDUCATION

University of Alabama at Birmingham (UAB) 2007-present
PhD Candidate
Department of Pathology/ Division of Molecular and Cellular Pathology
Howard Hughes Medical Institute (HHMI) Med-Grad Predoctoral Fellow
Expected date of graduation, May 2013
GPA: 4.0/4.0

University of Wisconsin-Madison, College of Agricultural Life Science 2002-2006
B.S. in Natural Sciences
Major: Genetics
Graduated May 13th, 2006
GPA: 3.5/4.0 Dean's List 2003, 2004, 2005, 2006

Study Abroad-Universidad Complutense, Madrid, Spain 2005
Trans-Atlantic Student Science Exchange Program

Study Abroad-University of California, Santa Barbara 2003
Extended learning Wildland Studies in Melbourne, Australia

AWARDS

- UAB Comprehensive Cancer Center
  - John R. Durant Award for Excellence in Cancer Research-Graduate Student Category 2011
- UAB Department of Pathology
  - Betty Spencer Pritchett Award for Outstanding Cancer Research 2011
- Susan G. Komen for the Cure Travel Scholarship
  - AACR annual conference Chicago, IL 2012
  - AACR annual conference Washington, DC 2010
- Howard Hughes Medical Institute (HHMI)
  - Science Education Alliance Teaching Assistant Award 2011
  - Genomic Analysis Training Janelia Farm, travel award 2010
  - Translational Medicine Symposium, HHMI Peer Cluster Meeting, travel award 2010
  - AACR Annual Conference- Denver, CO, travel award 2009
  - HHMI Med-Grad Predoctoral Fellowship 2007-present
- Department of Defense Breast Cancer Research Program Predoctoral Fellowship 2011-present
- McNair Scholars Graduate School Program 2004-2006
- Pathway Scholars Program Summer Research Internship 2004
- Undergraduate Research Scholar 2002-2004
- College of Agricultural Life Science Honors Program 2002-2005
- Exxon Mobil Mathematics Youth Award-Hispanic Heritage Awards Foundation 2002
- POSSE Foundation Scholarship 2001-2006

RESEARCH EXPERIENCE

UAB School of Medicine Division of Radiation Biology 2009-present
Predoctoral work in the lab of Dr. Donald J. Buchsbaum
Treatment of Breast Cancer Stem Cells with TRA-8 anti-DR5 Monoclonal Antibody

UAB Department of Cellular Molecular Biology 2008
Spring Rotation with Dr. Christopher Klug
Development of a Luciferase-Expressing Pancreatic Cancer Cell Line
Gene Expression Profiling to Identify Novel Cell-Surface Antigens on Malignant Pancreatic Ductal Epithelium

UAB Department of Pathology Division of Molecular and Cellular Pathology 2008
Winter Rotation with Dr. Selvarangan Ponnazhagan
Chromatin Insulators and their Potential use in rAAV Transgene Expression

UAB Department Immunology and Molecular and Cellular Biology 2007
Fall Rotation with Dr. Zedenk Hel
Induction of Long-Term Immunity via Transplantation of Antigen- Expressing Hematopoietic Stem Cells

UAB- Gene Therapy Center 2007
Summer Rotation with Dr. David Curiel and Dr. Larisa Pereboeva
Overexpression of HOXB4 in Mesenchymal Stem Cells

University of Wisconsin-Madison, Department of Radiology and Human Oncology 2003-2006
Undergraduate research mentor, Dr. Jamey Weichert
Validation of non-invasive tissue distribution by MicroPET scanning in mice
PLD Expression in Breast Cancer Tumor cells versus Normal Cells
In vivo Cell Trafficking of Metastasis of Colon Cancer

University of Wisconsin-Madison, Department of Biochemistry 2002-2003
HHMI investigator Dr. Judith Kimble
The Role of F17A9.3 in Germ Line Development of C. Elegans

PUBLICATIONS

** Contributed equally*

1. **Londoño-Joshi AI**, Oliver PG, Forero-Torres A, LoBuglio AF, Buchsbaum DJ: Basal-like breast cancer stem cells are sensitive to combination treatment with anti-DR5 monoclonal antibody and chemotherapy. *In preparation*

2. Fauci J, **Londoño-Joshi AI***, Sellers JC, Oliver PG, Ferrone S, Straughn JM, Jr, Buchsbaum DJ: Combination treatment of B7-H3 with platinum chemotherapy on ovarian cancer stem cells. *In preparation*

3. Whitworth JM, **Londoño-Joshi AI***, Sellers JC, Oliver PG, Muccio DD, Atigadda VR, Straughn JM, Jr, Buchsbaum DJ: The impact of novel retinoids in combination with platinum chemotherapy on ovarian cancer stem cells. *Gynecol Oncol* In press, 2012.

4. Bevis KS, McNally LR, Sellers JC, Della Manna D, **Londoño-Joshi AI**, Amm H, Straughn JM, Jr, Buchsbaum DJ: Anti-tumor activity of an anti-DR5 monoclonal antibody, TRA-8, in combination with taxane/platinum-based chemotherapy in an ovarian cancer model. *Gynecol Oncol*, 121:193-199, 2011.

5. **Londoño-Joshi AI**, Oliver PG, Li Y, Lee CH, Forero-Torres A, LoBuglio AF, Buchsbaum DJ: Basal-like breast cancer stem cells are sensitive to anti-DR5 mediated cytotoxicity. *Breast Cancer Res Treat*, Epub ahead of print, 2011.

ORAL PRESENTATIONS

[*]Invited Speaker

1. [*]Investigation of death receptor-5 mediated apoptosis breast cancer stem cells. Hematology and Oncology Seminar Series, Birmingham AL, 2010.

2. [*]Investigation of death receptor-5 mediated apoptosis in basal-like breast cancer stem cells. Science Hour, Department of Radiation Oncology, Birmingham AL, 2010.

3. [*]Sensitivity of basal-like breast cancer stem cells to death receptor-5 mediated apoptosis. Komen Breast Cancer Research Roundtable, Birmingham AL, 2010.

4. Treatment of triple negative metastatic breast cancer stem cells with TRA-8 anti-death receptor 5 monoclonal antibody. Graduate Student Research Day, Birmingham AL, 2009.

5. Treatment of triple negative metastatic breast cancer with TRA-8 anti death receptor 5 (DR5) monoclonal antibody. Pathology Seminar, Birmingham AL 2009.

6. Development of a luciferase-expressing pancreatic cancer cell line. Pathology Seminar, Birmingham AL, 2008.

7. Chromatin insulators and their potential use in rAAV transgene expression. Pathology Seminar, Birmingham AL, 2008.

8. Induction of long-term immunity via transplantation of antigen- expressing hematopoietic stem cell. Pathology Seminar, Birmingham AL, 2007.

9. Validation of non invasive tissue distribution by MicroPET scanning in mice, Undergraduate Symposium, Madison, WI, 2006.

10. Phospholipase-D expression in breast cancer tumor cells versus normal cells. Pathways Scholars Symposium, Madison, WI, The National McNair Scholars Symposium, Berkeley, CA, 2004.

11. Evaluating metastasis characteristics of CT-26 Cell with florescence labeled NM404. The Undergraduate Symposium, Madison, WI, 2004.

12. F17A9.3 role in C. elegans germ line development. The Undergraduate Research Scholar presentations, Madison, WI, 2002.

POSTER PRESENTATIONS

1. **Londoño-Joshi AI**, Forero-Torres A, Fineberg NS, Oliver PG, Zhou T, LoBuglio AF, Buchsbaum DJ: Basal-like breast cancer stem cells are sensitive to combination treatment with anti-DR5 monoclonal antibody and chemotherapy.
   - *Center for Clinical and Translational Science*, Birmingham, AL, 2011
   - *Proc Comprehensive Cancer Center 2011 Annual Research Retreat*, Birmingham, AL, 2011
   - *Pathology Research Day*, Birmingham, AL, 2011

2. **Londoño-Joshi AI**, Monti DL: Reading, writing, and arithmetic in the UAB phage explorations lab. *Science Education Alliance*, Janelia Farm, VA 2001

3. **Londoño-Joshi AI**, Forero-Torres A, Fineberg NS, Oliver PG, Zhou T, LoBuglio AF, Buchsbaum DJ: Sensitivity of breast cancer stem cells to TRA-8 anti-DR5 monoclonal antibody.
   - *American Association for Cancer Research National Conference*, Washington DC, 2010.
   - *HHMI Southern Regional Conference*, Chapel Hill, NC, 2010

4. **Londoño-Joshi AI**, Forero-Torres A, Fineberg NS, Oliver PG, Zhou T, LoBuglio AF, Buchsbaum DJ: Treatment of triple negative breast cancer stem cells with TRA-8 anti-DR5 monoclonal antibody and 4HPR. Molecular and Cellular Pathology Research Day, Birmingham AL, 2009.

5. **Londoño-Joshi AI**, Forero-Torres A, Fineberg NS, Oliver PG, Zhou T, LoBuglio AF, Buchsbaum DJ: Treatment of triple negative breast cancer stem cells with TRA-8 anti-DR5 monoclonal antibody. Stem Cell Symposium and Cancer Center Retreat, Birmingham AL, 2009.

6. **Orozco AI**, Klug K: Gene expression profiling to identify novel cell-surface antigens on malignant pancreatic ductal epithelium. Howard Hughes Med-to Grad Regional Conference, 2008.

