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Research Article **Oncology**

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Introduction

Metastatic progression of cancer is responsible for the majority of all human cancer deaths. For a tumor to metastasize, several complex biological processes must take place that enable tumor cells to detach from the primary tumor, invade the surrounding tissue, enter the bloodstream or lymphatic vessels, home to distant organs, and survive and proliferate at the secondary site (1, 2). To date, the molecular mechanisms that cause tumor invasion and metastasis are still not well understood; the identification of genes that regulate those mechanisms will certainly help in designing strategies aimed at preventing and treating the spread of cancer.

To achieve the various steps along the metastasis process, epithelial tumor cells may need to undergo a phenotypic conversion into mesenchymal, motile cells (3). The epithelial-mesenchymal transition (EMT) is a reversible process during which cells switch from a polarized, epithelial phenotype into a highly motile, mesenchymal phenotype (4). A key step during embryonic morphogenesis and wound repair in adult tissue, the EMT program is now being implicated in the progression of primary tumors toward metastases (5–7). At the biochemical level, the EMT program involves the downregulation of epithelial proteins such as E-cadherin and cytokeratins and the induction of mesenchymal proteins including fibronectin, N-cadherin, vimentin, and MMPs. E-cadherin is required for the formation of adherens junctions between epithelial cells, and the loss of E-cadherin is emerging as an indicator of EMT, tumor progression, and metastasis (8, 9). Moreover, the E-cadherin to N-cadherin switch (low E-cadherin and high N-cadherin) has been shown to be associated with cancer progression and cancer-related death (10). Numerous observations support the concept that the EMT process plays a role in the progression of tumors (11–13).

The EMT program triggered during tumor progression appears to be controlled by genes normally expressed in the early embryo, including *Twist*, *Snail*, *Slug*, *Goosecoid*, and *SIP1* (14–19). The transcription factors encoded by these genes can impart to tumor cells the traits of mesenchymal cells, including motility and invasiveness. The expression of Twist, for example, has been found to be elevated in various types of cancer, including breast, prostate, gastric, and melanoma (20). In prostate cancer, Twist expression has been positively correlated with Gleason grading and the presence of metastasis (21).

We previously demonstrated that the T-box transcription factor Brachyury (22–24) is highly expressed in various human tumors of epithelial origin and in human tumor cell lines derived from lung, colon, and prostate carcinomas, but not in most human normal adult tissues (25). We also demonstrated the potential of Brachyury as a target for T cell–mediated immunotherapy of cancer by identifying a $CD8^+$ T cell epitope of Brachyury that was able to expand Brachyury-specific T cells from the blood of cancer patients with the ability to lyse Brachyury-positive tumor cell lines. Our interest in this molecule as a potential target for cancer immunotherapy was further enhanced by its known role during development, where Brachyury is necessary for normal mesoderm formation (26, 27). However, to our knowledge, no reports exist so far demonstrating a role for Brachyury in tumor cells. Here, we sought to investigate whether Brachyury plays any role in the EMT process during tumor progression. Our results demonstrated that overexpression of Brachyury in tumor cells resulted in upregulation of mesenchymal markers and downregulation of epithelial markers, with a concomitant increase in tumor cell migration and ECM invasion. Moreover, Brachyury overexpression

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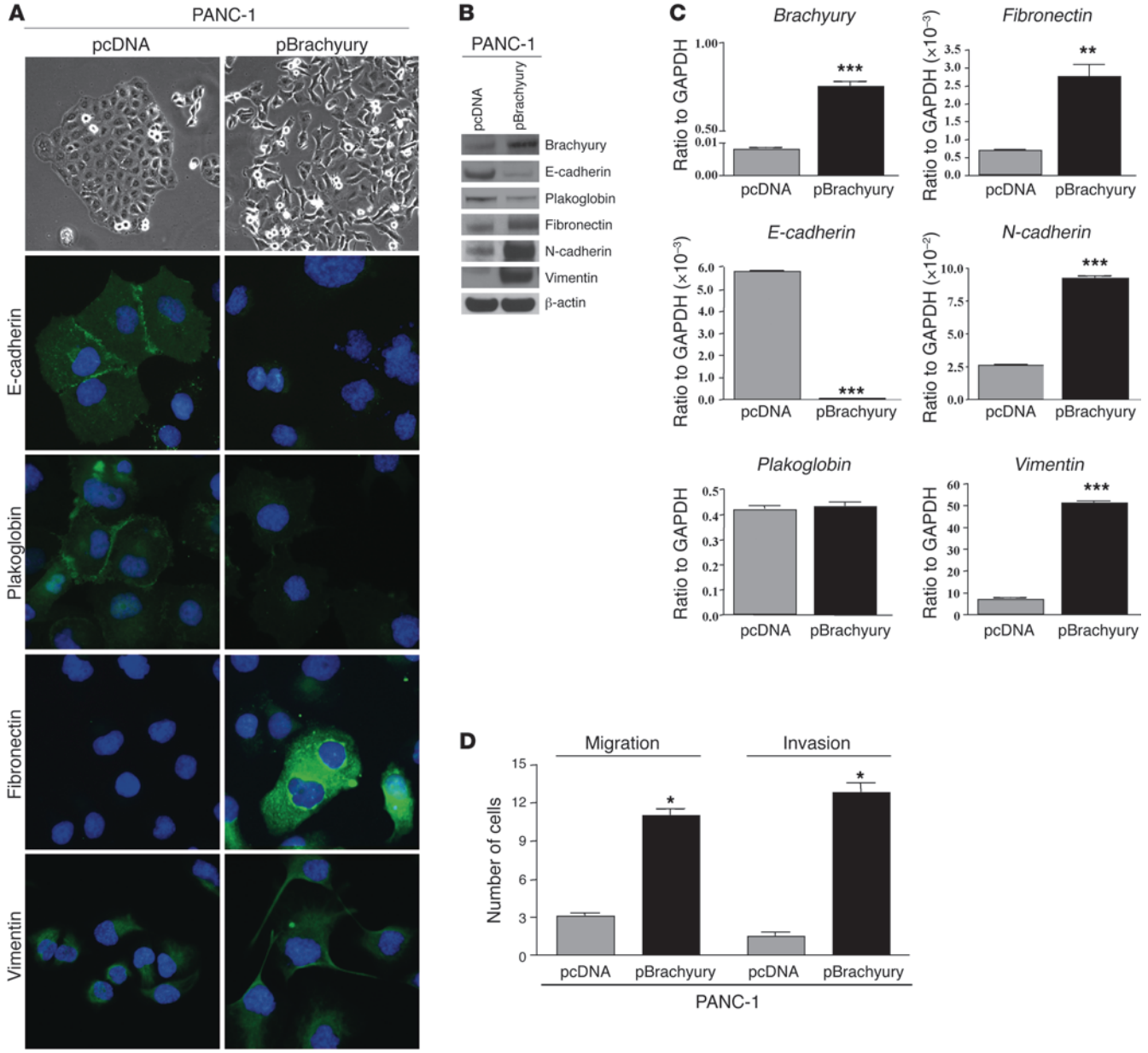

Figure 1

Brachyury alters EMT marker expression and induces a highly migratory and invasive phenotype in human epithelial tumor cells. (**A**) Bright-field images of PANC-1-pcDNA and PANC-1–pBrachyury cells grown on a plastic surface (top 2 panels: original magnification, ×10) and immunofluorescence analysis of EMT markers in cells grown on cover glasses (bottom panels: original magnification, ×40) are shown. The green signal represents the staining of the corresponding protein, and the blue signal represents the DAPI-stained nuclei. (**B**) Western blot analysis of Brachyury and EMT markers. β-Actin is shown as the protein loading control. (**C**) Real-time PCR analysis of cDNA from the above cells for *Brachyury* and EMT markers. (**D**) In vitro cell migration and ECM invasion assays. Results of 1 of 3 experiments are shown. Error bars indicate SEM of triplicate measurements. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ for pcDNA versus pBrachyury.

resulted in repression of E-cadherin transcription, and this effect was at least partly mediated by the transcriptional repressor Slug. Additionally, stable silencing of Brachyury expression in Brachyury-positive human carcinoma cells resulted in downregulation of mesenchymal markers and upregulation of epithelial markers, with concomitant loss of cell migration and ECM invasion. Brachyury expression also negatively modulated tumor cell cycle progression and correlated with low expression of cyclin D1. In vivo, Brachyury-inhibited human tumor cells had a diminished ability to form experimental lung metastases after intravenous injection in mice, as well as to disseminate from the primary, subcutaneous tumor to the site of metastases. Collectively, our results demonstrate that the transcription factor Brachyury confers on the tumor cells a mesenchymal phenotype as well as migratory and invasive abilities and attenuates tumor cell cycle progression. Analysis of multiple human lung tumor tissues showed that the percentage of tumors

