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, metastasis, 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" because of 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 antitumor 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. *Cancer Res; 74(7); 1945–57. ©2014 AACR.*

Introduction

Numerous reports have now demonstrated that the epithelial-to-mesenchymal transition (EMT) process is involved in solid tumor progression, invasion, and metastasis (1–7). Studies have also shown that cells undergoing EMT become more drug resistant (8–13). Several transcription factors have been implicated as drivers of EMT and metastatic progression, including Twist (12), Snail (5), 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" because of 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 before 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* (34). 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.

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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 (35–39) 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 antitumor activity. These and other studies have shown that yeast (even devoid of the tumor antigen) could efficiently activate murine and human dendritic cells via their Toll-like receptors (TLR) and consequently induce dendritic cells to produce high levels of type I cytokines such as interleukin (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 antitumor effects (35–39).

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 antitumor effect on lung metastases of the same mice. These antitumor effects were seen in (i) the unresected setting, (ii) in the neoadjuvant tumor setting where mice were vaccinated before the surgical resection of the primary tumor, and (iii) in the adjuvant setting where primary tumors were surgically removed before 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 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 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 gentleMACS M Tubes as per the manufacturer's recommendations (Miltenyi Biotec). Total RNA was isolated from tissue lysates and tumor cell lines using the RNeasy Extraction Kit (Qiagen), and reversed transcribed into cDNA using the Advantage RT-for-PCR Kit (Clontech). cDNA (2.5–10 ng) was used in quantitative real-time PCR reactions using the following probes specific for *Twist1* (Mm00442036_m1) and glyceraldehyde-3-phosphate

dehydrogenase (GAPDH; 4352339E). Fold change in relative mRNA expression was calculated as expression in 4T1 Twist short hairpin RNA (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) as per the manufacturer's directions. PCR was performed on the 7300 Real-Time PCR System (Applied Biosystems). Where indicated, values calculated as expression relative to GAPDH as previously described (2).

Immunohistochemistry

Female Balb/c mice ($n = 3$) were inoculated with 5×10^4 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) according to the manufacturer's instructions. Expression of mesenchymal markers was detected using rabbit antibody to N-cadherin and vimentin (Abcam). Epithelial marker expression was detected using rabbit antibody to E-cadherin (Cell Signaling). Entire slides were digitally scanned by an Aperio ScanScope CS scanning system and analyzed by Aperio ImageScope Viewer software (Aperio Technologies Inc.). The statistical analysis was composed of three 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) using a nucleofector device as per the manufacturer's recommendations (Lonza). Stable transfectants were selected utilizing growth media containing 2 µg/mL puromycin (Sigma-Aldrich).

Western blot analysis

Cells were washed twice with PBS and lysed in RIPA Lysis Buffer (Santa Cruz Biotech). Protein concentration was measured using a BCA Protein Assay Kit (Thermo Scientific). 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 3 times using PBS containing 0.05% Tween 20 (PBST), and incubated with appropriate IRDye-labeled secondary antibodies (LI-COR Biotechnology) at a 1:5,000 dilution in 5% milk in PBS for 1 hour at room temperature. Membranes were washed 3 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 (Sigma-Aldrich) was added per well at a final concentration of 0.5 mg/mL 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.). 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°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). Data are expressed as relative fluorescence units, 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) 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 (GlobeImmune; ref. 35). Expression of Twist protein from yeast-Twist was conformed by Western blot analysis (Supplementary Fig. S1).

Murine CD8$^+$ T-cell responses

Female Balb/c mice ($n = 5$) were vaccinated with PBS, wild-type yeast, or recombinant yeast-Twist vaccine at 1 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 μg/mL of Twist peptide (LYQVLQSDEL, CPC Scientific). 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 γ counter (Corba Autogamma; Packard Instruments). For IFN-γ production, the pooled splenocytes were stimulated for 7 days with 1 μg/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 μg/mL of either Twist peptide or control HIV peptide (AMQMLKETI, CPC Scientific) for 24 hours. Superna-

tant was collected and analyzed for murine IFN-γ by cytometric bead array (BD PharMingen) 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 cocultured with irradiated syngeneic spleen cells (APC) and Twist peptide (QQPASGKRGARKRRS, CPC Scientific) at 0.4 μg/mL. After 4 days of incubation, 1 μCi of [^{3}H]-thymidine (Perkin-Elmer) 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). FoxP3 antibody with appropriate isotype was purchased from eBioscience 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 Biosciences).

Antitumor vaccination

Female Balb/c mice ($n = 8$–10) were injected with 5×10^4 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 1,000 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 mice ($n = 3$) were injected with 5×10^4 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 1,000 mm^3. Primary tumors and lungs were harvested and T-cell infiltrates were detected using rabbit-polyclonal antibody to CD3 (Dako) 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 mice ($n = 8$–10) were injected with 5×10^4 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 to 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) intraperitoneal once per week. Tumor volume was recorded twice/week.