ABSTRACTS: non-presenting author

1. **Londoño-Joshi AI**, Della Manna DL, Ravi S, Oliver PG, Gillespie Y, Buchsbaum DJ: Treatment of glioblastoma cells with TRA-8 and AT-406. *The 2012 Brain SPORE Roundtable Meeting*, AZ, 2012.
2. Ravi S, **Londoño-Joshi AI**, Oliver PG, Gillespie Y, Buchsbaum DJ: Characterization and treatment of glioma stem-like cells with temozolomide and TRA-8 combination therapy. *Patho-biology and Molecular Medicine Symposium*, Birmingham, AL, 2011.
3. Whitworth JM, **Londoño-Joshi AI**, Sellers JC, Oliver PG, Muccio DD, Atigadda VR, Straughn JM, Jr, Buchsbaum DJ: The impact of novel retinoids in combination with platinum chemotherapy on ovarian cancer stem cells. *Proc Comprehensive Cancer Center 2011 Annual Research Retreat*, Birmingham, AL, 2011.
4. Aristizabal A, **Londoño-Joshi AI**, Forero-Torres A, LoBuglio AF, Buchsbaum DJ: Characterization of $CD44^{+}/CD24^{-}/ALDH^{+}$ Marker expression as a model for drug development against basal-like breast cancer tumor initiating cells. *Undergraduate Research Expo*, Birmingham, AL, 2011.
5. Ahmed A, Cain G, Clark D, Duckworth M, Dunkerley C, Johnston C, Mentreddy A, Nance C, **Londoño-Joshi AI,** Monti DL: Characterization of novel mycobacteriophages isolated from Alabama environmental samples. *Undergraduate Research Expo*, AL, 2011.
6. Alakija O, Blair P, Chang M, Layfield S, Lee P, Liu M, Patel M, Thomas S, **Londono-Joshi AI**, Monti DL: Genomic analysis of the novel B1 mycobacteriophage, OSmaximus. *Undergraduate Research Expo*, AL, 2011.
7. Forero-Torres A, Oliver PG, **Londoño-Joshi AI**, Zhou T, LoBuglio AF, Buchsbaum DJ: Death receptor 5, a therapeutic target for triple negative breast cancer. *The 2010 Breast Cancer Symposium*, National Harbor, MD, 2010.
8. Buchsbaum DJ, Oliver PG, **Londoño-Joshi AI**, Zhou T, LoBuglio AF, Forero-Torres A: Death receptor 5 (DR5), a therapeutic target for triple negative breast cancer (TNBC). *The 2010 Breast SPORE Roundtable Meeting*, National Harbor, MD, 2010.
9. **Londoño-Joshi AI**, Oliver PG, LoBuglio AF, Buchsbaum DJ: Treatment of triple negative breast cancer stem cells with TRA-8 anti-DR5 monoclonal antibody. *Proc Comprehensive Cancer Center 2009 Annual Research Retreat*, 2009.

PATENT

- Targeting Cancer Stem Cells March 2010. US Provisional Patent Application (# 61/315,413)

PUBLISHED GENOME SEQUENCES

- Mycobacterium phage OSmaximus, complete genome. GenBank: JN006064.1
- Mycobacterium phage Wee, complete genome. GenBank: HQ728524.1

TEACHING AND MENTORING EXPERIENCE

HHMI Science Education Alliance Phage Exploration, Professor Dr. Denise Monti
National Genomics Research Initiative

BY213 Phage Genomics I
Teaching Assistant
Fall 2010, Fall 2011, 4 credits lectured and taught lab for 2 months while professor was on leave
16 students (primarily honors biology majors)

BY214 Phage Genomics II
Teaching Assistant
Spring 2011, 4 credits lecture and lab
16 students (primarily honors biology majors)

McNair Scholars Program
Summer Research Peer Mentor
Summer 2009, 2010 and 2011
5 students (McNair Scholars undergraduate students)

Tutor Undergraduate Students
Fall 2009 Molecular Genetics, Spring 2010 Cancer Biology, and Fall 2010 Genetics

Research Mentoring
Supervised Undergraduate Student Research
Summer 2009, 2010, 2011
Andres Aristizabal; 2011 third place winner at state-wide poster competition

Supervised Graduate Student Rotation
Saranya Ravi, Fall 2011; *Amber Guidry*, Spring 2010

Supervised Gyn-Oncology Medical Fellow Research
Keri Bevis, 2010; *Jenny Whitworth*, 2011; *Janelle Fauci*, 2012

LEADERSHIP EXPERIENCE

Associate Member Council for the American Association for Cancer Research (AACR)	
Programming, Advocacy Professional Advancement Series	2011-present
Sub-committee, Associate Membership and Recruitment	2011-present
Toastmasters International Club	
President	2011-2012
Vice-President of Education	2010-2011
Founding Officer	2010
Graduate Student Association	
Treasurer	2010
Budget Committee Chair	2010
Cultural Activities Committee Co-Chair	2008-2010
Senator- Pathology Department	2008-2011
Howard Hughes Med to Grad	
Peer Cluster Regional Southern Conference Coordinator	2008
Volunteering Co-Chair	2008
La Colectiva (Hispanic Student Organization), President	2003-2006
Coalition of South American Students, Co-Chair	2003-2006
International Student Services Committee- Board member	2002-2006

EXTRACURRICULAR ACTIVITIES

- UAB Graduate Biomedical Sciences
  - Cancer Biology Admission Committee 2010-2012
- Pancreatic Action Network Birmingham Affiliate
  - Helped organized first annual Purple Stride 5k, 1K race 2011
  - Organized lab tour for pancreatic cancer patients and advocates 2011
- Hispanic Interest Coalition of Alabama: Medical translator, Birmingham AL 2008-present
- McNair Oral and Poster Presentation for Undergraduate Students: Judge, Birmingham, AL 2008-2011
- POSSE Foundation Undergraduate Student Scholarship: Selection committee, Chicago, IL 2006
- Bi-monthly weekend outings with handicapped and mentally disabled children, Madrid, Spain 2005
- Assisted terminally ill patients at Clinico Moncloa, Madrid Spain 2005
- American Multicultural Student Leadership Conference, Madison, WI 2004
- University of Wisconsin Hospital Burn Unit: Medical translator, Madison, WI 2004
- Boys and Girls Club: Holiday workshops and mentoring, Madison, WI 2003-2006
- World Health Day: Medical translator, Madison, WI 2003-2006
- Extended Learning Services, Little River Earth Sanctuary Environmental Project, Melbourne, Australia 2003

WORK EXPERIENCE

Hyde Park Dermatology- Medical Assistant/Insurance Coder 2007
Latin Solutions Marketing- Brand Ambassador 2006-2007
University of Wisconsin Health Service- Student Health Advocate 2003-2004
Southport Health Center- Assistant Manager 2000-2002
Instituto Cervantes of Chicago- Library Assistant 2001-2002
Illinois Public Interest Research Group- Canvasser 2001

CERTIFICATES

Toastmasters International Speaking and Leadership Certificates
Competent Communicator
Competent Leadership
Small rodent procedures:
Pancreatic cancer orthotopic surgery. Mammary-fat-pad orthotopic injections. Intravenous, intracardiac and intraperitoneal injections
Health Insurance Probability and Accountability Act (HIPAA)
PADI Open Underwater Scuba Diver

PROFESSIONAL MEMBERSHIPS

Society for Advancement of Chicanos and Native Americans in Science (SACNAS)
Center for Clinical and Translational Science (CCTS)
American Association for Cancer Research (AACR)
Birmingham International Center (BIC)
Toastmasters International (TMI)

