Vaccine-Mediated Immunotherapy Directed Against
a Transcription Factor Driving the Metastatic Process

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Abstract

Numerous reports have now demonstrated that the epithelial-to-mesenchymal transition (EMT) process is involved in solid tumor progression, metastases, and drug resistance. Several transcription factors have been implicated as drivers of EMT and metastatic progression, including Twist. Overexpression of Twist has been shown to be associated with poor prognosis and drug resistance for many carcinomas and other tumor types. The role of Twist in experimental cancer metastases has been principally studied in the 4T1 mammary tumor model, where silencing of Twist in vitro has been shown to greatly reduce in-vivo metastatic spread. Transcription factors such as Twist are generally believed to be “undruggable” due to their nuclear location and lack of a specific groove for tight binding of a small molecule inhibitor. An alternative approach to drug therapy targeting transcription factors driving the metastatic process is T-cell–mediated immunotherapy. A therapeutic vaccine platform that has been previously characterized consists of heat-killed recombinant Saccharomyces cerevisiae (yeast) capable of expressing tumor-associated antigen protein. We report here the construction and characterization of a recombinant yeast expressing the entire Twist protein, which is capable of inducing both CD8+ and CD4+ Twist-specific T-cell responses in vivo. Vaccination of mice reduced the size of primary transplanted 4T1 tumors and had an even greater anti-tumor effect on lung metastases of the same mice, which was dependent on Twist-specific CD8+ T cells. These studies provide the rationale for vaccine-induced T-cell–mediated therapy of transcription factors involved in driving the metastatic process.
Introduction

Numerous reports have now demonstrated that the epithelial-to-mesenchymal transition (EMT) process is involved in solid tumor progression, invasion, and metastases 1-7. Studies have also shown that cells undergoing EMT become more drug resistant 8-11. Several transcription factors have been implicated as drivers of EMT and metastatic progression, including Twist 12, Snail 13, Slug 14 and Brachyury 2, 15, among others; perhaps the most studied of these is Twist. Overexpression of Twist has been shown to be associated with poor prognosis for the following human carcinomas: breast 16, 17, colorectal 18, 19, cervical 20, lung 21, esophageal 22, hypopharyngeal 23, prostate 24, gastric 25, and bladder 26, as well as human melanoma 27, glioblastoma 28, gastrointestinal stromal tumor 29, and osteosarcoma 30. Overexpression of Twist has also been shown to be associated with hormone and drug resistance 17, 21 and the suppression of senescence programs 31.

The role of Twist in cancer metastases has been principally studied in the 4T1 mammary tumor model, where 4T1 cells are transplanted into the mammary fat pad and spontaneously metastasize to the lung. Silencing of Twist in vitro has been shown to greatly reduce in-vivo metastatic spread 32, 33. Transcription factors such as Twist are generally believed to be “undruggable” due to their nuclear location and lack of a specific groove for tight binding of a small molecule inhibitor. One study 9 has shown that pretreatment of 4T1 cells in vitro with the antibiotic salinomycin prior to transplant resulted in a 4-fold reduction of metastases, a decrease in the epithelial marker E-cadherin and an increase in the mesenchymal marker vimentin. Pretreatment of 4T1 cells with paclitaxel, however, induced the opposite effects.

An alternative approach to drug therapy to target transcription factors driving the metastatic process is T-cell–mediated immunotherapy. A recent study has shown that T cells
from mice vaccinated with a 10-mer Twist peptide could lyse 4T1 tumor cells in vitro. This provided evidence that Twist polypeptides could be processed through the cytoplasm and transported in the context of class I major histocompatibility complex (MHC) as a 9-10 peptide-MHC complex on the cell surface for T-cell recognition.

A therapeutic vaccine platform that has been previously characterized consists of heat-killed recombinant *Saccharomyces cerevisiae* (yeast) capable of expressing tumor-associated antigen (TAA) protein. Recombinant yeast-CEA vaccine was shown to efficiently activate murine and human T cells, which are capable of lysing murine and human tumor cells, respectively, and recombinant yeast-CEA vaccination of mice resulted in anti-tumor activity. These and other studies have shown that yeast (even devoid of the tumor antigen) could efficiently activate murine and human dendritic cells (DCs) via their Toll-like receptors (TLRs) and consequently induce DCs to produce high levels of type I cytokines such as IL-2, TNF-α, and IFN-γ. Thus the “yeast component” of the recombinant yeast is an integral part of the vaccine platform in its ability to activate the innate immune system and has been shown previously to contribute in part to anti-tumor effects.

We report here the construction and characterization of a recombinant yeast expressing the entire Twist protein, which is capable of the induction of both CD8+ and CD4+ Twist-specific T-cell responses in a 4T1 mammary breast cancer model. Vaccination of mice reduced the size of primary transplanted 4T1 murine mammary tumors and had an even greater anti-tumor effect on lung metastases of the same mice. These antitumor effects were seen in a) the unresected setting, b) in the neoadjuvant tumor setting where mice were vaccinated prior to the surgical resection of the primary tumor, and c) in the adjuvant setting where primary tumors were surgically removed prior to the administration of vaccine. These studies provide the
rationale for vaccine-induced T-cell–mediated therapy of transcription factors involved in driving the metastatic process.

Materials and Methods

Animals

All mice were housed and maintained in microisolator cages under specific pathogen-free conditions and in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines. All experimental studies were carried out under approval of the NIH Intramural Animal Care and Use Committee.

Tumor cells

4T1 murine mammary and P815 mouse lymphoblast-like mastocytoma cell lines were purchased from American Type Culture Collection (Manassas, VA) and maintained in the recommended medium.

