CHAPTER 2

A Twist–Snail axis critical for TrkB-induced EMT-like transformation, anoikis resistance and metastasis

A Twist–Snail axis critical for TrkB-induced EMT-like transformation, anoikis resistance and metastasis

Marjon A. Smit1#, Thomas R. Geiger1#, Ji-Ying Song2, Inna Gitelman3 and Daniel S. Peeper1

1 Division of Molecular Genetics, 2 Department of Experimental Animal Pathology, The Netherlands Cancer Institute, Amsterdam, The Netherlands 3 Department of Developmental Molecular Genetics, Faculty of Medicine, Ben Gurion University of the Negev, Beer Sheva, Israel

# These authors contributed equally to this work

In a genome-wide 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 induces also a strong morphological transformation resembling epithelial-mesenchymal transition (EMT). This required TrkB kinase activity, a functional MAPK pathway, suppression of E-cadherin and induction of Twist, a transcription factor contributing to EMT and metastasis. RNAi-mediated Twist depletion blocked TrkB-induced EMT-like transformation, anoikis suppression and growth of tumor xenografts. 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 EMT-like transformation and anoikis suppression induced by TrkB, but in contrast to Twist depletion, it failed to inhibit tumor growth. Instead, Snail RNAi specifically impaired formation of lung metastases. Epistasis experiments suggested that Twist acts upstream of 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 further light on the epistatic relationship of Twist and Snail, two key transcriptional regulators of EMT and metastasis.

Introduction

The success of 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 improving cancer treatment in the future. Metastasis is a multi-step process in which tumor cells have to overcome several barriers to form a secondary tumor at a distant anatomical site (Gupta & Massague, 2006; Eccles & Welch, 2007). 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 neigh-
boring tissue or vessels, epithelial tumor cells must acquire a more flexible and migratory phenotype, similar to that of mesenchymal cells (Thiery, 2002; Christofori, 2006). This can be achieved by an epithelial-mesenchymal transition (EMT), a process that was initially described in embryogenesis (Thiery & Sleeman, 2006; Yang & Weinberg, 2008). 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 (Grunert et al., 2003). At the same time, mesenchymal proteins are often induced, including smooth muscle actin (Masszi et al., 2003), fibronectin, N-cadherin or vimentin (Jechlinger et al., 2003; Berx et al., 2007). This is mediated (either directly or indirectly) by transcription factors like Twist, E12/E47, and members of the Snail and ZEB protein families (Huber et al., 2005). In vitro, EMT can be induced by activated oncogenes like RASV12, or by several receptor tyrosine kinases, such as MET or the EGF receptor (EGFR), often in cooperation with transforming growth factor β (TGFβ (Grunert et al., 2003)). However, the extent of up- and down-regulation of the epithelial and in particular of the mesenchymal markers varies between 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 (Grunert et al., 2003). In vivo, in the course of tumor cell invasion and metastasis, EMT is thought to occur mainly in a transient and reversible way, under influence of the tumor stroma (Massague, 2008; Yang & Weinberg, 2008).

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 lack of, cell adhesion) (Meredith et al., 1993; Frisch & Francis, 1994). Apart from its role in tissue homeostasis (Hall et al., 1994; Boudreau et al., 1995), anoikis conceivably also restricts the spread of tumor cells through tissues and via the circulation (Liotta & Kohn, 2004). In an attempt to identify new mediators of metastasis, we previously have used anoikis suppression as the basis for a genome-wide functional screen. In this way we identified the neurotrophic receptor tyrosine kinase 2 (Ntrk2/TrkB) as a potent anoikis suppressor (Douma et al., 2004). 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 (Klein et al., 1993; Ernfors et al., 1994; Jones et al., 1994). Consistent with the premise that anoikis forms a barrier to metastasis, TrkB-expressing epithelial cells form metastatic tumors in vivo (Douma et al., 2004). We recently showed that the ability of TrkB to suppress anoikis and induce metastasis requires its kinase function to be intact (Geiger & Peeper, 2007). Supporting the notion that TrkB may play an important role also in human cancer, it is found overexpressed in several human malignancies, including neuroblastoma (Nakagawara et al., 1994; Brodeur, 2003), prostate cancer (Dionne et al., 1998) and pancreatic cancer (Miklyoczki et al., 1999) (reviewed in (Geiger & Peeper, 2005)). In line with this, TrkB-interfering agents are currently being developed and tested for anticancer activity (Ruggeri et al., 1999; Desmet & Peeper, 2006). However, the
molecular mechanisms of 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 critically required for TrkB-induced anoikis suppression and metastasis, with a focus on EMT.

**Materials and methods**

**Vector constructs**  
Mouse pBabe-Hygro (pBH-)BDNF was described before (Douma et al., 2004). Mouse TrkB (GenBank accession number X17647) in the pBabe-Puro (pBP-) vector was subcloned into pMSCV-blast using the EcoRI restriction site. Human BDNF, human TrkB	extsuperscript{wt} and the human kinase inactive point mutant TrkB	extsuperscript{K588M} were described before (Geiger & Peeper, 2007). Mouse E-cadherin (X06115) in the pBP-IRESGFP vector was a gift from C. Niessen (Gottardi et al., 2001). 3’-HA-tagged mouse Snail (NM011427) in the pRV-IRESGFP vector was a gift from A. Munoz (Palmer et al., 2004). Mouse Twist1 (M63649) was a gift from R. Weinberg (Yang et al., 2004) and was subcloned by PCR into the pLZRS-MS-IRESGFP (pLSIE-) vector and pBP vector, adding a 5’-HA-tag and a Kozak sequence. Forward primer was: 5’-CGGGATCCGCCGCCATGGCTTACCCATACGATGTTCCAGATTACGCTATGCACGTGTCCAGC-3’, reverse primer was: 5’-GGAATTCCTAGTGGGACGCGGACATGG-3’. Short hairpin (sh)RNAs were expressed from pRetroSuper (Brummelkamp et al., 2002) with the following targeting sequences: EGFP: 5’-GCTGACCCTGAAGTTCATC-3’, rat Twist1 #1: 5’-AGACCAAATTTCACAAATTTCACAAGAA-3’, rat and human Twist1 #2: 5’-GGATCAAACTGGCCTGCAA-3’, human Twist1 #3: 5’-GAACACCTTTAGAAATAAA-3’, rat Snail #1: 5’-GAATGTCCTTGCTCCACAA-3’ and rat Snail #2: 5’-ACAGCTGCTTTGAGCCATGA-3’.

