WEE1 Inhibition Alleviates Resistance to Immune Attack of Tumor Cells Undergoing Epithelial–Mesenchymal Transition

Duane H. Hamilton, Bruce Huang, Romaine I. Fernando, Kwong-Yok Tsang, and Claudia Palena

Abstract

Aberrant expression of the T-box transcription factor brachyury in human carcinomas drives the phenomenon of epithelial–mesenchymal transition (EMT), a phenotypic modulation that facilitates tumor dissemination and resistance to conventional therapies, including chemotherapy and radiotherapy. By generating isogenic cancer cell lines with various levels of brachyury expression, we demonstrate that high levels of brachyury also significantly reduce the susceptibility of cancer cells to lysis by both antigen-specific T cells and natural killer cells. Our results indicated that resistance of brachyury-high tumor cells to immune-mediated attack was due to inefficient caspase-dependent apoptosis, manifested as inefficient nuclear lamin degradation in the presence of activated effector caspases. We correlated this phenomenon with loss of cell-cycle–dependent kinase 1 (CDK1), which mediates lamin phosphorylation. In support of a causal connection, pretreatment of tumor cells with a specific inhibitor of WEE1, a negative regulator kinase of CDK1, could counter the defective apoptosis of tumor cells expressing high levels of brachyury. Thus, our findings suggested that reconstituting CDK1 activity to threshold levels may be sufficient to restore immunosurveillance of mesenchymal-like cancer cells that have escaped previous immune detection or eradication. Cancer Res; 74(9); 2510–9. ©2014 AACR.

Introduction

The progression of carcinomas remains a poorly understood phenomenon. A recently recognized mechanism involved in tumor metastasis is the epithelial–mesenchymal transition (EMT), a phenotypic modulation that promotes the loss of tumor epithelial features and the simultaneous acquisition of mesenchymal-associated traits, including tumor motility and invasiveness (1, 2). In addition to promoting metastasis, EMT has also been associated with the acquisition of resistance to cell death (3), a characteristic that may favor the survival of mesenchymal cancer cells not only as they disseminate from the primary tumor, but also in response to chemotherapy, radiotherapy, and some small-molecule–targeted therapies (4, 5). As a result, cancer therapies may select for tumor cells undergoing EMT, a phenomenon first demonstrated in vivo with breast cancer recurrences (6).

Our laboratory has characterized the T-box transcription factor brachyury as a driver of EMT in human carcinoma cells (7, 8). We have shown that various types of tumors overexpress brachyury (8, 9) and that its levels of expression positively correlate with resistance to chemotherapy or radiotherapy (10, 11). We also demonstrated that circulating, brachyury-specific cytotoxic CD8+ T cells (CTL) can be detected in the blood of patients with carcinoma (12, 13), an observation that led us to propose a T-cell–based immunotherapeutic approach, rather than conventional therapies, as a means to specifically target tumor cells undergoing brachyury-mediated EMT.

In recent years the role of the immune system in tumor eradication and prognosis has gained increased recognition (14, 15) and immune evasion is now included as an "emerging hallmark of cancer" (16). To date, however, it is not clearly understood whether EMT contributes to the escape of tumors from host immunosurveillance and immune-mediated rejection. In the present study, human carcinoma cells undergoing EMT via brachyury overexpression were compared with their epithelial counterparts in terms of their susceptibility to immune-mediated attack. Our results demonstrate that high levels of brachyury reduce the susceptibility of carcinoma cells to either antigen-specific, CD8+ CTLs or innate natural killer (NK), and lymphokine-activated killer (LAK) cells by decreasing the contribution to cell death of caspase-dependent pathways, while leaving unaffected the caspase-independent tumor cytosis involving perforin. Analysis of apoptotic markers showed that resistance of brachyury-high tumor cells to caspase-mediated cell death is due to the absence of nuclear lamin degradation in the presence of normal levels of activated effector caspases, a defect that seems to be related to the loss of the cell-cycle–dependent kinase 1 (CDK1, also known as p34cdc2), a kinase involved in lamin phosphorylation and subsequent caspase-mediated lysis (17, 18). Pretreatment of tumor cells with a specific inhibitor of WEE1, a negative regulator kinase of CDK1, was shown to fully counter the
defective apoptosis of tumor cells with high levels of brachury in vitro, therefore indicating that reconstitution of CDK1 activity to a threshold level may be sufficient to improve immune-mediated attack of mesenchymal-like, brachury-high tumor cells.