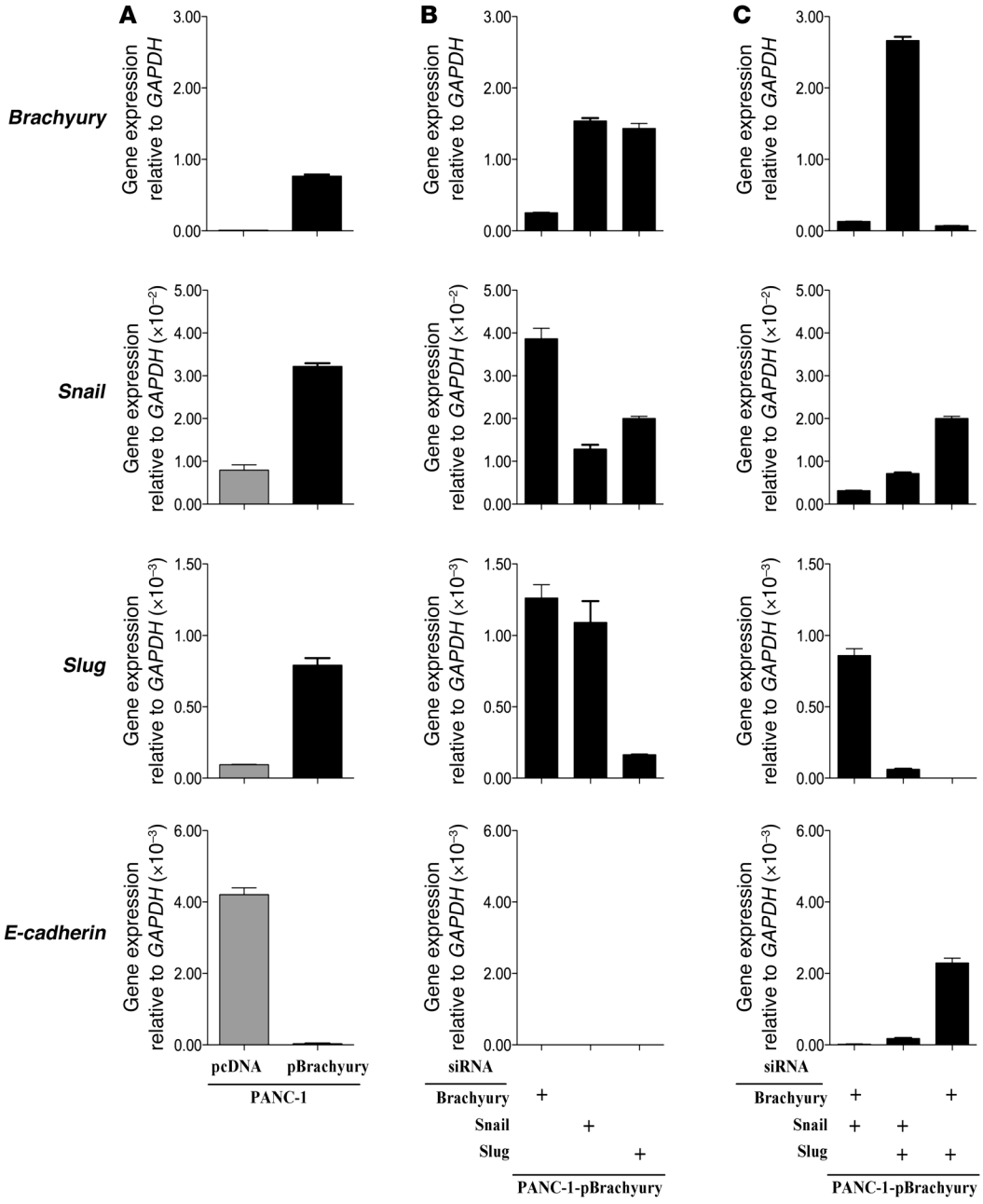

Figure 2
Control of E-cadherin expression in PANC-1–pBrachyury cells. Expression of *Brachyury*, *Snail*, *Slug*, and *E-cadherin* was analyzed by real-time PCR in PANC-1-pcDNA versus PANC-1–pBrachyury cells that were untransfected (**A**) or transiently transfected with various single (**B**) or combined (**C**) siRNAs, as indicated. The experiment was repeated twice, with comparable results; data from 1 experiment are shown. Error bars indicate SEM of triplicate measurements.

positive for *Brachyury* expression increases with tumor stage. The high expression of Brachyury in tumor cells and its potential role in the metastatic conversion of tumors make Brachyury an attractive target candidate for antitumor therapies.

Results

Brachyury induces a mesenchymal phenotype in epithelial cancer cells and enhances tumor cell migration and invasion. To elucidate whether Brachyury expression plays any role in the EMT process in tumor cells, we first overexpressed human Brachyury in an epithelial cancer cell line with nearly undetectable endogenous expression of Brachyury. The human pancreatic tumor cell line PANC-1, which is positive for the epithelial marker E-cadherin and has low levels of the mesenchymal protein vimentin, was chosen as a model of an epithelial cancer cell. PANC-1 cells were transfected with a vector encoding for human Brachyury, and a population of cells was further selected in the presence of zeocin. By single-cell cDNA analysis we determined that about 70% of cells in the mixed PANC-1–

