Cancer Cell
Article

Induction of EMT by Twist Proteins as a Collateral Effect of Tumor-Promoting Inactivation of Premature Senescence

Stéphane Ansieau,[1] Jeremy Bastid,[1,2,6] Agnès Doreau,[1,6] Anne-Pierre Morel,[3,6] Benjamin P. Bouchet,[1,2] Clémence Thomas,[1,2] Frédérique Fauvet,[3] Isabelle Puisieux,[1] Claudio Doglioni,[4] Sara Piccinin,[5] Roberta Maestro,[5] Thibault Voeltzel,[1] Abdelkader Selmi,[1,2] Sandrine Valsesia-Wittmann,[1] Claude Caron de Fromentel,[1] and Alain Puisieux[1,2,3,*]

[1]Inserm, U590, Lyon, F-69008, France
[2]Université de Lyon, Lyon 1, Institut des Sciences Pharmaceutiques et Biologiques, Lyon, F-69008, France
[3]Centre Léon Bérard, FNCLCC, Laboratoire de Recherche Translationnelle, Lyon, F-69008, France
[4]Belluno City Hospital, Belluno, I-32100, Italy
[5]CRO IRCCS, Aviano National Cancer Institute, Aviano, I-33081, Italy
[6]These authors contributed equally to this work
*Correspondence: puisieux@lyon.fnclcc.fr
DOI 10.1016/j.ccr.2008.06.005

SUMMARY

Twist1 and Twist2 are major regulators of embryogenesis. Twist1 has been shown to favor the metastatic dissemination of cancer cells through its ability to induce an epithelial-mesenchymal transition (EMT). Here, we show that a large fraction of human cancers overexpress Twist1 and/or Twist2. Both proteins override oncogene-induced premature senescence by abrogating key regulators of the p53- and Rb-dependent pathways. Twist1 and Twist2 cooperate with Ras to transform mouse embryonic fibroblasts. Interestingly, in epithelial cells, the oncogenic cooperation between Twist proteins and activated mitogenic oncoproteins, such as Ras or ErbB2, leads to complete EMT. These findings suggest an unanticipated direct link between early escape from failsafe programs and the acquisition of invasive features by cancer cells.

INTRODUCTION

Twist proteins are highly conserved basic helix-loop-helix (bHLH) transcription factors that have important regulatory functions during embryogenesis. In *Drosophila*, the ancestral Twist protein (named DTwist) is crucial for proper gastrulation and mesoderm formation (Simpson, 1983; Thisse et al., 1987). In mammals, two Twist-like proteins, Twist1 and Twist2, share high structural homology (Li et al., 1995; Wolf et al., 1991). Gene deletion experiments have shown that *TWIST1* is required for closure of the neural tube during mouse development (Chen and Behringer, 1995), while *TWIST2* knockout mice display elevated expression of proinflammatory cytokines causing perinatal death (Sosic et al., 2003). Interestingly, this phenotype is also found in individuals doubly heterozygous for *TWIST1* and *TWIST2* alleles, reflecting some functional redundancy (Bialek et al., 2004). While Twist proteins are only expressed in a subset of mesodermally and ectodermally derived tissues, *TWIST1* is overexpressed in various human solid tumors including numerous types of carcinomas as well as sarcomas, gliomas, neuroblastomas, and melanomas (Yang et al., 2004; Kwok et al., 2005; Mironchik et al., 2005; Zhang et al., 2007; Ohuchida et al., 2007; Entz-Werle et al., 2005; Elias et al., 2005; Valsesia-Wittmann et al., 2004; Hoek et al., 2004). The role of Twist1 in tumor progression has been convincingly associated with the metastatic process (Yang et al., 2004). Exogenous overexpression