Materials and Methods

Immune effector cells
Peripheral blood from healthy donors and patients with cancer was obtained under the appropriate Institutional Review Board approval and informed consent. Antigen-specific, HLA-A2–restricted CTLs were expanded from peripheral blood mononuclear cells (PBMC) from three different patients with cancer using the following HLA-A2–binding peptides as previously described: brachury, WLLPGTSTL (12); mucin-1 (MUC1), ALWGQDVTSV (19); and carcinoembryonic antigen (CEA), YLSGADLNL (20). Briefly, dendritic cells (DC) were prepared from PBMCs by culture in the presence of granulocyte macrophage colony–stimulating factor and interleukin (IL)-4, subsequently pulsed with the corresponding peptides and used to stimulate autologous T cells in vitro. CD8+ T cells were isolated with a magnetic CD8+ Isolation Kit (Miltenyi Biotech). Murine H-2Kb–restricted gp70–specific CTLs were established as previously described against peptide p15e (clone 160; 18). Lung A549 and H460; breast MDA-MB-231; and colon SW480. Cell lines were authenticated by short tandem repeat profiling. Cell culture

The following human carcinoma cell lines were obtained from the American Type Culture Collection: pancreatic PANC-1; lung A549 and H460; breast MDA-MB-231; and colon SW480. Cell lines were authenticated by short tandem repeat profiling. Expression vectors and transfection strategies were previously described (7). Silencing of CDK1 expression was achieved by using an ON-TARGET SMARTpool siRNA; control cells were transfected with a nontargeting siRNA pool as per the manufacturer’s recommendations (Thermo Scientific). All Western blots were imaged and quantified using the Odyssey Infrared Imaging System (LI-COR Biotechnology).

Cytotoxicity assay
Cytotoxicity assays were performed as previously described (12). To inhibit the function of perforin/granzyme, NK or T cells were preincubated with 200 nmol/L concanamycin A (CMA; Sigma) for 2 hours at 37°C. Caspase-dependent lysis was inhibited by preincubating target cells with 50 μmol/L Z-VA-D-FMK (Calbiochem) for 1 hour before the assay. Where indicated, apoptosis was initiated by cross-linking of the FAS receptor (CD95) with 12.5 μg/mL anti-CD95 (clone DX2) antibody (BD Pharmingen) and 0.5 μg/mL protein G (GE Healthcare) or incubation with 250 ng/mL of a recombinant, active multimeric Superkiller TRAIL (Enzo Life Sciences). When indicated, tumor cells were treated with 100 nmol/L of the WEE1 inhibitor MK-1775 (ChemieTeck) for 72 hours before the assay.

RNA isolation and real-time PCR
RNA isolation and real-time PCR assays were performed as previously described (7) utilizing the probes (Applied Biosystems) listed in Supplementary Table S1. Expression of CEA and gp70 mRNA was analyzed by PCR using a Titanium Taq PCR Kit (Clontech) with 50 ng cDNA using CEA or gp70–specific primers as listed in Supplementary Table S1. PCR products were quantified with an Agilent Bioanalyzer (Agilent Technologies).