LANGUAGES

Native proficiency in English and Spanish

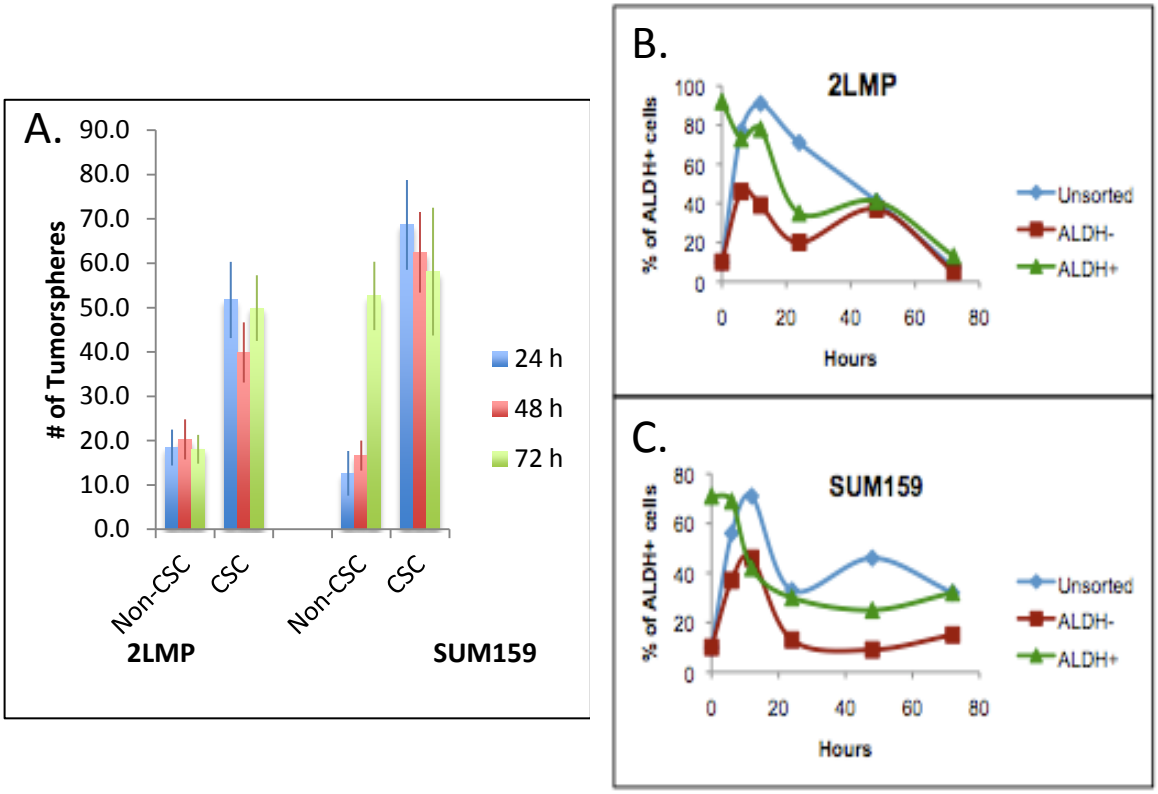

Figure 1. A) Tumorsphere forming ability after sorting for BrCSC. $CD44^{+}/CD24^{-}ALDH^{+}$ (CSC) and $CD44^{-}/CD24^{+}/ALDH^{-}$ (non-CSC) 2LMP and SUM159 cells were sorted and plated in low attachment plates in mammosphere media and visually counted at 24, 48, and 72 h time points. CSC were better able to form tumorspheres then non-BrCSC, however over time the SUM159 non-BrCSC were able to form tumorspheres. **B)** and **C)** Unsorted, $ALDH^{-}$ and $ALDH^{+}$ populations were analyzed at 0, 6, 12, 24, 48, and 72 h after initial sort. Unsorted populations rapidly gained ALDH enzymatic expression for the 1^{st} 24 h. However ALDH expression receded back to pre-sorted levels after 48 h. $ALDH^{-}$ cells initially gained marker expression but returned to original % of BrCSC enrichment. Interestingly, unsorted cells also rapidly gained ALDH expression in these conditions reaching ~80% enrichment by 12 h.

Phenotype	Cell Line	Tumorsphere forming ability	% $ALDH^+$	% $ALDH^+$/ $CD44^+/CD24^-$
Basal B	**SUM149**	**S/A**	**8**	**7**
	HCC38	**S/A**	**76**	**16**
	2LMP	**S/A**	**6**	**5**
	SUM159	**S/A**	**5**	**3**
	MB-436	**A**	**8**	**6**
	BT-549	**S/A**	**10**	**2**
Basal A	**HCC1187**	**A**	**60**	**2**
	BT20	**S**	**11**	**1**
	HCC70	**S/A**	**87**	**8**
	HCC1143	**S/A**	**10**	**7**

Table 1. Ten Basal-like breast cancer cell lines were analyzed for tumorsphere forming ability and BrCSC marker expression. Attached cells were sorted for for $ALDH^+/CD44^+/CD24^-$ marker expression and plated in low attachment plates in mammosphere media. Cells were allowed to grow for 48 h and then visually assessed for ability to form spheres or aggregates. All of the BLBC cells lines were able to at least form grape-cluster like aggregates. Only one line BT20 was able to form smooth spheroid-like morphology over a 48 h time point.

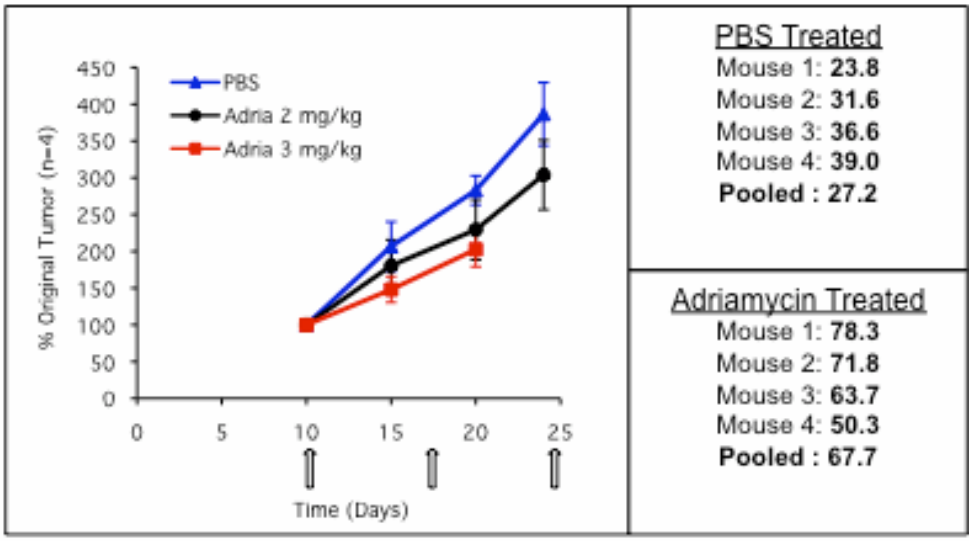

Figure 2. *In vivo* enrichment for CSC in Adriamycin treated 2LMP orthotopic NOD/SCID model**.** $2x10^6$ 2LMP cells were implanted into the MFP of 12 NOD/SCID mice. Tumors were allowed to grow for 10 days (till tumors reached 5x5 mm). On day 10, 17 and 24 Adriamycin was administered IP, at 2 mg/kg or 3 mg/kg. Tumors were measured on days 15, 20, and 24 and harvested on day 25 for analysis of ALDH expression. Mice treated with 3 mg/kg died after second dose of Adriamycin due to side effects of chemotherapy. Mice treated with 2 mg/kg did not show tumor regression. However, tumors were smaller and enriched for ALDH activity. Equal number of cells were pooled from each mouse to result in a 68% ALDH enrichment compared to 27% for PBS treated controls.

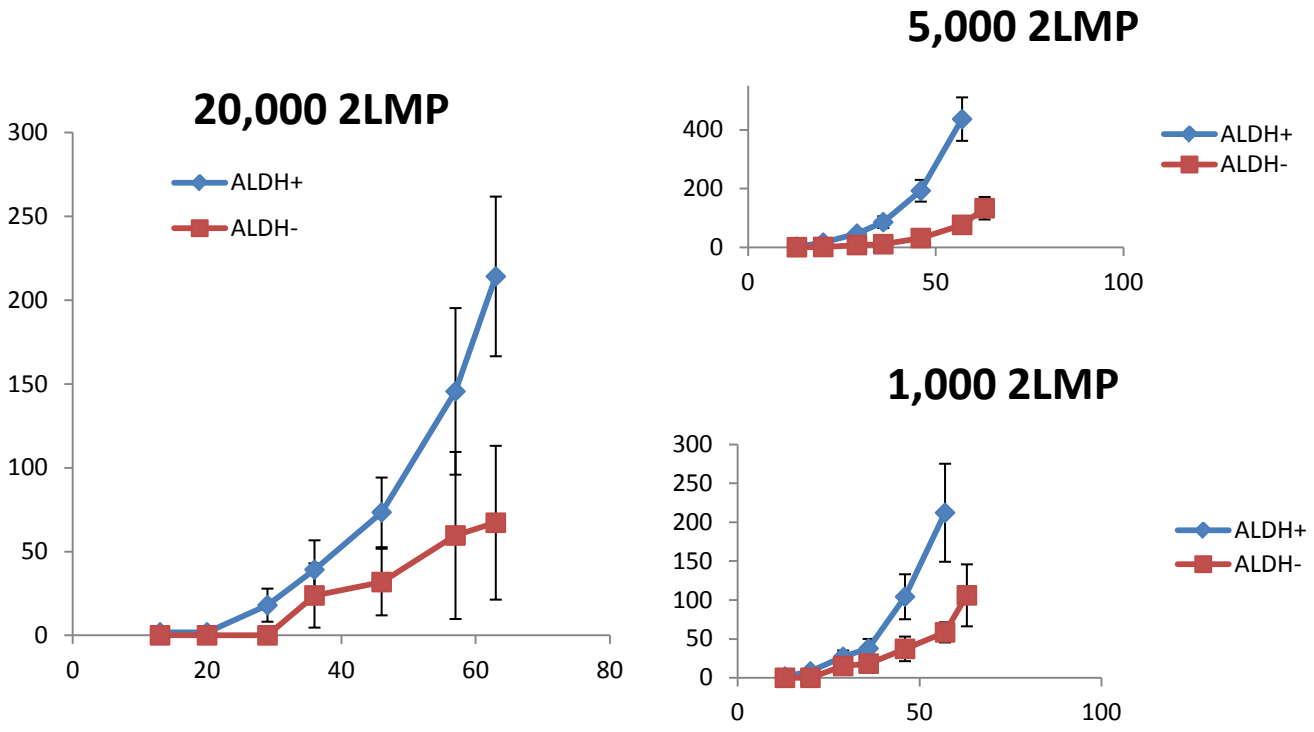

Figure 3. ALDH^{+} sorted cells form larger tumors at a faster rate compared to ALDH^{-} sorted cells. 2MLP cell lines were sorted for BrCSC markers and implanted into the MFP of NOD/SCID mice. Both ALDH$^{+/-}$ populations developed tumors, however, the BrCSC^{-} tumors were delayed and smaller, while the BrCSC^{+} grew larger and faster.