**Cell culture and retroviral transduction**  
Rat intestinal epithelial RIE-1 cells (a kind gift from R.D. Beauchamp and K.D. Brown, (Blay & Brown, 1984)) and E1A-immortalized rat kidney RK3E cells (ATCC) were cultured in Dulbecco’s modified Eagle’s Medium (DMEM) (Gibco) supplemented with 9% fetal calf serum (PAA laboratories GmbH) and penicillin+streptomycin (Gibco). Human breast epithelial MCF10A cells expressing the ecotropic receptor were cultured in DMEM-F12 (1:1) (Gibco) supplemented with 15 mM Heps buffer, 5% equine serum, 1% penicillin+streptomycin, 0.5 μg/ml fungizone, 10 μg/ml insulin, 20 ng/ml EGF, 100 ng/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 viral supernatant in the presence of 3.5 μg/ml polybrene (Sigma). Cells were selected (112.5 μg/ml for RIE-1 and 75 μg/ml for RK3E and MCF10A) with hygromycin B and subsequently infected with wild type or mutant TrkB viral supernatant. Stable cell lines were established by selection with
blasticidin (5 μg/ml for RIE-1 cells, 2.5 μg/ml for RK3E cells and MCF10A cells) or with puromycin (MCF10A, 1.0 μg/ml). RK3E cells expressing TrkB and BDNF were subsequently transduced with pBP-HA-Twist or pRV-IRES-GFP-Snail-HA viral supernatant. To generate cell lines expressing shRNAs, pBH-BDNF-expressing RK3E, RIE-1 and MCF10A cell pools were infected with pRS-Twist, pRS-Snail or pRS-EGFP. Cells were selected with 1.0 μg/ml puromycin (1.5 μg/ml for RIE-1 cells) and subsequently infected with pMSCV-blast-TrkB. To generate stable cell clones, cells were seeded at clonal densities and selected in medium containing 2.5 μg/ml blasticidin. One week later, single clones were picked and expanded. For re-introduction of E-cadherin, RK3E cells expressing MSCV-blast-TrkB + pBH-BDNF (‘RK3E\textsuperscript{TB}’ cells) were transduced with pBP-GFP-E-cadherin and seeded at low density in medium containing 1.0 μg/ml puromycin to obtain independent cell clones. For functional rescue experiments, RK3E\textsuperscript{TB} cell clones expressing shRNAs against Twist or Snail were infected with pRV-IRES-GFP-Snail-HA or pLSIE-HA-Twist and subjected to fluorescence-activated cell sorting (FACS). For cells expressing dominant negative RAC\textsuperscript{N17} (Ridley et al., 1992), RK3E cells were transduced first with pBH-BDNF (selection 75 μg/ml blasticidin), then with LZRS-mycRAC\textsuperscript{N17}-IRES-ZEO (selection with 100 μg/ml zeocin) and subsequently with MSCV-TrkB (selection 2.5 μg/ml blasticidin).

**Pharmacological inhibition of TrkB, MAPK, PI3K and RAC1 pathways**

Cells expressing empty vector control or TrkB+BDNF were treated with 20 μg/ml U0126 (Cell Signaling), 1 μM CI-1040 (Axon Medchem), 500 nM PI-103 (Echelon) or 75 μM NSC23766 (Calbiochem) for indicated time points. Cells were harvested in the presence of phosphatase inhibitors (1 mM sodium pyrophosphate, 2 mM sodium fluoride, 10 mM beta-glycophosphate and 2 mM sodium orthovanadate). For BDNF stimulation, cells were serum starved for 4 hours, pre-treated with the inhibitor for ½ hour and stimulated with 50 ng/ml recombinant human BDNF (Peprotech) for 5 minutes. Cells were harvested in the presence of phosphatase inhibitors. RK3E cells expressing vector control, TrkB only, TrkB+BDNF or RAS\textsuperscript{V12} were treated with 300 nM K252a (Calbiochem) or 20 μM GW441756 (Tocris) overnight. Cells were trypsinized and resuspended in trypan blue / PBS 1:1. Percentages of dead cells were counted using trypan blue exclusion. For western blot analysis cells were harvested in the presence of phosphatase 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, TrkB 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 removal of BDNF.

**Anoikis, soft agar, migration and invasion assays**

To induce anoikis we seeded 4*10\textsuperscript{5} freshly trypsinized cells into Ultra Low Cluster six-well cell culture dishes (Costar). Plates were scanned on an Epson Perfection 4990 Photo scanner 4 days later. For quantification of anoikis suppression, total protein amounts in each well were measured by cell lysis in RIPA buffer (50 mM
tris pH=8.0, 150 mM NaCl, 1% nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS) and BioRad Protein Assay. For soft agar assays, 1000 trypsinized cells were seeded in 0.4% low melting agarose (Sigma) on top of a 1% agarose layer and scans were taken 11 days later. Numbers of macroscopic colonies were determined using ImageJ software (http://rsb.info.nih.gov/ij/index.html). For migration and invasion assays, 250.000 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 hours noninvaded cells were removed and invaded cell were stained with crystal violet. Quantification was performed by counting invaded cells on 5 independent pictures of the well. All pictures of adherent cells were taken at 50x magnification.

