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A Twist-Snail Axis Critical for TrkB-Induced Epithelial-Mesenchymal Transition-Like Transformation, Anoikis Resistance, and Metastasis

Marjon A. Smit, Thomas R. Geiger, Ji-Ying Song, Inna Gitelman, and Daniel S. Peeper

In a genomewide anoikis suppression screen for metastasis genes, we previously identified the neurotrophic receptor tyrosine kinase TrkB. In mouse xenografts, activated TrkB caused highly invasive and metastatic tumors. Here, we describe that TrkB also induces a strong morphological transformation, resembling epithelial-mesenchymal transition (EMT). This required TrkB kinase activity, a functional mitogen-activated protein kinase pathway, suppression of E-cadherin, and induction of Twist, a transcription factor contributing to EMT and metastasis. RNA interference (RNAi)-mediated Twist depletion blocked TrkB-induced EMT-like transformation, anoikis suppression, and growth of tumor xenografts. By searching for essential effectors of TrkB-Twist signaling, we found that Twist induces Snail, another EMT regulator associated with poor cancer prognosis. Snail depletion impaired the formation of lung metastases. Epistasis experiments suggested that Twist acts upstream from Snail. Our results demonstrate that TrkB signaling activates a Twist-Snail axis that is critically involved in EMT-like transformation, tumorigenesis, and metastasis. Moreover, our data shed more light on the epistatic relationship between Twist and Snail, two key transcriptional regulators of EMT and metastasis.

The successful treatment of cancer patients is inversely correlated with the occurrence of secondary tumors, or metastases. A better understanding of the molecular mechanisms underlying metastasis will conceivably help to improve cancer treatment in the future. Metastasis is a multistep process in which tumor cells have to overcome several barriers to form a secondary tumor at a distant anatomical site (21, 34). One such barrier is imposed by the epithelium (the origin of most solid tumors), a highly organized structure with strong cell-cell adhesions and lined by a basement membrane composed of a dense extracellular matrix. To disseminate from the primary tumor and to invade neighboring tissue or vessels, epithelial tumor cells must acquire a more flexible and migratory phenotype, like that of mesenchymal cells (16, 73). This can be achieved by an epithelial-mesenchymal transition (EMT), a process that was initially described for embryogenesis (74, 79). EMT is characterized by the loss of polarized organization and a downregulation of epithelial proteins, including E-cadherin, γ-catenin/plakoglobin, α-catenin, and β-catenin (33). At the same time, mesenchymal proteins are often induced, including smooth muscle actin (47), fibronectin, N-cadherin, or vimentin (6, 41). This is mediated (either directly or indirectly) by transcription factors like Twist, E12/E47, and members of the Snail and ZEB protein families (38). In vitro, EMT can be induced by activated oncogenes, like RASV12, or by several receptor tyrosine kinases, such as MET or the epidermal growth factor receptor, often in cooperation with transforming growth factor β (TGF-β) (33). However, the extent of up- and downregulation of the epithelial markers and, in particular, of the mesenchymal markers varies among different cell lines and stimuli.

For this reason and also because intermediate forms of EMT with only a partial phenotype have been described, a precise definition of EMT is still under debate (33). In vivo, in the course of tumor cell invasion and metastasis, EMT is thought to occur mainly in a transient and reversible way, under the influence of the tumor stroma (46, 79).

Once tumor cells have left their original site and encounter new microenvironments during invasion, they are challenged by another barrier against metastasis: anoikis (apoptosis induced by inappropriate, or the lack of, cell adhesion) (28, 49). Apart from its role in tissue homeostasis (11, 36), anoikis conceivably also restricts the spread of tumor cells through tissues and via circulation (44). In an attempt to identify new mediators of metastasis, we have previously used anoikis suppression as the basis for a genomewide functional screen. In this way, we identified the neurotrophic tyrosine kinase receptor 2 (Ntrk2/TrkB) as a potent anoikis suppressor (20). TrkB and its ligand brain-derived neurotrophic factor (BDNF) play a crucial role in the development and function of the nervous system, including the promotion of neuronal survival (25, 42, 43). Consistent with the premise that anoikis forms a barrier to metastasis, TrkB-expressing epithelial cells form metastatic tumors in vivo (20). We recently showed that the ability of TrkB to suppress anoikis and induce metastasis requires its kinase function to be intact (29). Supporting the notion that TrkB may also play an important role in human...
cancer, it is found overexpressed in several human malignancies, including neuroblastoma (12, 53), prostate cancer (19), and pancreatic cancer (50; reviewed in reference 30). In line with this, TrkB-interfering agents are currently being developed and tested for anticancer activity (18, 68). However, the molecular mechanisms as to how TrkB signaling induces metastasis remain largely unknown.

Because improved understanding of the fundamental mechanistic aspects of TrkB signaling in metastasis is likely to be of preclinical relevance, we aimed here to reveal factors that are critically required for TrkB-induced anoikis suppression and metastasis, with a focus on EMT.