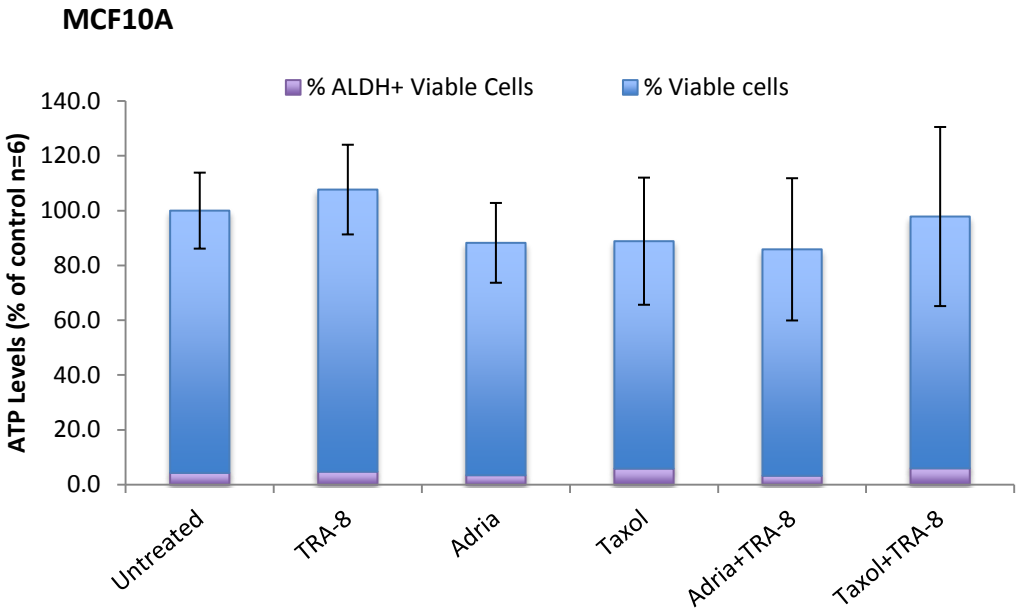

Figure 4. MCF10A sensitivity to TRA chemotherapy and ALDH expression after treatment. MCF10A mammospheres were treated with TRA-8 Adriamycin or Taxol. Cells were analyzed for viability and ALDH expression after treatment. The blue bars represent ATPlite cell viability and the purple bars represent % of $ALDH^+$ cells in surviving cells. There is no affect of TRA-8 on viability or ALDH activity. This indicates that TRA-8 is a safe treatment with no affect on normal breast cells.

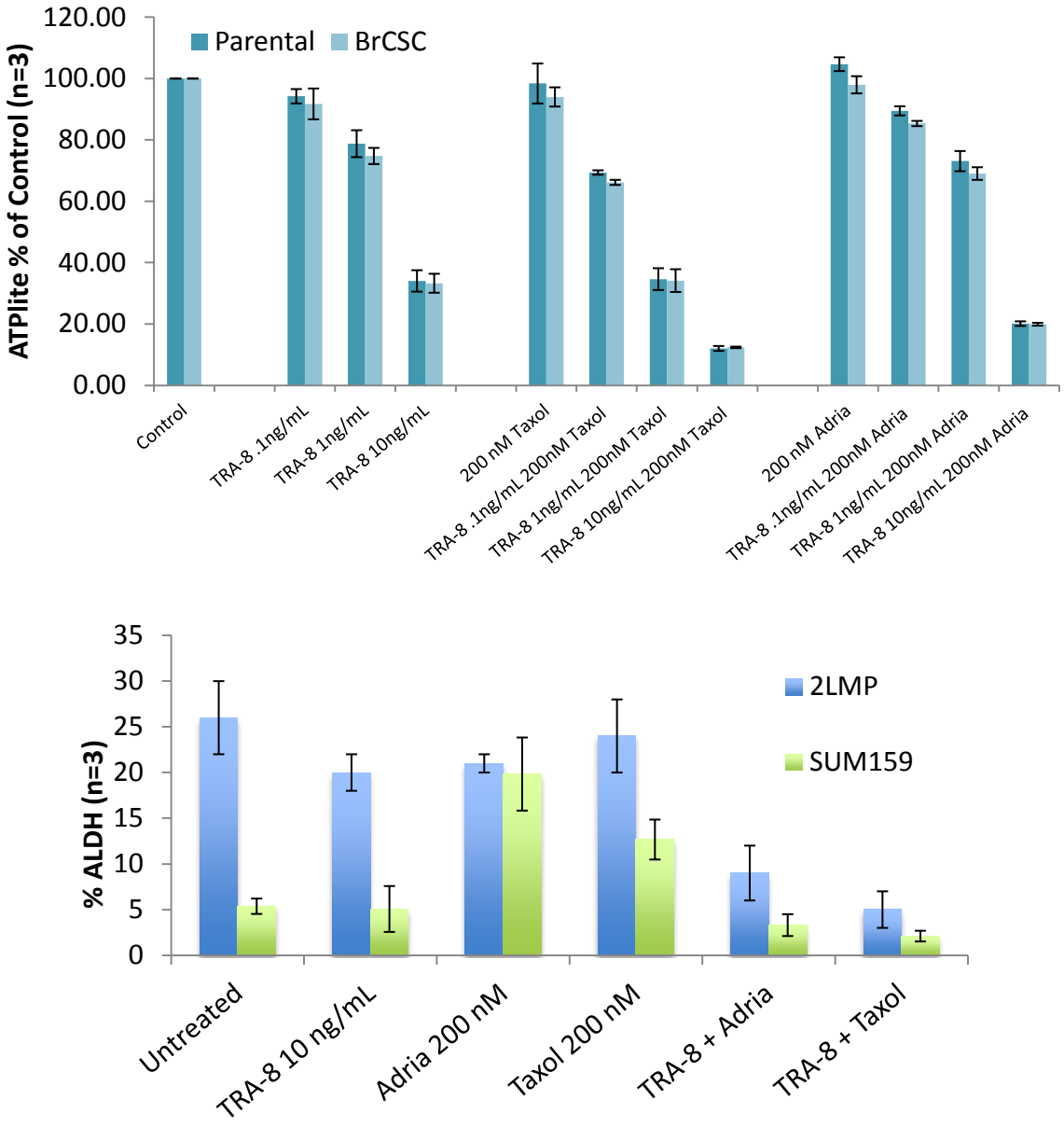

Figure 5. Combination treatment resulted in enhanced cytotoxicity and reduction in BrCSC marker expression. **A.** 2LMP cells were sorted using flow cytometry for $CD44^{+}/CD24^{-}/ALDH^{high}$. Cells were pre-treated with Adriamycin or Taxol for 24 h followed by 24 h treatment with TRA-8. **B.** 2LMP and SUM159 cells were analyzed for ALDH activity after treatment. * $p<0.001$

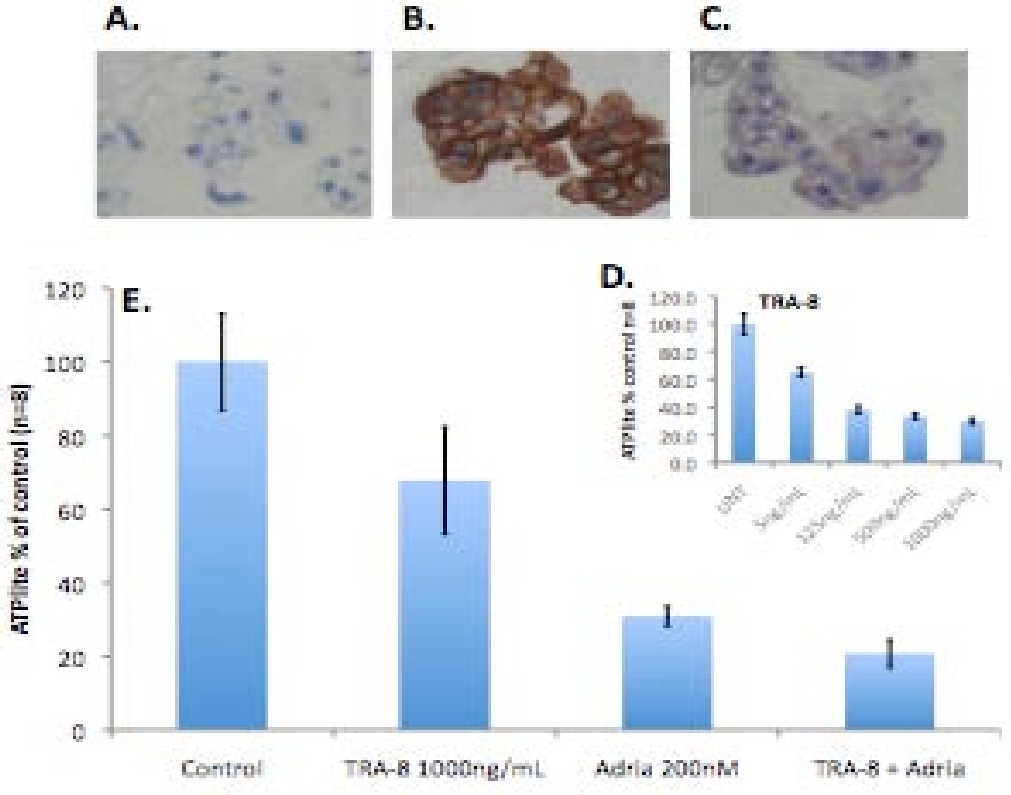

Figure 6. A patient pleural effusion from a triple negative breast cancer (TNBC) patient, was sensitive to TRA-8 and combination treatment. Sample was collected by slow centrifugation and characterized by IHC; **A**. (negative control) **B**. positive Moc31 (Adenocarcinoma) and **C**. negative for Calretinin (non-mesothelial). **D.** Clusters were dissociated, plated 2,000 cells/well and analyzed for 48 h TRA-8 sensitivity. **E**. Clusters were plated in a 96-well format ~ 100 clusters per well, pre-treated with Adriamycin for 24 h followed by 24 h incubation with TRA-8. Cells were analyzed for viability using ATPlite assay.