Western blot analysis
Commercial lysates from human lung and breast tumor biopsies were purchased from Idgenex. The following antibodies were used: pan-actin (clone Ab-5; Neo Markers), β-2 microglobulin (β2M; clone BBM1; Santa Cruz Biotechnology), caspase-8 (clone Ab-3; Calbiochem), brachury, lamin B1, lamin A/C (Abcam), and CDK1 and caspase-3 (Cell Signaling Technology). Where indicated, protein lysates were run on a 7% acrylamide gel supplemented with 25 μmol/L Phos-Tag (Wako Pure Chemical Industries), following the manufacturer’s recommendations. All Western blots were imaged and quantified using the Odyssey Infrared Imaging System (LI-COR Biotechnology).

Immunofluorescence and immunohistochemistry
Carcinoma cells were grown on coverslips and stained as previously described (7) with monoclonal antibodies recognizing CDK1 (Cell Signaling Technology), brachury (Abcam), and MUC1 (clone DF3; 1/50 dilution). Where indicated, tumor cells were incubated with LAK cells at 37°C for 1 hour, and stained for F-actin utilizing Alexa Fluor 488 phalloidin as per the manufacturer’s recommendations. Images were captured utilizing a Leica Fluorescent microscope. Confocal images were obtained utilizing a Zeiss LSM 510 META Confocal Microscope.

Lung tumor tissue arrays were purchased from US Biomax, Inc. Sections of paraffin-embedded, formalin-fixed tissues were tested with a brachury (Abcam) or a CDK1 antibody (EMD Millipore) as previously described (9), and counterstained with hematoxylin.

Murine studies
All animal studies were carried out in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. Experimental studies were carried out under approval of the NIH Intramural Animal Care and Use Committee. Murine colon carcinoma MC38 cells were stably transfected with an empty vector (pCMV) or a brachury-encoding (pBr) vector as previously described (22). Tumor cells (5 × 10^6 MC38-pCMV or MC38-pBr) were subcutaneously implanted into female C57BL/6 mice on day 0. Beginning on day 4, animals were vaccinated weekly with either Hank’s Balanced Salt Solution or 50 μg of a gp70 peptide (p15e) emulsified in Montanide ISA-51-VG (Seppic) at a 1:1 ratio. Where indicated, gp70–specific T cells were admixed with either 5 × 10^5 MC38-pCMV or MC38-pBr cells at a 1:1 ratio of effector cells to tumor cells and subcutaneously implanted into female C57BL/6 mice.
**Statistical analysis**

Data were analyzed using GraphPad Prism (version 4; GraphPad Software). Data points in graphs represent the mean ± SEM and \( P < 0.05 \) was considered significant.

**Results**

**Brachyury expression associates with resistance to immune-mediated lysis**

To investigate whether brachyury-mediated EMT could induce resistance to immune-mediated killing, an isogenic tumor cell pair was generated by stably transfecting pancreatic PANC-1 cells, characterized by low endogenous levels of brachyury, with a control vector (pCMV) or a vector encoding the full-length human brachyury protein (designated hereafter as pBr; Fig. 1A). Tumor lysis was evaluated using an HLA-A2-restricted brachyury-specific CTL line established against an epitope of the brachyury protein as previously described (12).

As shown in Fig. 1A, the cytotoxic lysis of PANC-1–pBr cells was significantly reduced compared with that of control PANC-1–pCMV cells, despite of greater levels of the target brachyury being expressed by the PANC-1–pBr cells. Impaired tumor lysis was also observed with human CTLs directed against an HLA-A2–restricted epitope of the tumor antigen MUC1 (19). As shown in Fig. 1B, the levels of MUC1 were not decreased as a result of brachyury overexpression, yet the PANC-1–pBr cells were lysed less efficiently than PANC-1–pCMV cells at all effector-to-target cell ratios evaluated. Comparable results were obtained with breast MDA-MB-231 cancer cells that exhibited decreased MUC1-specific T-cell lysis after brachyury overexpression (Supplementary Fig. S1A), or with PANC-1 cells induced into a mesenchymal phenotype by treatment with the EMT inducer TGF-\( \beta \) (Supplementary Fig. S1B), compared with their epithelial cell counterparts.