**MATERIALS AND METHODS**

**Vector constructs.** Mouse pBabe-Hygro-BDNF (pBH-BDNF) was described before (20). Mouse TrkB (GenBank accession number X17647) in the pBabe-Puro (pBP) vector was subcloned into pMSCV-blunt using the EcoRI restriction site. Human BDNF, human wild-type TrkB, and the human kinase inactive point mutant TrkB(K588M) were described before (29). Mouse E-cadherin (N06115) in the pBP-ires-Green fluorescent protein (GFP) vector was a gift from C. Niewiesk (32), 3′-hemagglutinin (HA)-tagged mouse Snail (NM011427) in the pRv-ires-GFP vector was a gift from A. Munoz (62). Mouse Twist1 (M63609) was a gift from R. Weinberg (78) and was subcloned into the pLRZS-Ms-ires-EGFP (pLISE) vector and pBP vector, adding a 5′ HA tag and a Kozak sequence. Forward primer used was 5′-CGAAGTCGACCGCGAGACTTATTGAGCTGC-3′; and reverse primer used was 5′-GAAGATCTGATTGGAAGCCGACAGCTG-3′. Short hairpin RNAs (shRNAs) were expressed from pReTroSuper (13), with the following targeting sequences: EGFP, 5′-GCTGACCACTGAGTTTCTATTCA-3′ (sh-EGFP); rat Twist1 #1, 5′-AGAAGGACTTACAACCAAGA-3′ (sh-Twist #1); rat and human Twist1 #2, 5′-GGATCAAATGCTGCGAGACTGAA-3′ (sh-Twist #2); human Twist1 #3, 5′-GAACACCTTGGACTCAAA-3′ (sh-Twist #3); rat Snail #1, 5′-GAATGTCCCTGTCACCATA-3′ (sh-Snail #1); and rat Snail #2, 5′-ACACGCTGCTCGACAGCA-3′ (sh-Snail #2).

**Cell culture and retroviral transduction.** Rat intestinal epithelial 1 (RIE-1) cells (a kind gift from R. D. Beauchamp and K. D. Brown [9]) and E1A-immortalized rat kidney RK3E cells (ATCC) were cultured in Dulbecco’s modifed Eagle’s medium (Gibco) supplemented with 9% fetal calf serum (FCS; PAA Laboratories GmbH) and penicillin-streptomycin (Gibco). Human breast epithelial MCF10A cells expressing the ectopic receptor were cultured in Dulbecco’s modified Eagle’s medium-F12 (1:1 dilution) (Gibco) supplemented with 15 mM HEPES buffer, 5% equine serum, 1% penicillin-streptomycin, 0.5 µg/ml amphotericin B, 10 µg/ml insulin, 20 ng/ml epidermal growth factor, 100 µg/ml cholera toxin, and 0.5 µg/ml hydrocortisone.

Ecotropic retrovirus was produced in Phoenix packaging cells (http://www.stanford.edu/group/nolan/retroviral_systems/phx.html). We transduced RIE-1, RK3E, and MCF10A cells first with pBH-BDNF (selection with 75 µg/ml hygromycin) and subsequently infected with pMSCV-blast-TrkB. To generate stable cell lines, cells were seeded at low density in medium containing 1.0 µg/ml puromycin to obtain independent cell clones. For functional rescue experiments, RK3E(TM) cells expressing shRNAs against Twist or Snail were infected with pRv-ires-GFP (Snail-HA or pLISE-HA-Twist and subjected to fluorescence-activated cell sorting. For cells expressing dominant-negative RAC(N17) (66), RK3E cells were transduced first with pBH-BDNF (selection with 75 µg/ml blasticidin), then with LZR5-mycRAC(N17)-ires-ZEO (selection with 100 µg/ml zeocin), and subsequently with MSCV-TrkB (selection with 2.5 µg/ml blastidicin).

**Pharmacological inhibition of TrkB, mitogen-activated protein kinase (MAPK), PI3 kinase (PI3K), and RAC1 pathways.** Cells expressing the empty vector control or TrkB and BDNF were treated with 20 µM U0126 (Cell Signaling Technology), 1 µM CI-1040 (Axon Medchem), 500 nM PI-103 (Echelon), or 75 µM NSC23766 (Calbiochem) for the indicated time points. Cells were harvested in the presence of phosphate inhibitors (1 mM sodium pyrophosphate, 2 mM sodium fluoride, 10 mM beta-glycophosphate, and 2 mM sodium orthovanadate). For BDNF stimulation, cells were starved for 4 h, pretreated with the inhibitor for 30 min, and stimulated with 50 ng/ml recombinant human BDNF (Peprotech) for 5 min. Cells were harvested in the presence of phosphate inhibitors.

RK3E cells expressing the vector control, TrkB only, TrkB and BDNF, or RAS(V12) were treated with 300 nM K252a (Calbiochem) or 20 µM GW44176 (Tocris Bioscience) overnight. Cells were trypsinized and resuspended in trypan blue–phosphate-buffered saline (PBS), with a 1:1 dilution. Percentages of dead cells were determined using trypan blue exclusion. For Western blot analysis, cells were harvested in the presence of phosphate inhibitors.

For transient TrkB activation, RK3E cells expressing TrkB were treated with 50 ng/ml recombinant human BDNF (PeproTech) for 2 days (medium was changed daily) and cultured for another 3 days without BDNF. Alternatively, transient expressing RK3E cells were cultured in 1% FCS, treated with 50 ng/ml recombinant BDNF for 2 days (medium was changed daily), and harvested 1 day after the removal of BDNF.

**Anoikis, soft agar, migration, and invasion assays.** To induce anoikis, we seeded 4 × 10^4 freshly trypsinized cells into ultra-low cluster (ULC) six-well cell culture dishes (Costar). Plates were scanned with an Epson Perfection 4990 photo scanner 4 days later. For quantification of anoikis suppression, total protein amounts in each well were measured by the Pierce BCA assay.

For soft agar assays, 1,000 trypsinized cells were seeded in 0.4% low-melting-point agarose (Sigma) on top of a 1% agarose layer, and scans were taken 11 days later. The numbers of macroscopic colonies were determined using ImageJ software (http://rsh.info.nih.gov/ji/index.html).

For migration assays, 20,000 freshly trypsinized cells were seeded on control inserts (for migration) or Matrigel (for invasion) in medium without FCS. The lower compartment contained 9% FCS. After 24 h, noninvaded cells were removed, and invaded cell were stained with crystal violet. Quantification was performed by counting invaded cells on five independent pictures of the well. All pictures of adherent cells were taken at a magnification of ×50.

