Introduction

The epithelial–mesenchymal transition (EMT) is a reversible process that promotes the phenotypic transformation of epithelial cells into mesenchymal cells, a phenomenon characterized by loss of cell polarity and epithelial markers, along with increased expression of mesenchymal markers and gain of migratory and invasive capacities (1–4). Although being crucial for normal gastrulation and organ development, EMT is also thought to contribute to the progression of carcinomas by promoting the dissemination of cancer cells and the establishment of metastasis (1–4). Numerous observations also indicate that EMT is involved in the acquisition of tumor resistance to chemotherapeutic interventions (5, 6), as well as with the acquisition of a stem cell–like phenotype by carcinoma cells (7, 8), a phenotypic change linked to drug resistance (9). Thus, targeting and modulating the molecular machinery that regulates EMT may provide alternative strategies for improving therapeutic interventions against metastasis and drug resistance (10).

During embryogenesis and tumor development, the phenomenon of EMT is controlled by the expression of several transcription factors, including Twist (11), Snail (12), Slug (13), or ZEB1/2 (14, 15), among others. Our laboratory showed that Brachyury (16–18), a molecule crucial for mesoderm differentiation, is overexpressed in a variety of human tumor tissues and cell lines but rarely found in normal adult tissues (19–21). Brachyury overexpression has been shown to drive EMT and to enhance the migratory and invasive features of human cancer cells in vitro (12), while promoting their metastatic dissemination in vivo (22, 23). By using a 9-mer epitope of the Brachyury protein that specifically binds to the HLA-A2 molecule, Brachyury-specific CD8+ T cells were expanded from the blood of patients with cancer that were able to lyse Brachyury-positive cancer cells in an MHC-restricted manner. These results formed the basis for the development of a recombinant yeast-Brachyury vaccine which is currently undergoing phase I clinical testing in patients with advanced carcinomas (24).

TGF-β is a pleiotropic cytokine that regulates cellular proliferation, differentiation, and survival (25–27). While...
functioning as a suppressor of cell growth and proliferation in most normal epithelial, endothelial, and hematopoietic cells, TGF-β stimulates cancer cells to proliferate and to acquire a more aggressive, metastatic phenotype during the later stages of tumor development (28). Multiple studies have also shown that TGF-β can induce EMT in normal and malignant cells, an effect first observed with cultures of normal mammary epithelial cells (MEC) that were induced into a fibroblast-like phenotype by exposure to TGF-β (29). TGF-β signaling relies on its binding to a heterotetrameric receptor composed of two TGF-β type I (TGF-βRI) and two TGF-β type II (TGF-βRII) receptor molecules (30–32). Binding of TGF-β to TGF-βRII induces the recruitment and activation of TGF-βRI which, in turn, leads to the propagation of the signal through the phosphorylation of Smad2/3 proteins resulting in the activation or suppression of target genes (32).

Although numerous studies have shown that Smad-dependent TGF-β induction of EMT in tumor cells results in the upregulation of various EMT transcription factors, including Snail, Slug, and Twist (33), no previous study has shown the regulation of Brachyury by TGF-β in the context of a tumor cell. In the present study, we sought to investigate the possible interaction between TGF-β and Brachyury. We report here for the first time that Brachyury is induced by TGF-β and that Brachyury and TGF-β partake in a positive feedback loop to sustain the mesenchymal characteristics of tumor cells that have undergone EMT. Furthermore, small-molecule–targeted inhibition of TGF-β signaling decreased Brachyury expression while simultaneously reversing the mesenchymal phenotype of various human cancer cells. It is also shown here for the first time that this reversion of EMT mediated by TGF-β inhibition markedly enhances cancer cell susceptibility to chemotherapy. These findings suggest that combinatorial therapies with chemotherapy and TGF-β inhibition may provide alternative strategies to combat cancer in the clinical setting.

Materials and Methods

Cell culture

Cell lines were purchased from American Type Culture Collection between 2005 and 2012: H460, H520, H596, SK-Lu-1, H441, SW480, SW620, DU145, LNCaP, and PC3. Cells were banked and subsequently passaged in our laboratory for periods less than 6 months. The H460 cell line and its derivatives were tested and authenticated by the NCI/DCCB. The ONYCAP23 line was provided by Onyx Ltd. and propagated in RPMI-1640 medium, containing 10% FBS, 2 mmol/L glutamine, 1 NaHCO3, 1× sodium pyruvate, and 1× nonessential amino acids (Invitrogen).