**RAC1 activity assay**

To determine RAC1 activity levels, cells were harvested in lysis buffer (0.5% nonidet P-40, 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 10% glycerol and protease inhibitors) and sheared 3x through a G25 syringe. Total protein amounts were determined using a BioRad protein assay. Samples (900 μg protein) were incubated for ½ hour with biotin-Pak-CRIB peptide and streptavidin-magnetic beads (Invitrogen). The Pak-CRIB peptide binds RAC in its active conformation only (Price et al., 2003). Samples were washed 2x 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 BioRad Protein Assay. Immunoblot analysis was performed using standard techniques, either on 7% polyacrylamide-SDS gels, or on 4-12% bis-tris precast gels (NuPAGE) for Twist and Snail. Antibodies used were: pan-Trk (C14, Santa Cruz), BDNF (N20, Santa Cruz), Twist hybridoma supernatant (Gitelman, 1997), Snail (H130, Santa Cruz for detection of overexpressed mouse Snail, hybridoma supernatant from K. Becker Sn9H2, (Rosivatz et al., 2006) for endogenous human Snail), E-cadherin, P-cadherin, N-cadherin, α-catenin, β-catenin, γ-tubulin, fibronectin, vimentin, RAS (all from BD), smooth muscle actin (1A4, Sigma), CDK4 (C22, Santa Cruz), β-actin (AC74, Sigma), γ-tubulin (DM 1A, Sigma) and RAC (Upstate). All antibodies were diluted 1:2000 in 4% Protifar plus (Nutricia), except antibody for TrkB: 1:1000, RAC 1:1000, BDNF: 1:500, Snail H130: 1:1000, Twist: 1:3 in PBS + 0,2% Tween, and Snail: 1:10 in PBS + 0,2% Tween. Antibodies for pERK and ERK (p44/42 MAPK, both Cell Signaling) were diluted 1:1000 in 4% BSA, antibodies against pAKT (Cell Signaling) and AKT (Santa Cruz) were diluted 1:1000 in 4% BSA + 1:50 western blot blocking reagent (Roche). Protein detection for western blotting was done with ECL reagent (Amersham) and exposed films were scanned on an Epson Perfection 4990 Photo scanner.
**Immunofluorescence**

Cells were grown on glass coverslips, fixed in 4% formaldehyde in PBS (in 70% ethanol for cells expressing pBP-iRES-GFP and pBP-iRES-GFP-E-cadherin to inactivate 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) in blocking solution for 1 hour at room temperature. After washing with PBS + 0.2% Tween we incubated the cells for 1 hour with Alexa Fluor 488 goat-anti-mouse secondary antibody (diluted 1:1000) and for 15 min with TO-PRO (diluted 1:500; both from Molecular Probes). Coverslips were mounted with AquaPolymount (Polysciences, Inc) and analyzed by confocal microscopy on a Leica TCS NT (Leica Microsystems, Heidelberg, Germany) confocal system, equipped with an Ar/Kr laser. Images were taken using a 100x NA 1.32 objective with standard filter combination(s) and Kalman averaging.

**Quantitative reverse transcriptase PCR**

Total RNA was isolated using Trizol (Invitrogen) and treated with DNase for 1 hour at 37°C (Promega). qRT-PCR was performed using the reverse transcriptase kit from Invitrogen. Primers were designed using Primer Express software. Used primers were: Twist-forward: 5’-CGCTGAACGAGGCATTTGC-3’, Twist-reverse: 5’-CCAGTTTGAGGGTGCTGAATC-3’, Snail-forward: 5’-CCAGTTTGAGGGTGCTGAATC-3’, Snail-reverse: 5’-TGCTGGAGGTGGGCACGTA-3’, TBP-forward: 5’-GATG TGAAGTTCCCCCATAGGC-3’, TBP-reverse: 5’-GATG TGAAGTTCCCCCATAGGC-3’, N-cadherin-forward: 5’-AGGGCCCTAAACGTGCTGACA-3’, N-cadherin-reverse: 5’-TCATAGTCGAAGAC- TAAAAGGGAGTGCATAT-3’, E-cadherin-forward: 5’-TGAGCATGCCCCAGTATCG-3’, E-cadherin-reverse: 5’-CTGCCCTTCAGGTTTTTCATCGA-3’, Slug-forward: 5’-CCAACTACAGCGAACCTGGGACAC-3’, Slug-reverse: 5’-TCTCACTGGATGATGGAGAAATGATC-3’, CTGF-forward: 5’-GTCTTCTTCTGCGACTTCGGCT-3’, CTGF-reverse: 5’-CATCTTTTGCAGTGACCAG-3’, BMP-4-forward: 5’-CTCAAGGGAGTGGAAATTGGG-3’, BMP-4-reverse: 5’-CATCGTGCCCAAAGTGCACC-3’. Detection was done with SYBRgreen master mix (Applied Biosystems) on an ABIPRISM700 thermal cycler (Applied Biosystems). RNA levels were normalized against rat TATA box binding protein (TBP).

**In vivo assays**

Eight-14 weeks old female Balb/c nude mice were subcutaneously injected with 1*10^5 cells into both flanks. Mice were inspected twice a week and euthanized by CO2 when tumors reached a volume of 1 cm^3 or started to ulcerate, according to a protocol approved by the Institutional Animal Experiment Ethics Committee. Tumor size was measured with a caliper and tumor volume calculated by the formula (a*b^2)/2, with a being the longest diameter and b the respective perpendicular diameter of the tumor. Metastatic lesions in the lungs were counted by visual inspection of hematoxylin-eosin (HE) stained histological tissue sections. Sections were analyzed on an Axiocomet-S100 microscope system with a color CCD camera and Axio Vision software (Zeiss). The sizes of metastases and the area of lung tissue per section were determined with ImageJ software. Total lung area analyzed was...
19.5 cm² for sh-EGFP #1, 17.3 cm² for sh-EGFP #2, 11.6 cm² for sh-Snail #1 and 19.1 cm² for sh-Snail #2. For experimental metastasis, 1*10⁶ cells were injected into the tail veins of 8-14 weeks 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 in SPSS 14.0.