**RAC1 activity assay.** To determine RAC1 activity levels, cells were harvested in 1% (0.5% Nonidet P-40, 50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM MgCl2, 10% glycerol and protease inhibitors) and shear washed through a G25 syringe. Total protein amounts were determined using a Bio-Rad protein assay. Samples (900 µg protein) were incubated for 30 min with biotin-Pak-CRIB peptide and streptavidin magnetic beads (Invitrogen). The Pak-CRIB peptide binds RAC in its active conformation only (65). Samples were washed twice with lysis buffer and analyzed for RAC1 by Western blotting.

**Immunoblotting and antibodies.** Cell pellets were lysed in RIPA buffer, and protein concentration was determined using the Bio-Rad protein assay. Immuno blot analysis was performed using standard techniques, either on 7% sodium dodecyl sulfate-polyacrylamide gels or on 4 to 12% bis-Tris precast gels (NuPAGE) for Twist and Snail. The antibodies used were pan-Trk (C14; Santa Cruz); BDNF (N20; Santa Cruz); Twist hybridoma supernatant (31); Snail (H130 [Santa Cruz] for the detection of overexpressed mouse Snail, hybridoma supernatant 6H2 from K. Becker [67] for endogenous human Snail); E-cadherin, Phospho-ERK1/2, Phospho-MAPK, PI3 kinase (PI3K), and RAC1 pathways.

All antibodies were diluted 1:2,000 in 4% Protifar Plus (Nutricia), except for the rabbit polyclonal a-tubulin (DM1A; Sigma); BDNF (N20; Santa Cruz)–hybridoma supernatant (31); Snail (H130 [Santa Cruz] for the detection of overexpressed mouse Snail, hybridoma supernatant 6H2 from K. Becker [67] for endogenous human Snail); E-cadherin, Phospho-ERK1/2, Phospho-MAPK, PI3 kinase (PI3K), and RAC1 pathways.

For Western blot analysis, the signal was detected for Western blotting was done with ECL reagent (Amersham), and autoradiographs were scanned with a Bio-Rad VersaDoc 4000 MP digital imaging system.
Immunofluorescence. Cells were grown on glass coverslips, fixed in 4% formaldehyde in PBS (in 70% ethanol for cells expressing pBP-IRE-9F-GFP and pBP-IRE-GFP-E-cadherin to inactivate the GFP signal), permeabilized with 0.2% Triton X-100, and blocked in 5% normal goat serum in PBS-0.2% Tween for 30 min. Coverslips were incubated with E-cadherin antibody (1:200 dilution) in blocking solution for 1 h at room temperature. After washing the cells with PBS-0.2% Tween, we incubated the cells for 1 h with Alexa Fluor 488 goat anti-mouse secondary antibody (diluted 1:1,000; Molecular Probes) and for 15 min with TO-PRO (diluted 1:500; Molecular Probes). Coverslips were mounted with Aqua-Poly/Mount (Polysciences, Inc) and analyzed by confocal microscopy on a Leica TCS NT confocal system (Leica Microsystems, Heidelberg, Germany), equipped with an Ar/Kr laser. Images were taken using a 100×, 1.32-numerical-aperture objective lens, with standard filter combination(s) and Kalman averaging.

qRT-PCR. Total RNA was isolated using Trizol (Invitrogen) and treated with DNease for 1 h at 37°C (Promega). Quantitative reverse transcriptase PCR (qRT-PCR) was performed using the reverse transcriptase kit from Invitrogen. Primers were designed using Primer Express software. The primers used were as follows: Twist-forward, 5'-CGCTGAAAGCAGATTTGCTC-3'; Twist-reverse, 5'-CCAGTTGAGGTTGCTGAAT-3'; Snail-forward, 5'-CCACACTGTTGAAAGCTTTT-3'; Snail-reverse, 5'-GTCGGAGGTTGGCCACGTA-3'; TBP-forward, 5'-GATGTGAAGTTCCCCATAAGGC-3'; TBP-reverse, 5'-TCTGGCCATGACTGCTACTGAACGTCG-3'; N-cadherin-forward, 5'-AGGGCCCTTTAAGTCGTAACA-3'; N-cadherin-reverse, 5'-CTATAGTCCGAGATACAAAAAGGACTATAT-3'; E-cadherin-forward, 5'-TGAGCTAGCAGCCCTAGATC-3'; E-cadherin-reverse, 5'-CGCTGAACGAGGCATTTGC-3'; Gata-3-forward, 5'-TGCGTCGCTTCAGGTTTTCATCGA-3'; Gata-3-reverse, 5'-GTGAAGTTCCCCATAAGGC-3'; Gata-2-forward, 5'-GTGAAGTTCCCCATAAGGC-3'; Gata-2-reverse, 5'-GTGAAGTTCCCCATAAGGC-3'; Gata-1-forward, 5'-GTGAAGTTCCCCATAAGGC-3'; Gata-1-reverse, 5'-GTGAAGTTCCCCATAAGGC-3'; P-cadherin-forward, 5'-GTCTTCTTGGACTGACTTGCTG-3'; P-cadherin-reverse, 5'-CATCTTGTCGGTGCAACGACG-3'; BMP-4-forward, 5'-CTCAAGGAGGTGGAAATGGG-3'; BMP-4-reverse, 5'-CATCTCGGGCAGGAATGAC-3'. Detection was done with SYBR green master mix (Applied Biosystems) on an ABI Prism 7000 thermal cycler (Applied Biosystems). RNA levels were normalized against rat TATA box binding protein (TBP).