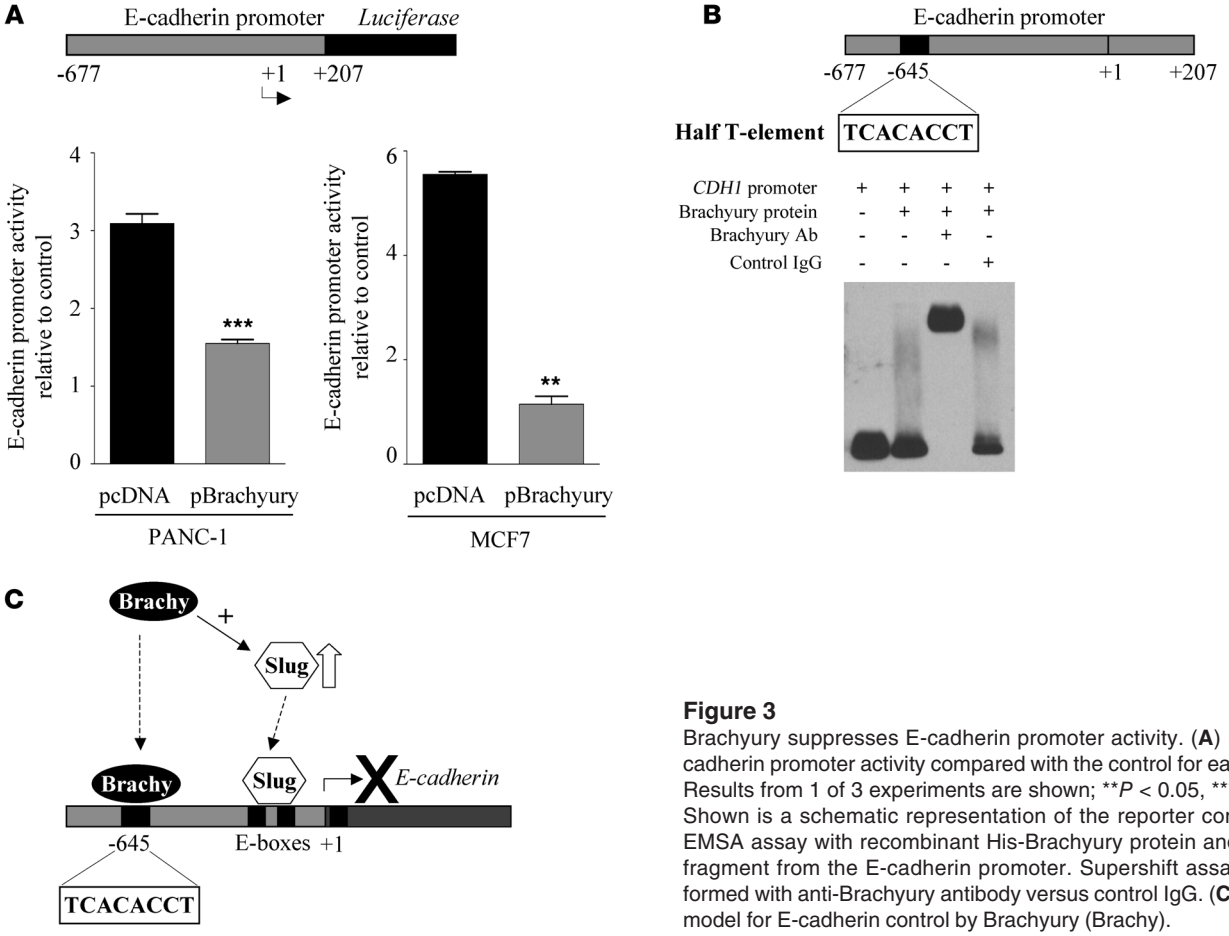

Figure 3
Brachyury suppresses E-cadherin promoter activity. (**A**) Relative E-cadherin promoter activity compared with the control for each cell line. Results from 1 of 3 experiments are shown; $^{**}P < 0.05$, $^{***}P < 0.001$. Shown is a schematic representation of the reporter construct. (**B**) EMSA assay with recombinant His-Brachyury protein and a labeled fragment from the E-cadherin promoter. Supershift assay was performed with anti-Brachyury antibody versus control IgG. (**C**) Proposed model for E-cadherin control by Brachyury (Brachy).

pBrachyury population had *Brachyury* mRNA levels at least 50-fold higher than the control PANC-1-pcDNA cells, while the remaining cells showed about a 35-fold upregulation of *Brachyury* mRNA (data not shown). Increased expression of Brachyury (3.5- and 95.6-fold at the protein and mRNA levels, respectively, for the mixed population) resulted in morphological changes indicative of EMT (Figure 1A). These cells grew as loose colonies of elongated cells, in contrast to the cuboidal, tightly attached cells of control pcDNA-transfected cells. Distinctive of EMT is also the loss of epithelial markers with simultaneous gain of proteins that are normally expressed on mesenchymal cells. While PANC-1-pcDNA cells stained positive for the epithelial markers E-cadherin and plakoglobin (γ-catenin) at the areas of cell-to-cell contact established among neighboring cells, E-cadherin was undetectable, and plakoglobin expression was dimmed and disorganized in PANC-1–pBrachyury cells (Figure 1A). Reduced levels of E-cadherin and plakoglobin proteins in PANC-1–pBrachyury cells were also confirmed by immunoblotting, as shown in Figure 1B. Conversely, enhanced levels of the mesenchymal proteins fibronectin, N-cadherin, and vimentin were observed in PANC-1 cells overexpressing Brachyury (Figure 1, A and B). Most of the changes in epithelial and mesenchymal marker expression induced by Brachyury in PANC-1 cells were also evident at the mRNA level (Figure 1C); a significant decrease in *E-cadherin* mRNA with simultaneous several-fold upregulation of *Fibronectin*, *N-cadherin*, and *Vimentin* mRNA characterized PANC-1–pBrachyury cells.

Further evidence of the ability of Brachyury to promote a mesenchymal phenotype in epithelial tumor cells was the emergence of in vitro cell motility and invasiveness in PANC-1–pBrachyury cells, as compared with control vector–transfected cells (Figure 1D). These findings were extended using the human breast carcinoma cell line MCF7, which showed acquisition of a mesenchymal phenotype (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI38379DS1) and enhanced migratory and invasive characteristics in vitro after Brachyury overexpression (Supplemental Figure 1B). Together, these data demonstrated that the transcription factor Brachyury endows epithelial tumor cells with the traits of mesenchymal cells, including motility and invasiveness.

Control of E-cadherin transcription in Brachyury-overexpressing carcinoma cells. Reduction of E-cadherin expression has been reported as a common event during the course of EMT (9). Two members of the Snail family of zinc finger transcription factors, Snail and Slug, have been previously shown to efficiently repress E-cadherin expression during the course of an EMT (16, 18, 19). Here we investigated whether Brachyury overexpression in PANC-1 cells is able to simultaneously drive the overexpression of these transcription factors. As shown in Figure 2A, while control PANC-1 cells showed some expression of *Snail* and very little *Slug* mRNA, the expression of both, and particularly that of *Slug*, was enhanced in the PANC-1–pBrachyury cells. Expression of Twist, another well-characterized EMT regulator, was very low in the PANC-1 cells

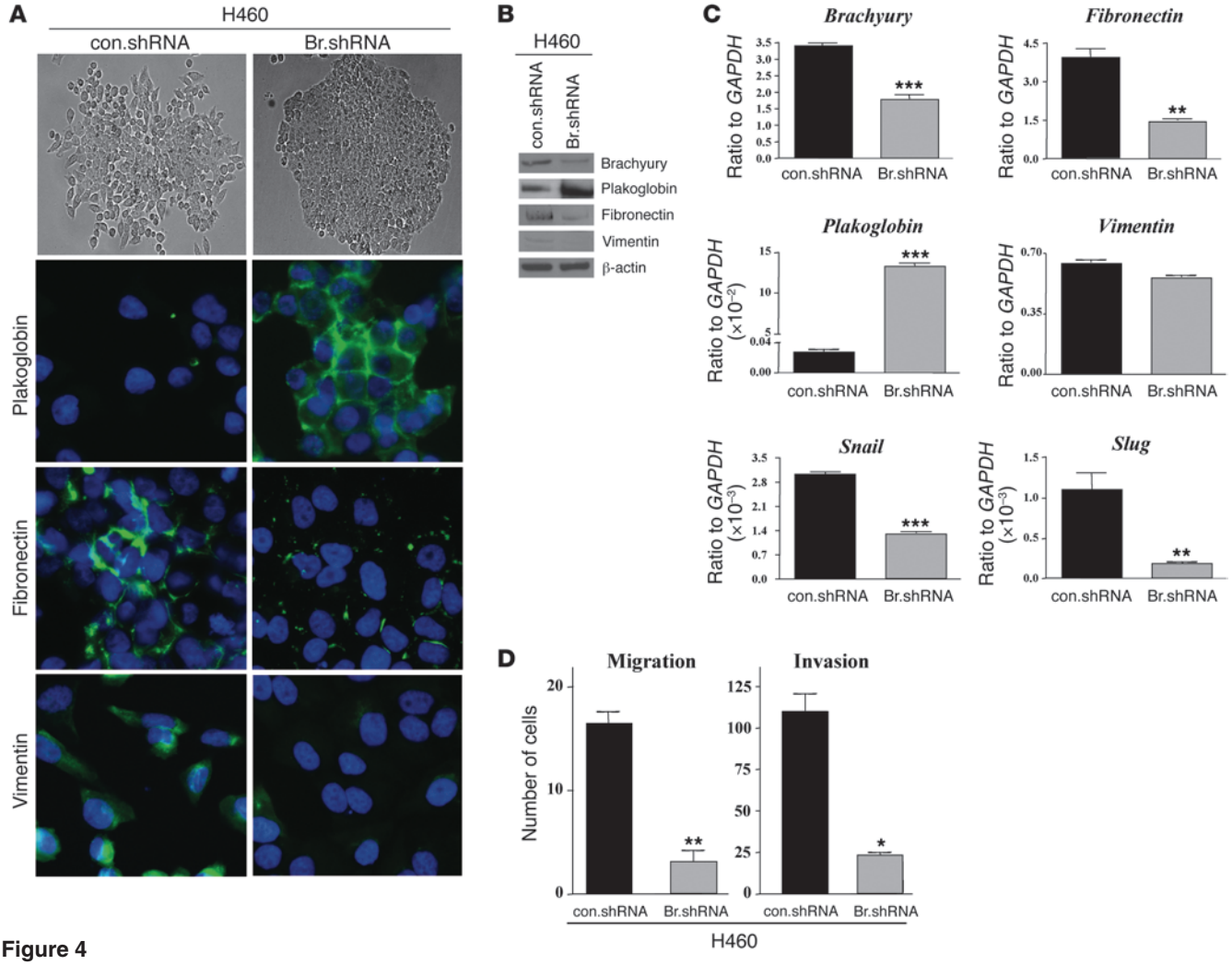

Figure 4

Brachyury inhibition reduces expression of mesenchymal markers, migration, and invasion of human lung carcinoma cells. (**A**) Bright-field images of H460-con.shRNA and H460-Br.shRNA cells grown on a plastic surface (top 2 panels: original magnification, ×10) and immunofluorescence analysis of EMT markers (bottom panels: original magnification, ×20) are shown. The green signal represents the staining of the corresponding protein, and the blue signal represents the DAPI-stained nuclei. (**B**) Western blot analysis of Brachyury and EMT markers. β-Actin is shown as a protein loading control. (**C**) Real-time PCR analysis of cDNA for *Brachyury* and EMT markers. (**D**) In vitro cell migration and ECM invasion assays. Results from 1 of 3 experiments are shown. Error bars indicate SEM of triplicate measurements. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ for con.shRNA versus Br.shRNA.