Loss-of-function experiments were also conducted to examine the impact of reducing brachyury expression on the...
susceptibility of colon SW480 carcinoma cells to immune-mediated cytotoxicity. Although brachyury silencing, indicated as Br short hairpin RNA (shRNA; Fig. 1C), had no significant impact on the level of expression of the tumor-associated antigen CEA (Fig. 1C), the susceptibility of SW480 cells to lysis by HLA-A2–restricted CEA-specific CTLs generated as previously described from the blood of a patient with cancer (20) was greatly enhanced (Fig. 1C). These results indicated that, at high levels of expression, brachyury might impair T-cell–mediated lysis regardless of the antigen specificity of the effector T cells.

**Defective lysis of brachyury-high tumor cells is not due to defective antigen presentation**

A well-recognized mechanism of tumor resistance to antigen-specific T-cell lysis is the downregulation of components of the antigen-presentation and/or -processing machinery. The PANC-1 tumor cell pair showed no differences about the levels of MHC class I or β2M (Supplementary Fig. S2A and S2B). Brachyury-high tumor cells also demonstrated a significant enhancement, rather than a reduction, in the expression of various components of the class I antigen-presentation machinery, including TAP1 and TAP2, tapasin, and the proteosome subunits LMP2 and LMP7 (Supplementary Fig. S2C). Furthermore, the defective lysis of PANC-1–pBr cells by MUC1–specific CTLs was not reversed when antigen presentation was fully bypassed via the addition of exogenous MUC1 versus control HIV peptide (Fig. 1D). On the basis of these results, it was evident that the reduced lysis of tumor cells with high levels of brachyury was not due to inefficient antigen presentation. This conclusion was further reinforced by the observation that reduced susceptibility of brachyury-high tumor cells to immune-mediated lysis was not restricted to antigen-specific T cells but also observed with antigen-nonspecific LAK cells. As shown in Fig. 1E, LAK-mediated lysis of A459–pBr cells was significantly reduced compared with that of A549–pCMV cells, even if a stable engagement between the effectors (LAK) and the tumor cells occurred, irrespective of the level of brachyury, as demonstrated by equivalent polar actin polymerization observed at the interacting surface area (arrows, Fig. 1E). Similar results were observed with the PANC-1 tumor cell pair (not shown).

**Degradation of nuclear lamins is defective in brachyury-high tumor cells**

To understand the mechanism(s) involved in the resistance of brachyury-high cells to immune-mediated attack, lysis of tumor cells following engagement of the immune-related, surface-associated death receptors FAS and TRAIL-R was first evaluated. As shown in Fig. 2A, brachyury overexpression induced a profound resistance to FAS and TRAIL-mediated lysis in PANC-1 cells, whereas stable silencing of brachyury significantly enhanced the susceptibility of SW480 cells to both apoptotic triggers (Fig. 2B). This resistance was not related to the level of expression of functional or decoy FAS and TRAIL receptors (Supplementary Fig. S2D and S2E), neither was associated with the expression of FAS-associated phospha-

tase-1 (FAP-1; Supplementary Fig. S2F), a protein previously reported to be involved in the attenuation of FAS-mediated cell death (23).

The association of brachyury with resistance to the extrinsic apoptotic pathway was further investigated with three single-cell–derived populations of the PANC-1–pBr cell line (designated as PANC-1–pBr clones Hi, Int, and Lo), characterized by high, intermediate, and low levels of brachyury expression, respectively (Fig. 2C). As shown in Fig. 2D, a strong inverse correlation was observed between the level of brachyury and the susceptibility of the tumor cells to TRAIL-mediated lysis ($r = -0.999$). Unexpectedly, the degree of cleavage of both the initiator caspase-8 and the effector caspase-3 was equivalent among the clones, regardless of the level of brachyury expression (Fig. 2E). The degradation of nuclear lamins, however, was markedly different among the clones. As shown in Fig. 2F, cleavage of lamin B1 and, to a lower extent lamin A/C, was profoundly defective in tumor cells with high levels of brachyury (PANC-1–pBr clone Hi), compared with that observed in the brachyury-low clone (PANC-1–pBr clone Lo).
Defective lysis of brachyury-high cells associates with loss of CDK1 and can be restored by WEE1 inhibition