In vivo assays. Eight- to 14-week-old female BALB/c nude mice were subcutaneously injected with 1 × 10^6 cells into both flanks. Mice were inspected twice a week and euthanized by CO2 when tumors reached a volume of 1 cm^3 or when clinical symptoms became apparent. Tumor size was measured with a caliper, and tumor volume was calculated by the formula (1/2) × width × length^2, with width being the longest dimension and length the respective perpendicular diameter of the tumor. Metastatic lesions in the lungs were counted by visual inspection of hematoxylin and eosin-stained histological tissue sections. Sections were analyzed with an Axiovert S100 microscope system, with a color charge-coupled-device camera and AxioVision software (Zeiss). The sizes of metastases and the area of lung tissue per section were determined with ImageJ software. The total lung area analyzed was 19.5 cm^2 for sh-EGFP #1, 17.3 cm^2 for sh-EGFP #2, 11.6 cm^2 for sh-Snail #1, and 19.1 cm^2 for sh-Snail #2. For experimental metastasis, 1 × 10^5 cells were injected into the tail veins of 8- to 14-week-old female BALB/c nude mice. Mice were inspected daily and euthanized by CO2 when clinical symptoms became apparent. Kaplan-Meier survival curves were generated using SPSS 14.0.

RESULTS

TrkB induces EMT-like transformation in epithelial cells. Consistent with our previous observations (20, 29), overexpression of TrkB and BDNF in several nonmalignant epithelial cell lines induced a striking morphological transformation, characterized by a spindle-shaped morphology and the loss of cell-cell contacts (Fig. 1A). This effect was observed in the following three different nonmalignant epithelial cell lines, originating from different tissues and species: RIE-1 cells, E1A-transformed rat kidney epithelial cells (RK3E cells), and to a somewhat lesser extent, human breast epithelial cells (MCF10A cells). To determine whether this morphological transformation represents EMT, we analyzed the levels of several epithelial and mesenchymal proteins (see the introduction). We observed a downregulation by activated TrkB of E-cadherin, α-catenin, β-catenin, γ-catenin, and P-cadherin in both rat epithelial cell lines, whereas in MCF10A cells, α-catenin and P-cadherin were slightly suppressed (Fig. 1B). In the latter cell type but not in the rodent cells, induction of fibronectin and vimentin was prominent (Fig. 1B). EMT markers are often differentially regulated across cell lines (33). To investigate a larger number of EMT-associated genes, we compared the gene expression profile of TrkB- and BDNF-expressing RK3E cells (referred to as RK3ETB cells) (C. Desmet and D. Peeper, submitted for publication) to the reported profile of RASV12-expressing and TGF-β1-treated EpH4 mouse mammary epithelial cells (EpH4RasTGF-β) (41), a classical system for studying EMT (33, 56). This analysis showed that 67% of the genes significantly deregulated in both RK3ETB cells, and EpH4RasTGF-β cells changed the expression level in the same direction.

The EMT-associated “cadherin switch” from E-cadherin to N-cadherin (16) was present in both rodent cell lines upon expression of TrkB and BDNF (Fig. 1B and C). Several transcription factors, including Twist and members of the Snail protein family, are known to repress E-cadherin, via E boxes in the E-cadherin promoter (38, 54). When we assessed the mRNA levels of Twist (Twist1), Snail (Snail1), and Slug (Snail2), we observed a marked induction of Twist and a small but reproducible induction of Snail in TrkB- and BDNF-expressing RIE-1 (RIE-1TB) and RK3ETB cells (Fig. 1D). Slug was not induced in this setting. Furthermore, two genes, BMP-4 and CTGF, which are downregulated in the EMT profile of EpH4RasTGF-β cells (41), were also downregulated at the mRNA level by activated TrkB (Fig. 1E). Together, our analyses demonstrate that in epithelial cells, TrkB induces a strong morphological transformation resembling EMT. As EMT is a complex and dynamic process involving a plethora of factors, we will refer to this process here conservatively as EMT-like transformation.

Continuous TrkB signaling is required for EMT-like transformation and survival of TrkB-expressing RK3E cells. Previously, we have shown that TrkB kinase activity is required for anoikis suppression and tumor formation in rat epithelial cells (29). Therefore, we speculated that kinase activity is also required for TrkB-induced EMT-like transformation. Indeed, a kinase-inactive point mutant, TrkBK588M (23, 35, 29) was unable to morphologically transform RK3E cells and to downregulate E-cadherin (Fig. 2A and B). In line with this observation, stimulation of TrkB-expressing cells with recombinant BDNF for 2 days induced a spindle-shaped morphology and the loss of E-cadherin, which was reverted upon withdrawal of BDNF for another 3 days (Fig. 2C and D). We then repeated this experiment in a more clinically relevant setting, with pharmacological inhibition of TrkB using the K252a alkaloid (72). RK3ETB cells treated with K252a induced a strong apoptotic response, as revealed by trypan blue exclusion (Fig. 2E) and accumulation of cleaved caspase 3 (Fig. 2F). This was not due to the unspecific toxicity of the inhibitor, because RASV12-transformed RK3E cells did not die upon treatment with K252a (Fig. 2E and F). Furthermore, we obtained similar results using another Trk inhibitor, GW 441756 (77) (see Fig. S1 in the supplemental material). K252a and GW 441756 each blocked BDNF-induced autophosphorylation of TrkB and subsequent phosphorylation of ERK but not RASV12-induced ERK phosphorylation (Fig. 2F; see also Fig. S1 in the supplemental material). Notably, parental RK3E cells were largely

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Insensitive to the Trk inhibitors and so were cells in which TrkB was not activated by BDNF stimulation. Lastly, when we treated serum-starved, TrkB-expressing RK3E cells with BDNF for 2 days and subsequently removed it from the medium, we observed massive cell death (Fig. 2G). These results demonstrate that continuous TrkB signaling is required for EMT-like transformation. Furthermore, they show that RK3E cells become addicted to activated TrkB and that they do so within a short period of time.