RNA isolation, quantitative RT-PCR, and EMT array

Tissues were collected from naïve mice, and cell lysates were obtained utilizing gentleMACSTM M Tubes as per the manufacturer’s recommendations (Miltenyi Biotec, Auburn, CA). Total RNA was isolated from tissue lysates and tumor cell lines using the RNeasy extraction kit (Qiagen, Valencia, CA), and reversed transcribed into cDNA using the Advantage RT-for-PCR kit (Clontech, Mountain View, CA). cDNA (2.5 – 10 ng) was used in quantitative real-time PCR reactions using the following probes specific for Twist1 (Mm00442036_m1) and GAPDH (4352339E). Fold change in relative mRNA expression was calculated as expression in
4T1 Twist shRNA cells relative to control shRNA cells. Relative mRNA expression levels of 84 genes involved in the EMT process were assessed using an EMT PCR array (SA Biosciences, Valencia, CA) as per the manufacturer’s directions. PCR was performed on the 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Where indicated, values calculated as expression relative to GAPDH as previously described 2.

**Immunohistochemistry**

Female Balb/C (n = 3) were inoculated with 5 x 10⁴ 4T1 cells on the mammary fat pad on day 0. On day 30, mice were euthanized, primary tumors and lung tissues were harvested. Twist expression was detected via immunohistochemistry using rabbit-polyclonal antibody to Twist (EMD Millipore, Billerica, MA) according to the manufacturer’s instructions. Expression of mesenchymal markers was detected using rabbit antibody to N-cadherin and vimentin (Abcam, Cambridge, MA). Epithelial marker expression was detected using rabbit antibody to E-cadherin (Cell Signaling, Danvers, MA). Entire slides were digitally scanned by an Aperio ScanScope CS scanning system and analyzed by Aperio ImageScope Viewer software (Aperio Technologies Inc., Vista, CA). The statistical analysis was composed of 3 independent murine tumors, within each contained a complete stained tumor section. Positive tumor regions were measured using the Positive Pixel Count v9 algorithm. Negative controls included omission of primary antibody with PBS and matched rabbit isotype antibody.

**Generation of Twist knockdowns**

4T1 cells were transfected with 1 μg of purified control shRNA or the Twist1-specific targeting shRNA (Origene, Rockville, MD) using a nucleofector device as per the
manufacturer’s recommendations (Lonza, Walkersville, MD). Stable transfectants were selected utilizing growth media containing 2 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO).

**Western blot analysis**

Cells were washed twice with PBS and lysed in RIPA Lysis Buffer (Santa Cruz Biotech, Dallas, TX). Protein concentration was measured using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Aliquots containing 40 μg of protein were run on an SDS-PAGE, and transferred to a nitrocellulose membrane. Membranes were blocked for 1 hour at room temperature with 5% milk in PBS and probed overnight at 4°C using antibodies specific for pan-actin (clone Ab-5, Neo Markers) or Twist (clone 2c1a, Abcam) at the concentrations recommended by the respective manufacturers. Membranes were washed three times using PBS containing 0.05% Tween20 (PBST), and incubated with appropriate IRDye-labeled secondary antibodies (LI-COR Biotechnology, Lincoln, NE) at a 1:5000 dilution in 5% milk in PBS for 1 hour at room temperature. Membranes were washed three times with PBST. All western blots were imaged using the Odyssey Infrared imaging system (LI-COR Biotechnology).

**Proliferation**

Five hundred tumor cells were seeded per well in 96-well trays, and cell viability was assessed by MTT. Briefly, MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich) was added per well at a final concentration of 0.5 mg/ml final concentration for 2 hours at 37°C. Medium was removed and the converted dye was solubilized in ice-cold isopropanol and measured at 560 nm on a micro plate reader (Bio-TEK Instruments). To evaluate the effect of radiation or chemotherapy on the growth of tumor cells, cells were
exposed in vitro to 5 Gy radiation dose or 1 ng/ml docetaxel in complete media or were left untreated. Cells were harvested 24, 48, or 72 hours after exposure, and the total number of adherent viable cells was determined by trypan blue exclusion.

Migration and invasion assays

In vitro cell migratory and invasive abilities were assessed using CytoSelect™ 96-well cell migration and invasion assays as per the manufacturer’s instructions (Cell Biolabs, Inc, San Diego, CA). Briefly, $10^5$ tumor cells in serum-free media were seeded into the upper chamber and media containing 20% FBS was placed into the lower chamber. After 24-hour incubation at $37^\circ\text{C}$, cells that had moved into the lower chamber were lysed and quantified utilizing the CyQuant GR dye solution; fluorescence was measured at 480/520 nm utilizing a micro plate reader (Bio-TEK Instruments, Winssski, VT). Data is expressed as relative fluorescence units (RFU), as per the manufacturer’s instructions.

F-actin staining

Cells were grown on glass coverslips, and stained for F-actin utilizing Alexa Fluor-488 labeled phalloidin (Invitrogen, Life Technologies, Grand Island, NY) as per the manufacturer's recommendations.

Vaccine constructs

Recombinant *S. cerevisiae* yeast constructs without antigen (wild-type yeast) or expressing the Twist 1 gene protein (referred to here as recombinant yeast-Twist) were engineered by methods similar to those previously described 35 (GlobeImmune, Louisville, CO).
Expression of Twist protein from yeast-Twist was conformed by western blot analysis (Supplemental Fig. 1).