SIGNIFICANCE

Because cells are frequently subjected to abnormal growth signals, multicellular organisms develop two major safeguard programs, senescence and apoptosis, that can eliminate potentially deleterious cells at early stages of tumor development. The mechanisms by which precancer cells escape these protective barriers remain to be determined. Herein, we identify Twist proteins as decisive early drivers of tumorigenesis. Indeed, Twist1 and Twist2 abrogated oncogene-induced senescence by inhibiting key regulators of this safeguard program. Strikingly, this deleterious effect was associated with complete epithelial-mesenchymal transition (EMT), a process associated with the acquisition of invasive potential. These observations suggest that some metastatic capabilities of cancer cells can be acquired during malignant conversion as a side effect of the inactivation of primary failsafe mechanisms.

of Twist1 increases the invasive and metastatic abilities of human cancer cells by promoting the downregulation of E-cadherin and the induction of an epithelial-mesenchymal transition (EMT) (Yang et al., 2004; Kwok et al., 2005; Mironchik et al., 2005). EMT, which was first recognized as a feature of embryogenesis, converts epithelial cells into mesenchymal cells and promotes cell motility through profound disruption of cell-cell junctions and extensive reorganization of the actin cytoskeleton (Hay, 1995).

The results presented herein indicate that *TWIST2*, similarly to *TWIST1*, is overexpressed in a large variety of human primary tumors and cancer cell lines. We next demonstrate that both Twist1 and Twist2 inhibit premature senescence in cancer cells, a process identified as an initial barrier to tumor development. Indeed, senescence occurs in vivo in precancerous lesions in response to aberrant mitogenic signaling, and its inactivation is required for progression toward malignancy (Chen et al., 2005; Michaloglou et al., 2005; Collado et al., 2005). We further show that this property allows Twist proteins to cooperate with mitogenic oncoproteins, resulting in full transformation of murine cells. Interestingly, in human epithelial cells, escape from premature senescence mediated by Twist1 or Twist2 is associated with complete EMT. Altogether, these findings suggest an as yet undescribed link between early escape from failsafe programs and acquisition of metastatic features.

RESULTS

Twist1 and Twist2 Override Oncogene-Induced Senescence in Murine and Human Cancer Cells

Twist1 has been shown to play a major role in breast cancer progression by promoting EMT and favoring the metastatic process (Yang et al., 2004). To further evaluate the role of both Twist proteins in carcinoma progression, we first took advantage of the MMTV-*ErbB2/Neu* transgenic mouse model (unactivated rat *ErbB2/Neu* gene under the transcriptional control of the mouse mammary tumor virus promoter/enhancer). *ERBB2* is a major oncogene involved in human breast tumorigenesis, and this mouse model is considered to be an appropriate tool for deciphering the molecular pathways involved in breast cancer progression. After a long latency, which is believed to represent the time required for mammary epithelial cells to acquire additional cooperative events, MMTV-*ErbB2/Neu* transgenic mice stochastically develop focal mammary tumors that can eventually metastasize to the lungs (Guy et al., 1992). Analysis of *TWIST1* and *TWIST2* expression in twenty independent spontaneous mammary tumors demonstrated a frequent activation of the *TWIST2* gene (Figure 1). Indeed, whereas *TWIST1* expression levels remained weak in all tumors, *TWIST2* was significantly upregulated ($p < 0.0001$) in 12 of them (60%), suggesting that Twist2 might be involved in tumor progression.

In an initial approach to define potential Twist2 oncogenic functions, cancer cell lines derived from either *TWIST2*-positive or -negative tumors from MMTV-*ErbB2/Neu* transgenic mice were established, and the consequences of *TWIST2* depletion by RNA interference were evaluated. Surprisingly, knockdown of *TWIST2* in *TWIST2*-expressing cell lines invariably triggered cellular senescence characterized by flattened cytoplasm, G1 growth arrest, senescence-associated β-galactosidase