Plasmids and transfections

A Brachyury-overexpressing vector (pBrachyury) and Brachyury-specific short hairpin RNA (shRNA) constructs were used for stable transfections as previously described (22).

Treatment with TGF-β1 or SD-208

Tumor cells were treated with indicated concentrations of human TGF-β1 (BD Biosciences) prepared in 0.1% bovine serum albumin/1× PBS, or the TGF-β receptor type I (ALK5) kinase inhibitor SD-208 (Sigma-Aldrich) dissolved in dimethyl sulfoxide.

Chemotherapy treatment

Cells were seeded in 96-well plates (5 × 104 cells per well in 100 μL media), allowed to attach overnight and treated as indicated. Docetaxel (Sanofi-Aventis), cisplatin (APP Pharmaceuticals), and vinorelbine (Bedford Laboratories) were added to the cultures for 6 hours, followed by media replacement. Cultures were maintained for designated time periods; cell survival was evaluated by the MTT assay. Survival for treated wells was calculated as a percentage of the values representing wells of untreated cells.

Real-time PCR

Total RNA was prepared with the RNeasy Kit (Qiagen), followed by reverse transcription with Advantage RT-for-PCR (Clontech). Real-time PCR was conducted as previously described (22, 23) using 10 to 50 ng cDNA with Gene Expression Master Mix and the following TaqMan gene expression assays (Applied Biosystems): Brachyury (Hs00610080), Snail (Hs00159959), Slug (Hs00161904), E-cadherin (Hs01013959), Fibronectin (Hs00415006), Plakoglobin (Hs01158408), TGF-β1 (Hs00998133), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 4326317E).

Western blot analysis, immunofluorescence, and immunohistochemistry

For Western blot analysis, cells were lysed in radioimmunoprecipitation assay buffer (RIPA) (Santa Cruz Biotechnology, Inc.). Proteins (25–35 μg) were resolved on SDS-PAGE, transferred onto nitrocellulose membranes, and probed with antibodies for fibronectin, E-cadherin, ZO-1 (BD Biosciences), β-actin (Neomarkers), and Brachyury, Snail, Slug (Abcam, Cambridge) at 4°C overnight. Detection was carried out using the Odyssey Infrared imaging system (LI-COR Biosciences). A Phospho-Smad Antibody Sampler Kit was used (Cell Signaling), following the manufacturer’s instructions. Immunofluorescence analysis of tumor cells cultured on glass coverslips was conducted as previously described (22). H460 control.shRNA and Brachyury.shRNA cells (4–7.5 × 105) were injected in the flank of nude mice. Fixed tumor tissues were analyzed by immunohistochemistry with anti-TGF-β1 (clone tgfβ17, Abcam). All mice were housed and maintained in microisolator cages under pathogen-free conditions and in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines; experimental studies were carried out under approval of the NIH Intramural Animal Care and Use Committee.
Cell migration and invasion

Assays in Blind Well Chambers (Neuro Probe Inc.) were conducted as previously described (22). Chambers were incubated for 12 to 24 hours at 37°C. Filters were fixed and stained with Dif-Quik. Cells on the bottom side of the filters were counted in 5 random × 100 microscope objective fields. Experiments were carried out in triplicate for each cell line.

ELISA

Briefly, 1 × 10^6 cells in 1 mL serum-free medium were plated in 6-well plates and incubated for 48 hours at 37°C. Culture supernatants were collected and assayed in triplicate with the human active TGF-β1 or TGF-β2 ELISA Kits (R&D Systems), according to the manufacturer’s instructions.

RNA interference

ON-TARGETplus SMARTpool siRNA for Brachyury and a nontargeting control siRNA were purchased from Dharmacon. DU145 cells were transfected following the manufacturer’s protocol. Six hours after transfection, cells were treated with 1 ng/mL of TGF-β1 or TGF-β2 and a nontargeting control siRNA were purchased from Dharmacon. DU145 cells were transfected following the manufacturers’ protocol. Six hours after transfection, cells were treated with 1 ng/mL of TGF-β1 for 48 hours. Treated cells were either harvested for RNA extraction or evaluated in an invasion assay.