Appendix C

Breast Cancer Res Treat
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PRECLINICAL STUDY

Basal-like breast cancer stem cells are sensitive to anti-DR5 mediated cytotoxicity

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Abstract Breast cancer stem cells (BrCSC) are resistant to common therapeutic modalities including chemotherapy, radiation, and hormonal agents. They are thought to contribute to treatment resistance, relapse, and metastases. This study examines the effect of a monoclonal anti-DR5 antibody (TRA-8) and chemotherapy (adriamycin, taxol) on BrCSC populations from basal-like breast cancer cell lines. Doubly enriched BrCSC ($CD44^+$, $CD24^-$, $ALDH^+$) cells were exposed to TRA-8 and control reagents and examined for cytotoxicity, caspase activation, tumorsphere formation and tumorigenicity. Doubly enriched BrCSC populations expressed cell surface DR5 and were sensitive to TRA-8 mediated cytotoxicity with induction of caspase 8 and 3 activation. TRA-8 at sub-nanomolar concentrations inhibited 2LMP and SUM159 BrCSC tumorsphere formation and was more than 50-fold more inhibitory than TRAIL or anti-DR4 at equimolar concentrations. Chemotherapy treatment of 2LMP and SUM159 cell lines resulted in a relative increase of BrCSC, whereas TRA-8 produced a decrease in the percentage of BrCSC. TRA-8 exposure to 2LMP and SUM159 BrCSC preparations produced significant inhibition of tumorigenicity. DR5 maybe a therapeutic target on the surface of basal-like BrCSC which is amenable to agonistic monoclonal anti-DR5 therapy.

Keywords Anti-DR5 · Tigatuzumab · Basal-like breast cancer · Breast cancer stem cells · Tumor initiating cells · Tumorspheres · Death receptor 5

Introduction

Basal-like breast cancer accounts for about 15% of all breast cancer [1]. It is characterized by a unique mRNA profile with CK5/6 expression, inactivation of *BRCA1* and commonly lacks estrogen receptor, progesterone receptor, and HER-2 amplification [1–3]. They are further categorized into basal A and basal B subtypes and appear to commonly have substantial numbers of breast cancer stem cells (BrCSC) or tumor initiating cells [4–6].

The cancer stem cell hypothesis suggests that tumors, similar to normal tissue, are organized in a cellular hierarchy, with cancer stem cells (CSC) at the top, as the only cells with potentially limitless proliferation abilities which are capable of driving tumor growth [7]. The more 'differentiated' descendants, which account for the majority or bulk of the tumor population, may also be able to proliferate, but regenerative ability is limited [7]. Cancer stem cells were first described in patients with acute leukemia and subsequently in a variety of solid tumors [8, 9]. In breast cancer, CSC were first reported in 2003 by Muhammad Al-Hajj using $CD44^+$ and $CD24^-$ surface expression [10]. Since then BrCSC have been characterized

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based on other cell surface antigens ($EpCAM^+$, $CD133^+$, $CD90^+$) and by functional activities including enhanced efflux pumping of a Hoechst dye (side population), overexpression of aldehyde dehydrogenase (ALDH, ALDEFLUOR assay), retention of the lipophilic dye PKH26, and tumorsphere-forming ability [11–14]. BrCSC are also called tumor initiating cells that are described as having the ability to self-renew, induce tumors at low cell numbers, have low rates of cell division, exhibit chemotherapy and radiation resistance, and have gene expression profiles which differ from the more differentiated cancer cell counterparts [15]. The concept of solid tumor and particularly BrCSC is controversial with several alternative explanations for stem-like cell behaviors [11, 16].

CSC are generally reported to be resistant to chemotherapy and radiation and BrCSC commonly lack "targetable" receptors like ER or HER2 [17–19]. Thus, there is considerable interest in finding therapeutic agents targeted to BrCSC. The presence of substantial numbers of BrCSC in basal-like breast cancer cell lines [10] provided the opportunity to examine the effects of TRA-8 (anti-DR5) on BrCSC enriched populations in terms of anti-DR5 mediated cytotoxicity, inhibition of tumorsphere formation in vitro, and tumorigenicity in vivo. TRA-8 is an agonistic monoclonal anti-DR5 antibody with cytotoxicity and antitumor activity in a variety of human tumor cell lines and murine tumor xenografts [20–23] including basal-like breast cancer cell lines [24].

Materials and methods

Drugs and antibodies

Adriamycin and taxol were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO) and prepared as 10 mM stock solutions in distilled H_2O or DMSO, respectively. Purified TRA-8 (IgG1) mAb was provided by Tong Zhou at the University of Alabama at Birmingham (UAB) as described previously [25]. Isotype-specific IgG1 control antibody was obtained from Southern Biotechnology Associates (Birmingham, AL). Anti-DR4 mAb 2E12 (IgG1, k) was provided by Tong Zhou (UAB). Super Killer TRAIL™ was purchased from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA). Conjugated antibodies APC mouse anti-human CD44, PE-Cy7 rat anti-mouse CD44, and corresponding isotype control antibodies were purchased from BD Pharmingen (San Jose, CA). ALDEFLUOR kit including diethylaminobenzaldehyde (DEAB) negative control was obtained from StemCell Technologies (Durham, NC). Cleaved caspase 8 rabbit mAb and cleaved caspase 3 rabbit mAb were purchased from Cell Signaling (Billerica, MA). Secondary antibodies, Alexa fluor 405 goat anti-rabbit IgG, and Alexa fluor 647 goat anti-mouse IgG1 were purchased from Invitrogen (Carlsbad, CA).

Cells and cell culture

The 2LMP subclone of the human breast cancer cell line MDA-MB-231 was obtained from Dr. Marc Lippman (University of Miami, Coral Gables, FL) and maintained in improved MEM supplemented with 10% FBS (Hyclone, Logan, UT). Basal-like cell lines HCC38, HCC1187, HCC1143, MDA-MB-436, BT-20, and BT-549 were obtained from American Type Culture Collection (Manassas, VA) and cultured according to supplier's directions with the exception of MDA-MB-436, which was grown in DMEM supplemented with 10 μg/ml insulin, glutathione, and 10% FBS. SUM159 was obtained from Asterand (Detroit, MI) and grown according to supplier's recommendation. All cell lines were maintained in antibiotic-free medium at 37°C in a 5% CO_2 atmosphere and routinely screened for Mycoplasma contamination. Sorted cells and tumorspheres were maintained in MEGM medium (Lonza, Walkersville, MD).

Doubly enriched BrCSC isolation by flow cytometry

Basal-like cell lines were plated in T75 flasks (Costar, Cambridge, MA) in corresponding media and harvested at 75% confluence. Cells were harvested with trypsin and labeled with 1 μl of ALDEFLUOR reagent in 100 μl ALDEFLUOR buffer per 5×10^6 cells and incubated at 37°C for 30 min. Cells were then labeled with APC-CD44 (1:25) and PE-CD24 (1:25) in 200 μl of ALDEFLUOR buffer on ice for 15 min. The ALDEFLUOR positive population was established by using 2×10^6 ALDEFLUOR labeled cells and 5 μl DEAB in 200 μl ALDEFLUOR buffer. The sorting gates were established using negative controls, DEAB and side scatter and forward scatter profiles were used to eliminate cell doublets [10, 11, 16]. Samples were sorted on a Becton–Dickinson-FACSAriaII™ or analyzed on Becton–Dickinson-LSRII™ flow cytometer (Chicago, IL). Data was evaluated using FlowJo software (Tree Star, Inc., Ashland, OR).

DR5 expression and functional caspase activation

2LMP, SUM159, and HCC1143 cell lines were harvested using cell stripper (Mediatech, Manassas, VA) to prevent cleavage of death receptor. Cells were incubated with ALDEFLUOR reagents for 30 min at 37°C. Cells were then labeled on ice with TRA-8 (IgG1) or IgG1 istotype control for 15 min. Cells were then incubated with CD44-PE-Cy7 (1:1,000), CD24-PE (1:100), and secondary

antibody (Alexa-647) (1:100) for 15 min on ice. Samples were analyzed by flow cytometry for DR5 expression on the $CD44^+/CD24^-/ALDH^+$ subpopulation. Analysis of caspase 8 and 3 activation of BrCSC was accomplished by harvesting cells using cell stripper and sorting for the $ALDH^+$ population. Sorted cells were treated for 2 h with TRA-8 or IgG1 control ($\sim 1 \times 10^6$ cells with 1 μg/ml TRA-8 or IgG1 in MEGM medium + 2% BSA). Cells were fixed with 1% paraformaldehyde for 5 min on ice and labeled with CD44-APC and CD24-PE (1:100) on ice for 15 min. Cells were then permeabilized using 3% BSA, 0.1% saponin in 200 μl PBS on ice for 15 min and labeled with cleaved caspase 3 or 8 (1:500) on ice for 15 min. Cells were incubated with secondary antibody Alexa-405 anti-rabbit (1:100) on ice for 15 min. Samples were kept in 0.1% saponin and analyzed by flow cytometry. Analysis was done on a minimum of three independent experiments.