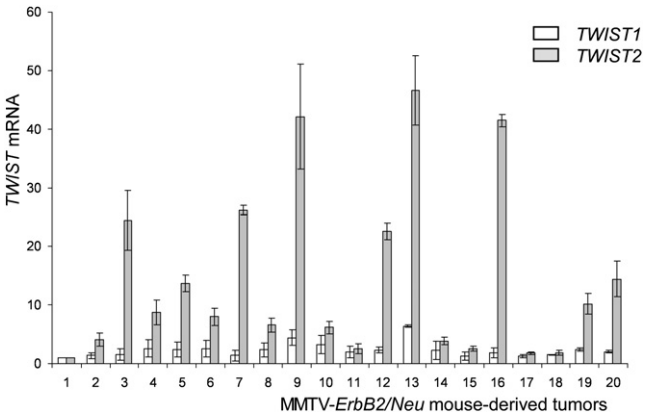

Figure 1. *TWIST2* Is Overexpressed in MMTV-*ErbB2/Neu*-Derived Mammary Tumors

TWIST1 and *TWIST2* gene expression in spontaneous tumors developed in MMTV-*ErbB2/Neu* transgenic mice as assessed by quantitative RT-PCR. Error bars represent mean ± SD of triplicate experiments.

(SA-β-gal) activity, and induction of the de novo marker of cellular senescence *DEC1* (Qian et al., 2008; Collado et al., 2005) (Figure 2). Similar results were obtained using two independent *TWIST2* shRNA sequences. As expected, these two shRNAs had no effect on *TWIST2*-negative cell lines, thus demonstrating the specificity of the observation.

As aberrant activation of ErbB2 in mammary epithelial cells triggers a premature senescence response (Trost et al., 2005), our observations suggested that Twist2 induction might override oncogene-induced senescence in vivo. This property was not limited to murine cells. In fact, *TWIST2* but also *TWIST1* depletion promoted senescence in human melanoma and breast cancer cell lines (Figure 3), linking Twist1 and Twist2 to the inactivation of this failsafe program.

TWIST1 and *TWIST2* Are Frequently Overexpressed in Human Cancers

Whereas the overexpression of *TWIST1* has already been described in a large variety of tumors, the status of *TWIST2* in malignancies remains largely unknown. We thus measured *TWIST1* and *TWIST2* mRNA levels in a wide range of human tumors ($n = 148$) and human tumor-derived cell lines ($n = 64$) encompassing eight different cancer types (Figure 4A; see also Figure S1 available online). Overall, *TWIST1* and *TWIST2* were significantly upregulated in primary tumor cells ($p = 0.002$ and $p = 0.04$, respectively) and in cancer cell lines ($p < 0.0001$ and $p = 0.0042$, respectively) compared to their normal counterparts. As shown in Figure 4A, *TWIST1* and *TWIST2* overexpression was particularly frequent in melanoma samples. Melanomas are malignant proliferation of cutaneous melanocytes that can develop from benign nevi. Premature senescence is a major safeguard mechanism preventing the progression from nevi to melanomas (Michaloglou et al., 2005). Although generally harboring activating mutations in the B-Raf oncoprotein (a major effector of Ras), nevi typically remain in a growth-arrested state for decades. Peeper and collaborators have demonstrated that cells within nevi display characteristic features of senescence (including expression of $p16^{Ink4a}$) and are growth arrested, whereas in