**Murine CD8\(^+\) T-cell responses**

Female Balb/C mice (n=5) were vaccinated with PBS, wild-type yeast or recombinant yeast-Twist vaccine at one yeast unit (YU) per site at 4 sites (4 YU total) on days 0, 7, 14 and 21. On day 35, mice were euthanized and spleens were removed, dispersed into single-cell suspensions, pooled and stimulated for 6 days with 1 \(\mu\)g/ml of Twist peptide (LYQVLQSDEL, CPC Scientific, Sunnyvale, CA). Bulk lymphocytes were recovered by centrifugation through a Ficoll-Hypaque gradient. For the assay of tumor-killing activity, the recovered lymphocytes and \(^{111}\)In-labeled target cells P815 pulsed with the Twist peptide were incubated for 5 hours and radioactivity in supernatants was measured using a gamma counter (Corba Autogamma; Packard Instruments, Downers Grove, IL). For IFN-\(\gamma\) production, the pooled splenocytes were stimulated for 7 days with 1 \(\mu\)g/ml of Twist peptide and bulk lymphocytes were recovered by centrifugation through a Ficoll-Hypaque gradient. Recovered T-cells were restimulated with fresh, irradiated, naive syngeneic splenocytes (as antigen processing cells, APC) and 1 \(\mu\)g/ml of either Twist peptide or control HIV peptide (AMQMLKETI, CPC Scientific) for 24 hours. Supernatant was collected and analyzed for murine IFN-\(\gamma\) by cytometric bead array (BD PharMingen, San Jose, CA) according to the manufacturer's instructions. Results were background-corrected for HIV peptide.
Murine CD4+ T-cell proliferation and flow cytometry

Female Balb/C mice (n=5) were injected with PBS, or vaccinated wild-type yeast or yeast-Twist vaccine at 1 YU per site at 4 sites (4 YU total) weekly for 3 or 4 weeks. Fourteen days after the last vaccination, animals were euthanized and splenocytes were collected and pooled. CD4+ T cells were purified by negative selection using a magnetic separation system (Miltenyi Biotec). To measure Twist-specific proliferation, CD4+ T cells were co-cultured with irradiated syngeneic spleen cells (APCs) and Twist peptide (QQPASGKRGRKRRS, CPC Scientific) at 0.4 µg/ml. After 4 days of incubation, 1 µCi of [3H]-thymidine (Perkin-Elmer, Waltham, MA) was added per well; plates were harvested after 16 hours of incubation and thymidine incorporation was measured using a 1450 Betaplate reader (Perkin-Elmer). To analyze T-cell activation markers, cells were harvested on day 5 and multicolor cytometric analyses for T-cell activation were performed using an LSR-II (BD Biosciences, San Jose, CA). FoxP3 antibody with appropriate isotype was purchased from eBioscience (San Diego, CA) and the remaining antibodies and their respective isotype control antibodies were purchased from BD Biosciences. Supernatant was also collected and analyzed for IFN-γ by cytometric bead array (BD Bioscience).

Antitumor vaccination

Female Balb/C (n=8-10) were injected with 5x10^4 4T1 mammary tumor cells. Four days or 11 days after tumor implantation, mice were injected with PBS or with wild-type yeast or yeast-Twist at a dose of 1 YU at 4 sites (4 YU total) and subsequently weekly for the duration of the experiment. Tumor growth was measured twice/week and recorded. Mice were euthanized either on day 21 or when the average tumor volume in each group reached 1000 mm^3. To
enumerate lung metastasis, lungs were harvested and dispersed into single-cell suspension and plated in the presence of 6-TG (Sigma-Aldrich). Ten days later, cells were fixed with methanol, stained with 0.03% methylene blue and the number of clonogenic metastatic cells was counted.

**Analysis of tumor T-cell infiltration**

To determine the presence of T-cell infiltrates in the primary tumors and lung tumor tissues, female Balb/C (n=3) were injected with 5x10⁴ 4T1 mammary tumor cells. Four days after tumor implantation, mice were injected weekly with PBS or with wild-type yeast or yeast-Twist at a dose of 1 YU at 4 sites (4 YU total). Mice were euthanized when the average tumor volume in each group reached 1000 mm³. Primary tumors and lungs were harvested and T-cell infiltrates were detected using rabbit-polyclonal antibody to CD3 (Dako, Carpinteria, CA) according to the manufacturer’s instructions and analyzed by Aperio ImageScope Viewer software (Aperio Technologies Inc.) as earlier described. Negative controls included omission of primary antibody with PBS and matched rabbit isotype.

**T-cell/NK depletion study**

Female Balb/C (n=8-10) were injected with 5x10⁴ 4T1 mammary tumor cells. Four days after tumor implantation, mice were vaccinated weekly with either PBS or wild-type yeast or recombinant yeast-Twist at a dose of 1 YU at 4 sites (4 YU total) and subsequently weekly for the duration of the experiment. On days 4–7 and every week thereafter, animals were depleted of CD4+ T cells, CD8+ T cells, or both CD4+ and CD8+ T cells by intraperitoneal administration of a daily dose (100 μg) of anti-CD4 (GK 1.5) and/or anti-CD8 (Lyt 2.2) hybridomas in PBS. For natural killer (NK) cell depletion, animals were injected with 25 μl anti-asialo-GM1 antibody
(Cedarlane, Burlington, Ontario, Canada) i.p. once/week. Tumor volume was recorded twice/week.

**Tumor resection and antitumor vaccination**

To model the neoadjuvant tumor setting, female Balb/C (n = 8-10) were injected with $5 \times 10^4$ 4T1 mammary tumor cells. Four days after tumor implantation, mice were vaccinated weekly with either PBS or wild-type yeast or yeast-Twist at a dose of 0.3 YU at 1 site (0.3 YU total). Tumors were allowed to grow and metastasize to distant site. On day 10, primary tumors were removed using sterile technique. To enumerate lung metastasis, mice were euthanized on day 40, lungs were harvested and the number of clonogenic metastatic cells was counted. To model the adjuvant tumor setting, female Balb/C (n=20) were injected with $5 \times 10^4$ 4T1 mammary tumor cells. On day 14, primary tumors were surgically removed using aseptic technique and after 1 week, mice were vaccinated with PBS or yeast-Twist at a dose of 1 YU at 4 sites (4 YU total) and weekly thereafter. Mice were observed and their survival was recorded.

**Toxicology study**

Female Balb/C mice (n = 5) were vaccinated with either PBS or wild-type yeast or recombinant yeast-Twist at a dose of 1 YU at 4 sites (4 YU total) weekly for 18 weeks. One week following the last injection, animals were assessed for any potential toxicities utilizing the following parameters: in-life body weight, CBC (20 parameters: WBC, LY, MO, EO, RBC, Hb, HCT, MCV, PLT), serum chemistry (5 parameters: BUN, CRE, AST, ALT, ALK), and autoimmuninity (5 parameters: Histone, n-RNP, dsDNA, ssDNA, CIC antibodies), as described previously 40.
Statistical analysis

Data were analyzed using GraphPad Prism (Version 5; GraphPad Software, La Jolla, CA). Data points in graphs represent the mean SD.