Results

TrkB induces EMT-like transformation in epithelial cells
Consistent with our previous observations (Douma et al., 2004; Geiger & Peeper, 2007), overexpression of TrkB and BDNF in several non-malignant epithelial cell lines induced a striking morphologic transformation, characterized by a spindle-shaped morphology and the loss of cell-cell contacts (Fig. 1A). This effect was observed in three different non-malignant epithelial cell lines, originating from different tissues and species: rat intestine epithelial cells (RIE-1), E1A-transformed rat kidney epithelial cells (RK3E), and, to a somewhat lesser extent, in human breast epithelial cells (MCF10A). To determine whether this morphologic transformation represents EMT, we analyzed the levels of several epithelial and mesenchymal proteins (see 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 (Grunert et al., 2003). To investigate a larger number of EMT-associated genes we compared the gene expression profile of TrkB+BDNF-expressing RK3E cells (from here on referred to as RK3E<sup>TB</sup> cells) (Desmet and Peeper, submitted) to the reported profile of RAS<sup>V12</sup>-expressing plus TGFβ1-treated EpH4 mouse mammary epithelial cells (EpH4<sup>RasTgfβ</sup>) (Jechlinger et al., 2003), a classical system for studying EMT (Oft et al., 1996; Grunert et al., 2003). This analysis showed that 67% of the genes significantly deregulated in both RK3E<sup>TB</sup> cells and EpH4<sup>RasTgfβ</sup> cells changed the expression level in the same direction.

The EMT-associated “cadherin switch” from E-cadherin to N-cadherin (Christofori, 2006) was present in both rodent cell lines upon expression of TrkB+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 (Nieto, 2002; Huber et al., 2005). When we assessed the mRNA levels of Twist (Twist1), Snail (Snai1) and Slug (Snai2), we observed a marked induction of Twist and a small but reproducible induction of Snail in RIE-1<sup>TB</sup> and RK3E<sup>TB</sup> 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 EpH4<sup>RasTgfβ</sup> cells (Jechlinger et al., 2003), were also downregulated at the mRNA level by activated TrkB (Fig. 1E). Together, our analyses demonstrate that in epithelial cells TrkB induces a strong morphologic transformation resembling EMT. As EMT is a complex and dynamic
process involving a plethora of factors, we will refer to this process here conserva-
tively 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 suppres-
sion and tumor formation in rat epithelial cells (Geiger & Peeper, 2007). There-
fore, we speculated that kinase activity is required also for TrkB-induced EMT-like
transformation. Indeed, a kinase-inactive point mutant, TrkB<sup>K588M</sup> (Eide et al., 1996);
(Haapasalo et al., 2002) (Geiger & Peeper, 2007) was unable to morphologically
transform RK3E cells and to downregulate E-cadherin (Fig. 2A and B). In line with

![Figure 1](image_url)

**Figure 1. TrkB induces EMT-like transformation in epithelial cells.** (A) Mesenchymal morphology induced
by overexpression of TrkB+BDNF in epithelial RIE-1, RK3E and MCF10A cells. (B) Effect on expression of epil-
ithelial and mesenchymal markers by TrkB+BDNF as analyzed by western blotting. V=Vector, TB=TrkB+BDNF,
sm-actin=smooth muscle actin. β-actin serves as loading control. (C) TrkB+BDNF induces downregulation
of E-cadherin mRNA and upregulation of N-cadherin mRNA levels in RIE-1 and RK3E cells (measured by qRT-PCR, n=3, error bar represents standard deviation (SD)). (D) Effect of TrkB+BDNF on the transcription
factors Twist, Snail and Slug in RIE-1 and RK3E cells as measured by qRT-PCR (n=3, error bar represents SD).
(E) Downregulation of BMP-4 and CTGF mRNA levels by TrkB+BDNF as measured by qRT-PCR (n=3, error bar
represents SD).
this observation, stimulation of TrkB-expressing cells with recombinant BDNF for two days induced a spindle-shaped morphology and loss of E-cadherin, which was reverted upon withdrawal of BDNF for another three 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 (Tapley et al., 1992). RK3E<sup>TB</sup> 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 unspecific toxicity of the inhibitor, because RAS<sup>V12</sup>-transformed RK3E cells did not die upon treatment with K252a (Fig. 2E and F). Furthermore, we obtained similar results using another Trk inhibitor, GW441756 (Wood et al., 2004) (Fig. S1). K252a and GW441756 each blocked BDNF-induced autophosphorylation of TrkB and subsequent phosphorylation of ERK, but not RAS<sup>V12</sup>-induced ERK phosphorylation (Fig. 2F and Fig S1). Notably, parental RK3E cells were largely 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 (Behrens et al., 1991). Therefore, we next investigated whether E-cadherin corresponds to a critical target for TrkB function by re-introducing E-cadherin into spindle-shaped RK3E<sup>TB</sup> 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). Similar to our previous observations in RIE-1 and MCF10A cells (Douma et al., 2004; Geiger & Peeper, 2007), active TrkB suppressed anoikis also in RK3E cells (Fig. 3D). E-cadherin restoration impaired anoikis suppression by TrkB (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 (Yang et al., 2004), 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 and Fig. S2A). As expected, kinase-inactive TrkB failed to induce Twist. (Fig. S2B). To assess the requirement of Twist for TrkB function in
Figure 2. Continuous TrkB signaling is required for EMT-like transformation and survival of RK3E \( \text{TB} \) cells. (A) Kinase-inactive TrkB\(^{K588M} \) does not change epithelial morphology of RK3E cells. (B) Kinase-inactive Trk\(^{-} \) does not affect E-cadherin protein levels as shown by western blot analysis. (C) Stimulation of TrkB-expressing RK3E cells with recombinant BDNF induces morphological transformation within 2 days, which is reverted back to an epithelial morphology after removal of BDNF for 3 days. (D) Western blot analysis of the cells described in (C). (E) RK3E \( \text{TB} \) cells treated overnight with the Trk inhibitor K252a show increased cell death as measured by trypan blue exclusion. Average of three independent experiments is shown, error bars indicate SD. TB=TrkB+BDNF. (F) RK3E \( \text{TB} \) cells treated overnight with the Trk inhibitor K252a show cleaved caspase 3; K252a inhibits autophosphorylation of TrkB and BDNF-induced phosphorylation of ERK, all determined by western blot analysis. β-actin serves as loading control for all western blots. TB=TrkB+BDNF. (G) Cells growing in serum-reduced medium (1% FCS) were treated with BDNF for 2 days. Cells were harvested 1 day after BDNF was removed from the medium. Apoptotic cells in the supernatant were included in the analysis for panels E, F and G.
Figure 3. Loss of E-cadherin is an essential feature of TrkB function. (A) Western blot analysis of E-cadherin, TrkB and BDNF from independent cell clones expressing indicated cDNAs. β-actin serves as loading control. (B) Overexpressed E-cadherin localizes at the cell membrane, as shown by indirect immunofluorescence and confocal microscopy. TO-PRO stains DNA. (C) Epithelial morphology induced by overexpression of E-cadherin in RK3E<sup>B</sup> cells. (D) E-cadherin restoration impairs TrkB-mediated anoikis suppression. Vector- or RK3E<sup>B</sup> cells and derived cell clones overexpressing E-cadherin or vector control were cultured on ultra low cluster plates for 4 days and scanned at 1x magnification (left panel) or quantified by measuring protein levels (right panel, n=3, error bar represents SD). * indicates a measured value of 0. (E) E-cadherin restoration impairs TrkB-mediated anchorage-independent growth. 1000 cells expressing indicated cDNAs were grown in 0.4% agarose for 11 days; scan of 1x magnification is shown (left panel) and its quantification (right panel, error bar represents SD of an experiment done in triplicate).
this regard, we generated stable cell lines expressing TrkB, BDNF as well as a short hairpin (sh)RNA against Twist (or against EGFP, as a control). To rule out off-target effects (Echeverri et al., 2006), we used two independent, non-overlapping shRNAs against Twist. Upon expression of sh-Twist in polyclonal RK3ETB 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 (Fig. S2C). Consistently, Twist depletion caused a marked reduction in anoikis suppression (Fig. S3A), cell migration, invasion (Fig. S3B) and anchorage-independent growth in soft agar (Fig. S3C) induced by TrkB, which was not due to inhibition of cell proliferation (Fig. S3D).