Promoter reporter assay

GoClone Reporter constructs of TGF-β1 (S721675) and GAPDH (721624) promoters and an empty vector (S790005, SwitchGear Genomics) were used. Cells (1 × 10^6) were plated on white 96-well plates, allowed to attach overnight and transfected using FuGENE HD (Promega) at 3:1 ratio to DNA. Luciferase activity was assayed using LightSwitch Luciferase assay reagent (LS010, SwitchGear) according to the manufacturer’s instructions.

Statistical analysis

Data were analyzed using GraphPad Prism (version 4; GraphPad Software). Two-tailed, unpaired t test was used. One-way ANOVA test was used for comparison across multiple groups. Data points in graphs represent mean ± SEM and P < 0.05 is considered significant.

Results

Upregulation of Brachyury in human carcinoma cells undergoing TGF-β1-induced EMT

To investigate whether TGF-β controls Brachyury expression in human carcinomas, tumor cell lines with an epithelial phenotype and low levels of Brachyury were treated with a range of concentrations of human TGF-β1. DU145 prostate carcinoma cells assumed a mesenchymal-like phenotype characterized by a spindle-like morphology (Fig. 1A), decreased expression of epithelial E-cadherin, and increased expression of mesenchymal fibronectin, both at the mRNA (Fig. 1B) and protein levels (Fig. 1C), after treatment with various concentrations of TGF-β1. The expression of Brachyury mRNA (Fig. 1D) and protein (Fig. 1E) showed a bell-shaped response curve with maximum upregulation in response to 1 ng/mL TGF-β1, whereas the effect was lost or diminished with 10 ng/mL TGF-β1. Additional prostate and lung carcinoma cell lines were evaluated; five out of seven lines also showed a significant enhancement of Brachyury expression after treatment with 1 ng/mL (but not 10 ng/mL) TGF-β1. All further experiments were carried out with 1 ng/mL TGF-β1, unless indicated.

Upregulation of Brachyury protein in response to TGF-β1 was predominantly observed in the nuclei of DU145 cells, as expected for a transcription factor, although weak cytosolic staining was also detected (Fig. 2A). Functionally, treatment of DU145 cells with TGF-β1 also resulted in a significant enhancement of the migratory and invasive capacity of these cells in vitro (Fig. 2B). In light of these results, we investigated the contribution of Brachyury to the invasive phenotype of TGF-β1-treated tumor cells. DU145 cells were left untreated or transfected with a pool of Brachyury-specific versus a nontargeting pool of siRNAs and subsequently incubated with TGF-β1 for 48 hours. As shown in Fig. 2C, Brachyury upregulation in response to TGF-β1 was efficiently prevented by transfection with Brachyury siRNA which, in turn, simultaneously inhibited the emergence of an invasive phenotype in response to TGF-β1 (Fig. 2D). Control siRNA–treated cells showed an increase of Brachyury mRNA in response to TGF-β1, as compared with untransfected DU145 cells (Fig. 2C). Although the reason for this enhancement is unknown, we speculate it could be due to generic effects of siRNA transfection including, for example, enhanced secretion of IFN-α and interleukin-8 (34), the latter known to be an inducer of Brachyury in tumor cells (23).

Consistent with the idea that a variety of transcription factors may act in concert to elicit the changes distinctive of EMT (8), we further investigated whether TGF-β1 treatment of carcinoma cells simultaneously upregulates the expression of Brachyury, Snail, and Slug and whether Brachyury overexpression is required for Snail and/or Slug upregulation to take place. Both Snail and Slug were upregulated in response to TGF-β1 treatment (Fig. 2E). Silencing of Brachyury before TGF-β1 treatment reduced Snail expression by 46% and 43% compared with the levels in control siRNA–treated cells, at the mRNA (Fig. 2F) and protein levels (Fig. 2G), respectively, while decreasing Slug to a lesser extent than Snail (19% at the protein level, Fig. 2G).