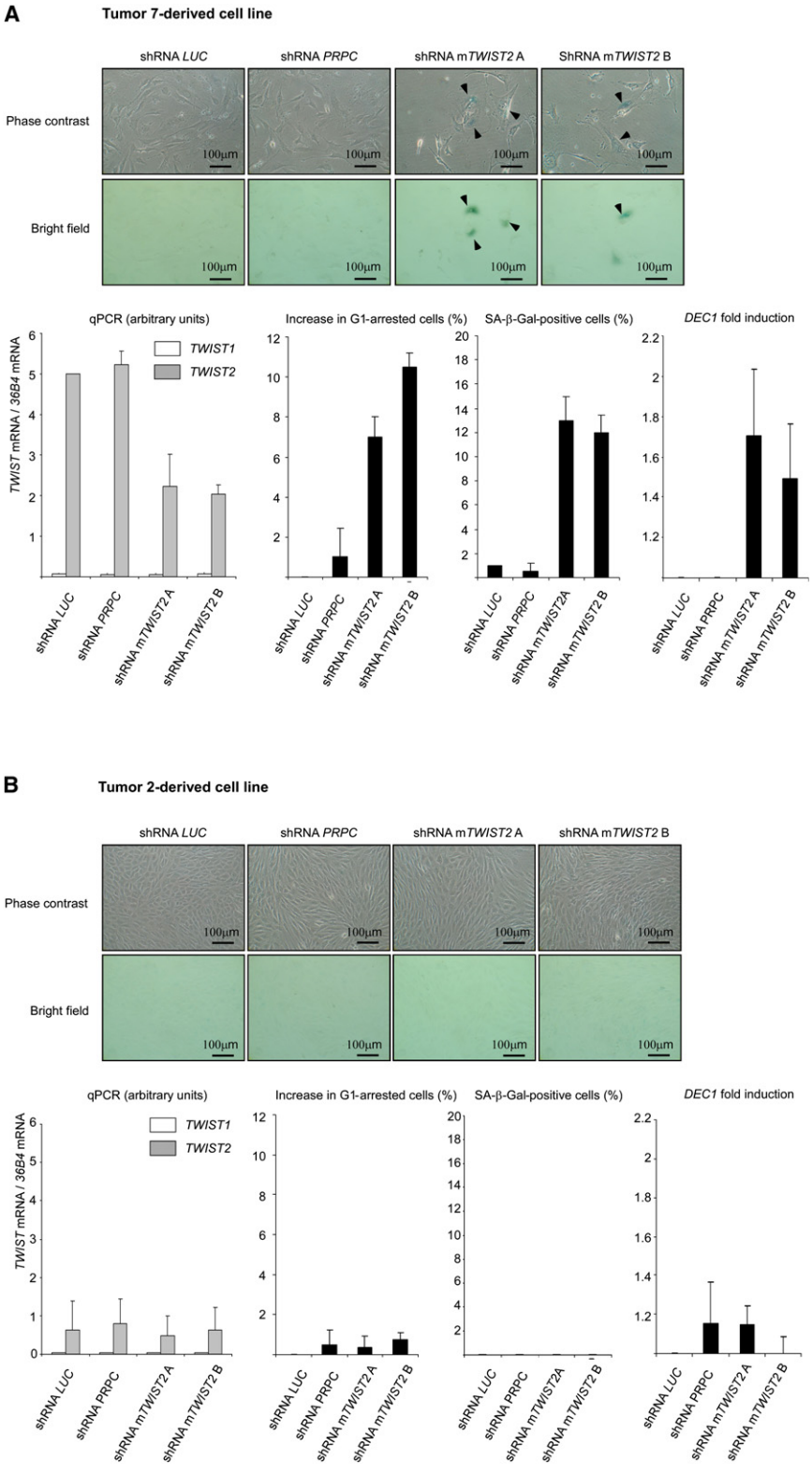

Figure 2. *TWIST2* Depletion Induces Senescence in MMTV-*ErbB2/Neu*-Derived Tumor Cells

Cells derived from MMTV-*ErbB2/Neu* transgenic mouse tumors either overexpressing *TWIST2* (tumor 7-derived cell line, [A]) or with no endogenous expression of *TWIST2* (tumor 2-derived cell line, [B]) were transiently infected with retroviral constructs expressing either shRNAs directed against the murine *TWIST2* gene (shRNA m*TWIST2* A and shRNA m*TWIST2* B) or shRNAs directed against the *luciferase* gene (shRNA *LUC*) or the prion pRpC protein (shRNA *PRPC*) as controls.

Upper panels: representative photomicrographs of cells obtained by phase-contrast microscopy and of SA-β-galactosidase staining observed in bright field.