and did not change as a result of Brachyury overexpression (data not shown). To elucidate the degree of involvement of either Snail or Slug in the reduction in *E-cadherin* expression, we further analyzed PANC-1–pBrachyury cells that were transiently transfected with siRNA specific for *Brachyury*, *Snail*, *Slug*, or combinations of them, as indicated in Figure 2, B and C. As shown in Figure 2B (bottom panel), *E-cadherin* expression remained deficient in PANC-1–pBrachyury cells inhibited for the expression of each individual transcription factor. Moreover, coinhibition of Brachyury and Snail, or Snail and Slug (Figure 2C), was unable to significantly restore *E-cadherin* expression. Interestingly, simultaneous silencing of Brachyury and Slug was needed to restore *E-cadherin* expression up to 52% of the level in the control PANC-1-pcDNA cells (Figure 2C), and this outcome was not improved by the concurrent inhibition of all 3 transcription factors (data not shown). Control, nontargeting siRNA did not have any detectable effect on the expression of any gene analyzed. These results suggested that the effects of Brachyury overexpression on *E-cadherin* are at least partially mediated by Slug, but not Snail.

To investigate whether the reduced level of E-cadherin in Brachyury-overexpressing cells was a result of transcriptional silencing, PANC-1–pBrachyury and MCF7-pBrachyury cells were transiently transfected with a reporter construct containing the *luciferase* gene under the control of an 884-bp fragment of the human E-cadherin promoter (position –677 to +207). As shown in Figure 3A, the reporter activity was significantly repressed in both PANC-1–pBrachyury and MCF7-pBrachyury compared with control cells, hence indicating that Brachyury directly or indirectly regulates *E-cadherin* transcription in these cells. It is known that Slug directly binds to the E-cadherin promoter. We investigated binding of Brachyury to the E-cadherin promoter by performing an EMSA assay; as shown in Figure 3B, a full consensus half-site for T-box

Table 1
Genes downregulated after Brachyury inhibition in H460 cells

Gene symbol	Full name	Fold downregulation[A]
Cell migration		
ITGA11	Integrin, alpha 11	119
IL8RB	Interleukin 8 receptor, beta	8
Extracellular matrix remodeling		
MMP24	Matrix metalloproteinase 24	29
MMP2	Matrix metalloproteinase 2, gelatinase A	11
Negative regulators of cell cycle		
CUL1	Cullin 1	6
p21	Cyclin-dependent kinase inhibitor, p21	3

[A]Fold change in gene expression in Brachyury-inhibited versus control shRNA-transfected H460 cells.

binding is present at –645 from the transcription start site in the E-cadherin promoter. In vitro, a purified, full-length recombinant Brachyury protein was able to bind to an 884-bp fragment of this promoter, though with low efficiency. The addition of a specific antibody against Brachyury, however, was able to efficiently shift the Brachyury-DNA complexes into a distinct, high-molecular-weight band (Figure 3B), and this effect was not observed when an irrelevant control IgG was used. Together these results indicated that overexpression of Brachyury in epithelial tumor cells induces the simultaneous enhancement of Slug expression and that Brachyury itself could bind to the E-cadherin promoter to silence *E-cadherin* expression acting in concert with the transcriptional repressor Slug (Figure 3C).

Brachyury inhibition results in loss of mesenchymal markers and reduced migration and invasion of tumor cells. The levels of *Brachyury* mRNA expression were analyzed by real-time PCR in various human tumor cell lines derived from lung, colon, and prostate carcinomas. Expression of *Brachyury* mRNA was remarkably high in certain lung cancer cell lines (Supplemental Figure 2). The Brachyury-positive, E-cadherin–negative non–small cell lung carcinoma cell line H460 has been previously shown to be highly migratory and invasive in vitro, as well as metastatic in vivo (28, 29). This cell line was selected for subsequent loss-of-function studies to further investigate the role of Brachyury in the mesenchymal transition and the metastatic behavior of epithelial tumor cells. H460 cells were stably transfected with a control nontargeting shRNA (designated con.shRNA) or 5 different Brachyury-specific shRNA-encoding vectors. Stable transfectants from 1 construct (designated Br.shRNA), showing 73% and 60% reduction in the levels of Brachyury protein and mRNA, respectively, were selected for further studies. While control shRNA–transfected H460 cells showed very few cell-cell associations in culture (Figure 4A), H460 cells inhibited for the expression of Brachyury grew as clusters of closely packed epithelial cells surrounded by empty spaces. Therefore, reduction of Brachyury expression induced a morphological change reminiscent of a mesenchymal-epithelial (MET) transition. The appearance of tight associations among Brachyury-inhibited tumor cells led us to investigate the expression of the epithelial markers E-cadherin and plakoglobin. Immunofluorescence and immunoblotting analysis failed to demonstrate upregulation of E-cadherin in Brachyury-inhibited H460 cells; *E-cadherin* mRNA was also undetectable by real-time RT-PCR. As shown in Figure 4C, expression of both *Snail* and *Slug* was significantly reduced in the Brachyury-inhibited H460 cells; the decrease, however, was not sufficient to restore E-cadherin expression. Conversely, intense staining of plakoglobin, a marker of desmosomal cell-cell adhesion structures, was observed at the areas of cell-to-cell contact in Brachyury-inhibited H460 cells (Figure 4A). The upregulation of plakoglobin expression was also confirmed by immunoblotting (Figure 4B) and at the mRNA level (Figure 4C). Reduction of Brachyury expression also resulted in a marked decrease in the expression of the mesenchymal markers fibronectin and vimentin (Figure 4, A and B). At the RNA level, the expression of *Fibronectin*, but not that of *Vimentin*, also showed a significant decrease as a result of Brachyury inhibition (Figure 4C). H460 cells tested negative for N-cadherin by all methods employed.

By performing in vitro migration and invasion assays, we investigated whether inhibition of Brachyury expression would also modify the functional characteristics of the H460 cells. As shown in Figure 4D, Brachyury-inhibited H460 cells showed significantly reduced migratory and invasive properties, as compared with the control cells. These results were also confirmed by use of an additional lung carcinoma cell line, H1703, which showed reduced vimentin expression and significantly reduced migratory and invasive characteristics in vitro after Brachyury inhibition (Supplemental Figure 1, C and D).

To investigate whether the absence of E-cadherin expression in the H460-Br.shRNA cells was due to the partial downregulation of Brachyury in the mixed population, 3 single-cell clones were isolated in which *Brachyury* mRNA levels were reduced by about 75%–85% compared with control cells (Supplemental Figure 3A). With this higher degree of Brachyury inhibition, all 3 clones showed a marked reduction in *Fibronectin* and had significantly decreased invasiveness (Supplemental Figure 3, A and B) but still failed to show upregulation of the epithelial marker E-cadherin. Together, these results indicated that the reduction of Brachyury expression in H460 lung carcinoma cells induces morphologic and biochemical changes indicative of a MET transition, even in the absence of E-cadherin expression.