Tumor resection and antitumor vaccination

To model the neoadjuvant tumor setting, female Balb/c mice ($n = 8$–10) were injected with 5×10^4 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 mice ($n = 20$) were injected with 5×10^4 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 autoimmunity (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). 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
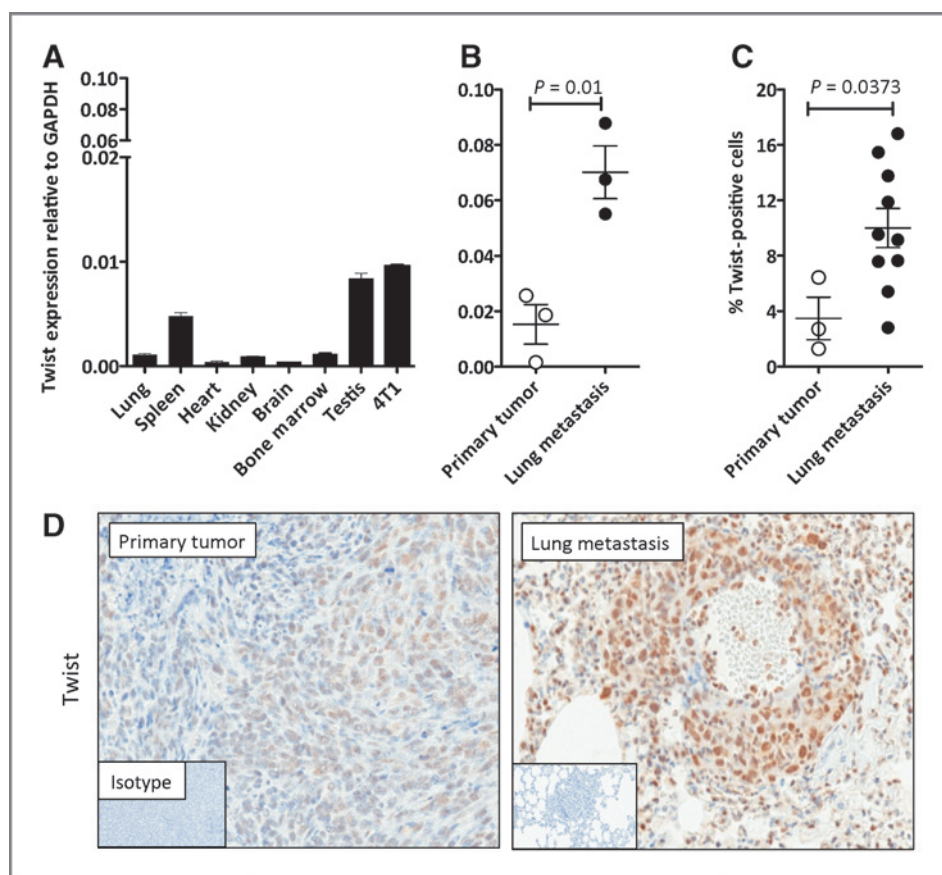
Figure 1. Expression of *Twist* in normal tissues and tumors. A, real-time PCR was performed to evaluate *Twist* RNA expression in several normal murine tissues and the 4T1 breast cancer cell line. Female Balb/c mice ($n = 3$) were injected with 5×10^4 4T1 cells in mammary fat pad. On day 30, primary 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 ± SEM from triplicate measurements. Data are representative of two independent experiments. C and D, Twist protein expression in primary tumor and lung metastases was detected by immunohistochemistry (magnification, ×20) and quantified by positive pixel quantification analysis. Statistical analyses were done by the Student *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. C and D, *in vitro* cell migration (C) and invasion (D) of the 4T1 cell line pair. Error bars indicate mean ± SEM 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, ×20). The blue signal represents the DAPI-stained nuclei and the green signal represents the staining of the F-actin.
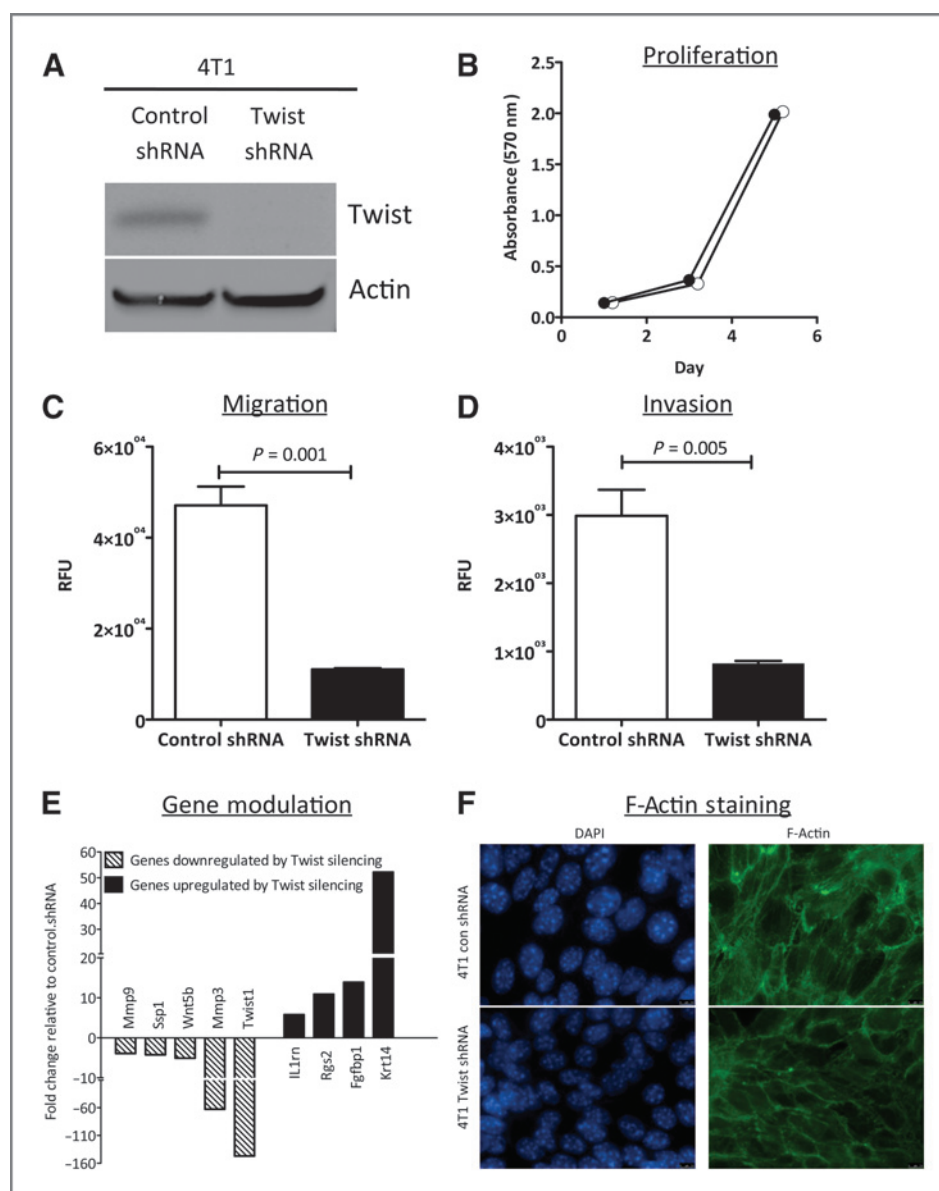
of *Twist* mRNA expression were seen in 4T1 cells and testis, generally considered to be an immunologically privileged site, with lower levels detected in spleen. Some other tissues evaluated showed detectable, albeit low, levels of *Twist* mRNA. Contrary to *Twist*, expression of *Brachyury* mRNA, a 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 because of 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 versus 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