Lower panels: *TWIST1* and *TWIST2* expression levels as assessed by real-time quantitative RT-PCR (qPCR; relative mRNA levels are indicated), % of increase in G1-arrested cells, % of SA-β-galactosidase (SA-β-Gal)-positive cells, and fold induction of the senescence marker *DEC1* are shown. Error bars represent mean ± SD of triplicate experiments.

melanoma, senescence is absent and cells proliferate (Michaloglou et al., 2005). Based on these observations, we evaluated Twist1 and $p16^{Ink4a}$ expression using immunohistochemistry in a series of 12 melanocytic nevi and 10 melanomas. (Of note, there is no reliable antibody against Twist2 currently available.) Strikingly, all nevi contained $p16^{Ink4a}$-expressing cells (50%–100% positive cells in 8 out of 12 cases), whereas no Twist1-expressing cells were observed. In contrast, high levels of Twist1 were detected in 5 out of 10 cases of melanoma. Consistent with a role of Twist1 in the inhibition of premature senescence, $p16^{Ink4a}$ immunoreactivity was either undetectable or low in these samples, with an exclusive pattern when compared with Twist1 immunoreactivity (Figure 4B).

Twist1 and Twist2 Cooperate with Activated Ras for Malignant Transformation and Disrupt Both p53 and Rb Tumor Suppressor Pathways

The induction of premature senescence following constitutive activation of the Ras/Raf mitogenic pathway is particularly well documented (Serrano et al., 1997). To further study the biological effects of Twist1 and Twist2, standard cooperation assays were performed in primary murine embryonic fibroblasts (MEFs) using an activated form of H-Ras ($H\text{-}Ras^{V12}$). As expected, MEFs infected only with $H\text{-}Ras^{V12}$ immediately stopped growing and underwent senescence characterized by flattened cytoplasm, induction of SA-β-gal activity, and G1 growth arrest (data not shown). In contrast, cells sequentially infected with $H\text{-}Ras^{V12}$ and either Twist1