Results

Expression of Twist in normal tissues and tumors

To evaluate Twist as a potential target of carcinoma therapy, Twist mRNA expression was analyzed in a range of normal murine tissues and the 4T1 tumor cell line (Fig. 1A). High levels of Twist mRNA expression were seen in 4T1 cells and testis, generally considered to be an immunologically privileged site, with lower levels detected in spleen. Some other tissues evaluated showed detectable, albeit low, levels of Twist mRNA. Contrary to Twist, expression of Brachyury mRNA, a different EMT regulator, was negative in all samples evaluated, with the exception of testis (data not shown). The 4T1 line was chosen for further study due to its ability to metastasize spontaneously to the lung, its involvement in EMT demonstrated in previous studies, and the fact that Twist was shown to be a mediator of metastatic spread in the 4T1 model 33. Figure 1B shows the significantly higher level of expression of Twist in biopsies of 4T1 pulmonary metastases vs. primary tumors by RT-PCR. This finding was further confirmed by immunohistochemistry (Fig. 1D) where the expression of Twist in pulmonary metastases was significantly elevated compared to primary tumors ($P=0.0373$, Fig. 1C). Since Twist is expressed in some normal organs, the results shown in Figure 1A also define Twist as a “self-antigen” to which Balb/c mice are supposedly immunologically ‘tolerant’.
Silencing Twist expression is associated with a decreased migratory phenotype

To confirm and extend previous observations that Twist is a driver of metastasis in this 4T1 model, it is shown that the reduced level of Twist in Twist shRNA–transfected 4T1 cells (Fig. 2A) reduced the level of migration (Fig. 2C) and invasion (Fig. 2D). However, Twist shRNA-treated cells grow in vitro at an identical rate to the control shRNA-treated cells (Fig. 2B). Expression of 84 genes involved in the process of EMT was analyzed by real-time PCR in 4T1 cells stably silenced for the expression of Twist1 (4T1 Twist shRNA) compared to control cells (control shRNA). Shown in Figure 2E is the selected group of genes whose expression was either upregulated or downregulated greater than 3-fold in Twist-silenced tumor cells. For example, Mmp-3 and Mmp-9 expression was markedly downregulated; Mmp-3 and Mmp-9 encode for the matrix metallopeptidases-3 and -9, respectively, enzymes known to participate in the breakdown of the extracellular matrix 41. Silencing of Twist1 in 4T1 cells also resulted in a marked (4.3-fold) decrease in the expression of Spp1, the gene encoding for the secreted sialoprotein osteopontin, previously described as a regulator of adhesion, motility, tumor growth and metastasis 42. The gene most greatly upregulated (50-fold) following Twist1 silencing in 4T1 cells was Krt14, which encodes for the epithelial marker protein keratin 14. Also markedly upregulated were the genes encoding for fibroblast growth factor binding protein 1 (Fgfbp1) and regulator of G-protein signaling 2 (Rsg2). The former is a protein involved in binding and activation of fibroblast growth factors, while regulator of G-protein signaling 2 may inhibit G protein-coupled receptor (GPCR)–mediated signaling via inactivation of G proteins. Additionally, silencing Twist expression in 4T1 cells also mediated re-organization of the actin cytoskeleton as indicated by F-actin staining depicted in Figure 2F. While tumor cells with high levels of Twist presented F-actin filaments organized into actin stress fibers, silencing of Twist
in 4T1 cells promoted cortical organization of F-actin in the areas of cell-to-cell contact. Although Twist was previously implicated in apoptotic evasion\(^1\), in the 4T1 model, however, silencing Twist did not improve cells’ sensitivity to radiation or docetaxel treatment (data not shown). Altogether, our data indicate that Twist plays a functional role in the 4T1 model on the remodeling of the extracellular matrix as well as on the events of tumor cell migration and invasion.

**Vaccination with recombinant yeast-Twist induces Twist-specific T-cell responses**

To determine whether CD8+ and/or CD4+ Twist-specific immune responses could be elicited in vivo, Balb/C mice were vaccinated four times at weekly intervals with recombinant yeast-Twist vaccine, wild-type yeast or buffer (PBS). Mice were euthanized on day 35. As mentioned above, wild-type yeast have previously been shown to stimulate DCs via TLRs which, in turn, can activate T cells to produce high levels of Type 1 cytokines; the yeast component of the vaccine is thus considered an integral part of the vaccine platform. Splenic T cells from mice in each of the vaccinated groups were pulsed with a 10-mer Twist peptide to activate CD8+ T cells. The splenocytes from mice vaccinated with the recombinant yeast-Twist produced 2-fold more IFN-\(\gamma\) and displayed significantly greater (> 3-fold, \(P=0.02\)) lytic activity against 4T1 cells than splenocytes from the wild-type yeast group or no treatment group (Fig. 3A, B). A novel 15-mer Twist peptide was also employed to activate CD4+ T cells, and significantly greater proliferative activity and increased IFN-\(\gamma\) production were also seen from splenocytes in the recombinant yeast-Twist vaccinated mice (Fig. 3C, D). These data, taken together, indicate that the recombinant yeast-Twist vaccine can break tolerance to this self-antigen. Analyses of populations of CD4+ memory T cells after *in vitro* re-stimulation revealed a
7- and 1.6-fold increase of CD4+ T memory stem cells (Tscm) in Twist-vaccinated mice compared to PBS- or yeast wild-type treated mice, respectively (Fig. 3E). This recently described Tscm population was thought to generate potent anti-tumor activity 44.

