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Introduction

Breast cancer

Breast cancer is the most commonly diagnosed cancer among U.S. women (approximately 28% or more than 1 in 4) with approximately 1 in 8 women expected to develop this malignancy over the course of her lifetime. In 2010, there are an estimated 207,000 newly diagnosed cases of invasive breast cancer resulting in about 39,800 deaths in the United States, a mortality rate higher than all other malignancies, except lung cancer. The 5-year survival rate hovers around 75-90 % for women diagnosed in the early stages with a dismal 15% for late stage breast cancer. While there are now more than 2.5 million breast cancer survivors in the U.S., in part due to better diagnostic and therapeutic efficacies, there is still plenty of room for improvement to reduce the mortality rates. Clearly, this is an important disease for women's health and significant resources are focused on developing novel and more effective treatment options.

Adoptive T-Cell Immunotherapy

We proposed supplementing current breast cancer treatment options with T-cell immunotherapy which can treat malignancies resistant to conventional therapies such as surgery, chemotherapy, and radiation. As breast carcinomas grow despite a functioning immune system they develop strategies to avoid immune recognition and elimination by T cells. Gene reprogramming bestow T cells with tumor targeting capabilities and early-phase clinical trials are currently testing efficacies of T cells with redirected specificity. In addition to redirecting specificity through the expression of a chimeric antigen receptor (CAR) against c-Met, which is highly expressed in aggressive breast cancer cells, we have engineered T cells to exploit tumor hypoxia as a conditional stimulus for activation and proliferation. By programming T cells to activate only in pathological tumor hypoxia (1% O₂), our approach seeks to avoid deleterious off-target effects, bypassing physiological normoxic (20% O₂) and normal c-Met⁺ tissues.

Hypoxic tumor microenvironment and c-Met

We selected c-Met, a hypoxia-inducible antigen widely expressed in breast cancer, as the target for redirected T-cell specificity for the following reasons:

- 1) Poor prognosis of c-Met⁺ breast tumors
- 2) Widely expressed in chemo-resistant and hypoxic breast tumors

3) c-Met signaling pathway significantly affects breast tumor survival

Our approach is to exploit the hypoxic breast tumor microenvironment to devise a robust tumor specific T-cell immunotherapy. Relapsed breast tumors are hypoxic and resistant to conventional chemotherapy and radiotherapy regimens. These hypoxic tumors are prone to metastasis leading to widespread treatment failure. Hypoxic breast tumors express high levels of c-Met, a hypoxia-inducible tyrosine kinase receptor for hepatocyte growth factor/scatter factor (HGF/SF), and a multipotent cytokine that induces invasive and metastatic growth. c-Met activation triggers mitogenic, morphogenic, and angiogenic changes in breast tumors, invariably associated with extremely poor prognosis, reduced survival, and high metastatic risks.

Body

We hypothesize that a combination of immunotherapy which exploits the tumor microenvironment would probably be a innovative and novel alternative to supplement current treatment modalities

Goals

To develop chimeric antigen receptors (CARs) to target antigens on breast cancer cells. Our approach focuses on exploiting the hypoxic tumor microenvironment to conditionally activate reprogrammed T cells specific for the c-Met antigen. Specifically, we want to (1) develop a CAR specific for c-Met antigen to reprogram T cells for antigen-dependent killing of breast cancer cells, and (2) develop a CAR to target breast cancer cells that can be conditionally expressed under hypoxia.

CAR constructs

c-Met specific CAR was constructed by fusing the single-chain variable fragments (scFv) of a mouse anti-human c-Met antibody to the human IgG4 Fc domain and signaling endodomains of human CD28 and CD3 (CD247). In addition, to render the CAR oxygen-sensitive, an oxygen-dependent degradation domain from Hypoxia Inducible Factor-1 (HIF-1, the master transcriptional regulator of cellular hypoxia response) was incorporated into the c-Met CAR yielding c-Met-ODDD CAR, which is oxygen-sensitive. Both the c-Met CAR and c-Met-ODDD CAR were cloned into *Sleeping Beauty* (SB) transposition vector (transposon DNA, schematic in Appendix). The expression cassettes were flanked by inverted

repeats/direct repeats (IR/DR) for specific recognition by SB transposase, which cuts and pastes the transposon DNA into genomic T cell DNA.