Loss of E-cadherin is an essential feature of TrkB function. E-cadherin is regarded as a major player in EMT (5). Therefore, we next investigated whether E-cadherin corresponds to a critical target for TrkB function by reintroducing E-cadherin into spindle-shaped RK3ETB cells. E-cadherin was expressed to relatively high levels in two independent cell clones (Fig. 3A) and correctly localized to the cell membrane (Fig. 3B). Importantly, restoration of E-cadherin levels reverted the cell morphology to an epithelial phenotype (Fig. 3C). Like our previous observations in RIE-1 and MCF10A cells (20, 29), active TrkB also suppressed anoikis in RK3E cells (Fig. 3D) as well as anchorage-independent growth in soft agar (Fig. 3E). These findings demonstrate that the loss of E-cadherin is an essential feature of the mechanism by which TrkB activates an EMT-like program and suppresses anoikis.

Twist is required for TrkB-induced EMT-like transformation, anoikis suppression, and tumorigenesis. In view of the important role of E-cadherin in TrkB-induced EMT and anoikis resistance, we next addressed how TrkB downregulates E-cadherin. As Twist can induce EMT and plays a critical role in metastasis (78), we first focused on this basic helix-loop-helix transcription factor, investigating whether it corresponds to a critical TrkB target in E-cadherin repression. In support of a role for Twist in this setting, activated TrkB induced the expression of Twist both at the mRNA (Fig. 1D) and protein levels (Fig. 4A; see also Fig. S2A in the supplemental material). As expected, kinase-inactive TrkB failed to induce Twist (see Fig. S2B in the supplemental material). To assess the requirement of Twist for TrkB function in this regard, we generated stable cell lines expressing TrkB, BDNF, and shRNAs against Twist (or against EGFP, as a control). To rule out off-target effects (22), we used two independent, nonover-
lapping shRNAs against Twist. Upon expression of sh-Twist in polyclonal RK3E TB cell pools, EMT was partially reverted (data not shown). To enhance this effect, we first knocked down Twist in RK3E cells expressing BDNF, subsequently transduced the cells with TrkB-encoding retrovirus, and then established independent stable clonal cell lines. Indeed, this led to a robust block to TrkB-induced EMT-like transformation (Fig. 4B). As anticipated, this correlated well with restoration and correct subcellular localization of E-cadherin (Fig. 4C and D). This was seen also for RIE-1TB cells (see Fig. S2C in the supplemental material). Consistently, Twist depletion caused a marked reduction in anoikis suppression (see Fig. S3A in the supplemental material), cell migration, invasion (see Fig. S3B in the supplemental material), and anchorage-
independent growth in soft agar (see Fig. S3C in the supplemental material) induced by TrkB, which was not due to inhibition of cell proliferation (see Fig. S3D in the supplemental material).