**Antitumor and antimetastatic effect of recombinant yeast-Twist vaccination**

Anti-tumor activity was evaluated in vaccinated tumor-bearing mice. 4T1 cells were transplanted in mammary fat pads on day 0 and mice were vaccinated three times at weekly intervals starting on day 4. Mice were euthanized on day 21 and lung metastases were enumerated. As seen in Figure 4A, on day 21 there was a significant reduction ($P=0.0199$) in primary tumor volume in the recombinant yeast-Twist vaccinated group vs. the wild-type yeast vaccinated group or PBS vaccinated group (n=8–10 mice/group). There was also a statistical difference ($P=0.005$) in the number of lung metastases in the Twist vaccinated group vs. the PBS vaccinated group and a trend but a non-statistical difference ($P=0.16$) between Twist vaccinated and wild-type yeast vaccinated groups (Fig. 4B).

Other studies have shown that immunosuppressive factors, correlating with the size of the primary tumor, can influence the growth of secondary lesions. To control for the possibility that the size of the primary tumor was influencing the number of lung metastases, mice were transplanted with 4T1 in fat pads on day 0 and vaccinated weekly starting at day 4 with recombinant yeast-Twist, wild-type yeast or buffer; primary tumor volumes were then measured. Mice in each group were euthanized when the average tumor volume reached 1,000 mm$^3$ (Fig. 4C). As seen in Figure 4D, there was a reduction in the number of lung metastases in the recombinant yeast-Twist vaccinated group vs. the wild-type yeast vaccinated group ($P=0.03$) and vs. the PBS vaccinated group ($P=0.004$). Moreover, the percentage of mice with ≤150
clonogenic metastatic cells was 12.5% in the control group, 16% in the wild-type yeast vaccinated group, and 58% in the recombinant yeast-Twist vaccinated group.

Analysis of splenic immune populations in tumor-bearing mice receiving no treatment of yeast-Twist vaccine indicated that the yeast-Twist vaccine did not alter the relative percentage or mean expression of Tregs or myeloid-derived suppressor cells (MDSCs, data not shown). Similar observations were attained in non-tumor-bearing mice receiving PBS, yeast-WT, or yeast-Twist vaccine. Independently of the presence of tumor, yeast-Twist vaccination also did not alter the relative percentage of CD4+ and CD8+ T cells, and NK, B, or DC and also did not alter the expression of CD28 on both CD4+ and CD8+ T cells (data not shown). The primary tumors and lung tumor tissues were subjected to immunohistochemistry staining to detect the presence of CD3+ T-cell infiltrates. The quantification of T-cell infiltrates revealed similar numbers of CD3+ T-cell infiltrates in the primary tumors from mice treated with PBS, wild-type yeast and yeast-Twist (Fig. 5B). However, an intense staining of CD3+ T-cell infiltrates was detected in lung tumors harvested from mice that received yeast-Twist when compared to other groups (Fig. 5A). Positive pixel quantification confirmed a significant increase of CD3+ T-cell infiltrates in lung tumor tissues harvested from mice vaccinated with yeast-Twist vs. PBS-treated ($P=0.0007$) and vs. wild-type yeast vaccinated group ($P=0.0005$) (Fig. 5C). Immune depletion studies were conducted to determine which immune cell subset(s) was most responsible for the anti-tumor effect. As seen in Figure 5D, CD4 or NK cell depletion had minor effects (non-statistical) on anti-tumor activity. Depletion of CD8+ T cells, however, completely abrogated the anti-tumor effect of the recombinant yeast-Twist vaccination.
Antitumor and antimetastatic effect of recombinant yeast-Twist vaccination in the neoadjuvant 4T1 mammary tumor model

The prior studies were conducted with mice vaccinated with 1 yeast unit (YU) at 4 sites weekly for 3 weeks, with either recombinant yeast-Twist or wild-type yeast. As was mentioned and shown above, wild-type yeast vaccination was expected to induce some level of anti-tumor activity. In an attempt to minimize the effect of wild-type yeast vaccination in this model, mice were vaccinated three times at weekly intervals with reduced levels of both wild-type yeast and recombinant yeast-Twist: 0.3 YU at a single site (13-fold lower dose than previously used dose of 4 YU). To model the neoadjuvant tumor setting, mice were transplanted with 4T1 tumor cells in the fat pad at day 0, and were vaccinated weekly starting at day 4 throughout the study. On day 14, primary tumors were removed, and mice were euthanized on day 40. As seen in Figure 6A, there was no statistical difference in tumor volume of mice vaccinated with recombinant yeast-Twist, wild-type yeast or PBS in the size of primary tumors removed at day 10. Analyses of lungs removed from mice on day 40 (Fig. 6B), however, showed a clear difference in number of lung metastases ($P=0.02$) between mice vaccinated with recombinant yeast-Twist vaccine vs. wild-type yeast, and a difference between the PBS vaccinated group and the recombinant yeast-Twist vaccinated group ($P<0.0001$). The percentage of mice with ≤1,000 clonogenic lung metastatic cells was 0% in the control group, 25% in the wild-type yeast vaccinated group, and 71% in the recombinant yeast-Twist vaccinated group. These studies provide evidence for a difference in the therapeutic effect of the recombinant yeast-Twist vaccine for metastatic lesions vs. the primary tumor lesion, and provide additional evidence that the size of the primary tumor did not affect the ability of the recombinant yeast-Twist vaccine to reduce the number of lung metastases.
Antitumor and antimetastatic effect of recombinant yeast-Twist vaccination when vaccine administration was delayed and in the adjuvant 4T1 mammary tumor model