Previous reports have demonstrated that lamin phosphorylation is a required step for degradation of the nuclear lamina to take place during mitosis and apoptosis (18). We, therefore, hypothesized that inadequate lamin phosphorylation in brachyury-high tumor cells could be responsible, at least in part, for the inefficient degradation of the nuclear laminas, even in the presence of normal levels of activated effector caspases. As shown in Fig. 3A, TRAIL treatment of tumor cells with low levels of brachyury (PANC-1–pBr clone Lo) resulted in efficient phosphorylation and concurrent cleavage of lamin B1, an effect that was not observed in the brachyury-high clone (PANC-1–pBr clone Hi).

One of the kinases known to be involved with the phosphorylation of nuclear lamins is the CDK1 (24). Western blot analysis of CDK1 expression in the single-cell clonal populations of PANC-1–pBr (Fig. 3B) demonstrated a strong inverse correlation between the levels of brachyury and CDK1 protein (r = −0.909). Additional immunofluorescence and Western blot analyses conducted with the PANC-1 tumor cell pair corroborated the marked reduction of CDK1 protein in brachyury-high tumor cells (Fig. 3C). This decrease in CDK1 protein was not associated with changes in mRNA levels (Supplementary Fig. S3A), but instead with a marked reduction of CDK1 protein stability, as demonstrated by the rapid degradation of CDK1 in brachyury-high cells (PANC-1–pBr and H460 Con shRNA) as compared with brachyury-low cells (PANC-1–pCMV and H460 Br shRNA, respectively, Fig. 3D and Supplementary Fig. S3B).

The role of CDK1 loss in the acquisition of resistance of brachyury-high tumor cells to immune-mediated attack was further investigated by evaluating the impact of CDK1 silencing on the susceptibility of the tumor to lysis by NK cells. As shown in Fig. 4A, silencing of CDK1 led to a significant reduction in the susceptibility of A549 cells to NK-mediated lysis. As this effect was not seen with tumor cells pretreated with the pan-caspase inhibitor Z-VAD-FMK, the results suggested that reduced levels of CDK1 impair caspase-dependent apoptosis. In additional experiments, CDK1 was overexpressed in tumor cells with high levels of brachyury (H460-pCDK1), a manipulation that resulted in enhanced NK-mediated lysis, compared with control H460-pCMV cells (Fig. 4B).

On the basis of the above results, we postulated that tumor sensitization to immune-mediated attack could be achieved by restoring the activity of remaining CDK1 protein to an adequate level for efficient nuclear apoptosis to proceed. The approach was investigated by inhibiting the WEE1 kinase, a cell-cycle kinase that negatively regulates CDK1 activity. As demonstrated in Fig. 4C, pretreatment of PANC-1–pBr tumor cells with MK-1775, a WEE1-specific small-molecule inhibitor, was sufficient to fully reconstitute the lysis of PANC-1–pBr clone Hi cells to the levels observed with the PANC-1–pBr clone Lo. We observed similar results in the ability of MK-1775 to restore the lysis of A549–pBr to levels comparable with A549–pCMV cells in response to NK- and TRAIL-mediated cell death (Supplementary Fig. S4). As expected, pretreatment with MK-1775 was associated with increased phosphorylation of laminB1 in PANC-1–pBr clone Hi cells treated with TRAIL (Fig. 4D).