Similar to our previous findings in RIE-1 cells (Douma et al., 2004; Geiger & Peeper, 2007), RK3ETB cells (but not parental RK3E cells, which are devoid of tumorigenic potential; data not shown and (Ruppert et al., 1991)) 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. 4E and S3E). In a previous study, using a different cell system, knockdown of Twist did not affect tumor growth but inhibited only metastasis (Yang et al., 2004), which probably reflects differences in the cellular context or origin. In view of the observed difference in primary tumor growth as a function of Twist expression, we considered this setting not suitable to study the requirement of Twist in TrkB-induced metastasis. Therefore, we injected cells intravenously into nude mice. Although this experimental metastasis assay precludes an assessment of the early steps of tumor progression (tumor growth, invasion, intravasation into vessels), it does allow measuring the capacity of tumor cells to colonize the lungs. Silencing of Twist in RK3ETB prolonged the survival of the mice (Fig. 4F), which is consistent with previous findings (Yang et al., 2004). These results suggest that Twist plays an important role both in TrkB-induced oncogenicity and metastasis.

TrkB-induced EMT-like transformation and induction of Twist is mediated via the MAPK pathway

To shed further light on how TrkB upregulates Twist and triggers EMT-like transformation, we assessed on which signaling pathways downstream of TrkB it depends. Several canonical signal transduction pathways are stimulated by TrkB, including MAP-kinase (MAPK) pathway, PI3-kinase (PI3K) pathway and RAC1 GTPase signaling (Huang & Reichardt, 2003; Miyamoto et al., 2006). Therefore, we used pharmacological inhibitors of MEK (U0126 (Favata et al., 1998)), PI3K (PI-103, (Fan et al., 2006)). To inhibit RAC1, we used NSC23766 (Sanz-Moreno et al., 2008) and a dominant negative variant, RACN17 (Ridley et al., 1992)). Inhibition of the MAPK pathway with U0126 was confirmed by measuring phospho (p) ERK levels (Fig. 5A). U0126
Figure 4. Twist is required for TrkB-induced EMT and tumorigenesis. (A) Induction of Twist protein levels in TrkB+BDNF–expressing RK3E cells analyzed on western blot. V=Vector, TB=TrkB+BDNF. CDK4 serves as loading control. (B) sh-Twist prevents morphologic transformation of RK3E cells by TrkB+BDNF. (C) sh-Twist prevents downregulation of E-cadherin by TrkB+BDNF in RK3E cells, as shown by western blot analysis for proteins as indicated. β-actin serves as loading control. (D) E-cadherin localizes at the cell membrane of RK3E\textsuperscript{TB} 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 into both flanks each with 1*10^5 RK3E\textsuperscript{TB} + indicated shRNAs. Growth curves for average tumor volumes are shown: n=8 tumors for sh-EGFP #1, sh-EGFP #2 and sh-Twist #1, n=11 tumors for sh-Twist #2. Error bar represent standard error of the mean (SEM). * indicates a p value < 0.01 in a two-sided student’s t-test. (F) Effect of sh-Twist on experimental metastasis. Mice were intravenously injected with 1*10^6 RK3E\textsuperscript{TB} + indicated shRNAs. Mice were euthanized when clinical symptoms became apparent; n=4 mice for 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.
Figure 5. TrkB-induced EMT-like transformation is mediated via the MAPK pathway. (A) Downregulation of phospho (p) ERK upon treatment with U0126. Cells were serum starved for 4 hours, pre-treated with 20 µM U0126 for ½ hour and stimulated with 50 ng/ml BDNF for 5 minutes. Cells were harvested in the presence of phosphatase inhibitors. (B) Induction of Twist in RK3E<sup>T</sup>B 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. This figure panel contains two parts derived from the same gel. (C) TrkB-induced EMT-like transformation is dependent on the MAPK pathway. RK3E<sup>T</sup>B cells were treated with 20 µM U0126 for 2 days and analyzed by western blot. (D) Morphology of the cells described in (C). (E) HA-Twist or Snail-HA overexpression in RK3E<sup>T</sup>B cells partially prevented the reversion to an epithelial morphology induced by U0126 treatment for 2 days. (F) Western blot analysis of cells described in (E). (G) Downregulation of pAKT upon treatment with PI-103. Cells were serum starved for 4 hours, pre-treated with 500 nM PI-103 for ½ hour and stimulated with 50 ng/ml BDNF for 5 minutes. Cells were harvested in the presence of phosphatase inhibitors. (H) Induction of Twist in RK3E<sup>T</sup>B cells is not dependent on the PI3K pathway. Cells were treated overnight with 500 nM PI-103 and analyzed on western blot. (I) TrkB-induced EMT-like transformation is not dependent on PI3K pathway. Cells were treated with 500 nM PI-103 for 2 days and analyzed on western blot. (J) Morphology of cells described in (I). β-actin serves as loading control for all western blots.
treatment abolished the induction of Twist by TrkB (Fig. 5B) and restored E-cadherin levels in RK3ETB cells (Fig. 5C), which was accompanied by a reversion of the spindle-shaped cell morphology towards an epithelial appearance (Fig. 5D). To rule out off-target effects of the U0126 inhibitor, we used a second MEK inhibitor, CI-1040 (Barrett et al., 2008), which gave identical results (Fig. S4). The effects of MEK inhibition on E-cadherin levels 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 either Twist, E-cadherin or cell morphology (Fig. 5H, I and J), despite the fact that pAKT levels could be effectively downregulated (Fig. 5G). Likewise, a RAC1N17 dominant negative mutant failed to significantly affect cell morphology, E-cadherin or Twist levels. Nor did it impede the migratory or invasive properties of RK3ETB cells (Fig. S5B-D). This was in spite of the observation that RAC1N17 suppressed RAC1 activity below that of control RK3E cells, as shown by RAC1 pull down assay (Fig. S5A). In line with this, treatment with the RAC1 inhibitor NSC23766 altered neither cell morphology nor E-cadherin levels (Fig. S5E and F).