Unlike passive therapies, vaccine induced therapeutic responses require multiple vaccinations at appropriate intervals to maximize immune responses. We have previously shown that multiple vaccinations of recombinant yeast vaccines are required. We have previously shown that multiple vaccinations of recombinant yeast vaccines are required for optimal antitumor responses. 4T1 tumors are rapidly growing, and mice bearing unresected 4T1 tumors reach ethical limits for experiments by approximately day 28. If vaccines were initiated on day 14, one could not administer the minimum of three vaccinations required, since mice would be required to be sacrificed at or prior to the third vaccination. Our data demonstrate that administration of yeast-Twist 4 days after tumor implantation was able to elicit a robust antitumor and antimetastatic effect (Fig. 4, Fig. 6). To further strengthen this finding, we performed an additional study in a more rigorous setting where vaccine was given when primary tumor is more established and consequently permitting more tumor cells to metastasize to distant site. Briefly, female Balb/C mice (n=20 per group) were inoculated with 5x10⁴ 4T1 mammary tumor cells in the mammary pad on day 0. On day 11, primary tumors were recorded, the tumors were palpable and the average tumor volume was 113.4 mm³ (Fig. 7A inset). Mice were then randomized to receive either PBS or yeast-Twist and the primary tumor volumes were measured. As seen in Figure 7A, delaying administration of yeast-Twist until day 11 after tumor implantation, where primary tumors are established and thus provide a longer window for tumor cells to metastasize to distant site, still generated a significant primary antitumor effect, albeit less pronounced when compared to earlier vaccine administration. Mice in each group were then
euthanized when the average tumor volume reached 1,000 mm³. As seen in Figure 7B, there was a reduction in the number of lung metastases in the recombinant yeast-Twist vaccinated group vs. PBS vaccinated group ($P=0.03$).

To model adjuvant setting, we performed additional studies where 4T1 cells were transplanted in mammary fat pads on day 0. On day 14, primary tumor volume was surgically removed and 1 week later (day 21 after initial tumor implantation), mice were vaccinated with PBS or yeast-Twist vaccine. Survival curve analyses revealed that mice that received yeast-Twist vaccine experienced greater overall survival, presumably by reduced pulmonary metastases burden, compared to mice that received PBS ($P=0.01$, not shown).

In light of the fact that Twist is expressed in some normal Balb/C tissues (Fig. 1), it was possible that the anti-tumor effect of the recombinant yeast-Twist vaccine could also elicit an autoimmune and/or other toxic effect. Balb/C mice were each vaccinated 18 times at weekly intervals with recombinant yeast-Twist, wild-type yeast, or unvaccinated control. Groups were age matched. Mice were euthanized at 19 weeks. There were no significant differences among any of the groups in (a) body weight, (b) 20 different blood chemistry parameters, (c) seven different clinical chemistry parameters, and (d) five different autoimmunity panels (Supplemental Table 1).

**Discussion**

The studies reported here show for the first time that a vaccine can induce a therapeutic response targeting a transcription factor that is a driver of the metastatic process. While both CD4+ and CD8+ Twist-specific T-cell responses were induced by the recombinant yeast-Twist vaccine (Fig. 3), antibody-mediated depletion studies showed that CD8+ T cells were primarily
responsible for the anti-tumor effects (Fig. 5D). These studies also lend further support to previous studies 2, 34, 45 that T cells can be generated to transcription factors. While transcription factors such as Twist and Brachyury are active in the nucleus, these studies provide evidence that transcription factors can be processed in the cytoplasm as polypeptides and transported to the cell surface in the form of 9-10 mer peptide–MHC class I molecule complexes or 15 mer–MHC class II molecule complexes. These peptide-MHC complexes can in turn be recognized by CD8+ and CD4+ T cells, respectively. The studies reported here also demonstrate that Twist is expressed in some normal murine tissues (Fig. 1) and that the vaccine used can break tolerance to the Twist self antigen. More importantly, these studies demonstrate that T-cell–mediated anti-tumor effects can be induced in the absence of any apparent autoimmunity or other toxic effects (Supplemental Table 1).

It is unclear at this time why the recombinant yeast-Twist vaccine was more effective in the control of the pulmonary metastases vs. the primary tumor. One possible explanation is the observation that there is more Twist expression in the metastases than the primary tumor (Fig. 1 B-D). This correlates with the significant increase of T-cell infiltrates in the lung tissues from mice treated with yeast-Twist when compared to other treatment groups, whereas similar numbers of T-cell infiltrates were detected in the primary tumors among all groups (Figs. 5A-C). These data suggest that the T-cell infiltrates in the lung tumor tissues are Twist-specific. Another explanation is the differences in the tumor microenvironments of the primary vs. metastatic lesions.

While the recombinant yeast-Twist vaccine is shown to be more efficacious in the induction of Twist-specific immune responses and anti-tumor activity than the wild-type yeast vector (Figs. 3, 4, 6, 7), the experiments reveal some degree of immune and anti-tumor effects
with the wild-type yeast vector. Previous studies have shown that wild-type yeast can induce a strong innate immune response; treatment of murine or human immature DCs was shown to efficiently mature DCs and produce levels of type I cytokines analogous to the use of TLR agonists, which are being evaluated as potential cancer therapeutics 35-39. Thus it is confounding to consider the wild-type yeast vector as a true control for any immune response. As seen in the neoadjuvant tumor setting (Fig. 6), however, when the number of yeast units was reduced for both the recombinant yeast-Twist vaccine and the wild-type yeast vector, the differential anti-tumor effect for the recombinant yeast-Twist vaccine became more pronounced.