In order to delineate how Brachyury might promote migration and invasion of H460 cells, we have conducted a detailed analysis of gene expression in Brachyury-inhibited versus control shRNA–transfected H460 cells, with emphasis on genes involved in cell motility, metastasis, and invasion. Of 226 genes analyzed, 16 genes showed at least a 3-fold change in expression in response to Brachyury inhibition. Of particular interest were 2 genes known to regulate cell migration in response to chemotactic gradients, integrin-alpha 11 (*ITGA11*) and the IL8 receptor, beta (*IL8RB*), that were downregulated 119- and 8-fold, respectively, in response to Brachyury inhibition (Table 1). In migration assays with control H460 cells, a specific, blocking anti–IL8RB antibody was able to reduce the migratory response of these cells by 86% compared with that of cells treated with a control IgG (R.I. Fernando, unpublished observations), suggesting that expression of IL8RB plays an important role in the migration of H460 cells. Among genes known to participate in the degradation of the ECM, the MMP-encoding genes *MMP2* and *MMP24* were downregulated 11- and 29-fold, respectively, in Brachyury-inhibited H460 cells (Table 1). Changes

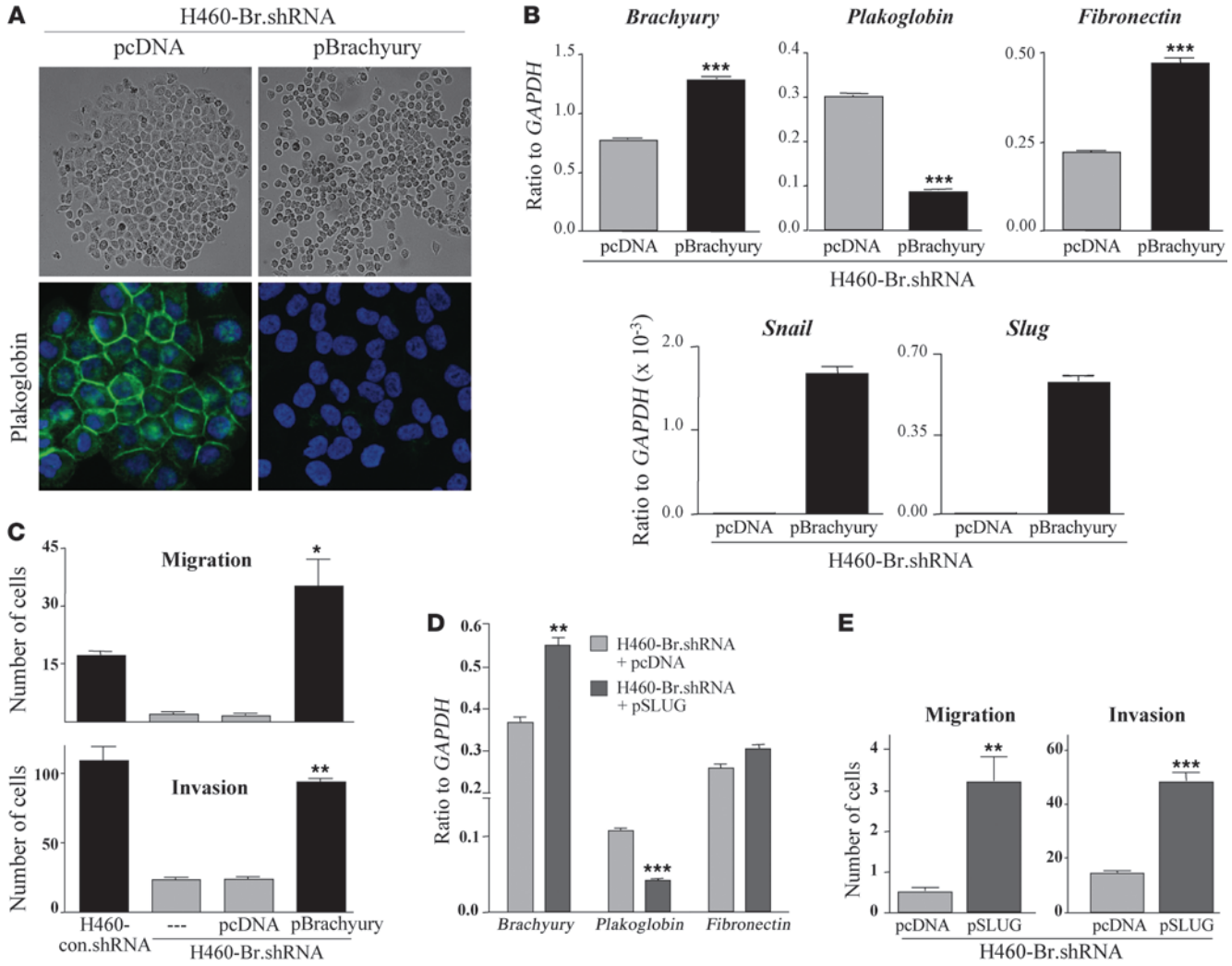

Figure 5

H460-Br.shRNA cells rescued by Brachyury or Slug overexpression. (**A**) Bright field images of cells grown on plastic surface (top: original magnification, ×10) and immunofluorescence analysis of Plakoglobin (bottom: original magnification, ×20) are shown. The green signal represents the staining of the corresponding protein, and the blue signal represents the DAPI-stained nuclei. (**B**) Real-time PCR analysis for *Brachyury* and EMT markers and (**C**) cell migration and ECM invasion assay with H460-Br.shRNA coexpressing empty vector (pcDNA) or a human Brachyury-encoding vector (pBrachyury). (**D**) Expression of Brachyury and EMT markers and (**E**) cell migration and invasion assays with H460-Br.shRNA cells transiently transfected with control (pcDNA) or a human Slug-expressing vector (pSLUG). Error bars indicate SEM of triplicate measurements. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$ for pcDNA versus pBrachyury or pSLUG.

in the expression of these genes were consistent with the loss of invasive characteristics by Brachyury-inhibited H460 cells.

To confirm that the observed results were not due to an off-target effect, we conducted a rescued expression experiment in which Brachyury-inhibited H460 cells were stably cotransfected with a vector encoding for the human *Brachyury* gene. Restoration of Brachyury expression in H460-Br.shRNA cells resulted in the disappearance of cell-to-cell contact areas, with concomitant loss of plakoglobin (Figure 5, A and B). Moreover, reappearance of the mesenchymal marker fibronectin (Figure 5B) and reacquisition of a migratory and invasive phenotype (Figure 5C) to the levels of the control cells were observed in Brachyury-rescued cells. The levels of *Snail* and *Slug* mRNA were also enhanced after Brachyury reexpression in these cells (Figure 5B).

Collectively, these studies demonstrated for the first time to our knowledge a role for the T-box transcription factor Brachyury in the maintenance of a mesenchymal tumor phenotype at the biochemical and functional levels, in human tumor cell lines.

Slug restores a mesenchymal phenotype in H460 cells inhibited for the expression of Brachyury. As a further demonstration of the role of Slug on the effects mediated by Brachyury in tumor cells, we also transiently transfected H460-Br.shRNA cells with a vector encoding for human Slug. Overexpression of Slug in the Brachyury-deficient H460 cells was able to induce a significant enhancement of *Brachyury* expression and a decrease in the levels of *Plakoglobin* mRNA, without a significant effect on the levels of *Fibronectin* mRNA (Figure 5D). The migratory and invasive abilities of the H460-Br.shRNA cells were also rescued by Slug transfection (Figure 5E), indicating