Brachyury and TGF-β1 participate in a positive feedback loop

Our findings that TGF-β1 induces the expression of Brachyury in human carcinoma cells led us to investigate whether Brachyury, in turn, is able to induce TGF-β1 expression. Stable overexpression of Brachyury in DU145 cells significantly increased the levels of secreted TGF-β1 protein, compared with control vector-transfected cells (Fig. 3A). Likewise, stable silencing of Brachyury caused a consistent and significant decrease in TGF-β1 levels in
culture supernatants from SW620 and H460 cells, compared with control cells (Fig. 3B). A rescue experiment was also carried out with H460 Brachyury.shRNA cells transfected with an empty vector versus a vector encoding Brachyury (pBrachyury). As shown in Fig. 3C, single reconstitution of Brachyury expression was able to restore secretion of TGF-β1, therefore indicating that TGF-β1 and Brachyury participate in a positive feedback loop in tumor cells undergoing EMT.

The correlation between Brachyury and TGF-β1 expression in the H460 cell pair was further analyzed in vivo with xenografts grown subcutaneously in immunodeficient mice. Although TGF-β1 was almost undetectable in H460 Brachyury.shRNA tumors, positive TGF-β1 staining was detected in tumors formed by H460 control.shRNA cells, reinforcing the positive association between Brachyury and TGF-β1 (Fig. 3D). We have previously shown that silencing of Brachyury in H460 cells does not affect primary tumor growth, but instead suppresses the tumor cells’ ability to disseminate from the subcutaneous site to the lungs (22), an observation that could partly be due to decreased TGF-β1 secretion in Brachyury-silenced cells, as shown in this study.

Because of the tight association between Brachyury and TGF-β1 secretion, LNCaP and H520 cells with high-basal levels of Brachyury (Fig. 1F and G) were evaluated for cytokine secretion. Both cell lines secreted TGF-β1 in the ng/mL range (Supplementary Fig. S1A), however, addition of exogenous TGF-β1 to LNCaP cells upregulated fibronectin and decreased E-cadherin (Supplementary Fig. S1B), indicating that TGF-β signaling remains functional in these cells.
As the role of TGF-β2 in cancer progression is now recognized (35), we have also evaluated whether Brachyury expression correlates with TGF-β2 secretion. As shown in Fig. 3E, a direct association was observed between Brachyury and TGF-β2 in H460 cell lines with a range of Brachyury levels. Furthermore, experiments will be carried out to elucidate the potential role of TGF-β2 on the acquisition and maintenance of EMT features in Brachyury-high carcinoma cells.

**Brachyury induces transcriptional activation of the TGF-β1 promoter**

To further characterize the mechanism involved in the upregulation of TGF-β1 in Brachyury-high tumor cells, mRNA analysis was conducted. As shown in Fig. 4A and B, TGF-β1 mRNA expression positively correlated with the level of Brachyury in carcinoma cells, indicating a potential regulatory mechanism mediated by Brachyury at the transcriptional level. In addition, a promoter reporter assay using a vector encoding the luciferase gene under the control of a 910 bp fragment of the TGF-β1 promoter transfected into H460 control-shRNA versus H460 Brachyury-shRNA cells showed that Brachyury silencing results in almost complete abolishment of TGF-β1 promoter activity (Fig. 4C).

**Inhibition of TGF-β signaling decreases Brachyury and reverses EMT**

We hypothesized that the high levels of Brachyury expression observed in some human carcinoma cells may
partially result from the autocrine activity of TGF-β1 produced by the same tumor cells. Thus, it may be possible to reduce Brachyury expression by small-molecule–targeted inhibition of TGF-βRI. To evaluate this hypothesis, DU145 cells were treated with various concentrations of SD-208 (Fig. 5A), a small-molecule inhibitor of the kinase activity of TGF-βRI, before treatment with TGF-β1. Blockade of TGF-β1 signaling attenuated the phenotypic changes characteristic of EMT and blocked the upregulation of Brachyury in response to cytokine treatment (Fig. 5B). As expected, SD-208 completely abolished Smad-2 phosphorylation (Fig. 5C) and prevented the migratory and invasive behavior induced by TGF-β1 in DU145 cells (Fig. 5D). By using tumor cell models with high-basal levels of Brachyury and TGF-β1, we showed that treatment with SD-208 increases expression of the epithelial markers, ZO-1 and Plakoglobin (Fig. 5E and F), while greatly reducing the expression of mesenchymal fibronectin. Functionally, restriction of TGF-β signaling significantly impaired the ability of H460 tumor cells to migrate and to invade the ECM, in a dose-dependent manner (Fig. 5G).