Twist, on the other hand, has been shown to be a poor prognostic indicator for a wide range of human tumors 8, 16, 17, 19-22, 25, 29, 46, 47. It is unclear at this time, however, whether it will be a valid target for immunotherapy or other types of therapy due to its fairly high level of expression in several human normal adult tissues 15. It should also be pointed out that while numerous studies with human carcinoma cells have shown a clear correlation between biologic processes of EMT, such as migration, invasion, and phenotypic changes (such as conversion of epithelial markers to mesenchymal markers), this correlation is not as clear with murine carcinoma cell lines. For example, it has been shown 32 that 4T1 cells express E-cadherin but are migratory and invasive, while 6NR1 cells express vimentin and N-cadherin, and do not express E-cadherin, but fail to metastasize. In our studies, the expression of Twist corresponds to some, but not all features associated with EMT. *In-vitro*, silencing of Twist expression inhibited the ability of 4T1 cells to migrate and invade, modulated genes associated with EMT and mediated re-organization of the actin cytoskeleton (Fig. 2). *In-vivo*, primary tumors and pulmonary metastases of 4T1 cells were analyzed for 84 genes involved in the EMT process by RT-PCR; several genes that are classically upregulated during the EMT process, such
as Cdh2 (N-cadherin), Vim (vimentin) and Zeb2 (Zinc finger E-box binding homeobox 2), were overexpressed in the lung metastasis biopsies by 4.5-, 12- and 6.2-fold, respectively, when compared to primary tumors. In addition, an EMT array was performed on primary tumors harvested from Twist-vaccinated and non-treated tumors. Tumors from Twist-vaccinated mice demonstrated 3- to 6-fold decrease in expression of genes that are classically upregulated during EMT and the metastasis process, such as N-cadherin (Cdh2), SRY-box containing gene 10 (Sox10), Tissue inhibitor of metalloproteinase 1 (Timp1), and Zinc finger E-box binding homeobox 1 (Zeb1, not shown). However, we confirmed that the EMT process in this 4T1 murine model does not exhibit the phenotypic characteristics of EMT in human carcinoma cells. Using immunohistochemistry, we were able to evaluate the expression of classical epithelial marker E-cadherin and mesenchymal markers N-cadherin and vimentin on 4T1 primary tumors and lung metastases (Supplemental Figure 2). Although there was a significant decrease in E-cadherin expression in lung metastases compared to primary tumors (Supplemental Figure A), comparable N-cadherin and vimentin expression between primary tumors and lung metastases was also observed (Supplemental Figure 2). This confounding observation further confirmed that the EMT process in murine is not as well defined as in human carcinomas. Another transcription factor, Brachyury, has been shown to be a driver of EMT for a range of human carcinomas. Brachyury has been shown to be differentially expressed in human carcinomas vs. normal adult human tissue; it is not expressed in murine carcinomas. Brachyury-specific human T cells have been generated in vitro that are capable of lysing human tumor cells. A phase I trial is ongoing employing a recombinant Brachyury vaccine.

The 4T1 model, however, as has previously been shown 32, 33 and reported here, is an excellent model to study the biology of metastatic process. Numerous preclinical studies and
several clinical studies have now shown the anti-tumor synergy between vaccine therapy and chemotherapy, radiation therapy, hormonal therapy, or the use of small molecule targeted therapeutics 48-50. The studies reported here also support the use of the 4T1 model to further study interventions of the metastatic process by employing vaccines targeting the transcription factor Twist, as a monotherapy or in a range of combination therapies.

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Figure Legends

Figure 1. Expression of *Twist* in normal tissues and tumors. **A**, Real-time PCR was performed to evaluate *Twist* RNA expression in several normal murine tissues and the 4T1 breast cancer cell line. Female Balb/C mice (n=3) were injected with 5x10^4 4T1 cells in mammary fat pad. On day 30, primary tumors and lung metastases were harvested. **B**, Twist mRNA expression in primary tumor and lung metastases was evaluated by real-time PCR. Error bars indicate mean ± S.E.M. from triplicate measurements. Data is representative of two independent experiments. **C-D**, Twist protein expression in primary tumor and lung metastases was detected by immunohistochemistry (magnification 20X) and quantified by positive pixel quantification analysis. Statistical analyses were done by Student’s *t*-test.

Figure 2. Silencing Twist expression is associated with a decreased migratory/invasive phenotype. The functional role of Twist in 4T1 tumor cells was analyzed by gene knockdown. **A**, Western blot analysis of Twist protein in the 4T1 control and Twist shRNA cell lines. Actin is shown as the protein loading control. **B**, Proliferation of the 4T1 control and Twist shRNA cells was assessed by MTT assay after 1, 3, and 5 days of *in vitro* growth. *In vitro* cell migration (**C**) and invasion (**D**) of the 4T1 cell line pair. Error bars indicate mean ± S.E.M. from triplicate measurements. **E**, Fold change in relative mRNA expression in genes known to be involved in the EMT process in 4T1 Twist shRNA relative to control shRNA cells. **F**, F-actin staining of the 4T1 cell line pair (magnification 20X). The blue signal represents the DAPI-stained nuclei and the green signal represents the staining of the F-actin.
Figure 3. Vaccination with recombinant yeast-Twist induces Twist-specific T-cell responses.

Female Balb/C mice (n=5 per group) were injected with PBS or were vaccinated with 1 YU/site at four sites of yeast wild-type or yeast-Twist vaccine on days 0, 7, 14, and 21. On day 35, mice were euthanized and spleens were harvested. A, To evaluate lytic activity, lymphocytes were incubated for 5 hours with $^{111}\text{In}$-labeled target P815 cells pulsed with Twist peptide. Radioactivity in the supernatant was measured and specific lysis of 4T1 tumor cells was calculated. This lysis was done on P815 pulsed with the 10-mer Twist peptide. B, To evaluate Twist-specific CD$^{8+}$ T-cell responses, splenic cells were stimulated with Twist 10-mer peptide. Supernatants from these cultures were collected and analyzed for murine IFN-$\gamma$ by cytometric bead array. To evaluate CD$^{4+}$ T cell proliferation in response to Twist 15-mer peptide, CD$^{4+}$ T cells were purified and tested for proliferation by culturing with irradiated APCs and Twist peptide for 5 days. C, Proliferation in response to stimuli was measured by incorporation of $^3$H-thymidine, which was added during the final 18 hours. All experiments were done two times with similar results. Data shown are representative from one experiment. Statistical analyses were done by Student’s $t$-test. Error bars indicate mean ± S.E.M. from triplicate measurements. D, Supernatants from these cultures were collected and analyzed for murine IFN-$\gamma$ by cytometric bead array. E, Populations of CD$^{4+}$ memory T cells after in vitro re-stimulation. Isolated CD$^{4+}$ T cells were restimulated in vitro for 5 days with Twist 15-mer peptide or control LCMV peptide; CD$^{4+}$ T cell memory subsets were defined based on the expression of CD44 and CD62L, using matched isotype controls.