Expression of brachyury and CDK1 in tumor tissues

The expression of brachyury and CDK1 protein was compared by Western blot analysis in commercial protein lysates derived from lung (cases 1–7) and breast (cases 8–10) primary carcinoma tissues. As shown in Fig. 5A, there was an inverse correlation (r = −0.779) between the level of brachyury expression and that of CDK1 protein in lung tumor tissues. A trend was also observed in two of three breast cancer tissues evaluated (Fig. 5B) that showed low levels of CDK1 expression.
in correspondence with high levels of brachyury. Additional lung cancer tissues were also analyzed by immunohistochemistry (IHC) for the expression of brachyury and CDK1 protein. As shown in Fig. 5C, an inverse correspondence between the levels of brachyury and those of CDK1 could be observed in four of six lung carcinomas evaluated.

**Differential effect of brachyury on caspase-dependent versus perforin-dependent tumor lysis**

Immune effector cells have the capacity to kill target cells in absence of caspase activation through the perforin-dependent actions of granzymes. In light of the impact of brachyury expression on the caspase-dependent apoptotic pathway, we investigated whether high brachyury levels could also impair perforin-dependent lytic pathways. These studies were performed with two PANC-1–pBr clones (clone Hi vs. Lo with high vs. low levels of brachyury, respectively) that were left untreated or pretreated with CMA before the assay, a manipulation that ablates perforin-dependent lysis of target cells. As shown in Fig. 6A, single blockade of caspase activity significantly reduced the lysis of the PANC-1–pBr clone Lo but had no effect on the lysis of the PANC-1–pBr Hi clone. In contrast, blockade of perforin-dependent lysis via CMA treatment was able to significantly reduce the lysis of the PANC-1–pBr Hi but not that of the PANC-1–pBr Lo clone. Combined blockade of caspases and perforin activity, as expected, markedly reduced the lysis of both tumor cell lines. These results indicated that tumor cells with low levels of brachyury could be efficiently lysed after triggering of caspase-dependent pathways whereas lysis of tumor cells with high levels of brachyury seems to be exclusively dependent on the involvement of the perforin-mediated pathway.

**Tumor cells with high brachyury escape immune attack in vivo**

To investigate whether high levels of brachyury expression would drive tumor resistance to effector lysis in vivo, the murine colon carcinoma cell line MC38 was stably transfected with a control vector (MC38–pCMV) or a vector encoding the full-length brachyury protein (MC38–pBr; Fig. 6B). Similarly to our observations with human carcinoma cells, overexpression of brachyury in MC38 cells concomitantly decreased the levels of CDK1 protein (Fig. 6B) without affecting the expression of gp70, the envelope protein of an endogenous murine retrovirus previously described as a tumor antigen in the MC38 model (21). The effect of brachyury overexpression on tumor lysis by gp70-specific CTLs was then evaluated in vitro. As shown in Fig. 6C, gp70-specific CTLs were able to lyse MC38–pBr cells less efficiently than control MC38–pCMV cells at all effector-to-target ratios used and, in agreement with the results with human carcinoma cells, the defective lysis of MC38–pBr cells was more pronounced after ablation of perforin-mediated cytotoxicity. To test whether brachyury could also induce resistance to immune effector cells in vivo, gp70-specific CTLs were admixed in vitro at a ratio of 2:1 with MC38–pBr or MC38–pCMV tumor cells and subsequently injected subcutaneously into syngeneic C3H/HeJ mice. As shown in Fig. 6D, gp70-specific T cells were efficient at preventing growth of MC38–pCMV cells (tumors failed to grow in 3/3 mice) but failed to control the growth of MC38–pBr tumors in 3 of 3
Discussion

The activation of the EMT program in tumors has been proposed as a mechanism by which cancer cells bearing a mesenchymal-like phenotype may survive conventional anti-neoplastic interventions. The studies reported here indicate that acquisition of a mesenchymal-like phenotype via expres-

sion of high levels of the EMT regulator brachyury could also mediate resistance to immune-mediated attack, potentially contributing to tumor ignorance and failure of immune rejection of human tumor cells.