Cell viability assays using ATPLite

Sorted $CD44^+/CD24^-/ALDH^+$ cells were plated on ultra-low attachment plates (Costar) at 2,000 cells per 50 μl of MEGM medium. Bulk unseparated cells were collected from total viable gates established by forward and side scatter parameters to control for any variables introduced by sorting the cells. Cells from the bulk unseparated populations were plated in optically clear 96-well black plates (Costar) in corresponding media. Sorted and bulk cells were treated with (0.1, 1, 10, 100, or 1,000 ng/ml) of TRA-8 immediately after plating and incubated for 24 h at 37°C. TRA-8 was diluted in culture medium immediately before use. Cell viability was determined by measurement of cellular ATP levels using the ATPLite luminescence-based assay (Packard Instruments, Meriden, CT) described elsewhere [26]. The manufacturer's recommended protocol was followed with the exception that all reaction volumes (culture medium and reagents) were reduced by one-half. All samples were assayed in quadruplicate and IC_{50} values are reported as the median from a minimum of three independent experiments.

In vitro treatment of tumorspheres

2LMP and SUM159 cell lines were sorted for $ALDH^+$ cells. Approximately $\sim 1 \times 10^6$ cells were allowed to form primary spheres at a density of 100,000 cells/ml for 3–4 days in MEGM medium. Tumorspheres were mechanically dissociated and plated in ultra-low attachment 96-well plates (Costar) at 2,000 cells per well. TRA-8 (anti-DR5), 2E12 (anti-DR4), TRAIL, IgG isotype control, adriamycin, or taxol were added to the wells and incubated at 37°C for 48 h in quadruplicate. Tumorspheres were visually counted using a reticle eye piece. Mean tumorsphere inhibition was calculated relative to untreated control spheres. At least three independent experiments were conducted per cell line in quadruplicate.

Effect of drug treatment of breast cancer cells on BrCSC population

2LMP and SUM159 breast cancer cells were plated in 6-well well culture plates at 80,000 cells/well (Costar 3516). Cells were treated with adriamycin (200 nM) or taxol (200 nM) for 48 h, or TRA-8 (10 ng/ml) for 24 h. Cells in suspension after treatment along with attached cells were harvested and incubated with Aldefluor reagent for 30 min at 37°C following manufacturer's protocol. Cells were analyzed using a LSRII™ flow cytometer (Becton–Dickinson). Cells were gated based on forward and side scatter properties for single viable cells. The signal from autofluorescence of drug treatment was accounted for in the final analysis of BrCSC ALDH marker expression.

Ex vivo treatment of BrCSC and tumor implantation

$CD44^+/CD24^-/ALDH^+$ 2LMP and SUM159 cells (1×10^6) were sorted and allowed to recover for 13 h in MEGM medium in ultra-low attachment plates at 37°C. Cells (2×10^4) were separated into treatment groups and drug or antibody was added (IgG, 20 nM), 2E12 (20 nM), TRA-8 (20 nM), and adriamycin (500 nM). Cells were treated for 3 h at 37°C and then aliquoted in 200 μl (1:1 Matrigel) and injected into the mammary fat pad of 4 week old NOD/SCID mice (Harlan, Prattville, AL). Tumor size was determined by the product of two largest diameters. Two independent animal experiments were conducted for the 2LMP and SUM159 cell lines.

Statistical analysis

The IC_{50} is the drug concentration producing the median effect of 50% cell killing which was estimated based on the Hill Equation with nonlinear regression model for each assay [27]. Due to small number of replicate experiments, a nonparametric statistical method with Kruskal–Wallis test was used for the comparison between two groups, e.g., IC_{50}, percentage of tumorsphere number and ATP level [28]. The Generalized Linear Model (GLM) with PROC MIXED was used to compare the tumor size over time among experimental groups. Main effect and interaction between treatment groups and measurement time point were fitted in the model with appropriate variance and covariance structure selected. The statistical analysis was carried out with Statistical Analysis Software (SAS) version 9.2.

Results

Anti-DR5 (TRA-8) induced cytotoxicity to BrCSC enriched cells

Eight basal-like cell lines underwent dual BrCSC enrichment ($CD44^+/CD24^-/ALDH^+$) and these BrCSC enriched populations were compared with their unseparated parental cells in regard to sensitivity to anti-DR5 (TRA-8) mediated cytotoxicity (Table 1). As reported previously [24], the basal-like cell lines were quite sensitive ($IC_{50} < 100$ ng/ml) to TRA-8 mediated cytotoxicity except for HCC1143 which was moderately resistant (IC_{50} of 101–1,000 ng/ml). All the cell line BrCSC enriched cell preparations were very sensitive to anti-DR5 mediated cytotoxicity including cell line HCC1143. In 6/8 instances, the BrCSC enriched cells were significantly more sensitive than their parental cells.

DR5 expression of BrCSC enriched cells

The dual separated ($CD44^+/CD24^-/ALDH^+$) and unseparated cell preparations from 2LMP, SUM159, and HCC1143 underwent flow cytometry for analysis of cell membrane expression of DR5. As shown in Fig. 1, cells from all cell lines were strongly positive and the unseparated (blue lines) and dual enriched (red lines) had comparable expression of DR5.

Apoptosis of anti-DR5 treated BrCSC enriched cell populations

To determine that anti-DR5 can mediate apoptosis of the BrCSC enriched cells, the 2LMP and SUM159 $ALDH^+$ populations were incubated with anti-DR5 or control IgG for 2 h, and the $CD44^+$ $CD24^-$ cell population were tested for cellular expression of activated caspase 8 and activated caspase 3. Figure 2 illustrates that a substantial portion of the 2LMP (Fig. 2a) and SUM159 (Fig. 2b) BrCSC underwent caspase 8 and 3 activation (green lines) as compared with BrCSC exposed to control IgG (red line). This delineates that anti-DR5 triggers caspase activation and apoptosis of BrCSC doubly enriched cells over even short durations of 2–3 h.

Analysis of anti-DR5 effect on BrCSC tumorsphere formation

Tumorsphere formation has been reported as a measure of BrCSC presence in enriched cell populations [29]. Figure 3 provides the effects of anti-DR5 (TRA-8), anti-DR4 (2E12), TRAIL, adriamycin, taxol, control IgG, and control media on secondary tumorsphere formation of 2LMP (Fig. 3a), and SUM159 (Fig. 3b) cell lines. Impressive inhibition of secondary tumorsphere formation was caused by anti-DR5 at doses as low as 0.1 nM with 80% inhibition of 2LMP cells ($P = 0.019$) and 95% inhibition of SUM159 cells ($P = 0.02$). Anti-DR5 produced significantly more inhibition of tumorsphere formation at 0.1 nM than 5.0 nM TRAIL ($P = 0.019$) or 5.0 nM anti-DR4 ($P = 0.028$) in 2LMP cells. The SUM159 cells had comparable observations with 0.1 nM anti-DR5 producing more inhibition than 5.0 nM TRAIL ($P = 0.019$) or 5.0 nM anti-DR4 ($P = 0.02$). Adriamycin and taxol had modest or no inhibitory effects. Thus, anti-DR5 appears to be superior to other DR-mediated agents at tumorsphere formation inhibition.

Effect of drug exposure of breast cancer cell lines on BrCSC population

Drug or TRA-8 treatment of breast cancer cell lines may change the percentage of $ALDH^+$ cells (BrCSC) in the total cell population. Flow cytometry analysis of 2LMP basal-like cells after adriamycin or taxol treatment showed a 4.6-fold or 2.2-fold increase of $ALDH^+$ cells, respectively (Fig. 4). SUM159 cells treated with adriamycin or taxol had a 3.3-fold and 1.9-fold increase in the percentage

Table 1 Sensitivity of sorted BrCSC and unseparated parental cells to TRA-8 mediated cytotoxicity

Phenotype	Cell line	Sorted $CD44^+CD24^-$ $ALDH^+$ IC_{50} TRA-8 (ng/ml)	Unseparated parental IC_{50} TRA-8 (ng/ml)	*P* value sorted vs. unsorted
Basal B	HCC38	0.10[a]	0.74	0.127
	2LMP	0.65	1.06	0.008
	SUM159	0.88	5.54	0.049
	MDA-MB-436	0.62	0.31	0.248
	BT-549	0.63	5.55	0.02
Basal A	HCC1187	0.85	24.72	0.049
	BT-20	7.24	16.49	0.275
	HCC1143	77.12	628.43	0.049

[a] All samples were assayed in quadruplicate and are reported as the median from a minimum of three independent experiments