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) specifically required for metastasis in this system. The zinc finger transcription factor Snail is a direct repressor of E-cadherin (Batlle et al., 2000; Cano et al., 2000). Studies in Drosophila have shown that Twist can induce Snail (Ip et al., 1992). Consistent with this, ectopic expression of Twist led to a five-fold induction of Snail mRNA levels in RK3E cells (Fig. 8A). Furthermore, Snail was induced by overexpression of TrkB+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 pro-oncogenic and pro-metastatic functions of TrkB, we generated stable cell clones of RK3ETB and either of two independent shRNAs against Snail (Fig. 6B). Similar to 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), as well as 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, F and S4D, E).
Snail is required for TrkB-induced metastasis

In contrast to sh-Twist, however, subcutaneous injection of sh-Snail-expressing RK3ETbb cells into nude mice resulted in the formation of primary tumors with kinetics indistinguishable from those of tumor cells expressing control shRNA (Fig. 7A, Fig. S6). 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 RK3ETbb tumors metastasized to the lungs, which was strongly suppressed upon Snail depletion (Table 1). This was accompanied by, on average, a >5-fold 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 in the lungs of intravenously injected RK3ETbb cells (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.

Table 1. Silencing of Snail reduces the incidence of lung metastases from subcutaneous tumors.

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<tr>
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*RK3ETbb + indicated shRNAs.

b Number of mice that developed pulmonary metastases (> 0.1mm in size) out of total number of mice with subcutaneous tumors.

Figure 6. Snail is required for TrkB-induced EMT-like transformation, anoikis resistance and anchorage-independent growth. (A) Increased protein levels of Snail in TrkB+BDNF-expressing MCF10A cells as judged by western blotting for the indicated proteins. (B) Snail knockdown in RK3ETbb, as measured by qRT-PCR (n=3, error bars represent SD). (C) Sh-Snail prevents morphologic transformation of RK3E cells by TrkB+BDNF. Photographs in Figure 6C and Figure 4B are derived from the same experiment. (D) Sh-Snail prevents downregulation of E-cadherin by TrkB+BDNF in RK3E cells, as judged by western blotting for the indicated proteins. (E) Sh-Snail impairs TrkB-mediated anoikis suppression. RK3E cells expressing indicated cDNAs were cultured on ULC plates and scanned at 1x magnification after 4 days (left panel) or quantified by determining the total protein levels (right panel, n=3, error bar represents SD). Pictures in Figure 6F and S2A are derived from the same experiment. (G) Sh-Snail impairs TrkB-mediated migration and invasion as determined by migration and invasion assay. 250.000 freshly trypsinized cells were seeded on control inserts (for migration) or matrigel (for invasion) and cells that translocated towards a serum gradient were counted 24h later. Error bars represent SD of 3 independent experiments. Graphs in Figures 6G and S2B were derived from the same experiment. (H) Sh-Snail impairs TrkB-mediated anchorage-independent growth. 1000 RK3E cells expressing indicated cDNAs were grown in 0.4% agarose for 11 days; 1x magnification is shown (left panel) and quantification of the number of colonies (right panel). β-actin serves as loading control for all western blots.
Figure 7. Snail is required for TrkB-induced metastasis. (A) Sh-Snail does not affect TrkB-induced tumorigenesis. Balb/c nude mice were subcutaneously injected into both flanks each with $1 \times 10^5$ RK3E<sup>Tk+</sup> indicated shRNAs. Growth curves for average tumor volumes are shown: n=8 tumors for all cell lines. Error bars represent SEM. Curves in Figure 7A and 4E were derived from the same experiment. (B) H+E-stained histological sections of lungs from the mice described in (A), showing metastatic tumor lesions. (C) Quantification of lung metastases from mice described in (A). Lesions ≥ 0.1mm were counted from 17-23 slides per cell line. Bar diagram shows average values of three experiments, error bars represent SD. (D) Effect of Sh-Snail on experimental metastasis. Mice were intravenously injected with $1 \times 10^6$ RK3E<sup>Tk+</sup> expressing indicated shRNAs. Mice were euthanized when clinical symptoms became apparent; n=4 for each cell line. Significance values were obtained by first combining the data from both shRNAs against the same gene (EGFP or Snail) and subsequently performing a Log rank test. Curves in Figures 7D and 4F were derived from the same experiment.
Snail acts downstream of Twist