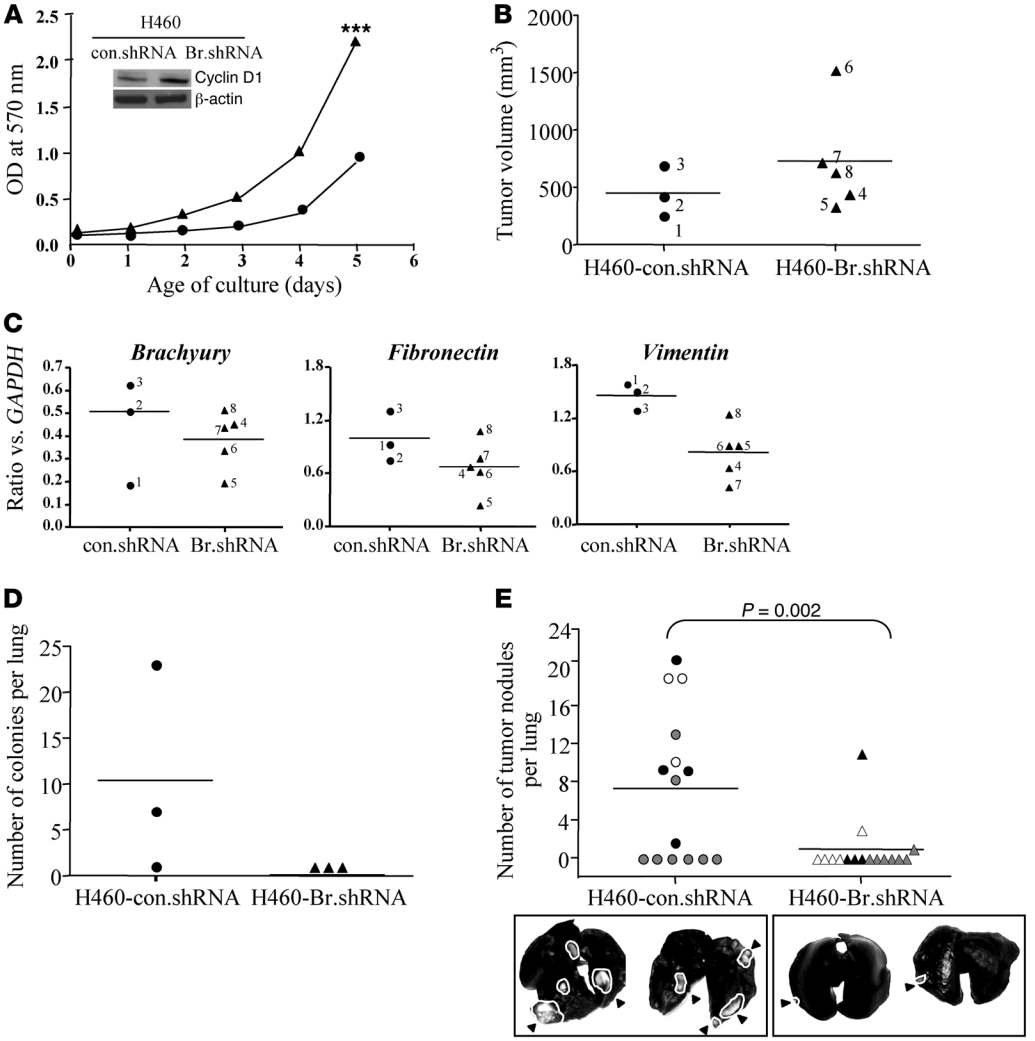

Figure 6

Brachyury inhibition reduces tumor metastasis. (**A**) H460 cells stably transfected with con.shRNA (circles) or Br.shRNA vectors (triangles) were assessed for cell proliferation and survival by MTT assay. Three independent experiments showed comparable results; results from 1 experiment are shown. ***$P = 0.0003$ for con.shRNA versus Br.shRNA. Western blot shows cyclin D1 and β-actin expression. (**B**) Subcutaneous tumors were induced by injection of 4×10^6 of H460 cells in HBSS admixed with 50% (v/v) Matrigel. Graph shows volumes from 8 tumors at day 15 after tumor implantation. (**C**) cDNA from tumors in **B** were analyzed by real-time PCR for indicated markers. (**D**) Lungs from animals bearing subcutaneous tumors were collected at day 15 after tumor implantation, homogenized, and cultured in puromycin-containing medium. Graph shows visible colony counts. (**E**) Mice were inoculated with 7.5×10^5 H460 cells transfected as indicated via tail vein. Forty-five days after tumor implantation, animals were euthanized, and lungs were evaluated for tumor nodules. Graph shows results from 3 independent experiments. Experiments 1–3 are denoted by black, gray, and white circles (con.shRNA) and triangles (Br.shRNA), respectively. Statistical difference between treatment groups was analyzed by pooled results of the above experiments. Two representative lungs from each group are shown for comparison. White outlines and black arrowheads indicate tumor masses.

that the effects of Brachyury on the mesenchymal transition in tumor cells are, at least in part, mediated by Slug.

Brachyury inhibition impairs establishment of metastasis in vivo. To elucidate the impact of Brachyury expression on the metastatic behavior of H460 tumor cells in vivo, we first analyzed the effect of Brachyury on tumor cell growth. In vitro, Brachyury-downregulated H460 cells proliferated at a higher rate than control vector H460 cells (Figure 6A). Increased cell proliferation in H460-Br.shRNA cells positively correlated with increased level of cyclin D1 protein (Figure 6A, inset) and, at the gene level, with downregulation (3- and 6-fold, respectively) of the CDK inhibitor *p21* and *CUL1*, a mediator of ubiquitin-dependent proteolysis of G_1 regulatory proteins (Table 1).

In vivo, H460 cells transfected with Brachyury shRNA grew similarly to control cells after subcutaneous implantation in nude mice (Figure 6B), thus indicating that Brachyury expression does not influence primary tumor growth. RNA expression of *Brachyury*, *Fibronectin*, and *Vimentin* was tested in samples obtained from primary tumors formed by either control or Br.shRNA H460 cells. As shown in Figure 6C, tumors formed by control H460 cells expressed the mesenchymal markers *Fibronectin* and *Vimentin*, and their expression was reduced in the Br.shRNA cells at 15 days after tumor implantation.

Examination of lungs from animals bearing either control or Brachyury shRNA–transfected subcutaneous H460 tumors failed to show macroscopic lung nodules in both groups at 15 days after

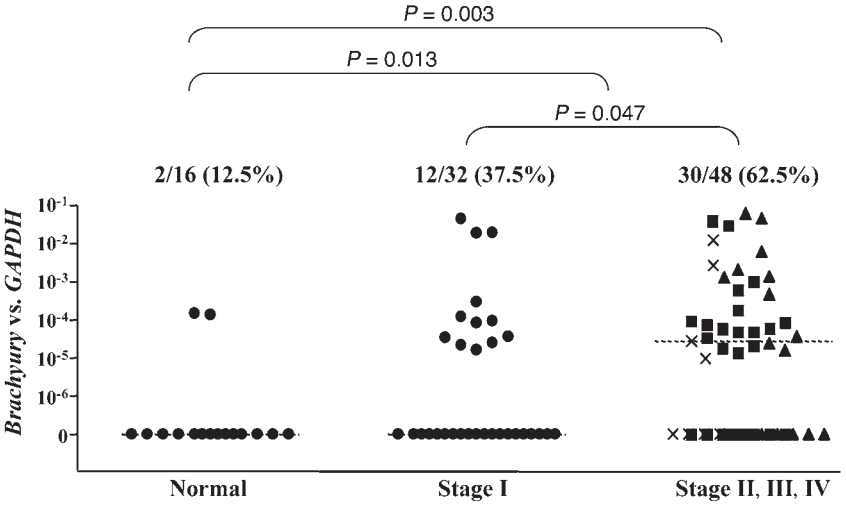

Figure 7
Brachyury expression in human lung tumor tissues. Real-time PCR was performed for *Brachyury* on lung tumor tissue cDNA from 80 lung cancer patients of the indicated stages of disease. The stage II, III, and IV cDNA samples are further represented by squares, triangles, and ×'s, respectively. As controls, 16 samples of normal lung cDNA were also analyzed, each obtained from a pathologically normal section of lung from a cancer patient. All values and the medians for each group are expressed as a ratio to the endogenous control *GAPDH*.

tumor implantation. We then cultured lung tissue homogenates and quantified the number of tumor cell colonies formed. As shown in Figure 6D, tumor cell colonies (1, 7, and 22 colonies) grew from the lungs of 3 of 3 (100%) animals in the H460-shRNA control group, while no tumor cell colonies grew from the lungs of 3 animals subcutaneously implanted with Brachyury-inhibited H460 cells. Therefore, in the H460 tumor model, Brachyury expression regulates the metastatic dissemination of cells from the primary, subcutaneous site to the lungs.

Additionally, experimental lung metastases were established by intravenous injection of H460 cells in nude mice. Forty-five days after tumor injection, the animals were euthanized, and lungs were macroscopically examined for the presence of metastatic lesions. As shown in Figure 6E, 9 of 15 (60.0%) animals developed lung tumor nodules in the H460-shRNA control group, while only 3 of 16 (18.8%) animals injected with Brachyury-inhibited H460 cells showed lung tumor nodules. Additionally, the size of lung nodules macroscopically observed in the animals receiving Brachyury-inhibited H460 cells was substantially smaller than that of nodules observed in animals receiving the control shRNA–transfected H460 cells (Figure 6E). Together, these results demonstrated that inhibition of Brachyury expression impairs the ability of H460 tumor cells to effectively establish lung metastasis.

Expression of Brachyury mRNA in human lung tumor samples. The magnitude of Brachyury expression by various human lung tumor cell lines was particularly striking (Supplemental Figure 2). We thus further analyzed the expression of *Brachyury* by real-time PCR in lung tumor tissue cDNA arrays representing 80 lung cancer patients at various stages of disease, as well as in control normal lung tissue cDNA obtained from histologically normal sections of lung from 16 cancer patients. For analysis, we compared lung tumor tissue samples from stage I, in which cancer cells have not invaded nearby lymph nodes or metastasized, with samples from the more invasive stages II, III, and IV. As shown in Figure 7, there was a significant difference in *Brachyury* mRNA expression between normal and tumor lung tissues ($P = 0.013$). Moreover, *Brachyury* mRNA was expressed in more lung tumor tissue samples from stages II, III, and IV (30 of 48 [62.5%]) than samples from stage I disease (12 of 32 [37.5%]) ($P = 0.047$) or from normal lung tissue (2 of 16 [12.5%]) ($P = 0.003$). The positive correlation between Brachyury expression and lung tumor stage suggests that Brachyury potentially associates with lung tumor progression and makes Brachyury an interesting target for lung cancer, particularly in the more aggressive stages of disease.