Growing of CAR on aAPC platform

Peripheral blood mononuclear cells (PBMC) were isolated from normal donors by venipuncture and Ficoll fractionation. These cells are nucleofected using Amaxa T cell transfection with SB transposase and transposon constructs. The cells were then stimulated using γ -irradiated artificial antigen presenting cells (aAPC), a K562-based HLA^{-ve} cell line constitutively expressing c-Met antigen, CD86, CD137L, IL-15, and CD64. These co-stimulatory molecules ensure optimal T cell expansion.

Testing of CAR expression based on oxygen condition

T cells were expanded on 1% (hypoxic condition) or 20% (normoxic condition) oxygen supplemented with 5% CO₂ in a humidified environment at 37°C. CAR expression were determined by Fluorescent-Activated Cell Sorting (FACS). Cell numbers were enumerated and fold expansion calculated over a 4-week (4 x 7 days stimulations) period.

Testing of CAR on breast cancer cell lines

c-Met expression was verified on breast cancer cell lines and co-cultures were performed to assay for CAR-directed T cells killing of target tumor cells under either low or normal oxygen conditions (1%, or 20% O₂).

Key Research Accomplishments

We capitalized on the triple associations of hypoxia, c-Met, and breast carcinoma to develop novel and specific immunotherapy for enhanced therapeutic efficacy. Successful targeting of breast cancer will involve not only redirected specificity, but also modifying T cells to overcome the hostile/hypoxic tumor microenvironment:

Research accomplishments include:

- (i) We can use the oxygen-dependent degradation domain (ODDD) to render the expression of red-fluorescent protein sensitive to hypoxia (Figure 1).

- (ii) We can fuse ODDD to c-Met-specific CAR to render T cells capable of only expressing the CAR-ODDD under conditions of hypoxia, but not normoxia (Figure 1). Control c-Met-specific CAR is expressed under hypoxia and normoxia.
- (iii) We can propagate c-Met-specific CAR-ODDD to large and clinically-sufficient numbers under hypoxia on γ -irradiated c-Met⁺ aAPC (Figure 2).
- (iv) We can demonstrate re-directed effector function of c-Met-ODDD⁺ T cells based on selected killing of hypoxic tumor cells expressing c-Met (Figure 3).