Like our previous findings in RIE-1 cells (20, 29), RK3ETB cells (but not parental RK3E cells, which are devoid of tumorigenic potential [69; data not shown]) were highly tumorigenic in nude mice (Fig. 4E). In contrast, subcutaneous injection of RK3ETB cells in which Twist had been depleted resulted in tumor growth that was significantly, albeit moderately, reduced.
FIG. 4. Twist is required for TrkB-induced EMT and tumorigenesis. (A) Induction of Twist protein levels in TrkB and BDNF-expressing RK3E cells, analyzed by Western blotting. V, vector; TB, TrkB and BDNF. CDK4 serves as the loading control. (B) sh-Twist prevents morphological transformation of RK3E cells by TrkB and BDNF. (C) sh-Twist prevents downregulation of E-cadherin by TrkB and BDNF in RK3E cells, as shown by Western blot analysis for the indicated proteins. β-actin serves as the loading control. (D) E-cadherin localizes at the cell membrane of RK3ETB cells upon Twist depletion, as shown by immunofluorescence (TO-PRO stains DNA). (E) sh-Twist impairs TrkB-mediated tumorigenesis. BALB/c nude mice were subcutaneously injected with 1 × 10⁵ RK3ETB cells plus the indicated shRNAs into each flank. Growth curves for average tumor volumes are shown, with 8 tumors for sh-EGFP #1, sh-EGFP #2, and sh-Twist #1 and 11 tumors for sh-Twist #2. Error bars represent the standard errors of the mean. An asterisk indicates a P value of <0.01 in a two-sided Student t test. (F) Effect of sh-Twist on experimental metastasis. Mice were intravenously injected with 1 × 10⁶ RK3ETB cells plus the indicated shRNAs. Mice were euthanized when clinical symptoms became apparent, with four mice from each cell line. Significance values were obtained by first combining the data from both shRNAs against the same gene (EGFP or Twist) and subsequently performing a log rank test.
FIG. 5. TrkB-induced EMT-like transformation is mediated via the MAPK pathway. (A) Downregulation of phospho-ERK (pERK) upon treatment with U0126. Cells were serum starved for 4 h, pretreated with 20 μM U0126 for 30 min, and stimulated with 50 ng/ml BDNF for 5 min. Cells were harvested in the presence of phosphatase inhibitors. (B) Induction of Twist in RK3E cells requires an intact MAPK pathway, as shown by Western blot analysis. Cells were treated overnight with 20 μM U0126 and analyzed by Western blot analysis. Two parts derived from the same gel are shown. TB, TrkB and BDNF. (C) TrkB-induced EMT-like transformation is dependent on the MAPK pathway. RK3E cells were treated with 20 μM U0126 for 2 days and analyzed by Western blotting. (D) Morphology of the cells described in the legend to panel C. (E) HA-Twist or Snail-HA overexpression in RK3E cells partially prevented the reversion to an epithelial morphology induced by U0126 treatment for 2 days. (F) Western blot analysis of cells described in the legend to panel E. (G) Downregulation of pAKT upon treatment with PI-103. Cells were serum starved for 4 h, pretreated with 500 nM PI-103 for 30 min, and stimulated with 50 ng/ml BDNF for 5 min. Cells were harvested in the presence of phosphatase inhibitors. (H) Induction of Twist in RK3E cells is not dependent on the PI3K pathway. Cells were treated overnight with 500 nM PI-103 and analyzed by Western blotting. (I) TrkB-induced EMT-like transformation is not dependent on the PI3K pathway. Cells were treated with 500 nM PI-103 for 2 days and analyzed by Western blotting. (J) Morphology of cells described in the legend to panel I. β-actin serves as the loading control for all Western blots.
FIG. 6. Snail is required for TrkB-induced EMT-like transformation, anoikis resistance, and anchorage-independent growth. (A) Increased protein levels of Snail in TrkB and BDNF-expressing MCF10A cells, as judged by Western blotting of the indicated proteins. (B) Snail knockdown in RK3E/TB cells, as measured by qRT-PCR (n = 3; error bars represent standard deviations). Rel., relative. (C) sh-Snail prevents morphological transformation of RK3E cells by TrkB and BDNF. Photographs shown here and in Fig. 4B are derived from the same experiment. (D) sh-Snail prevents downregulation of E-cadherin by TrkB and BDNF in RK3E cells, as judged by Western blotting of the indicated proteins. (E) E-cadherin localizes at the cell membrane of RK3E/TB cells upon Snail depletion, as shown by immunofluorescence (TO-PRO stains DNA). Pictures shown here and in Fig. 4D are derived from the same experiment. (F) sh-Snail impairs TrkB-mediated anoikis suppression. RK3E cells expressing the indicated cDNAs were cultured on ULC plates and scanned at a magnification of ×1 after 4 days (left) or quantified by determining the total protein levels (right) (n = 3; error bars represent standard deviations).
could be effectively downregulated (Fig. 5G). Likewise, a domination of Twist occurs at multiple levels. Inhibition of the PI3K effector Twist (Fig. 5E and F) (the remainder of these panels significantly by overexpression of the presumptive downstream inhibitor, CI-1040 (1), which gave identical results (see Fig. S4). To demonstrate that of the MAPK pathway with U0126 was confirmed by measuring phospho-ERK levels (Fig. 5A). U0126 treatment abolished the induction of Twist by TrkB (Fig. 5B) and restored E-cadherin levels in RK3E? cells (Fig. 5C), which was accompanied by a reversion of the spindle-shaped cell morphology toward an epithelial appearance (Fig. 5D). To rule out off-target effects of the U0126 inhibitor, we used a second MEK inhibitor, CI-1040 (1), which gave identical results (see Fig. S4 in the supplemental material). The effects of MEK inhibition on E-cadherin and cell morphology could be reverted significantly by overexpression of the presumptive downstream effector Twist (Fig. 5E and F) (the remainder of these panels will be discussed below). The partial effect suggests that activation of Twist occurs at multiple levels. Inhibition of the PI3K pathway failed to have this effect on Twist, E-cadherin, or cell morphology (Fig. 5H to J), despite the fact that pAKT levels could be effectively downregulated (Fig. 5G). Likewise, a dominant-negative RAC1N17 mutant failed to significantly affect cell morphology, E-cadherin, or Twist levels. Nor did it impede the migratory or invasive properties of RK3E? cells (see Fig. S5B to D in the supplemental material). This was in spite of the observation that RAC1N17 suppressed RAC1 activity below that of the control RK3E cells, as shown by the RAC1 pull-down assay (see Fig. S5A in the supplemental material). In line with this, treatment with the RAC1 inhibitor NSC23766 altered neither cell morphology nor E-cadherin levels (see Fig. S5E and F in the supplemental material). These results demonstrate that TrkB-induced EMT-like transformation and induction of Twist is mediated mainly via the MAPK pathway.

Snail is required for TrkB-induced EMT-like transformation, anoikis resistance, migration, invasion, and anchorage-independent growth. In view of these results, we set out to dissect the different functions of Twist, aiming to identify its downstream target(s) that is specifically required for metastasis in this system. The zinc finger transcription factor Snail is a direct repressor of E-cadherin (2, 14). Studies of Drosophila have shown that Twist can induce Snail (39). Consistent with this, ectopic expression of Twist led to a induction by fivefold of Snail mRNA levels in RK3E cells (see Fig. 8A). Furthermore, Snail was induced by overexpression of TrkB and BDNF in MCF10A cells (Fig. 6A). We were unable to assess endogenous Snail protein levels in RK3E and RIE-1 cells by any of the available Snail antibodies. To investigate the contribution of Snail to the prooncogenic and prometastatic functions of TrkB, we generated stable cell clones of RK3E? cells and two independent shRNAs against Snail (Fig. 6B). Like what was observed for Twist, silencing of Snail in RK3E cells prevented TrkB-induced EMT-like transformation (Fig. 6C). Again, this correlated well with restoration of E-cadherin levels and its correct localization at the cell membrane (Fig. 6D and E). Furthermore, Snail was required for TrkB-induced anoikis resistance (Fig. 6F), cell migration and invasion (Fig. 6G), and anchorage-independent growth (Fig. 6H). Of note, as we had observed for Twist, Snail overexpression also rescued the effects of MEK inhibition on cell morphology and E-cadherin levels (Fig. 5E and F; see also S4D and E in the supplemental material).

Snail is required for TrkB-induced metastasis. In contrast to sh-Twist, however, subcutaneous injection of sh-Snail-expressing RK3E? cells into nude mice resulted in the formation of primary tumors, with kinetics indistinguishable from those of tumor cells expressing control shRNA (Fig. 7A; see also Fig. S6 in the supplemental material). This allowed us to specifically address the role of Snail in TrkB-induced metastasis, using an experimental system comprising all steps of the metastatic cascade. Almost 100% of the subcutaneous RK3E? tumors metastasized to the lungs, which was strongly suppressed upon Snail depletion. For each cell line (RK3E? cells plus indicated shRNAs), the incidence of metastasis, measured by the number of mice that developed pulmonary metastases (>0.1 mm in size) out of the total number of mice with subcutaneous tumors, observed was as follows: sh-EGFP #1, 15/15; sh-EGFP #2, 14/15; sh-Snail #1, 6/14; and sh-Snail #2, 6/15. This was accompanied by, on average, a >fivefold drop in the number of metastatic pulmonary lesions (Fig. 7B and C).
Consistent with and extending these findings, Snail silencing also strongly delayed the outgrowth of intravenously injected RK3ETB cells in the lungs (Fig. 7D). Taken together, these results show that Snail is dispensable for TrkB-expressing cells to produce a primary tumor but strongly contributes to their capacity to metastasize.