Since it is unknown whether the Twist-Snail axis present in Drosophila (Ip et al., 1992) is operational also 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 of Snail predicts that the latter should be able to antagonize a phenotype that is altered as a function of the first. 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 RK3EBsh-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, also E-cadherin levels, which initially were high after Twist depletion, were suppressed strongly by overexpression of Snail-HA (Fig. 8E). These results suggest an epistatic relationship between Twist and Snail, with Twist acting upstream of 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 RK3ECsh-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+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 (Grunert et al., 2003). In line with a prototypic EMT, activated TrkB in rat epithelial cells induced a switch from E-cadherin to N-cadherin, 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 RK3EBsh cells to that of EpH4RasTGFβ cells, we observed that 67% of the significantly deregulated EMT-associated genes changed in the expected direction (Desmet
and Peeper, submitted and 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 E12/47 (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, and which depends on the presence of both Twist and Snail.

Figure 8. Snail acts downstream of Twist. (A) Induction of Snail mRNA by overexpression of HA-Twist in RK3E cells, as measured by qRT-PCR (n=3, error bar represents SD). (B) mRNA levels of Twist in RK3E cells overexpressing Snail-HA, measured by qRT-PCR (n=3, error bar represent SD). (C) Snail-HA and HA-Twist are both expressed to high levels and downregulate E-cadherin as showed by western blot analysis. (D) Functional rescue of cell morphology by overexpression of Snail-HA in RK3E cells expressing sh-Twist. (E) Western blot analysis for E-cadherin, Snail and BDNF of the cells used in (D). (F) No morphological change by overexpression of HA-Twist in RK3E + sh-Snail. (G) Western blot analysis for E-cadherin, Twist and other proteins as indicated of the cells used in (F). Open arrowhead indicates ectopic HA-Twist, filled arrowhead indicates endogenous Twist. (H) Snail protein levels are downregulated upon Twist knockdown in TrkB+BDNF expressing MCF10A cells as measured by western blot analysis. β-actin serves as loading control for all western blots.
We show that the EMT-like changes induced by TrkB via upregulation of Twist rely on a functional MAPK pathway. Similar to these findings, RAS\(^{V12}\) + TGFβ-induced EMT in EpH4 cells also is mediated mainly via MAPK pathway (Janda et al., 2002). 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 (Douma et al., 2004). Interestingly, whereas MEK inhibition did not affect the viability of RK3E\(^{TB}\) cells, treatment with the Trk inhibitors K252a or GW441756 induced a strong apoptotic response. The viability of RK3E cells expressing no or inactive TrkB hardly depended on Trk signaling, as both inhibitors induced little death in that setting. Remarkably, this was seen also 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’ (Weinstein & Joe, 2006). Only two 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 (Ip et al., 1992). 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 (Yang et al., 2004), 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, rather than in parallel, pathways. Furthermore, our results suggest that Twist acts upstream of 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 (Vesuna et al., 2008); a possible contribution of Snail
was not addressed in that study. It thus appears that E-cadherin is subject to two modes of Twist-dependent regulation: a direct one involving Twist binding to the promoter, and an indirect one involving Snail upregulation.

Whereas it has been proven difficult to provide histopathological evidence for EMT in human carcinomas (Yang & Weinberg, 2008), several reports have shown a correlation between Snail expression levels and the propensity to metastasize (reviewed in (Peinado et al., 2007)). For breast cancer particularly, Snail overexpression has been associated with lymph node metastasis (Blanco et al., 2002) and shorter overall survival (Elloul et al., 2005). Besides metastasis, Snail overexpression predicts also tumor relapse, which has functionally been confirmed in a breast cancer mouse model (Moody et al., 2005). Snail has also been associated with aggressive disease in other cancer types (for review, see (Becker et al., 2007)), including hepatocellular carcinoma (Sugimachi et al., 2003), ovarian cancer (Blechschmidt et al., 2007) and head and neck squamous cell carcinoma (Yang et al., 2007). 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 (Cano et al., 2000) and increased metastasis after orthotopic injection into nude mice (Yin et al., 2007). Conversely, RNAi-mediated inhibition of Snail impairs metastasis of subcutaneously injected HaCa4 cells (Olmeda et al., 2008). 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 (Olmeda et al., 2007a; Olmeda et al., 2007b; Olmeda et al., 2008). A similar phenomenon, albeit less dramatically, is shown in this paper for Twist. Therefore, it has not been straightforward to conclude whether delayed metastasis is due to metastasis-specific functions of Snail (e.g., cell invasion, anoikis suppression) or due to its more general function in 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 to compensate for the loss of Snail, something the soft agar conditions in vitro cannot. 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 on 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 (Zhou et al., 2004; Yook et al., 2006),
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 warranted also 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 (Batlle et al., 2000), playing an essential role in EMT (Behrens et al., 1989), tumor progression (Perl et al., 1998; Derksen et al., 2006) and metastasis (Oka et al., 1993; Mbalaviele et al., 1996; Onder et al., 2008). The loss of E-cadherin–mediated cell-cell adhesion is thought to promote cell migration and invasion (Birchmeier & Behrens, 1994). Furthermore, E-cadherin is part of a complex that regulates β-catenin signaling (Cavallaro & Christofori, 2001; Gottardi et al., 2001; Onder et al., 2008) and the activity of Rho GTPases (Noren et al., 2000), 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 (Onder et al., 2008). Furthermore, loss of E-cadherin induces Twist (Onder et al., 2008), 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 loss of E-cadherin resulted in anoikis suppression (Derksen et al., 2006). 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 induce also stem-cell like properties in non-tumorigenic, as well as transformed, human mammary epithelial cells (Mani et al., 2008). 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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References


Figure S1. Continuous TrkB signaling is required for survival of RK3ETB cells. Western blot analysis of control, TrkB, TrkB+BDNF or RASV12-expressing cells treated with GW441756 overnight. Apoptotic cells in the supernatant were included in the analysis.