In recent years, numerous tumor antigens have been identified and antigen-specific CD8+ T-cell immune responses have been detected in the blood of patients with cancer and in lymphocytic infiltrates of multiple types of tumors (15, 26). The acquisition of immune evasion mechanisms, however, allows cancer cells to grow and metastasize despite of the presence of a measurable antitumor immune response (27, 28). Defects in the antigen-processing and/or -presentation machinery, including decreased expression of HLA molecules and reduced levels of tumor antigens are well-recognized mechanisms of immune evasion. These phenomena, however, cannot explain all instances of immunologic resistance. Reports are now starting to implicate the phenomenon of EMT in tumor resistance to CTL-mediated lysis; Akalay and colleagues (29), for example, recently demonstrated that EMT might induce resistance to CTL lysis through the induction of autophagy. In this report, we have extended the understanding of the role of EMT in tumor resistance to immune attack by showing that brachyury decreases the ability of antigen-specific T cells to lyse tumor cells in the presence of effective levels of MHC, antigen or the various components of the antigen presentation machinery. Interestingly, tumor cells with high levels of brachyury showed an upregulation, rather than a downregulation, of the immunoproteosome subunits LMP2 and LMP7. This phenomenon, which is commonly seen in cellular responses to stress, may lead to enhanced generation and presentation of MHC class I antigenic epitopes. Thus, deficient antigen presentation can be ruled out as the mechanism of resistance of brachyury-high cells. Furthermore, we show that the impairment of immune effector-mediated lysis of brachyury-high cells could also be observed during antigen-independent cytotoxicity mediated by NK or LAK cells.

The results from this study indicate that the poor killing associated with high levels of brachyury is mainly due to inefficient caspase-dependent apoptotic death, and not with perforin-mediated lysis involving granzymes. Previous studies have investigated the mechanisms by which other EMT transcription factors could drive resistance to cell death. For example, the overexpression of snail has been shown to impair apoptosis in response to TNF-α by decreasing the activity of initiator caspase-8 and effector caspase-3 (3). Contrasting with those studies, the resistance of brachyury-high tumor cells to caspase-mediated cell death takes place in the presence of normal levels of fully activated effector caspasess. Instead, the major apoptotic defect identified here is the absence of degradation of nuclear lamins. The nuclear lamina is a protein mesh formed by the intermediate filament lamins A-type (lamins A and C) and B-type (lamins B1, B2; ref. 30), closely associated with the inner nuclear membrane and the chromatin. In addition to playing a key role in maintaining the nuclear envelope integrity and nuclear architecture, disassembly of the lamina is a required step during mitosis as well as for the induction of caspase-dependent apoptosis (18). We
demonstrate here a profound reduction in CDK1 levels in brachyury-high tumor cells, which, in turn, results in deficient lamin phosphorylation and defective degradation by effector caspases (31). Although the mechanism involved in the reduction of CDK1 protein in brachyury-high cells has not been investigated here, we have previously shown that brachyury expression reduces the levels of the cell-cycle regulator p21 (10), which, in turn, could promote the assembly and stabilization of cyclin B1/CDK1 kinase at the G2-M transition (32).

The WEE1 kinase inactivates CDK1 by phosphorylating Tyr15 (33). Previous reports have indicated a role for WEE1 in the decreased response of breast cancer cells to TRAIL-mediated apoptosis (34) or the resistance to radiotherapy in various types of tumor cells (35). Currently, a specific inhibitor of WEE1 is being tested in Phase II clinical trials for solid tumors in combination with chemotherapy. Our results demonstrate that WEE1 blockade by MK-1775 is able to fully revert the resistance of brachyury-high tumor cells to caspase-dependent cell death induced by TRAIL, NK, or LAK effector cells. This observed reconstitution of susceptibility to cell death is presumably achieved by restoring threshold levels of activated CDK1 in brachyury-high cells, which might then allow for the proper phosphorylation of lamins and their subsequent targeting for degradation by activated caspases (Supplementary Fig. S5).