Snail acts downstream from Twist. Since it is unknown whether the Twist-Snail axis present in Drosophila (39) is also operational in mammalian cells, we determined whether Twist induces Snail in rat epithelial cells. In support of this possibility, we observed that overexpression of HA-Twist in RK3E cells increased Snail mRNA levels by a factor of 5 (Fig. 8A). Conversely, Snail-HA overexpression hardly affected Twist mRNA levels (Fig. 8B), although HA-Twist and Snail-HA were both expressed to high levels, which were sufficient to downregulate E-cadherin (Fig. 8C). The model in which Twist
acts upstream from Snail predicts that the latter protein should be able to antagonize a phenotype that is altered as a function of the first one. Thus, we examined whether overexpression of Snail-HA rescues the reversion of TrkB-expressing cells to an epithelial morphology, owing to Twist depletion. Indeed, ectopic expression of Snail-HA in RK3E<sub>T</sub>-sh-Twist cells induced a dramatic EMT-like morphological transformation, changing the epithelial morphology back to a spindle-shaped appearance (Fig. 8D). Consistent with this observation, E-cadherin levels, which initially were high after Twist depletion,
interaction was confirmed by the reverse experiment, in which in the context of this experimental setting. This functional Twist and Snail, with Twist acting upstream from Snail, at least these results suggest an epistatic relationship between were suppressed strongly by overexpression of Snail-HA (Fig. 8E). These results suggest an epistatic relationship between Twist and Snail, with Twist acting upstream from Snail, at least in the context of this experimental setting. This functional interaction was confirmed by the reverse experiment, in which HA-Twist overexpression in RK3E\textsuperscript{sh-Snail} cells failed to affect cell morphology (Fig. 8F). In line with this, E-cadherin levels remained unchanged upon HA-Twist overexpression in Snail-depleted cells (Fig. 8G). Furthermore, in TrkB- and BDNF-expressing MCF10A cells, Snail levels were downregulated upon knockdown of Twist (Fig. 8H). This demonstrates that Twist is required for the induction of Snail by TrkB.

**DISCUSSION**

The results presented here show that TrkB induces an EMT-like transformation in epithelial cells and that it does so through a Twist-Snail signaling axis, which is dependent on the MAPK pathway. Furthermore, we demonstrate that Snail plays a critical and specific role in TrkB-mediated metastasis (Fig. 9).

The term EMT has been used quite broadly to describe processes that enable epithelial cells to acquire fibroblastoid properties (33). In line with a prototypic EMT, activated TrkB in rat epithelial cells induced a switch from E-cadherin to N-cadherin, the downregulation of several catenin proteins, and a spindle-shaped morphology with reduced cell-cell adhesion. However, in this system, TrkB failed to induce the mesenchymal proteins vimentin, smooth-muscle actin, and fibronectin. In human mammary epithelial MCF10A cells, most epithelial markers were not changed by TrkB expression, but the mesenchymal proteins were induced, which is consistent with the increasing notion that cellular context has to be taken into consideration when studying EMT. By comparing the gene expression profile of RK3E\textsuperscript{sh-Snail} cells to that of Eph4\textsuperscript{ras\textsuperscript{Tg60}} cells, we observed that 67% of the significantly deregulated EMT-associated genes changed in the expected direction (Desmet and Peepere); data not shown). Consistently, by assessing the mRNA expression levels of several known EMT mediators, we found an induction by TrkB of Twist and, to a smaller extent, of Snail. We show that both transcription factors are critically required for the EMT-like changes induced by TrkB, while two other mediators of EMT, Slug (Fig. 1D) and E12/E47 (data not shown), were not induced by TrkB. We infer from these results that TrkB activation changes the phenotype of epithelial cells in a way that strongly resembles EMT, which depends on the presence of both Twist and Snail.

We show that the EMT-like changes induced by TrkB via upregulation of Twist rely on a functional MAPK pathway. Like these findings, RAS\textsuperscript{V12}–TGF-\textbeta\textsuperscript{β}-induced EMT in Eph4 cells is also mediated mainly via MAPK pathway (40). The signaling pathways required for EMT and anoikis suppression are likely to be different across cell types, as TrkB-induced anoikis suppression in RIE-1 cells seems to be more dependent on PI3K signaling (20). Interestingly, whereas MEK inhibition did not affect the viability of RK3E\textsuperscript{sh-Snail} cells, treatment with the Trk inhibitors K252a or GW 441756 induced a strong apoptotic response. The viability of RK3E cells expressing no or inactive TrkB hardly depended on TrkB signaling, as both inhibitors induced little death in that setting. Remarkably, this was also seen for cells expressing activated RAS, implying some specificity for these inhibitors. This result also suggests that RK3E cells with sustained TrkB activation undergo “oncogene addiction” (76). Only 2 days of activation by BDNF was sufficient to induce this dependency. Our findings raise the possibility that certain tumors may critically rely on TrkB signaling, offering an opportunity for a TrkB-based anticancer therapy.