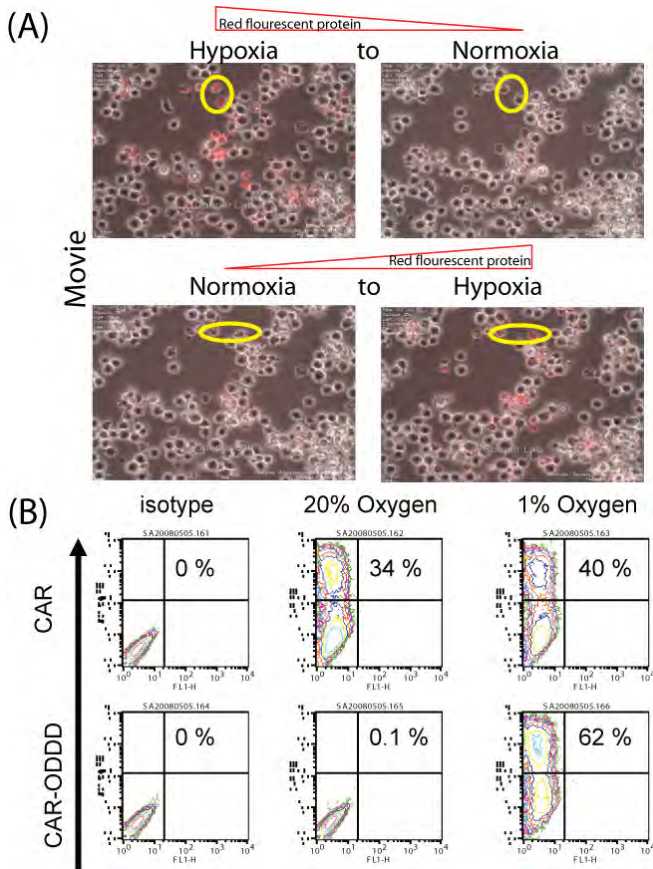


Figure 1: (A) Expression (in K562 cells) of the red-fluorescent protein AsRed-2, fused to ODDD, under hypoxia (1% O₂) and then loss of expression under normoxia (20% O₂), and followed by re-expression of AsRed2-ODDD as the oxygen tension was reduced. (In these preliminary data, not all the cells express AsRed2-ODDD.) Yellow circles highlight cell fluorescence indicating molecular sensing of O₂ and AsRed2 expression. (B) Expression of c-Met-specific CAR (without fusion to ODDD) under normoxia and hypoxia (top). The human HIF-1 ODDD was fused in frame to the 3' end of the c-Met-specific CAR (CAR-

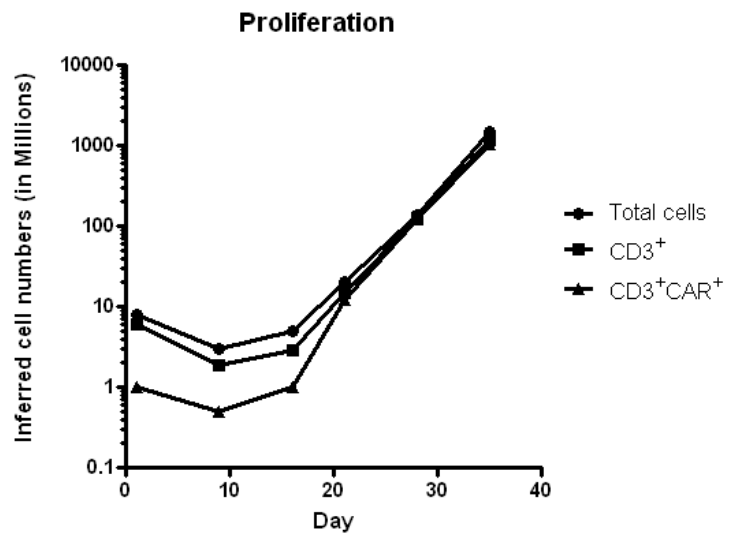


Figure 2: Selective propagation of c-Met-specific CAR-ODDD⁺ T cells can be numerically expanded on c-Met⁺ aAPC under hypoxia (1% O₂).