Discussion

The studies reported here identify Brachyury as a controller of the EMT process in tumor cells. This conclusion is based on the observation that Brachyury overexpression in human carcinoma cells induces a repertoire of biochemical, morphologic, and functional changes characteristic of EMT. Concomitant upregulation of at least 2 additional EMT transcription factors, Snail and Slug, in Brachyury-overexpressing cells is consistent with the notion that a variety of transcription factors can act in concert to elicit the changes distinctive of EMT (30).

Brachyury is a T-box transcription factor that plays an evolutionarily conserved function in vertebrate development as a gene required for mesoderm formation (31–33); ectopic expression of *Xbra*, the *Xenopus* homolog of *Brachyury*, is sufficient to direct early embryo cells into differentiated mesodermal tissues (34). Here we have shown that as a consequence of Brachyury overexpression, E-cadherin expression is silenced, a repression that is, at least partly, mediated by the transcriptional repressor Slug, while Snail does not appear to play a role toward this effect. Brachyury and other members of the T-box transcription family preferentially bind to the palindromic consensus element AATTTCACACCTAGGTGTGAAATT. A half-site (TCACACCT) of this consensus sequence is located at position –645 of the human E-cadherin promoter. Here we have demonstrated that Brachyury is able to bind to the E-cadherin promoter, although with low efficiency in vitro. These results are in agreement with previous observations demonstrating low binding of T-box proteins to a half consensus site, such as the one present in the E-cadherin promoter (35, 36). The stabilization mediated by addition of a Brachyury-specific antibody, however, would suggest that in vivo binding of Brachyury to the half-site on the E-cadherin promoter could be greatly improved by interactions with accessory proteins or cofactors. We propose a model whereby Brachyury overexpression in tumor cells induces a concurrent enhancement of Slug expression, followed by the effective silencing of *E-cadherin* transcription as a result of Brachyury and Slug association within the E-cadherin promoter region. A question remaining from this study is why only a partial restoration of *E-cadherin* mRNA was observed after simultaneous Brachyury and Slug silencing.

We hypothesize that this result could be due to (a) an incomplete silencing of the transcription factors after siRNA transfection or (b) perhaps other regulatory factors being also implicated in the control of E-cadherin expression in the PANC-1–pBrachyury cells.

The human pancreatic cell line PANC-1 was only used here as a model cell line to investigate the effect of Brachyury overexpression in an epithelial cancer cell. *Brachyury* mRNA expression is endogenously high, however, in various lung carcinoma cell lines. Loss-of-function experiments using the lung carcinoma H460 cell line complemented the gain-of-function experiments in elucidating the role of Brachyury in the acquisition by tumor cells of a mesenchymal phenotype, including motility and invasiveness. Inability to invade the ECM after Brachyury inhibition was concomitant with a reduction in the expression of genes encoding for MMP2 and MMP24, a membrane-bound metalloproteinase known to activate MMP2. Both proteins were previously shown to be overexpressed in tumors and to play a role in cancer progression in several neoplasia (37–39). Reduction in Brachyury expression resulted in morphological, biochemical, and functional changes indicative of a transition from a mesenchymal to an epithelial phenotype in H460 cells. Although no expression of E-cadherin was observed either at the protein or mRNA level after Brachyury silencing, striking changes in plakoglobin in inverse relation to Brachyury levels suggested a modulation of desmosomal junctions in H460 cells, thus promoting cell spreading. Interestingly, the transcription factor Slug, but not Snail, has been shown, in addition to repressing *E-cadherin* transcription, to control desmosomal disruption during the initial and necessary steps of EMT (40, 41). Induction of EMT by FGF-1 treatment or Slug overexpression in the rat bladder carcinoma cell line NBT-II is also characterized by dissociation of desmosomes, with no change in E-cadherin expression (41). In a similar way, our results indicated the acquisition of epithelial traits in H460 cells inhibited for Brachyury expression without reexpression of E-cadherin. It cannot be ruled out, however, that (a) downregulation of Brachyury in the H460-Br.shRNA cells might have been incomplete and insufficient to alleviate E-cadherin suppression, though these cells did gain other epithelial traits; (b) other mechanisms, such as promoter hypermethylation, may be mediating E-cadherin suppression in the H460 cells; and (c) other epithelial cadherins might mediate adherens junctions among H460 cells inhibited for Brachyury expression. As a further demonstration of the role of Slug on the effects mediated by Brachyury in tumor cells, we have shown here that overexpression of Slug in Brachyury-deficient H460 cells was able to reinstate the low levels of *Plakoglobin* typical of H460 control cells, as well as to rescue the migratory and invasive abilities of those cells. There was no effect of Slug expression, however, on the levels of *Fibronectin* mRNA. Interestingly, in agreement with those results, PANC-1–pBrachyury cells that have been treated with siRNA for Slug alone or a combination of Brachyury and Slug siRNA did not show reduced levels of *Fibronectin* mRNA (data not shown), thus reinforcing the idea that Slug is only partially responsible for the effects of Brachyury and that different transcription factors might be responsible for the multiple effects observed along the EMT transition.

The analysis of in vitro cell growth of H460 cells transfected with control versus Brachyury-specific shRNA revealed that Brachyury attenuates the proliferation of H460 cells in vitro. Our results suggest that Brachyury intervenes in the cell cycle progression most likely at the G_1-S transition both by suppressing cyclin D1 expression and by inhibiting the activity of cyclin/CDK complexes. The decrease in *Snail* levels after Brachyury inhibition could be of relevance in terms of the effects observed at the cell cycle level in these cells and is consistent with previous reports on the ability of Snail to trigger EMT while concomitantly downregulating cell cycle progression (42). Although tumor cell proliferation is necessary for tumor formation and growth, a segregation of invasive and proliferative phenotypes has been previously shown for malignant gliomas and astrocytomas (43, 44). Indeed, inhibition of Brachyury expression induced cell proliferation of H460 lung cancer cells in vitro, while significantly suppressing their ability to disseminate in vivo from primary tumor sites or to form lung metastasis after intravenous administration in nude mice.

The pathophysiological relevance of these findings in relation to tumor progression was analyzed here by comparing *Brachyury* mRNA levels in lung tumor samples and normal lung tissue derived from lung cancer patients. Brachyury was present in lung tumor samples from the more invasive stages II, III, and IV, but absent in most tumor tissue samples from stage I, in which cancer cells have not invaded nearby lymph nodes or metastasized, or in normal lung tissues. These results suggested that Brachyury expression is associated with late-stage lung tumor. While further analysis is needed to understand whether Brachyury expression levels correlate with decreased disease-free survival, this observation, together with the functional role described here, make Brachyury a potential target for therapeutic interventions against lung cancer, particularly in the more aggressive stages of the disease.

Brachyury is an attractive target because multiple signaling events may ultimately converge to upregulate its expression. In a previous report, we demonstrated that Brachyury-specific T cells could be expanded from the blood of cancer patients after in vitro stimulation with a 9-mer peptide of the Brachyury protein. We also showed that Brachyury-specific T cells have the ability to lyse tumor cells endogenously expressing Brachyury (25). Therefore, targeting of Brachyury-positive tumor cells via Brachyury-specific T cell immunotherapy could be a more beneficial strategy than the targeting of individual, redundant signaling pathways. In conclusion, the results reported here indicate that Brachyury is able to induce biochemical, morphologic, and functional changes characteristic of EMT in human carcinoma cells. Its overexpression in human lung carcinomas, together with the functional role described here, further support the potential of targeting *Brachyury* as a tumor-associated gene.

Methods

Cell culture. The following human carcinoma cell lines were obtained from ATCC: pancreatic PANC-1; lung H460, H1703, H441, H226, SW900, and H520; colon SW480, SW620, Colo201, Colo205, and T84; prostate LNCaP, DU145, and PC3; and breast MCF7. All cell lines were propagated as recommended by ATCC. The P4E6 and ONYCAP23 prostate carcinoma cell lines were provided by Onyvax Ltd. and were propagated in KSFM (Invitrogen) supplemented with 5% FBS, 2 mM glutamine, 1× antibiotic/antimycotic, and 5 ng/ml EGF (PeproTech Inc.).