Previous studies performed in *Drosophila* showed that Twist can bind to the Snail promoter and induce its expression (39). However, to date, it is unclear whether this epistatic relationship is conserved in mammalian cells. As Twist is known to induce EMT and to play a critical role in breast cancer metastasis (78), we determined whether a Twist-Snail signaling pathway is conserved in the rat epithelial cells. As depletion of either Twist or Snail each fully restored E-cadherin levels to those seen in parental RK3E cells (Fig. 4C and 6D), this suggests that these two factors act in one and the same pathway rather than in parallel pathways. Furthermore, our results suggest that Twist acts upstream from Snail both in regulating E-cadherin and in mediating EMT. Others have shown recently that in human breast tumor cells, Twist can also directly bind to the E-cadherin promoter (75); a possible contribution of Snail was not addressed in that study. It thus appears that E-cadherin is subject to the following two modes of Twist-dependent regulation: a direct one involving Twist binding to the promoter and an indirect one involving Snail upregulation.

**FIG. 9.** Model depicting a critical TrkB effector pathway contributing to EMT, anoikis suppression, and metastasis. TrkB activation leads to induction of both Twist and Snail. These transcription factors repress E-cadherin, thereby inducing EMT and anoikis suppression and facilitating metastasis. Twist, acting upstream from Snail, conceivably has additional targets, including those that are primarily required for tumor growth. For simplicity, only the functional and epistatic relationships that are addressed in this paper are indicated. We do not exclude other upstream and downstream factors and feedback loops that may be present.
Whereas it has been proven difficult to provide histopathological evidence for EMT in human carcinomas (79), several reports have shown a correlation between Snail expression levels and the propensity to metastasize (reviewed in reference 63). For breast cancer, particularly, Snail overexpression has been associated with lymph node metastasis (8) and shorter overall survival (24). Besides metastasis, Snail overexpression also predicts tumor relapse, which has functionally been confirmed in a breast cancer mouse model (52). Snail has also been associated with aggressive disease in other cancer types (for a review, see reference 3), including hepatocellular carcinoma (71), ovarian cancer (10), and head and neck squamous cell carcinoma (80). Although these observations imply that Snail is functionally involved in metastasis, the experimental evidence for this is still incomplete. Overexpression of Snail has been shown to induce EMT, cellular migration (14), and increased levels metastasis after orthotopic injection into nude mice (81). Conversely, RNA interference (RNAi)-mediated inhibition of Snail impairs metastasis of subcutaneously injected HaCa4 cells (59). However, a limitation of these as well as other tumor cell lines is that downregulation of Snail not only decreased metastasis but also dramatically impaired the growth of the primary tumor (58–60). A similar phenomenon, albeit less dramatic, is shown in this paper for Twist. Therefore, it has not been straightforward to conclude whether delayed metastasis is due to the metastasis-specific functions of Snail (e.g., cell invasion and anoikis suppression) or due to its more general function with in vivo tumor cell proliferation. The cell system used in our study is genetically better defined than tumor cell lines, because its oncogenic and metastatic potentials strictly depend on activated TrkB. Here, RNAi against Snail did not affect growth of the primary subcutaneous tumors, although knockdown of Snail partially impaired TrkB-mediated anoikis suppression and growth in soft agar. It thus appears that the microenvironment in the subcutaneous compartment in the mouse allows for compensation of the loss of Snail, something the soft agar conditions in vitro cannot allow. We can only speculate what the reason for this is. Conceivably, this involves ECM components as well as growth and/or survival factors provided by blood vessels. In contrast to primary tumor growth and consistent with the strong negative effect that a Snail knockdown had on cell migration and invasion, the formation of lung metastases was strongly impaired, thereby unmasking the specific requirement for Snail in spontaneous TrkB-driven metastasis. Our results, together with those discussed above, therefore raise the possibility that functional inhibition of Snail in cancer may impact tumor growth, tumor cell survival, and/or tumor cell metastasis. As transcription factors are not the favorite class of targets for pharmacological inhibition, upstream regulators of Snail, like GSK3β (82, 83), might provide better targets for therapeutic intervention. However, our knowledge about the (de)regulation and activation of Snail is still incomplete and should be further investigated, as is also warranted by this work.

This model leads to the question as to which of the targets of Snail are most relevant for its metastatic function. One of the best-studied Snail targets is E-cadherin (2), playing an essential role in EMT (4), tumor progression (17, 64), and metastasis (48, 57, 61). The loss of E-cadherin-mediated cell-cell adhesion is thought to promote cell migration and invasion (7). Furthermore, E-cadherin is part of a complex that regulates β-catenin signaling (15, 32, 61) and the activity of Rho GTPases (55), further impinging on cell migration and invasion. Others have demonstrated that RNAi-mediated downregulation of E-cadherin (but not overexpression of a dominant-negative mutant) is sufficient to induce EMT and metastasis (61). Furthermore, the loss of E-cadherin induces Twist (61), suggesting that a feed-forward loop could strengthen the maintenance of EMT. We show that restoration of E-cadherin expression in TrkB-expressing cells that have undergone EMT not only restored the epithelial morphology but also interfered with anoikis suppression (Fig. 3). This observation is consistent with the findings from a conditional E-cadherin knockout mouse model for breast carcinoma, in which the loss of E-cadherin resulted in anoikis suppression (17). Therefore, Twist, Snail, and E-cadherin conceivably provide a link between EMT and anoikis suppression, both of which contribute to metastasis. A recent report showed that Twist and Snail also induce stem cell-like properties in nontumorigenic, as well as transformed, human mammary epithelial cells (45). Taken together with the role of Twist and Snail in tumor expansion and metastasis, this raises the interesting possibility that genes from within the Twist/Snail transcriptome are involved in regulating both stem cell phenotype and oncogenic transformation. While further work should shed light on such a possible connection, here, starting from the observation that TrkB suppresses anoikis and promotes metastasis, we established that a Twist-Snail axis mediating EMT is critically required for these important cancer biological processes. Further elucidation of the factors and pathways involved in this program may reveal targets for therapeutic intervention.

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