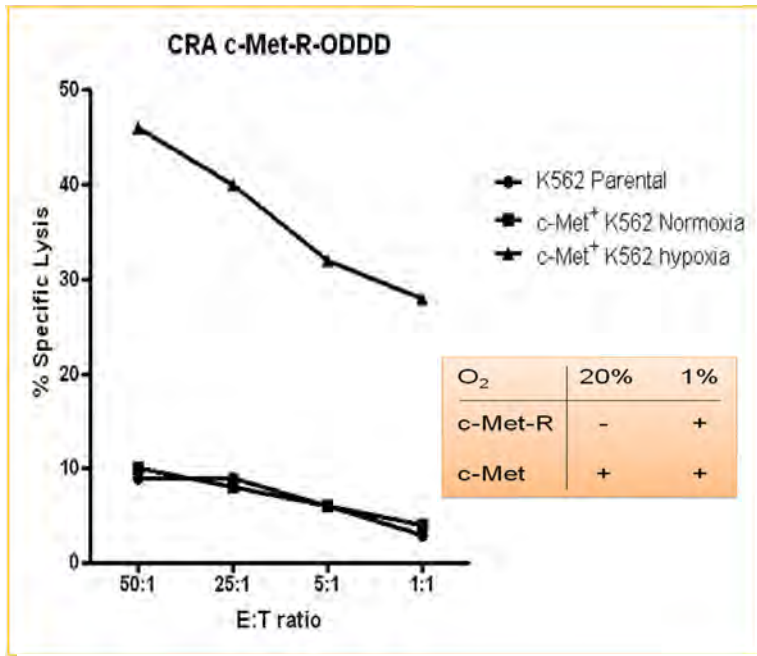


Figure 3: Chromium release assay of c-Met-specific CAR-ODDD⁺ T cells under conditions of normoxia and hypoxia. The tumor cells (K562) were transfected to enforce expression of c-met on the cell surface independent of O₂ concentration. The T cells conditionally express the CAR-ODDD under conditions of hypoxia.

Reportable Outcomes

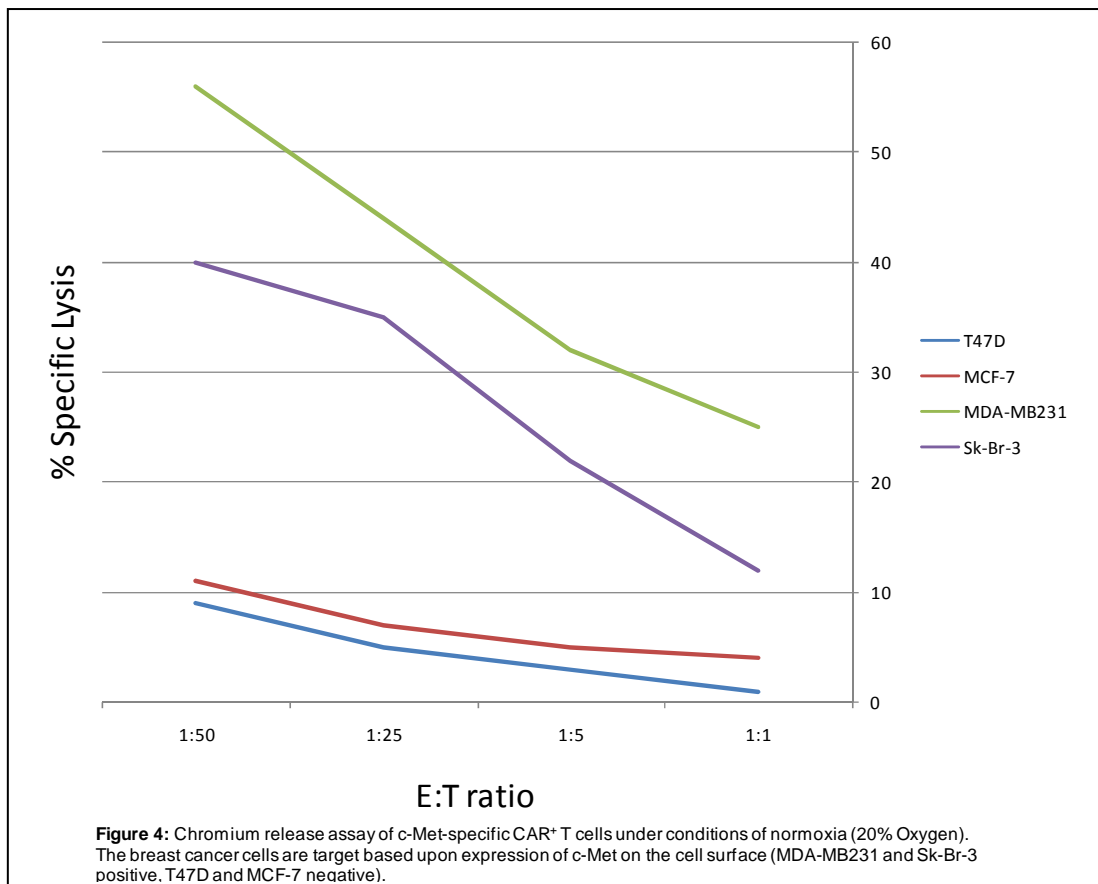
The ability to genetically manipulate T cells and expand them to clinically relevant numbers *ex vivo* provides new opportunities to enhance their biologic activity *in vivo*.