with primary tumors ($P = 0.0373$; Fig. 1C). Because Twist is expressed in some normal organs, the results shown in Fig. 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 with

control cells (control shRNA). Shown in Fig. 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, whereas regulator of G-protein signaling 2 may inhibit G protein–coupled receptor–mediated signaling via inactivation of G proteins. In addition, silencing Twist expression in 4T1 cells also mediated re-organization of the actin cytoskeleton as indicated by F-actin staining depicted in Fig. 2F. Although 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 (43), 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 4 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 dendritic cells 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-γ 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 and B). A novel 15-mer Twist peptide was also used to activate CD4^{+} T cells, and significantly greater proliferative activity and increased IFN-γ production were also seen from splenocytes in the recombinant yeast-Twist vaccinated mice (Fig. 3C and 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 restimulation revealed a 7- and 1.6-fold increase of CD4^{+} T memory stem cells (Tscm) in Twist-vaccinated mice compared with PBS- or yeast wild-type–treated mice, respectively (Fig. 3E). This recently described Tscm population was thought to generate potent antitumor activity (44).

Antitumor and antimetastatic effect of recombinant yeast-Twist vaccination

Antitumor activity was evaluated in vaccinated tumor-bearing mice. 4T1 cells were transplanted in mammary fat pads on day 0 and mice were vaccinated 3 times at weekly intervals starting on day 4. Mice were euthanized on day 21 and lung metastases were enumerated. As seen in Fig. 4A, on day 21 there was a significant reduction ($P = 0.0199$) in primary tumor volume in the recombinant yeast-Twist vaccinated group versus 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 versus the PBS vaccinated group and a trend but a nonstatistical 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 Fig. 4D, there was a reduction in the number of lung metastases in the recombinant yeast-Twist vaccinated group versus the wild-type yeast vaccinated group ($P = 0.03$) and versus 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 (data not shown). Similar observations were attained in non–tumor-bearing mice receiving PBS, yeast wild-type, 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 dendritic cells 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 with other groups (Fig. 5A).
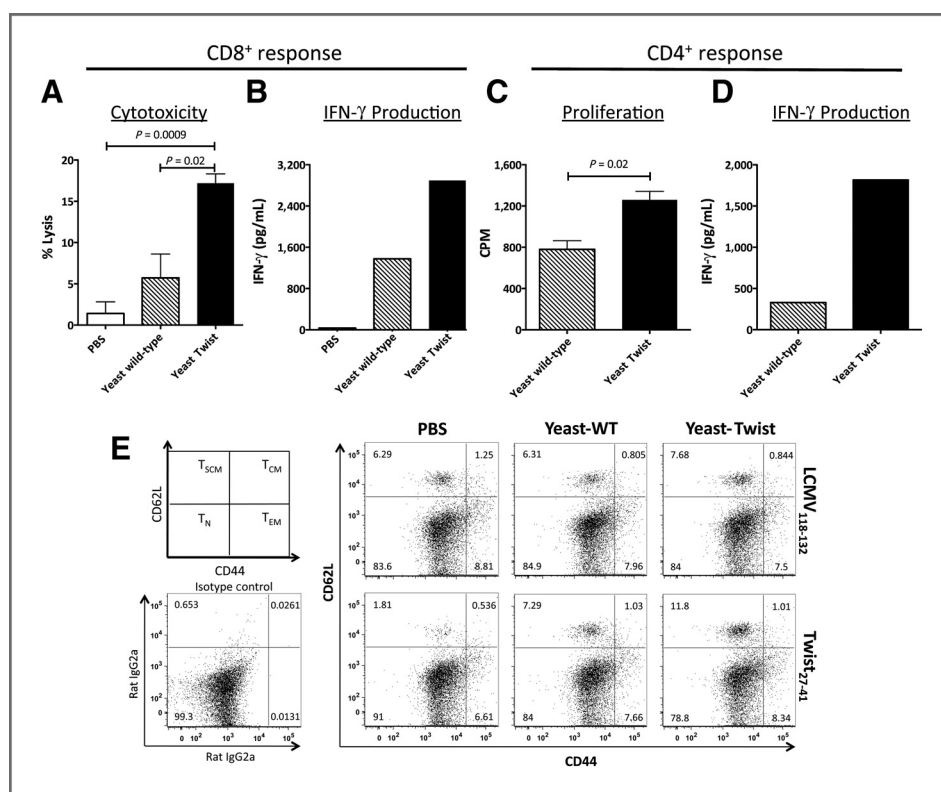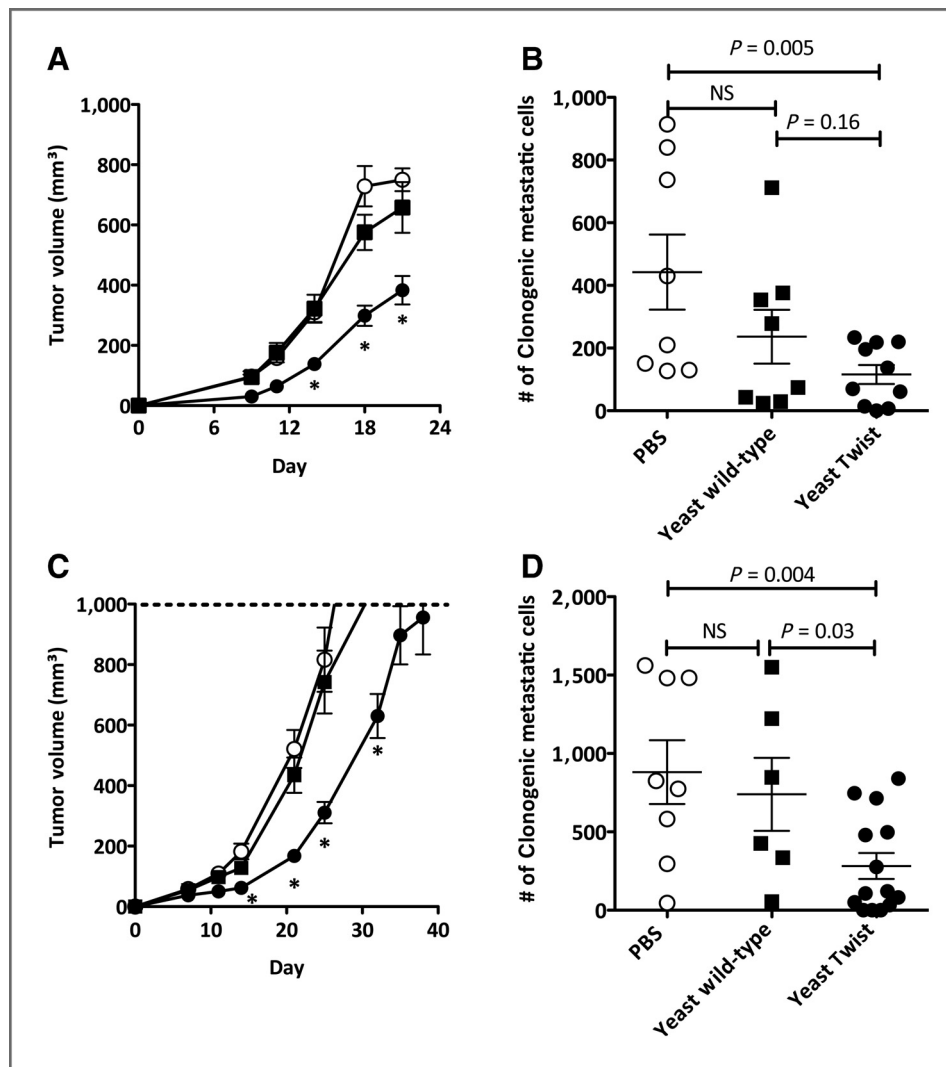