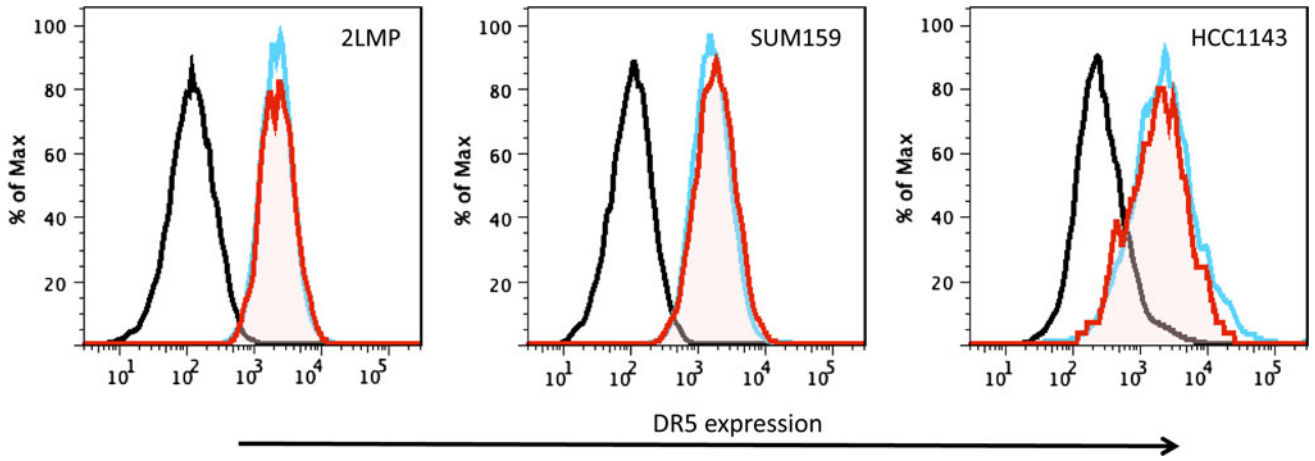

Fig. 1 Flow cytometry analysis of DR5 membrane expression on the $CD44^+/CD24^-/ALDH^+$ subpopulation of basal-like breast cancer cells. 2LMP, SUM159, and HCC1143 cells were labeled with ALDEFLUOR (FITC), CD44 (PE, Cy7), CD24 (PE), and anti-DR5 (TRA-8 Alexa-647) then analyzed by flow cytometry. DR5 membrane expression on total unsorted bulk population (*blue line*) or $CD44^+/CD24^-/ALDH^+$ (*red line*) subpopulation compared with isotype control (*black line*). 2LMP, SUM159, and HCC1143 $CD44^+/CD24^-/ALDH^+$ subpopulations had similar DR5 expression compared with the total unsorted cell population

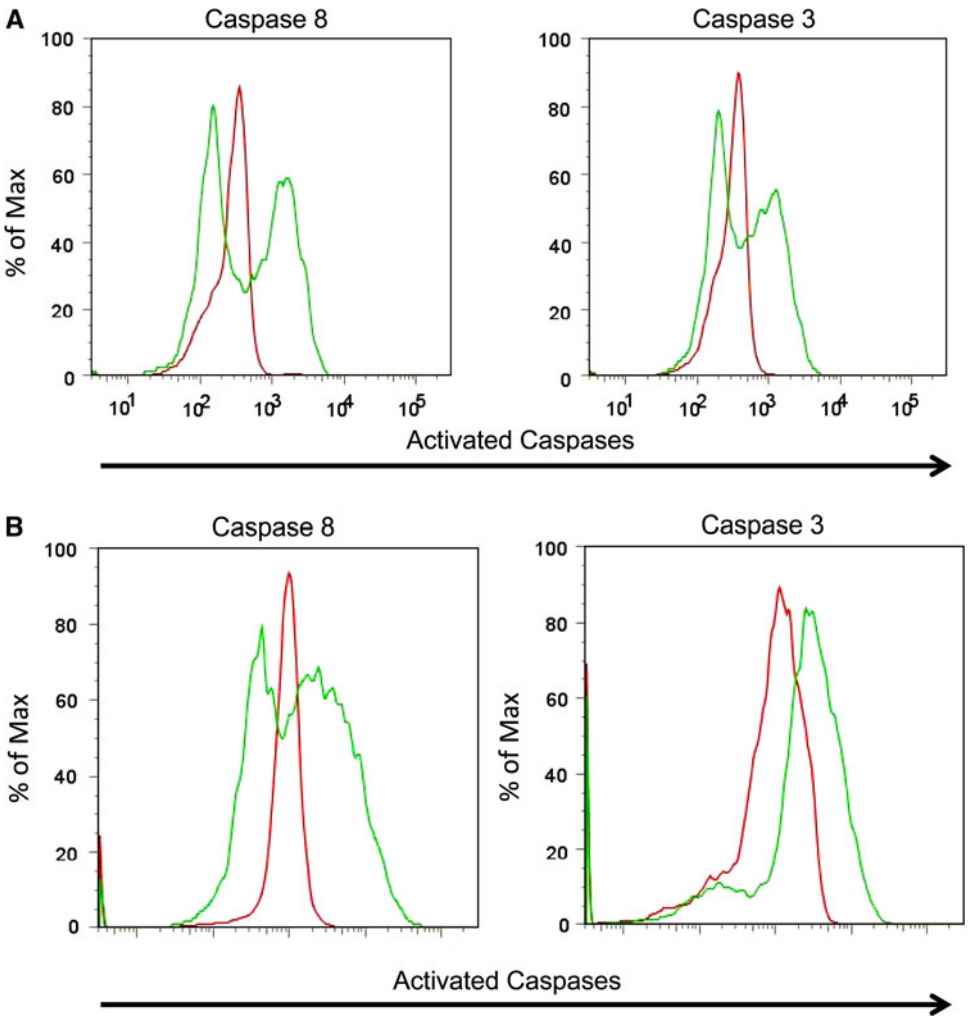

Fig. 2 Flow cytometry analysis of caspase activation in $CD44^+/CD24^-/ALDH^+$ subpopulation of 2LMP and SUM159 cells after treatment with TRA-8. 2LMP (**a**) and SUM159 (**b**) cells were sorted for the $ALDH^+$ subpopulation and then treated with TRA-8 or control IgG for 2 h. Cells were then fixed and stained for CD44 (APC), CD24 (PE), and activated caspases 3 or 8 (secondary Alexa 405). 2LMP and SUM159 BrCSC enriched cells had caspase 3 and caspase 8 activation (*green line*) after incubation with TRA-8 compared with IgG control (*red line*)

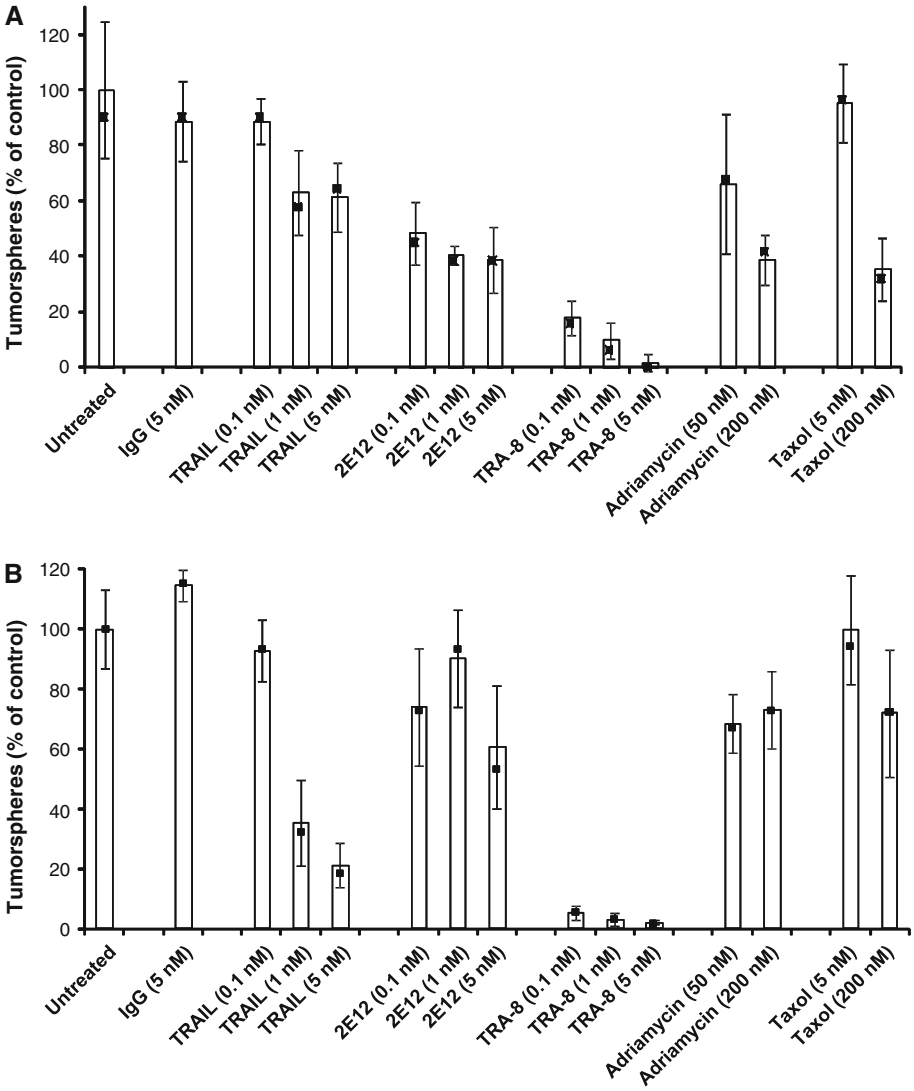

Fig. 3 Secondary tumorsphere formation inhibition by TRA-8. 2LMP cells (**a**) and SUM159 (**b**) cells were sorted using flow cytometry for $ALDH^+$ cells and allowed to form primary tumorspheres for 3 days. After tumorspheres were mechanically dissociated, single cells (2,000 cells/well) were plated in low attachment plates and treated with IgG, TRAIL, 2E12 (anti-DR4), TRA-8, adriamycin or taxol. After 48 h, tumorspheres ranging from 40 to 120 μm in size were visually counted using a reticle eye piece. Mean tumorsphere inhibition was calculated relative to untreated controls (*blue bars*) (*filled square*) represent median values. *Error bars* represent SD of the samples run in quadruplicate

of $ALDH^+$ cells, respectively. By contrast, 2LMP and SUM159 cells treated with TRA-8 resulted in a 1.8-fold and 1.7-fold decrease in $ALDH^+$ tumor cells compared with untreated cells, respectively. Thus, BrCSC appear to be resistant to chemotherapy but sensitive to anti-DR5.