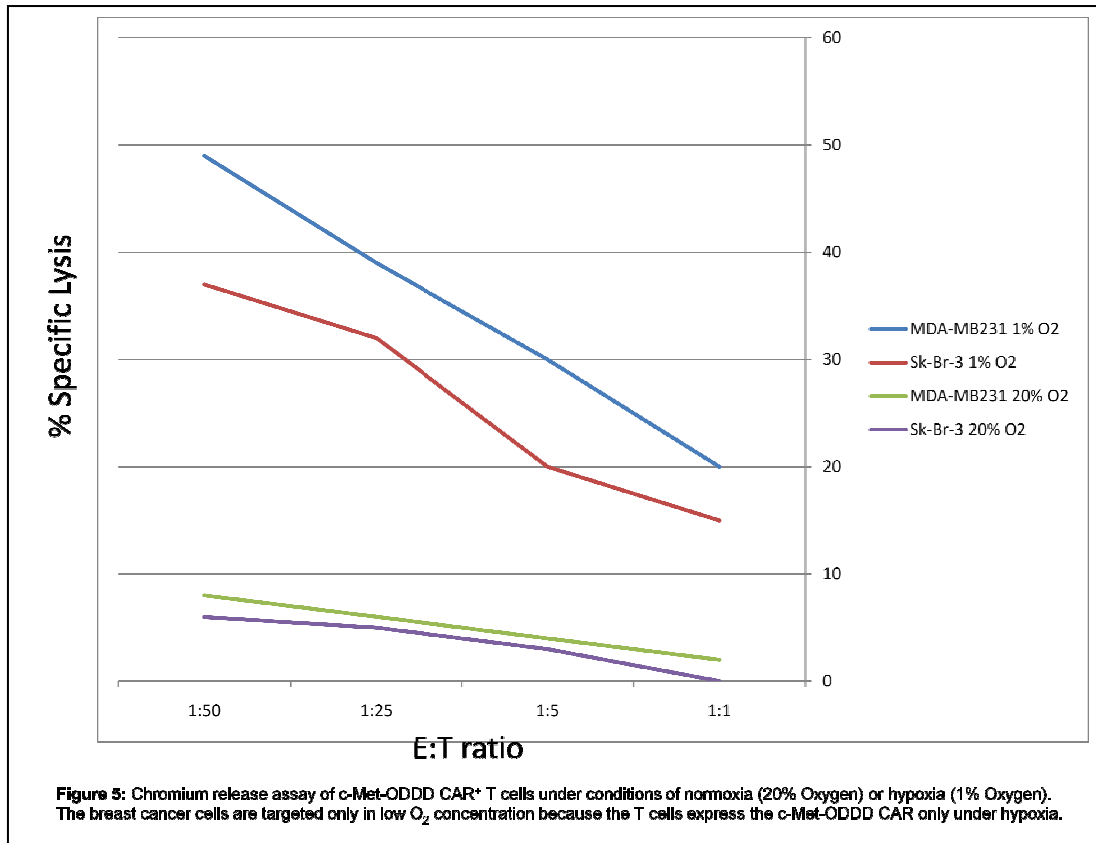
Our data demonstrate that:

- (i) c-Met CAR⁺ T cells are able to kill c-Met⁺ breast cancer cells only (Figure 4).
- (ii) c-Met ODDD CAR⁺ T cells are able to kill c-Met⁺ breast cancer cells only in low oxygen conditions, such as tumor environments (Figure 5).