To date, there is no clear understanding of what factors dictate the cytolytic mechanisms used by CTLs to kill tumor cells, either in vivo or in vitro. A study with murine renal carcinoma cells, for example, has shown that tumors that present low levels of MHC class I–associated antigens to the effector T cells are preferentially lysed by the FasL pathway, whereas at high levels of peptide the CTLs lose their preference for effector pathway usage (36). We demonstrate here that efficient lysis of brachyury-high tumor cells could only be achieved by effector cells capable of lysing via the granule/perforin pathway. This observation is consistent with the idea that during perforin-dependent apoptosis, granzymes A and B have been shown to directly cleave the nuclear lamins, thus being able to promote the disruption of the nuclear membrane even in the absence of proper lamin phosphorylation (37).

A phase I clinical trial of a recombinant yeast–brachyury vaccine (22) is currently ongoing in patients with advanced tumors (38). This vaccine is aimed at inducing a brachyury-specific T-cell immune response that could eliminate tumor cells undergoing brachyury-mediated EMT. The results from this study indicate that the effectiveness of an immune

Figure 6. Brachyury induces tumor immune resistance in vivo. A, lysis of PANC-1-pBr Lo and Hi clones left untreated or pretreated with the pan-caspase inhibitor Z-VA-D-FMK by NK cells left untreated or pretreated with CMA for inhibition of perforin-mediated lysis. B, Western blot analysis of brachyury and CDK1 expression in MC38 cells transfected with pCMV versus pBr, and expression of gp70 in the same cells (right). C, lysis of the MC38 tumor cell pair was conducted at indicated effector-to-target ratios with gp70-specific CTLs either untreated or pretreated with CMA for inhibition of perforin-dependent lysis. Shown is a representative result of two similar experiments. D, indicated tumor cells were admixed in vitro with gp70-specific T cells and subsequently implanted subcutaneously into C57BL/6 mice. Shown is the tumor volume at day 16 after implantation. E, tumor cells (MC38-pCMV vs. -pBr) were implanted subcutaneously on day 0; mice were vaccinated with gp70 peptide in adjuvant [vs. control Hank’s Balanced Salt Solution (HBSS)] at days 4, 11, and 18. Shown is the tumor volume on day 25 after tumor implantation. *, P < 0.05; **, P < 0.01.
response against tumor cells that express high levels of brachyury could be further enhanced by (i) reconstituting caspase-dependent cell death via inactivation of the WEE1 kinase, or (ii) by downregulating the expression of brachyury, which paradoxically can increase the susceptibility of the tumor to brachyury-specific (and other tumor-specific) T cells elicited by vaccination of patients with a brachyury-based vaccine. Although paradoxical, this strategy would allow for an alleviation of resistance mechanisms mediated by brachyury, and improve tumor lysis even in the presence of lower levels of the target antigen.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: D.H. Hamilton, C. Palena
Development of methodology: D.H. Hamilton, K.-Y. Tsang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.H. Hamilton, B. Huang, R.I. Fernando
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.H. Hamilton, B. Huang, R.I. Fernando, C. Palena
Writing, review, and/or revision of the manuscript: D.H. Hamilton, B. Huang, R.I. Fernando, K.-Y. Tsang, C. Palena
Study supervision: C. Palena

Acknowledgments
The authors thank Dr. Jeffrey Schlom for helpful discussions on the article, Dr. James W. Hodge for the gq70-specific T cells, Dr. Yvona Ward and the CCR’s CBRR Microscopy Core Facility for their assistance in obtaining confocal images, Margie Dubenstein, Marion Taylor, and Bertina Gibbs for technical assistance, and Debra Weingarten for editorial assistance.

Grant Support
This research was supported by the Intramural Research Program of the Center for Cancer Research, National Cancer Institute, NIH.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 9, 2013; revised February 12, 2014; accepted February 22, 2014; published OnlineFirst March 13, 2014.

References