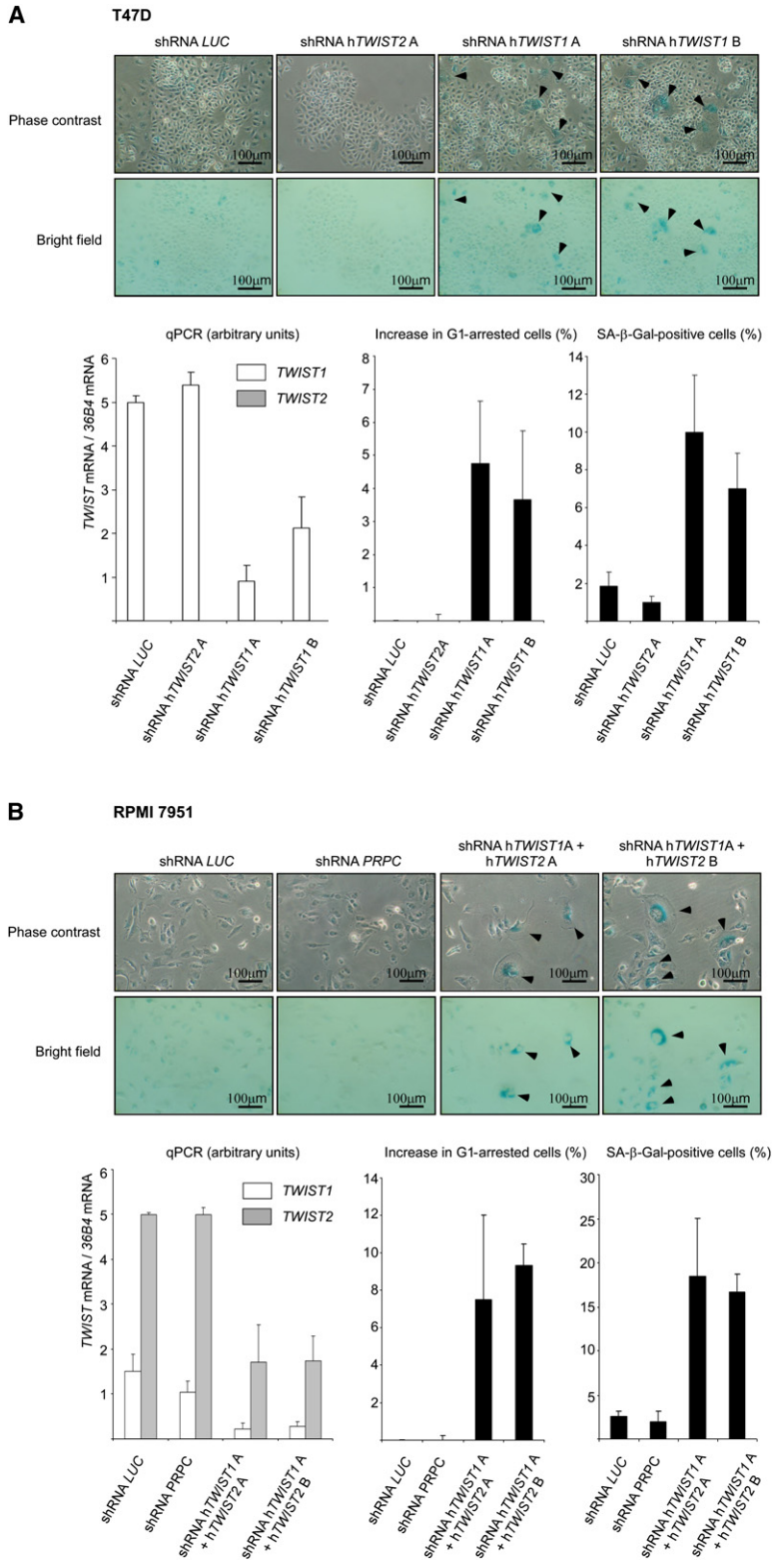

Figure 3. *TWIST1* and/or *TWIST2* Depletion Induces Senescence in Human Breast Cancer and Melanoma Cell Lines

T47D (human breast cancer cell line carrying four copies of *ERBB2*; [A]) and RPMI 7951 (human melanoma cell line carrying a mutant B-Raf^{V600E}; [B]) cells were doubly infected with lentiviral constructs expressing either shRNA directed against the human *TWIST1* (shRNA h*TWIST1* A and shRNA h*TWIST1* B) or *TWIST2* (shRNA h*TWIST2* A and shRNA h*TWIST2* B) genes as indicated or shRNAs directed against the *luciferase* gene (shRNA *LUC*) or the prion pRpC protein (shRNA *PRPC*) as controls.

Upper panels: representative photomicrographs of cells obtained by phase-contrast microscopy and of SA-β-galactosidase staining observed in bright field.

Lower panels: *TWIST1* and *TWIST2* expression levels as assessed by real-time qPCR (relative mRNA levels are indicated), % of increase in G1-arrested cells, and % of SA-β-Gal-positive cells are shown. Error bars represent mean ± SD of triplicate experiments.

or Twist2 continued proliferating (Figure 5A) and could be maintained in culture for months. MEFs overexpressing both H-Ras^{V12} and Twist1 or Twist2 exhibited hallmarks of malignant transformation such as loss of contact inhibition, growth in soft agar, and high tumorigenic potential as demonstrated after subcutaneous grafting into athymic *nude* mice (Figures 5B–5D; Figure S2). Although the time required for tumor growth varied slightly, clonogenic and tumorigenic capabilities were observed even in the presence of relatively low levels of ectopic Ras and/or Twist expression (data not shown). Upon histological examination, expanding xenografts displayed all of the features of aggressive sarcomas, with high levels of aberrant mitosis, anisokaryosis, and major angiogenic activity, further demonstrating a potent oncogenic cooperation between Twist proteins and Ras (Figure 5). Of note, although Twist proteins have been shown to regulate proinflammatory cytokine gene expression (Sosic et al., 2003), microarray analysis of MEFs and transformed MEFs did not show striking differences in expression of chemokines, interleukins, and their receptors (Table S1).