Table 1. c-Met expression in breast cancer cells

Breast cancer cell lines:	c-Met expression:
T47D	low / negative
MCF-7	low/ negative
MDA-MB231	positive
SkBr-3	positive





Conclusions

We have generated a CAR coupled to a molecular sensor for oxygen that is capable of targeting c-Met which can be useful in the treatment of breast cancer. Conditional activation of T cells can be a useful way of avoiding unwanted effects with antigens which have a widespread expression profile. Oxygen-sensitive CARs can be used to activate T cells only in tumor microenvironment leaving normal c-Met⁺ epithelial cells untouched.

c-Met overexpressions are also detected in other malignancies so our platform technology can also be useful in tumors with pathologic HGF/c-Met signaling axis. In addition, this technology can be adapted to target specific antigens of choice for other tumor types.

To our knowledge, no publication is found detailing therapeutic development conditionally targeting c-Met in breast carcinomas. We are therefore at the cutting edge forefront in our attempt to leverage our platform technology to conditionally reprogram T cells to target c-Met in breast cancer.

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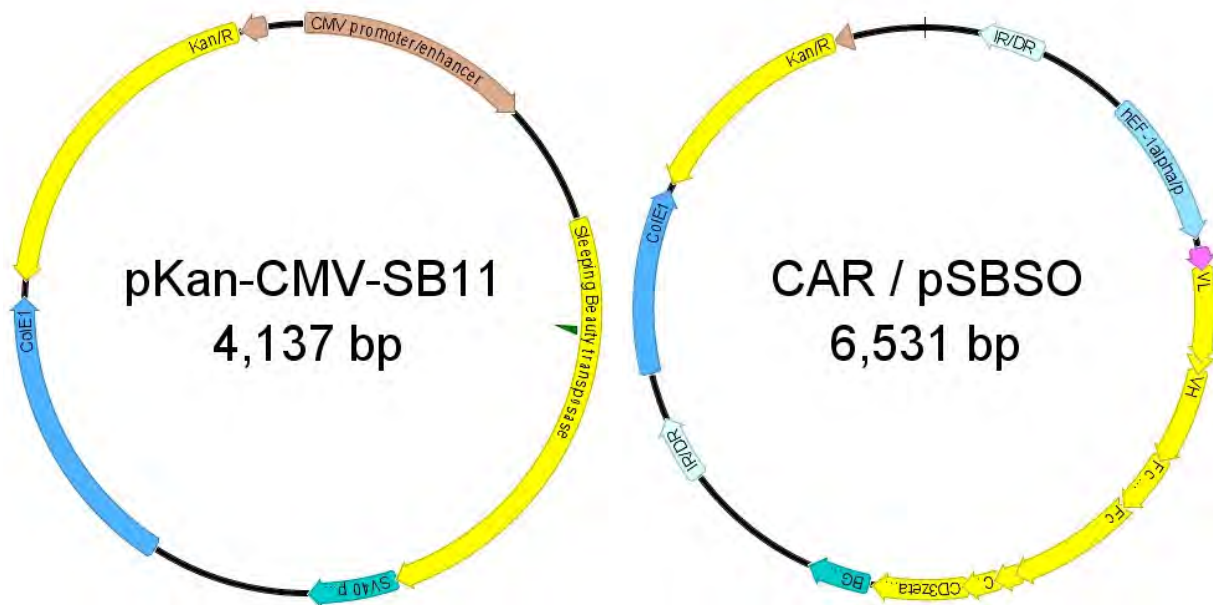
List of Personnel Supported by Grant

Sonny Oon Ang

Tijuan Mi

Appendices

Schematic of Sleeping Beauty Transposition system consisting of a transposase expression plasmid and a transposon DNA vector.



Left Panel: plasmid construct for SB11 transposase. Right Pane: DNA vector of c-Met specific chimeric antigen receptor (CAR)