Figure 4. Antitumor and antimetastatic effect of recombinant yeast-Twist vaccination. In Panels A-D, female Balb/C mice (n=8-10 per group) were inoculated in the mammary pad with 4T1
mammary tumor cells on day 0. Mice were injected with PBS (open circles) or vaccinated with 1 YU/site at four sites of yeast wild-type (closed squares) or recombinant yeast-Twist vaccine (closed circles). 

A, Mice were vaccinated on days 4, 11, and 18. Primary tumor volume was measured twice a week and recorded. 

B, On day 21, mice were euthanized and lungs were harvested and dispersed to single-cell suspension and plated. After 10 days incubation, total number of clonogenic metastatic cells was counted. 

C, Mice were vaccinated on day 4 and weekly thereafter. Primary tumor volume was measured twice a week and recorded. When the average of the primary tumor volume for each group reached 1000 mm³, mice were euthanized, lungs were harvested and dispersed to single-cell suspension and plated. 

D, After 10 days incubation, total number of clonogenic metastatic cells was counted. For Panels A–D, * indicates statistical significance (P < 0.05) between mice that received recombinant yeast-Twist vaccine and mice that received PBS or yeast wild-type. Statistical analyses were done by Student’s t-test. NS: No significance. This experiment was done two times with similar results. 

Data shown are representative from one experiment.

Figure 5. The detection of T-cell infiltrates in primary and lung tumor tissues. Female Balb/C (n = 3 per group) were inoculated with 5x10⁴ 4T1 in the mammary fat pad on day 0. Mice received either PBS or 1 YU/site at four sites of wild-type yeast or recombinant yeast-Twist vaccine alone started on day 4 and weekly thereafter. When the average of the primary tumor volume for each group reached 1000 mm³, mice were euthanized, and their primary tumors and lung tumor tissues were harvested and A, the presence of CD3+ T-cell infiltrates were detected by immunohistochemistry. 

B, Positive pixel quantification analysis of CD3+ T-cell infiltrates in primary tumor tissues. 

C, Positive pixel quantification analysis of CD3+ T-cell infiltrates in
lung tumor tissues. D, The effect of T-cell or NK depletion on tumor growth of mice receiving recombinant yeast-Twist vaccine. Female Balb/C (n = 10 per group) were inoculated with 5x10^4 4T1 in the mammary fat pad on day 0. Mice received either PBS or 1 YU/site at four sites of recombinant yeast-Twist vaccine alone started on day 4 and weekly thereafter, or recombinant yeast-Twist vaccine concurrently with NK cell\(^-\), CD4\(^+\) and/or CD8\(^+\) T-cell\(^-\) depleting antibodies. Tumor volume was measured twice a week and recorded. Statistical difference based on Student’s t-test as measured on day 18 post-tumor transplant.

**Figure 6.** Antitumor and antimetastatic effect of recombinant yeast-Twist vaccination in the neoadjuvant 4T1 mammary tumor model. Female Balb/C mice (n=8-10 per group) were inoculated with 5x10^4 4T1 mammary tumor cells in the mammary pad on day 0. Mice were vaccinated with PBS (open circles) or 0.3 YU/site at one site of yeast wild-type (closed squares) or recombinant yeast-Twist vaccine (closed circles) on day 4 and followed with weekly injections thereafter. Tumor volume was measured once a week and recorded on day 10 post-tumor implantation (A). On day 14, primary tumors were surgically removed. On day 40 post-tumor implantation, mice were euthanized and lungs were harvested, dispersed to single-cell suspension and plated. Ten days after initial incubation, total number of clonogenic metastatic cells was counted (D). Statistical analyses were done by Student’s t-test.

**Figure 7.** Antitumor and antimetastatic effect of recombinant yeast-Twist vaccine when vaccination was delayed and in the adjuvant 4T1 mammary tumor model setting. Female Balb/C mice (n=20 per group) were inoculated with 5x10^4 4T1 mammary tumor cells in the mammary pad on day 0. A, On day 11, primary tumor volume was palpable (inset) and mice were
randomized to receive either PBS (open circles) or 1 YU/site at four sites of yeast-Twist (closed circles) followed with weekly injection thereafter. Primary tumor volume was measured and recorded. When the average of the primary tumor volume for each group reached 1000 mm$^3$, mice were euthanized, lungs were harvested and dispersed to single-cell suspension and plated. 

**B.** After 10 days incubation, total number of clonogenic metastatic cells was counted. Statistical analyses were done by Student’s *t*-test. * indicates statistical significance (P < 0.05) between mice that received recombinant yeast-Twist vaccine and mice that received PBS.
Figure 1

A. Twist Expression relative to GAPDH

B. % Twist Positive Cells

C. 
P = 0.0373

D. Primary Tumor
Lung Metastasis

Isotype
Figure 5

A. **T-cell Infiltration (Lung Tumor)**

B. **Primary Tumor**

C. **Lung Tumor**

D. **Tumor Volume**
Figure 6

A. Tumor Volume (mm$^3$) vs. Treatment

B. Number of clonogenic metastatic cells vs. Treatment

- **PBS**
- **Yeast Wild-type**
- **Yeast Twist**

Statistical significance (P-values):

- **Tumor Volume**:
  - PBS vs. Yeast Wild-type: P = 0.0001
  - PBS vs. Yeast Twist: P = 0.02

- **Number of clonogenic metastatic cells**:
  - PBS vs. Yeast Wild-type: P = 0.0001
  - PBS vs. Yeast Twist: P = 0.02
Figure 7

A.

B.
Vaccine-Mediated Immunotherapy Directed Against a Transcription Factor Driving the Metastatic Process


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