The p53 and Rb tumor suppressor pathways are known to play a pivotal role in the onset of premature senescence (Serrano et al., 1996). To further investigate the mechanisms by which Twist proteins inhibit oncogene-induced failsafe programs, we compared the endogenous expression of p16^{Ink4a}, ARF, p53, and p21^{Cip1} in MEFs infected with H-Ras^{V12} alone or in combination with Twist1 or Twist2 retroviral expression constructs. As shown in Figure 6A, Twist1 or Twist2 expression was sufficient to completely abrogate p16^{Ink4a} and p21^{Cip1} induction by activated Ras without downregulating ARF and p53 levels. Identical inhibitory effects were observed in cell lines either generated through the MEF transformation assays (Ras + Twist1, Ras + Twist2) or derived from tumors induced in athymic *nude* mice (Figure 6A). To confirm that Twist per se was sufficient to abrogate p16^{Ink4A} and p21^{Cip1} activation, we

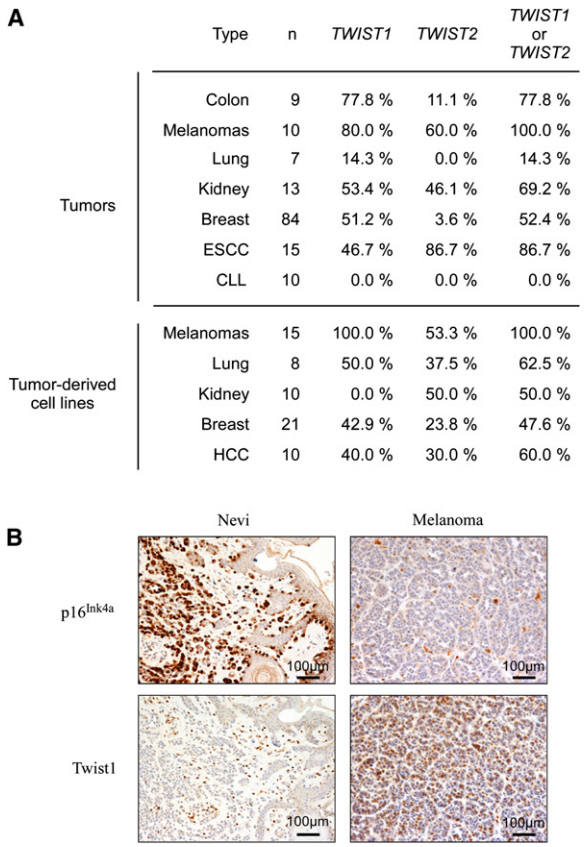

Figure 4. *TWIST1* and *TWIST2* Genes Are Overexpressed in Various Human Tumors and Tumor-Derived Cell Lines

(A) *TWIST1* and *TWIST2* gene expression was assessed by TaqMan real-time quantitative PCR analysis. Expression was compared with that of healthy tissue or normal cell counterparts. Percentages of samples harboring an overexpression of *TWIST1* or *TWIST2* genes (>2-fold) are shown. (More information is available in Figure S1 and the Supplemental Experimental Procedures.) ESCC, esophageal squamous cell carcinoma; CLL, chronic lymphoid leukemia; HCC, hepatocellular carcinoma.

(B) Immunohistochemical analysis of $p16^{Ink4a}$ and Twist1 protein levels in human nevi and melanomas. Brownish dots detected in the cytoplasm of nevus cells are melanin granules. The dark brown cells in nevi are fibroblasts revealing that dermal fibroblasts are reactive for Twist1.