Figure S2. (A) TrkB+BDNF induce Twist in MCF10A cells, as analyzed by western blot analysis. (B) Kinase-inactive TrkBK588M is unable to induce Twist protein levels, as shown by western blotting. Western blots are derived from the same experiment as Figure 2B. Asterisk indicates an aspecific band. (C) Twist is involved in TrkB-induced EMT-like transformation. sh-Twist prevents morphologic transformation of RIE-1 cells by TrkB+BDNF. β-actin serves as loading control for all western blots.
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TrkB + BDNF

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**B**

Relative protein levels

TrkB + BDNF

**C**

TrkB + BDNF

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TrkB + BDNF

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**E**

Percentage of mice not sacrificed

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Figure S4. TrkB-induced EMT-like transformation is mediated via MAPK signaling. (A) Downregulation of phospho (p) ERK upon treatment with CI-1040. Cells were serum starved for 4 hours, pre-treated with 1 µM CI-1040 for ½ hour and stimulated with 50 ng/ml BDNF for 5 minutes. Cells were harvested in the presence of phosphatase inhibitors. (B) Induction of Twist in RK3E<sup>TB</sup> cells requires an intact MAPK pathway, as shown by western blot analysis. Cells were treated overnight with 1 µM CI-1040 and analyzed by western blot analysis. Asterisk indicates an aspecific band. (C) TrkB-induced EMT-like transformation is dependent on MAPK signaling. RK3E<sup>TB</sup> cells were treated with 1 µM CI-1040 for 2 days and analyzed by western blot. (D) HA-Twist or Snail-HA overexpression in RK3E<sup>TB</sup> cells partially prevents the reversion of an epithelial morphology induced by CI-1040 treatment of 2 days. (E) Western blot analysis of cells described in (D).

Figure S3. Twist is involved in TrkB-induced anoikis resistance, migration, invasion and anchorage-independent growth. (A) RK3E cells expressing indicated cDNAs were cultured on ULC plates and scanned at 1x magnification after 4 days (left hand panel) or quantified by measuring total protein levels. Values are given relative to day 0 (right hand panel, n=3, error bars represent SD). Pictures were derived from the same experiment as Figure 6F. (B) Twist is involved in TrkB-induced migration and invasion as determined by Boyden chamber migration and invasion assay. 250,000 freshly trypsinized cells were seeded on control inserts (for migration) or matrigel (for invasion) and cells that translocated towards a serum gradient were counted 24h later. Error bars represent SD of 3 independent experiments. Graphs in Figure 6G are derived from the same experiment. (C) 1000 RK3E cells expressing indicated cDNAs were grown in 0.4% agarose for 11 days; 1x magnification (left hand panel) and quantification of macroscopic colonies (right hand panel, n=3 error bars represent SD) is shown. Pictures are derived from the same experiment as Figure 6H. (D) Sh-Twist does not affect cell proliferation in vitro. Equal numbers of RK3E<sup>TB</sup> cells expressing the indicated shRNAs were seeded on regular cell culture plates and counted every 2 to 3 days. (E) Sh-Twist impairs TrkB-mediated tumorigenesis, as shown by Kaplan-Meier survival curves. Balb/c nude mice were subcutaneously injected into both flanks each with 1*10<sup>5</sup> RK3E<sup>TB</sup> + indicated shRNAs. Mice were euthanized when tumors reached a size of 1 cm<sup>3</sup>. Three independent experiments were carried out with total n=15 (for each sh-EGFP #1, sh-EGFP #2), n=10 (sh-Twist #1), n=11 (sh-Twist #2). The experiment was terminated at 100 days. The corresponding values were used in the table. 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.
Figure S5. RAC1 is not involved in TrkB-induced EMT, migration and invasion. (A) Overexpression of a dominant negative mutant form of RAC1 (RAC1<sup>N17</sup>) in RK3E<sub>T8</sub> cells decreases RAC1 activity, as shown by a RAC1 pull down assay. Cells were lysed and incubated with Pak-CRIB peptide and bound to streptavidin-magnetic beads to determine the amount of active RAC1. Open arrowhead indicates mycRAC1<sup>N17</sup>, filled arrowhead indicates RAC1. Picture consists of two panels derived from the same blot. (B) No change in morphology by overexpressing RAC1<sup>N17</sup> in RK3E<sub>T8</sub> cells. (C) Western blot analysis of the cells described in (B). (D) RAC1 is not involved in TrkB-induced migration and invasion as determined by migration and invasion assay. 250,000 freshly trypsinized cells were seeded on control inserts (for migration) or matrigel (for invasion) and cells that translocated towards a serum gradient were counted 24h later. Error bars represent SD of an experiment done in triplicate. # indicates a p value > 0.1 in a two-sided students t-test. (E) No change in morphology of RK3E<sub>T8</sub> cells after 2 days treatment with RAC1 inhibitor NSC23766. (F) Western blot analysis of cells described in (E). β-actin serves as loading control for all western blots.

Figure S6. Snail depletion does not affect TrkB-induced tumorigenesis, as shown by Kaplan Meier survival curves. 1*10<sup>5</sup> RK3E<sub>T8</sub> cells expressing indicated shRNAs were subcutaneously injected into both flanks of Balb/c nude mice. Mice were euthanized when tumors reached a size of 1 cm<sup>3</sup>. Three independent experiments were carried out with total n=15 for each cell line. The experiment was terminated at 100 days and corresponding values were used in the Table. Significance values were obtained by first combining the data from both shRNAs against the same gene (EGFP or Snail) and subsequently performing a Log rank test.