In relation to the effect of TGF-β1 signaling blockade on the expression of Brachyury, treatment of H460 cells with SD-208 decreased Brachyury expression by approximately 50% at all concentrations tested (Fig. 6A). Additional carcinoma cell lines, SW480 and SW620, also showed a significant decrease of Brachyury in response to SD-208 treatment (Supplementary Fig. S2). These results indicated that SD-208 is able to induce phenotypic changes commonly attributed to a mesenchymal-epithelial transition (MET).
response to chemotherapy, an effect that was more pronounced in Brachyury-silenced cells compared with Brachyury-high H460 cells.

Discussion

The findings presented here show for the first time the existence of a positive feedback loop between the EMT regulator Brachyury and TGF-β1 signaling in human cancer cells. This stems from the observation that treatment of various human carcinoma cell lines with TGF-β1 induces Brachyury and, conversely, modulation of Brachyury expression in tumor cells directly affects, in a positive correlation, the secretion of TGF-β1. We also showed that treatment with an inhibitor of TGF-βRI is sufficient to downregulate Brachyury in various human cancer cells, and to simultaneously revert their mesenchymal features, including resistance to chemotherapy treatment.

Numerous studies have shown a dual role for TGF-β1 in cancer development (27, 32). Although suppressing tumor growth at early phases, TGF-β can stimulate tumor invasion, metastasis, and angiogenesis, and suppress antitumor immunoresponses at later tumor stages (28, 36–38). As a result, targeted inhibition of TGF-β signaling has been the focus of intense investigation for therapeutic interventions in cancer (39, 40). Regarding its role on tumor progression, TGF-β is able to regulate the transcriptional EMT program directly and through interactions with a variety of signaling networks, including Notch, Wnt, and others (37, 41). Once EMT is induced, it has also been shown that secretion of TGF-β and the expression of molecules involved in TGF-β signaling pathways are induced (42, 43). In agreement with those studies, we have shown here that treatment of human cancer cells with TGF-β1 induces the expression of Brachyury, a T-box protein that regulates EMT in human carcinoma cells (22, 23). Although the mechanism by which TGF-β1 upregulates Brachyury mRNA and protein expression was not studied here, the relevance of Brachyury expression during TGF-β1-induced EMT was shown by silencing Brachyury before cytokine treatment, a manipulation that abolished the acquisition of tumor invasiveness and prevented upregulation of Snail in response to TGF-β1. These results suggested that Brachyury is not just a bystander gene upregulated during EMT but rather a gene of relevance to the phenotypic changes associated with epithelial cell plasticity. We have shown in previous reports (22, 23) that overexpression of Brachyury drives upregulation of Snail in a variety of carcinoma cell lines. The results shown here indicate that Brachyury may also participate in the regulation of Snail expression in response to TGF-β1, and that both proteins are required for the acquisition of tumor invasiveness. As no DNA-binding site for Brachyury was observed within the promoter region of Snail, the positive influence of Brachyury on Snail expression seems to be indirect.

Expression of Brachyury in response to various doses of TGF-β1 followed a bell-shaped pattern of expression, with...
Brachyury being upregulated at low (but not high) concentrations of TGF-β1. These results are in agreement with previous reports showing that low concentrations of activin, a member of the TGF-β superfamily, activate Brachyury expression during Xenopus development, an effect that is lost with high concentrations (44, 45). This biphasic phenomenon has been attributed to the ability of high concentrations of activin to activate expression of Goosecoid that, in turn, represses the Brachyury promoter. We hypothesize that a similar regulatory phenomenon could mediate the differential regulation of Brachyury in carcinoma cells in response to treatment with low versus high doses of TGF-β1, where high doses of the cytokine may induce additional transcriptional regulators that could, in turn, repress Brachyury.