Figure 3. Vaccination with recombinant yeast-Twist induces Twist-specific T-cell responses. Female Balb/c mice ($n = 5$ per group) were injected with PBS or were vaccinated with 1 YU/site at 4 sites of yeast wild-type or yeast-Twist vaccine on days 0, 7, 14, and 21. On day 35, mice were euthanized and spleens were harvested. A, to evaluate lytic activity, lymphocytes were incubated for 5 hours with ^{111}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 CD8^{+} T-cell responses, splenic cells were stimulated with Twist 10-mer peptide. Supernatants from these cultures were collected and analyzed for murine IFN-γ by cytometric bead array. To evaluate CD4^{+} T-cell proliferation in response to Twist 15-mer peptide, CD4^{+} 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 the Student t test. Error bars indicate mean $\pm$ SEM from triplicate measurements. D, supernatants from these cultures were collected and analyzed for murine IFN-γ by cytometric bead array. E, populations of CD4^{+} memory T cells after *in vitro* restimulation. Isolated CD4^{+} T cells were restimulated *in vitro* for 5 days with Twist 15-mer peptide or control LCMV peptide; CD4^{+} T-cell memory subsets were defined based on the expression of CD44 and CD62L, using matched isotype controls.

Positive pixel quantification confirmed a significant increase of CD3^{+} T-cell infiltrates in lung tumor tissues harvested from mice vaccinated with yeast-Twist versus PBS-treated ($P = 0.0007$) and versus 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 antitumor effect. As seen in Fig. 5D, CD4 or NK cell depletion had minor effects (nonstatistical) on antitumor activity. Depletion of CD8^{+} T cells, however, completely abrogated the antitumor 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 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 antitumor activity. In an attempt to minimize the effect of wild-type yeast vaccination in this model, mice were

vaccinated 3 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 Fig. 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 versus 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

Figure 4. Antitumor and antimetastatic effect of recombinant yeast-Twist vaccination. In 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 4 sites of yeast wild-type (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 1,000 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. A–D: *, 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 the Student t test. NS, no significance. This experiment was done two times with similar results. Data shown are representative from one experiment.