Effect of anti-DR5 exposure on BrCSC enriched cell population tumorigenicity

The major requirement of BrCSC is the ability to generate fully constituted human breast cancer in immuno-compromised mice. BrCSC enriched cell populations were exposed to anti-DR5 (TRA-8), anti-DR4 (2E12), control IgG, or adriamycin for 3 h before injection of treated cells into the mammary fat pads of NOD/SCID mice ($n = 5$ for each group). Figure 5a illustrates that the control IgG, anti-DR4, and adriamycin treated cells generated 5/5 tumors. The IgG control mice were sacrificed on day 44 to comply

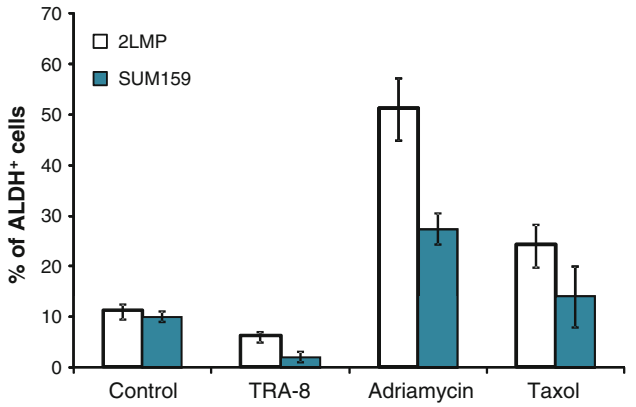

Fig. 4 In vitro treatment with TRA-8 decreased the percentage of $ALDH^+$ cells in 2LMP and SUM159 breast cancer cell lines. 2LMP and SUM159 cells (80,000 cells/well) were treated with TRA-8 (10 ng/ml) for 24 h, and adriamycin (200 nM) or taxol (200 nM) for 48 h in 6-well cell culture plates. Mean $ALDH^+$ cells after treatment from a minimum of three independent experiments are shown. *Error bars* denote SE

with IACUC guidelines with tumor sizes exceeding 175 mm^2. The anti-DR4 and adriamycin treated tumors were somewhat slower growing but not statistically different than the control. In contrast, the anti-DR5 treated cells developed measurable tumors in only 2/5 animals by day 50 and even these tumors were small and had severely retarded growth compared with the IgG control treated tumors ($P = 0.0001$). Figure 5b illustrates examples of the 2LMP tumors in the control and treated NOD/SCID mice. The tumors that grew in the four treated groups of mice had similar histology with poorly differentiated cells, high mitotic rates, and focal areas of necrosis. Similar studies with SUM159 cell line showed that the IgG treated BrCSC enriched cells generated 5/5 tumors compared with 0/5 mice observed in the TRA-8 treated group at day 105. Thus, it appears that TRA-8 can seriously impair the tumorigenicity of BrCSC enriched cell populations.

Discussion

There is considerable interest in finding therapeutic agents that could be targeted to CSC to enhance the efficacy of treatment regimens and potentially reduce tumor resistance and relapse. We have previously shown the anti-tumor activity of an agonistic monoclonal anti-DR5 antibody (TRA-8) to 2LMP and other basal-like cell lines in vitro and in vivo [24]. Others have shown DR-mediated cytotoxicity to basal B but not basal A breast cancer cell lines [30]. Given that basal-like cell lines are enriched in CSC [5, 18], it represented an opportunity to examine the effect of TRA-8 on BrCSC populations.

Doubly enriched BrCSC subpopulations ($CD44^+/CD24^-/ALDH^+$) of both basal-like A and B type were sensitive to TRA-8 mediated cytotoxicity. Further, DR5 expression on BrCSC subpopulations were identical to their unseparated parental population and brief interaction with TRA-8 triggered caspase 8 and 3 activation. Thus, it appears that basal-like BrCSC subpopulations share sensitivity to anti-DR5 mediated cytotoxicity similar to their parental cells and in some instances even have increased sensitivity. In vitro treatment of parental breast cancer cell lines with adriamycin or taxol increased the percentage of $ALDH^+$ cells, while TRA-8 produced a decrease in the percentage of $ALDH^+$ cells. These results indicate that the bulk cells were more sensitive to chemotherapy treatment than the BrCSC, whereas the BrCSC were more sensitive to TRA-8 treatment than the bulk cells. Similarly, anti-DR5 treatment decreased the percentage of CSC in pancreatic cancer [31].

A prior study had reported that TRAIL was able to mediate cytotoxicity to colon cancer CSC (dye efflux side population) and that this population was enriched for expression of DR4 [32]. We thus contrasted the effects of TRAIL, anti-DR4, and anti-DR5 on BrCSC tumorsphere formation. These studies demonstrated the superiority of TRA-8 over TRAIL and anti-DR4 in terms of inhibition of BrCSC tumorsphere formation. Similarly, TRA-8 was

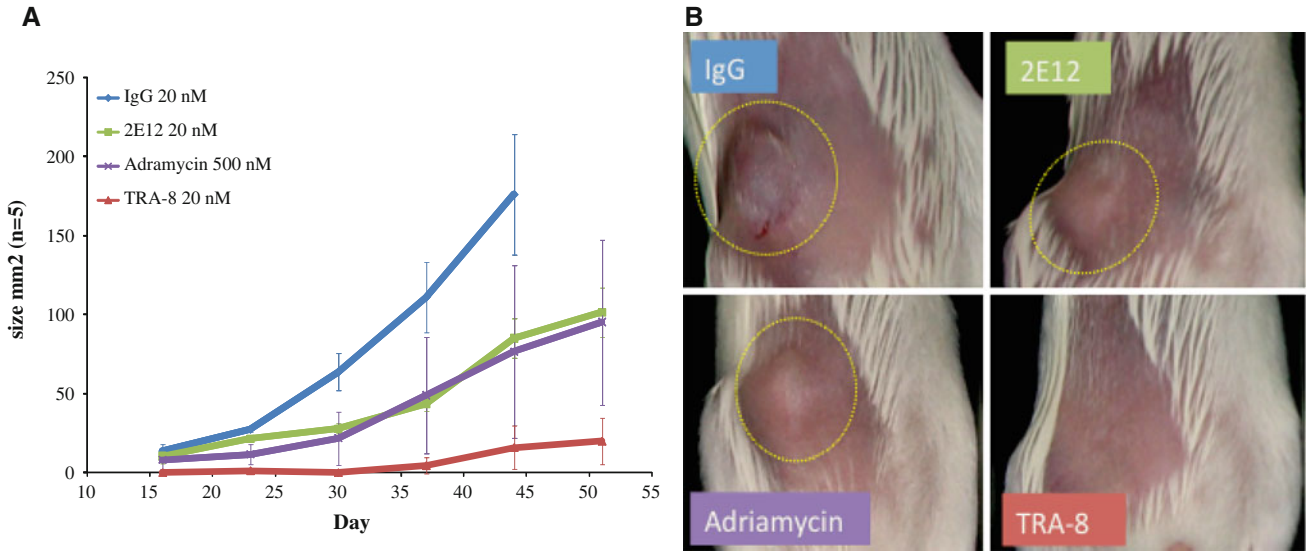

Fig. 5 Effect of ex vivo treatment of BrCSC enriched cells on tumorgenicity in NOD/SCID mice. 2LMP cells were sorted using flow cytometry for $CD44^+/CD24^-/ALDH^+$ BrCSC markers and the cells were allowed to recover for 13 h. Cells were treated with TRA-8, 2E12, adriamycin or IgG control for 3 h and implanted into the MFP of groups of five NOD/SCID mice. **a** *Graph* represents the average tumor size and number of tumors formed. Only 2/5 small, slow growing tumors were observed to develop within 50 days with TRA-8 treated cells while 5/5 tumors developed in the IgG, 2E12, and adriamycin treatment groups (P value <0.0001). **b** These are images taken of one representative mouse in each group at day 30 after implantation (*dotted circle* shows the tumor)

superior to anti-DR4 in inhibition of BrCSC tumorigenicity. This may reflect differences among CSC of different tumor types. Indeed, CSC from glioblastoma cell lines have been reported to be resistant to TRAIL mediated cytotoxicity [33].

These observations suggest that DR5 maybe a target on the surface of basal-like breast cancer cell lines and BrCSC by which an agonistic monoclonal anti-DR5 antibody could mediate anti-tumor activity/efficacy. Tigatuzumab is the CDR grafted, humanized version of TRA-8 which has entered clinical trials [34]. Because of these studies and others, the Translational Breast Cancer Research Consortium has recently opened a randomized phase II trial of abraxane ± tigatuzumab for metastatic triple negative breast cancer (ClinicalTrials.gov NCT01307891).

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