In multiple tumor cell models, it has been shown that TGF-β has the ability to function in an autocrine fashion to maintain the EMT phenotype (46, 47). We have shown here for the first time the existence of a feed-forward loop between Brachyury and TGF-β1, as high levels of Brachyury associated with high expression of TGF-β1 mRNA and protein, whereas silencing of Brachyury in lung carcinoma cells almost completely abolished the activity of a reporter of TGF-β1 promoter. The analysis of the TGF-β1 promoter sequence failed to identify the palindromic consensus element AATTTCACACCTAGTGTA or its half-site (TCACACCT) known to be required for Brachyury-binding to DNA. We hypothesized that Brachyury may indirectly promote TGF-β1 transcription either by inducing and/or repressing other transcriptional regulators that could directly control the activity of the TGF-β1 promoter, or by modifying their ability to efficiently bind and regulate transcription of TGF-β1.
SD-208 decreases Brachyury expression and enhances chemotherapy sensitivity of human carcinoma cells. A, H460 cells were treated with increasing concentrations of SD-208 for 72 hours and evaluated for Brachyury and β-actin expression by Western blot analysis. Bottom graph shows Brachyury levels normalized to β-actin. B, chemical structure of chemotherapeutics used. C, H460 cells were left untreated (0) or pretreated for 48 hours with indicated concentrations of SD-208 and subsequently exposed to chemotherapeutics (165/0.12 ng/mL cisplatin/vinorelbine, 165 ng/mL cisplatin, 0.12 ng/mL vinorelbine, and 0.07 ng/mL docetaxel). Survival was evaluated after 4 days by the MTT assay. D, expression of Brachyury and GAPDH in indicated tumor cells incubated for 48 hours with increasing doses of SD-208. E, survival of H460 control.shRNA and Brachyury.shRNA cells pretreated with indicated doses of SD-208 and subsequently treated as above with cisplatin/vinorelbine. Cis, cisplatin; vin, vinorelbine (ng/mL).
A variety of TGF-β signaling inhibitors are currently undergoing preclinical development (40). A dual TGF-βRII and TGF-βRII small-molecule inhibitor (LY2109761), for example, was previously shown to induce MET in a subpopulation of breast cancer cells with mesenchymal and stem cell–like properties (48). In the present study, we have shown that mesenchymal–like tumor cells naturally high for Brachyury can be converted into an epithelial phenotype by single blockade of TGF-β signaling with SD-208. The phenomenon of EMT has been previously associated with the acquisition of resistance to chemotherapy and radiation (8). Tumor cells undergoing EMT via overexpression of Brachyury have been shown to be more resistant to the cytotoxic effects of various chemotherapies and radiation (49). In the present study, we showed that reversion of EMT via treatment with SD-208 is able not only to reduce the levels of Brachyury but also to enhance in vitro tumor susceptibility to cisplatin, vinorelbine, and docetaxel. By using tumor cells silenced for the expression of Brachyury, we have also shown that Brachyury plays a direct role in chemotherapy resistance of H460 cells and that inhibition of TGF-β1 signaling further alleviates tumor resistance by mechanisms other than the downregulation of Brachyury.

The results presented here suggest that combinatorial approaches of conventional therapeutics and TGF-β signaling inhibition may provide a more effective strategy in the treatment of metastatic disease. In addition, our laboratory has developed a Brachyury–based cancer vaccine for the treatment of metastatic carcinomas. Ongoing studies are also exploring the potential benefit of using a TGF-βR inhibitor combined with anti-Brachyury–based immunotherapies for the treatment of metastatic carcinomas.

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

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Conception and design: D.H. Hamilton, C. Palena
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 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Larocca, J.R. Cohen, R.I. Fernando
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 Writing, review, and/or revision of the manuscript: C. Larocca, J.R. Cohen, R.I. Fernando, B. Huang, D.H. Hamilton, C. Palena
 Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Larocca
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Acknowledgments
The authors thank Dr. Jeffrey Scholm for his intellectual contributions, critical discussions, and support. The authors also thank Margie Dubenstein for technical assistance and Debra Weingarten for editorial assistance.

Grant Support
This work is supported by the funds from 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 October 16, 2012; revised April 4, 2013; accepted June 14, 2013; published OnlineFirst June 19, 2013.

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# Molecular Cancer Therapeutics

## An Autocrine Loop between TGF-β1 and the Transcription Factor Brachyury Controls the Transition of Human Carcinoma Cells into a Mesenchymal Phenotype

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