for a difference in the therapeutic effect of the recombinant yeast-Twist vaccine for metastatic lesions versus 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 inter-

vals to maximize immune responses. We have previously shown that multiple vaccinations of recombinant yeast vaccines are required for optimal antitumor responses (39). 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, because mice would be required to be sacrificed at or before 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 (Figs. 4 and 6). To further strengthen this finding, we
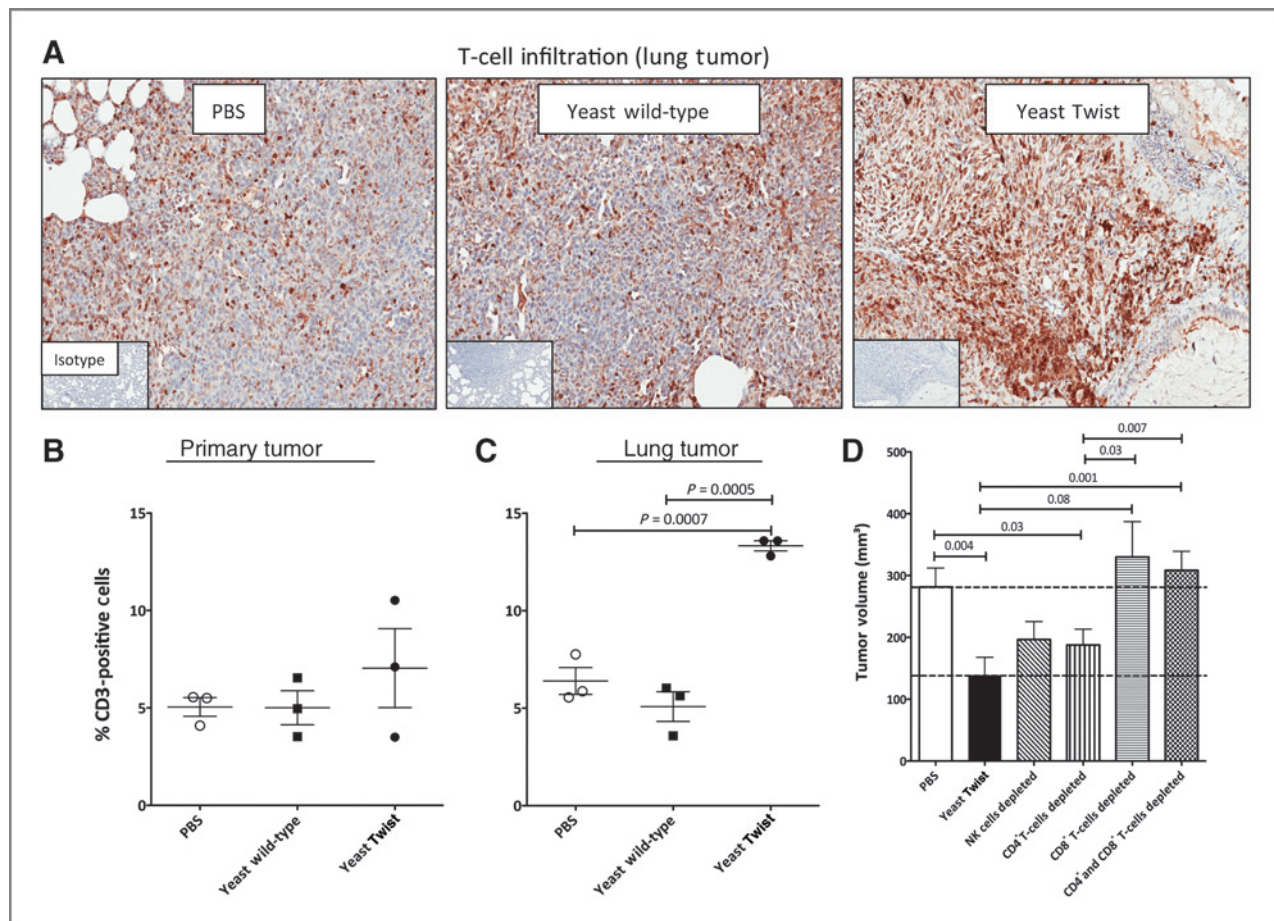
Figure 5. The detection of T-cell infiltrates in primary and lung tumor tissues. Female Balb/c mice ($n = 3$ per group) were inoculated with 5×10^4 4T1 in the mammary fat pad on day 0. Mice received either PBS or 1 YU/site at 4 sites of wild-type yeast or recombinant yeast-Twist vaccine alone started on day 4 and weekly thereafter. When the average of the primary tumor volume for each group reached 1,000 mm^3, mice were euthanized, and their primary tumors and lung tumor tissues were harvested, and the presence of CD3$^+$ T-cell infiltrates was detected by immunohistochemistry (A). 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 mice ($n = 10$ per group) were inoculated with 5×10^4 4T1 in the mammary fat pad on day 0. Mice received either PBS or 1 YU/site at 4 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 the Student t test as measured on day 18 posttumor transplant.

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 5×10^4 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 mm^3 (Fig. 7A, inset). Mice were then randomized to receive either PBS or yeast-Twist and the primary tumor volumes were measured. As seen in Fig. 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 with earlier vaccine administration. Mice in each group were then euthanized when the average tumor

volume reached 1,000 mm^3. As seen in Fig. 7B, there was a reduction in the number of lung metastases in the recombinant yeast-Twist vaccinated group versus PBS vaccinated group ($P = 0.03$).