Plasmids. Full-length human Brachyury was cloned into the pcDNA4/TO vector (Invitrogen); the resulting construct was designated pBrachyury. Brachyury-specific shRNA constructs (Br.shRNA) were obtained from Sigma-Aldrich. A control plasmid encoding for nontargeting shRNA (con.shRNA) and the pcDNA4/TO empty vector (pcDNA) were also used. Luciferase reporter constructs encoding for the E-cadherin promoter and corresponding random control sequence were purchased from SwitchGear Genomics. A plasmid encoding for full-length human Slug was purchased from OriGene Technologies Inc.

Tumor cell transfections. For stable transfections, 1×10^6 cells (H460 or H1703) were transfected with 1 μg of linearized con.shRNA or Br.shRNA

plasmid, using Nucleofector technology (Lonza). After 48 hours, cells were selected using puromycin (1 μg/ml) (Sigma-Aldrich). One million cells (MCF7 or PANC-1) were transfected following the same procedure with 1 μg of linearized pcDNA or pBrachyury vector. Cells were selected using 25 or 100 μg/ml Zeocin (Invitrogen) for MCF7 or PANC-1 cells, respectively. H460-Br.shRNA cells were transiently transfected with 1 μg pcDNA3.1 or pSLUG plasmid, using Fugene-6 (Roche); cells were used after 72 hours.

Real-time PCR. Total RNA prepared by using the RNeasy kit (QIAGEN) was reverse transcribed with Advantage RT-for-PCR (Clontech). cDNA (1–100 ng) was amplified in triplicate using Gene Expression Master Mix and the following TaqMan gene expression assays (Applied Biosystems): *Brachyury* (Hs00610080), *Snail* (Hs00195591), *Slug* (Hs00161904), *Twist1* (Hs00361186), *E-cadherin* (Hs01013959), *Fibronectin* (Hs00415006), *Plakoglobin* (Hs00158408), *Vimentin* (Hs00958116), and *GAPDH* (4326317E). Mean Ct values for target genes were normalized to mean Ct values for the endogenous control GAPDH [–ΔCt = Ct(GAPDH) – Ct(target gene)]. The ratio of mRNA expression of target gene versus *GAPDH* was defined as $2^{(-\Delta Ct)}$.

Expression of Brachyury mRNA in human lung tumor tissues. Real-time PCR was performed for *Brachyury* and *GAPDH* as described above on commercially available normal and tumor lung tissue cDNA panels (TissueScan Lung Cancer Tissue qPCR Arrays I and III, OriGene Technologies Inc.).

Western blot analysis. Cells were lysed in RIPA Lysis buffer (Santa Cruz Biotechnology Inc.). Proteins (10–25 μg) were resolved on SDS-PAGE, transferred onto nitrocellulose membranes, and probed with antibodies for fibronectin, vimentin, N-cadherin, E-cadherin, plakoglobin (BD Biosciences), cyclin D1, β-actin (Neomarkers), and Brachyury (gift from Aves Labs Inc.) at 4°C overnight. Detection was performed with the Odyssey Infrared imaging system (LI-COR Biotechnology). For membranes incubated with Brachyury antibody, anti-chicken–HRP secondary antibody (Aves Labs) was used, followed by autoradiography.

Cell migration and invasion assays. Blind Well Chambers (Neuroprobe) with 12-μm-pore polycarbonate filters were used. RPMI-1640 supplemented with 10% FBS was added to the lower chambers; cells (1×10^4 to 1×10^5) were added in serum-free medium onto the upper chambers. For invasion assays, filters were precoated with Matrigel (BD Biosciences). Chambers were incubated for 24–48 hours at 37°C. Filters were fixed and stained with Diff-Quik (Dade Behring Inc). Cells on the bottom side of the filters were counted in 5 random ×100 microscope objective fields. Experiments were conducted in triplicate for each cell line.

Cell growth and survival assays. MTT (Sigma-Aldrich) was used as described previously (45). Briefly, 1×10^3 cells were plated in triplicate in 24-well plates; MTT was added at 24-hour intervals. After 3 hours, the medium was removed, converted dye was solubilized in ice-cold isopropanol, and absorbency was measured at 560 nm on a microplate reader (Bio-TEK Instruments).

Gene expression profiling. Pathway-specific Oligo GEArrays (Microarrays for Human ECM and Adhesion Molecules, Human Cell Cycle, and Human Tumor Metastasis-related genes) were purchased from SABiosciences. Manufacturer's recommendations were followed for RNA labeling, membrane hybridization, chemiluminescence detection, and data extraction and analysis. Data for each gene were normalized to *GAPDH*.

RNA interference. ON-TARGETplus SMARTpool siRNA for Brachyury, Snail, Slug, and a nontargeting control siRNA were purchased from Dharmacon. Cells were incubated with siRNA (100 nM) and DharmaFECT reagent in antibiotic-free medium for 48 hours.

Immunohistochemistry. Cells cultured on glass coverslips were fixed with 4% formaldehyde for 15 minutes, permeabilized with 0.2% Triton-X for 10 minutes, and blocked with PBS containing 10% goat serum and 1% FBS for 1 hour. Coverslips were incubated overnight at 4°C with primary antibody dilutions (1:50 ratio) prepared in 1% FBS in PBS. After 3 washes with PBS, coverslips were incubated with Alexa Fluor 488–conjugated anti-mouse antibody (Invitrogen) for 1 hour at room temperature. Cells were washed 5 times with PBS under low light conditions and mounted using VECTASHIELD with DAPI (Vector Laboratories).

E-cadherin promoter assay. PANC-1 and MCF7 cells stably transfected with pcDNA or pBrachyury vectors were seeded in 96-well white tissue culture plates (E&K Scientific) at 3,000 cells per well in 100 μl. Clear 96-well plates were maintained in parallel to assess confluence. After 24 hours, cells were transfected with 50 ng E-cadherin promoter or control luciferase plasmid (SwitchGear Genomics) using Fugene-6 (Roche) in triplicate. Forty-eight hours later, cells were incubated with 100 μl ONE-Glo Luciferase substrate (Promega). Luciferase activity was measured using a 1450 Betaplate reader (Perkin-Elmer).

EMSA. Double-stranded DNA fragment encoding for the E-cadherin promoter (position –677 to +207) was labeled using Biotin 3′ End Labeling Kit (Thermo Scientific). Binding reactions (20 μl) were performed using the LightShift EMSA Kit (Thermo Scientific) with 15 ng of labeled DNA and 50 ng of a purified His-Brachyury protein, in 5 mM $MgCl_2$, 0.05% (v/v) NP40, and 5% (v/v) glycerol. For antibody supershift, 200 ng of Brachyury-specific antibody (Santa Cruz Biotechnology Inc.) or 1 μg of a control, purified IgG (Serotec) were added. Reactions were incubated for 30 minutes at room temperature, supplemented with 4 μl loading buffer, and immediately loaded onto a 1.2% (w/v) agarose gel in 0.5× TBE. After capillary transfer to nylon membranes, DNA was detected by autoradiography.

In vivo tumor metastasis. All mice were housed and maintained in microisolator cages under specific pathogen–free conditions, in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines. All experimental studies were carried out under approval of the NIH Intramural Animal Care and Use Committee. To establish subcutaneous tumors, 4×10^6 to 7.5×10^6 cells were injected in the flank of 5- to 6-week-old female athymic *nu/nu* mice. Fifteen days after tumor implantation, animals were euthanized, and lungs were harvested and washed with PBS and processed to obtain single-cell suspensions. Cells were washed 3 times with PBS, resuspended in 10 ml of culture medium containing puromycin (1 μg/ml), and plated onto T25 flasks. Cultures were maintained in a tissue culture incubator for 15 days without disturbing. To evaluate clonogenic metastatic colonies, cells were fixed with methanol and stained with 0.03% (w/v) methylene blue solution, and the colonies were counted. To establish experimental lung metastasis, we inoculated nude mice with 7.5×10^5 tumor cells/mouse via tail vein (in 100 μl HBSS) on day 0. At day 45 after tumor inoculation, animals were euthanized, and lungs were inflated with India ink. Macroscopic tumor nodules were counted.

Statistics. Data were analyzed using GraphPad Prism (version 4; GraphPad Software). Two-tailed, unpaired *t* test was used. Data points in graphs represent mean ± SEM, and *P* values less than 0.05 were considered significant.

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