To model the 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 with 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 antitumor effect of the recombinant yeast-Twist vaccine could also elicit an autoimmune and/or other toxic effects. Balb/c mice
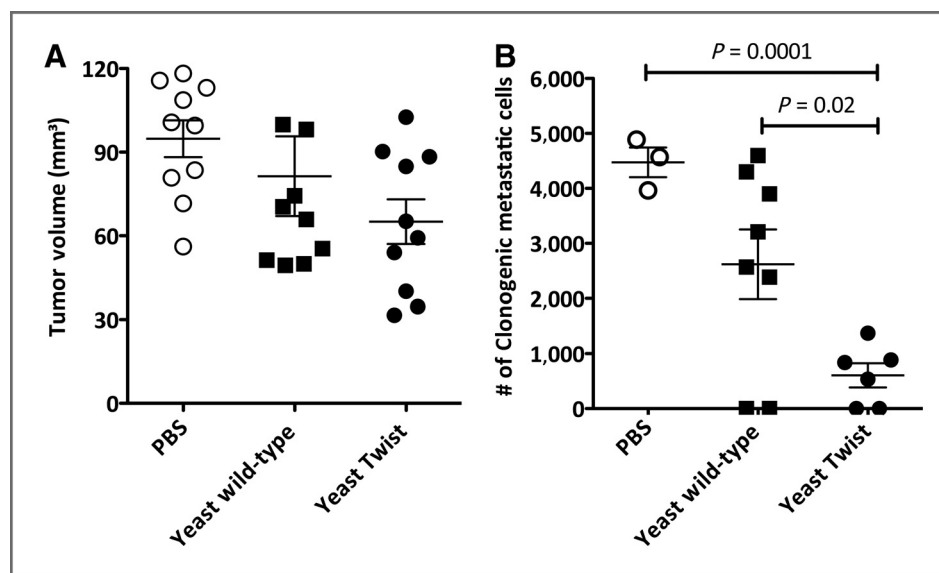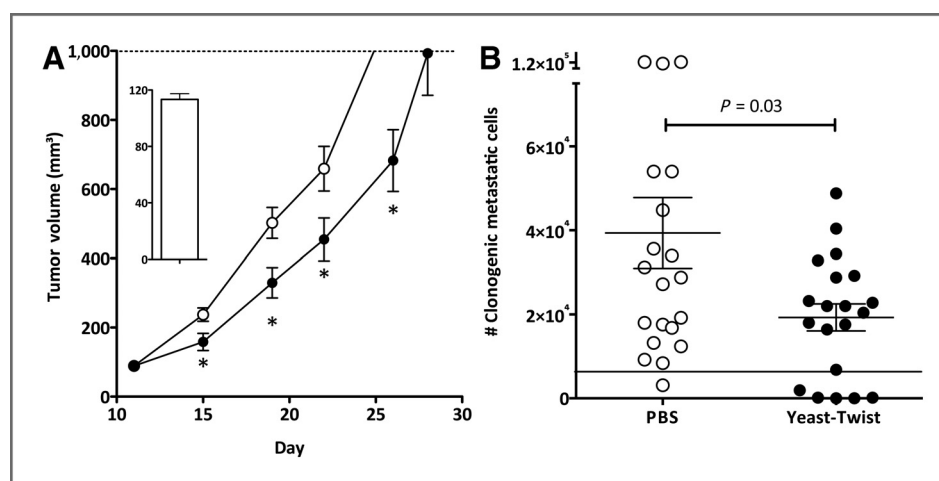
Figure 6. Antitumor and antimetastatic effect of recombinant yeast-Twist vaccination in the neoadjuvant 4T1 mammary tumor model. Female Balb/c mice ($n = 8–10$ per group) were inoculated with 5×10^4 4T1 mammary tumor cells in the mammary pad on day 0. Mice were vaccinated with PBS (open circles) or 0.3 YU/site at one site of yeast wild-type (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 posttumor implantation (A). On day 14, primary tumors were surgically removed. On day 40 posttumor 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 the Student t test.

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 (i) body weight, (ii) 20 different blood chemistry parameters, (iii) 7 different clinical chemistry parameters, and (iv) 5 different autoimmunity panels (Supplementary Table S1).

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. Although 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 antitumor effects (Fig. 5D).

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 5×10^4 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 4 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 1,000 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 the Student t test. *, statistical significance ($P < 0.05$) between mice that received recombinant yeast-Twist vaccine and mice that received PBS.

These studies also lend further support to previous studies (2, 34, 45) that T cells can be generated to transcription factors. Although 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 antitumor effects can be induced in the absence of any apparent autoimmunity or other toxic effects (Supplementary Table S1).

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

Although the recombinant yeast-Twist vaccine is shown to be more efficacious in the induction of Twist-specific immune responses and antitumor activity than the wild-type yeast vector (Figs. 3, 4, 6, and 7), the experiments reveal some degree of immune and antitumor 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 dendritic cells was shown to efficiently mature dendritic cells 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 YUs was reduced for both the recombinant yeast-Twist vaccine and the wild-type yeast vector, the differential antitumor effect for the recombinant yeast-Twist vaccine became more pronounced.

Twist 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 because of its fairly high level of expression in several human normal adult tissues (15). It should also be pointed out that although 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 mes-

enchymal 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, whereas 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 with primary tumors. In addition, an EMT array was performed on primary tumors harvested from Twist-vaccinated and nontreated 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 (Supplementary Fig. S2). Although there was a significant decrease in E-cadherin expression in lung metastases compared with primary tumors (Supplementary Fig. S2A), comparable N-cadherin and vimentin expression between primary tumors and lung metastases was also observed (Supplementary Fig. S2). 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 versus 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 using a recombinant Brachyury vaccine.

The 4T1 model, however, as has previously been shown (32, 33, 41) 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 antitumor 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 using vaccines targeting the transcription factor Twist, as a monotherapy or in a range of combination therapies.

Disclosure of Potential Conflicts of Interest

Authors' Contributions

Conception and design: A. Ardiani, S.R. Gameiro, C. Palena, D.H. Hamilton, T.H. King, J. Schlom, J.W. Hodge
Development of methodology: A. Ardiani, S.R. Gameiro, C. Palena, D.H. Hamilton, J. Schlom, J.W. Hodge
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Ardiani, S.R. Gameiro, C. Palena, D.H. Hamilton, A. Kwilas, J.W. Hodge
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Ardiani, S.R. Gameiro, C. Palena, D.H. Hamilton, J. Schlom, J.W. Hodge
Writing, review, and/or revision of the manuscript: A. Ardiani, S.R. Gameiro, C. Palena, D.H. Hamilton, J. Schlom, J.W. Hodge
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Ardiani
Study supervision: S.R. Gameiro, J. Schlom, J.W. Hodge

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References

1. Brabletz T, Hlubek F, Spaderna S, Schmalhofer O, Hiendlmeyer E, Jung A, et al. Invasion and metastasis in colorectal cancer: epithelial-mesenchymal transition, mesenchymal-epithelial transition, stem cells and β-catenin. Cells Tissues Organs 2005;179:56–65.
2. Fernando RI, Litzinger M, Trono P, Hamilton DH, Schlom J, Palena C. The T-box transcription factor Brachyury promotes epithelial-mesenchymal transition in human tumor cells. J Clin Invest 2010;120:533–44.
3. Gravdal K, Halvorsen OJ, Haukaas SA, Akslen LA. A switch from E-cadherin to N-cadherin expression indicates epithelial to mesenchymal transition and is of strong and independent importance for the progress of prostate cancer. Clin Cancer Res 2007;13:7003–11.
4. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest 2009;119:1420–8.
5. Lee MY, Chou CY, Tang MJ, Shen MR. Epithelial-mesenchymal transition in cervical cancer: correlation with tumor progression, epidermal growth factor receptor overexpression, and snail up-regulation. Clin Cancer Res 2008;14:4743–50.
6. Sarrio D, Rodriguez-Pinilla SM, Hardisson D, Cano A, Moreno-Bueno G, Palacios J. Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. Cancer Res 2008;68:989–97.
7. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2002;2:442–54.
8. Chen Y, Li L, Zeng J, Wu K, Zhou J, Guo P, et al. Twist confers chemoresistance to anthracyclines in bladder cancer through upregulating P-glycoprotein. Chemotherapy 2012;58:264–72.
9. Gupta PB, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA, et al. Identification of selective inhibitors of cancer stem cells by high-throughput screening. Cell 2009;138:645–59.
10. Huang B, Cohen JR, Fernando RI, Hamilton DH, Litzinger MT, Hodge JW, et al. The embryonic transcription factor Brachyury blocks cell cycle progression and mediates tumor resistance to conventional antitumor therapies. Cell Death Dis 2013;4:e682.
11. Kajiyama H, Shibata K, Terauchi M, Yamashita M, Ino K, Nawa A, et al. Chemoresistance to paclitaxel induces epithelial-mesenchymal transition and enhances metastatic potential for epithelial ovarian carcinoma cells. Int J Oncol 2007;31:277–83.
12. Kwok WK, Ling MT, Lee TW, Lau TC, Zhou C, Zhang X, et al. Up-regulation of TWIST in prostate cancer and its implication as a therapeutic target. Cancer Res 2005;65:5153–62.
13. Marshall JL, Hoyer RJ, Toomey MA, Faraguna K, Chang P, Richmond E, et al. Phase I study in advanced cancer patients of a diversified prime-and-boost vaccination protocol using recombinant vaccinia virus and recombinant nonreplicating avipox virus to elicit anti-carcinoembryonic antigen immune responses. J Clin Oncol 2000;18:3964–73.
14. Bolos V, Peinado H, Perez-Moreno MA, Fraga MF, Esteller M, Cano A. The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. J Cell Sci 2003;116:499–511.
15. Hamilton DH, Litzinger MT, Fernando RI, Huang B, Palena C. Cancer vaccines targeting the epithelial-mesenchymal transition: tissue distribution of brachyury and other drivers of the mesenchymal-like phenotype of carcinomas. Semin Oncol 2012;39:358–66.
16. Riaz M, Sieuwerts AM, Look MP, Timmermans MA, Smid M, Foekens JA, et al. High TWIST1 mRNA expression is associated with poor prognosis in lymph node-negative and estrogen receptor-positive human breast cancer and is co-expressed with stromal as well as ECM related genes. Breast Cancer Res 2012;14:R123.
17. Vesuna F, Lisok A, Kimble B, Domek J, Kato Y, van der Groep P, et al. Twist contributes to hormone resistance in breast cancer by down-regulating estrogen receptor-α. Oncogene 2012;31:3223–34.
18. Gomez I, Pena C, Herrera M, Munoz C, Larriba MJ, Garcia V, et al. TWIST1 is expressed in colorectal carcinomas and predicts patient survival. PLoS ONE 2011;6:e18023.
19. Okada T, Suehiro Y, Ueno K, Mitomori S, Kaneko S, Nishioka M, et al. TWIST1 hypermethylation is observed frequently in colorectal tumors and its overexpression is associated with unfavorable outcomes in patients with colorectal cancer. Genes Chromosomes Cancer 2010;49:452–62.
20. Kajiyama H, Shibata K, Umezu T, Mizuno M, Suzuki S, Yamamoto E, et al. Expression of Twist enhances risk of poor oncologic outcome in patients with stage Ib to II cervical carcinoma with lymphovascular space involvement. Hum Pathol 2013;44:181–8.
21. Nakajima TE, Yoshida H, Okamoto N, Nagashima K, Taniguchi H, Yamada Y, et al. Nucleostemin and TWIST as predictive markers for recurrence after neoadjuvant chemotherapy for esophageal carcinoma. Cancer Sci 2012;103:233–8.
22. Gao Y, Xuan XY, Zhang HY, Wang F, Zeng QR, Wang ZQ, et al. Relationship between TWIST expression and epithelial-mesenchymal transition of oesophageal squamous cell carcinoma. Cell Biol Int 2012;36:571–7.
23. Yu L, Lu S, Tian J, Ma J, Li J, Wang H, et al. TWIST expression in hypopharyngeal cancer and the mechanism of TWIST-induced promotion of metastasis. Oncol Rep 2012;27:416–22.
24. Gajula RP, Chettiar ST, Williams RD, Thiyagarajan S, Kato Y, Aziz K, et al. The twist box domain is required for twist1-induced prostate cancer metastasis. Mol Cancer Res 2013;11:1387–400.
25. Ru GQ, Wang HJ, Xu WJ, Zhao ZS. Upregulation of Twist in gastric carcinoma associated with tumor invasion and poor prognosis. Pathol Oncol Res 2011;17:341–7.
26. Yu Q, Zhang K, Wang X, Liu X, Zhang Z. Expression of transcription factors snail, slug, and twist in human bladder carcinoma. J Exp Clin Cancer Res 2010;29:119.

27. Weiss MB, Abel EV, Mayberry MM, Basile KJ, Berger AC, Aplin AE. TWIST1 is an ERK1/2 effector that promotes invasion and regulates MMP-1 expression in human melanoma cells. Cancer Res 2012;72: 6382–92.

28. Mikheeva SA, Mikheev AM, Petit A, Beyer R, Oxford RG, Khorasani L, et al. TWIST1 promotes invasion through mesenchymal change in human glioblastoma. Mol Cancer 2010;9:194.

29. Ding J, Zhang Z, Pan Y, Liao G, Zeng L, Chen S. Expression and significance of twist, E-cadherin, and N-cadherin in gastrointestinal stromal tumors. Dig Dis Sci 2012;57:2318–24.

30. Yin K, Liao Q, He H, Zhong D. Prognostic value of Twist and E-cadherin in patients with osteosarcoma. Med Oncol 2012;29:3449–55.

31. Tran PT, Shroff EH, Burns TF, Thiyagarajan S, Das ST, Zabuawala T, et al. Twist1 suppresses senescence programs and thereby accelerates and maintains mutant Kras-induced lung tumorigenesis. PLoS Genet 2012;8:e1002650.

32. Lou Y, Preobrazhenska O, auf dem Keller U, Sutcliffe M, Barclay L, McDonald PC, et al. Epithelial-mesenchymal transition (EMT) is not sufficient for spontaneous murine breast cancer metastasis. Dev Dyn 2008;237:2755–68.

33. Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell 2004;117:927–39.

34. Demaria S, Wang B, Yang AM, Santori F, Kawashima N, Matsumura S. Immunotherapy against metastatic breast cancer with a twist (abstract). Breast Cancer Res Treat 2007;106:S31.

35. Bernstein MB, Chakraborty M, Wansley EK, Guo Z, Franzusoff A, Mostbock S, et al. Recombinant Saccharomyces cerevisiae (yeast-CEA) as a potent activator of murine dendritic cells. Vaccine 2008; 26:509–21.

36. Boehm AL, Higgins J, Franzusoff A, Schlom J, Hodge JW. Concurrent vaccination with two distinct vaccine platforms targeting the same antigen generates phenotypically and functionally distinct T-cell populations. Cancer Immunol Immunother 2010;59:397–408.

37. Cereda V, Vergati M, Huen NY, di Bari MG, Jochems C, Intrivici C, et al. Maturation of human dendritic cells with Saccharomyces cerevisiae (yeast) reduces the number and function of regulatory T cells and enhances the ratio of antigen-specific effectors to regulatory T cells. Vaccine 2011;29:4992–9.

38. Remondo C, Cereda V, Mostbock S, Sabzevari H, Franzusoff A, Schlom J, et al. Human dendritic cell maturation and activation by a heat-killed recombinant yeast (Saccharomyces cerevisiae) vector encoding carcinoembryonic antigen. Vaccine 2009;27:987–94.

39. Wansley EK, Chakraborty M, Hance KW, Bernstein MB, Boehm AL, Guo Z, et al. Vaccination with a recombinant Saccharomyces cerevisiae expressing a tumor antigen breaks immune tolerance and elicits therapeutic antitumor responses. Clin Cancer Res 2008; 14:4316–25.

40. Hodge JW, Grosenbach DW, Aarts WM, Poole DJ, Schlom J. Vaccine therapy of established tumors in the absence of autoimmunity. Clin Cancer Res 2003;9:1837–49.

41. Tao K, Fang M, Alroy J, Sahagian GG. Imagable 4T1 model for the study of late stage breast cancer. BMC Cancer 2008;8:228.

42. Tuck AB, Hota C, Chambers AF. Osteopontin (OPN)-induced increase in human mammary epithelial cell invasiveness is urokinase (uPA)-dependent. Breast Cancer Res Treat 2001;70:197–204.

43. Jin HO, Hong SE, Woo SH, Lee JH, Choe TB, Kim EK, et al. Silencing of Twist1 sensitizes NSCLC cells to cisplatin via AMPK-activated mTOR inhibition. Cell Death Dis 2012;3:e319.

44. Gattinoni L, Restifo NP. Moving T memory stem cells to the clinic. Blood 2013;121:567–8.

45. Palena C, Polev DE, Tsang KY, Fernando RI, Litzinger M, Krukovskaya LL, et al. The human T-box mesodermal transcription factor Brachyury is a candidate target for T-cell-mediated cancer immunotherapy. Clin Cancer Res 2007;13:2471–8.

46. Jiang W, Pang XG, Wang Q, Shen YX, Chen XK, Xi JJ. Prognostic role of Twist, Slug, and Foxc2 expression in stage I non-small-cell lung cancer after curative resection. Clin Lung Cancer 2012;13:280–7.

47. Lee KW, Lee NK, Kim JH, Kang MS, Yoo HY, Kim HH, et al. Twist1 causes the transcriptional repression of claudin-4 with prognostic significance in esophageal cancer. Biochem Biophys Res Commun 2012;423:454–60.

48. Ardiani A, Farsaci B, Rogers CJ, Protter A, Guo Z, King TH, et al. Combination therapy with a second-generation androgen receptor antagonist and a metastasis vaccine improves survival in a spontaneous prostate cancer model. Clin Cancer Res 2013;19:6205–18.

49. Hodge JW, Ardiani A, Farsaci B, Kwilas AR, Gameiro SR. The tipping point for combination therapy: cancer vaccines with radiation, chemotherapy, or targeted small molecule inhibitors. Semin Oncol 2012; 39:323–39.

50. Schlom J. Therapeutic cancer vaccines: current status and moving forward. J Natl Cancer Inst 2012;104:599–613.

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

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