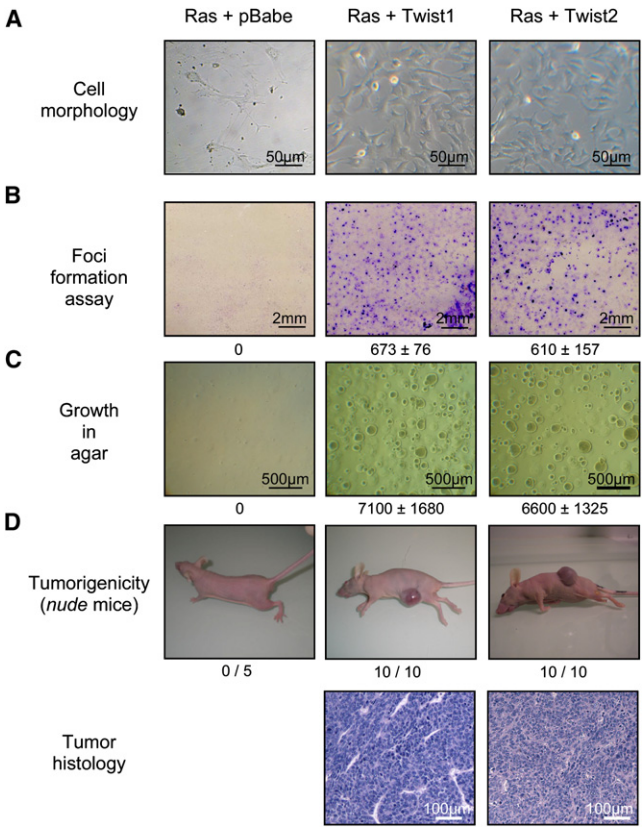

Figure 5. Twist Proteins Cooperate with Ras in MEF Transformation

Primary murine embryonic fibroblasts (MEFs) were sequentially infected with a retroviral vector encoding $H\text{-}Ras^{V12}$ (Ras) and a retroviral vector encoding Twist1, Twist2, or an empty vector (pBabe) as a control.

(A) Ras-infected cells underwent senescence, whereas Ras + Twist-infected cells are immortalized.

(B) Foci formation assays indicating that Ras + Twist-infected MEFs have lost cell contact inhibition. Foci numbers (for 3×10^3 cells) ± SD of triplicate experiments are indicated.

(C) Ras + Twist-infected MEFs grow on soft agar. Colony numbers (for 2×10^5 cells) ± SD of triplicate experiments are indicated.

(D) MEFs infected with Ras alone or with Ras + Twist1 or Twist2 were subcutaneously grafted into irradiated Swiss *nude* mice. Ras + Twist-transformed MEFs are tumorigenic. The number of mice developing tumors is indicated. The tumors display characteristics of aggressiveness (including high mitotic index and high vascular density).

knocked down *TWIST1* expression using RNA interference in Ras + Twist1-transformed MEFs. As shown in Figure 6B, inhibition of *TWIST1* expression led to a significant reinduction of the expression of both cyclin-dependent kinase inhibitors. Nevertheless, this reinduction was not sufficient to trigger senescence, which might be due to residual ectopic Twist1. To gain insight into the mechanisms underlying the regulatory roles of Twist proteins, the effect of Twist1 and Twist2 on the $p16^{INK4A}$ and $p21^{CIP1}$ genes was assessed in reporter assays. Consistent with the protein expression profile observed, Twist1 and Twist2 inhibited $p16^{INK4A}$ and $p21^{CIP1}$ promoter activation by $H\text{-}Ras^{V12}$ and p53 (Figures 6C and 6D). Although the molecular mechanisms involved in this effect are still under investigation, preliminary experiments suggest that the ability of Twist proteins to prevent the Ras-mediated activation of $p16^{INK4A}$ might be associated with their ability to interact with the Ras effector Ets2 (Figure S3).

Twist1 and Twist2 Overcome Premature Senescence and Cooperate Further with Mitogenic Oncoproteins to Promote EMT and Invasiveness

The ability of Twist1 and Twist2 to overcome oncogene-induced senescence was confirmed in a variety of human cells including primary mammary epithelial cells (HMECs) (Figure 7), IMR90 and MRC5 diploid fibroblasts (Figure S4), and immortalized BJ primary fibroblasts expressing an inducible version of $H\text{-}Ras^{V12}$ (data not shown). In all cases, expression of Twist1 or Twist2 completely abolished premature senescence induced by the activated mitogenic oncoprotein. We thereby confirmed that Twist1 or Twist2 overexpression was sufficient to override this crucial primary safeguard mechanism, thus providing a rationale for the observed oncogenic cooperation. Intriguingly, experiments with HMECs revealed a second level of cooperation. As

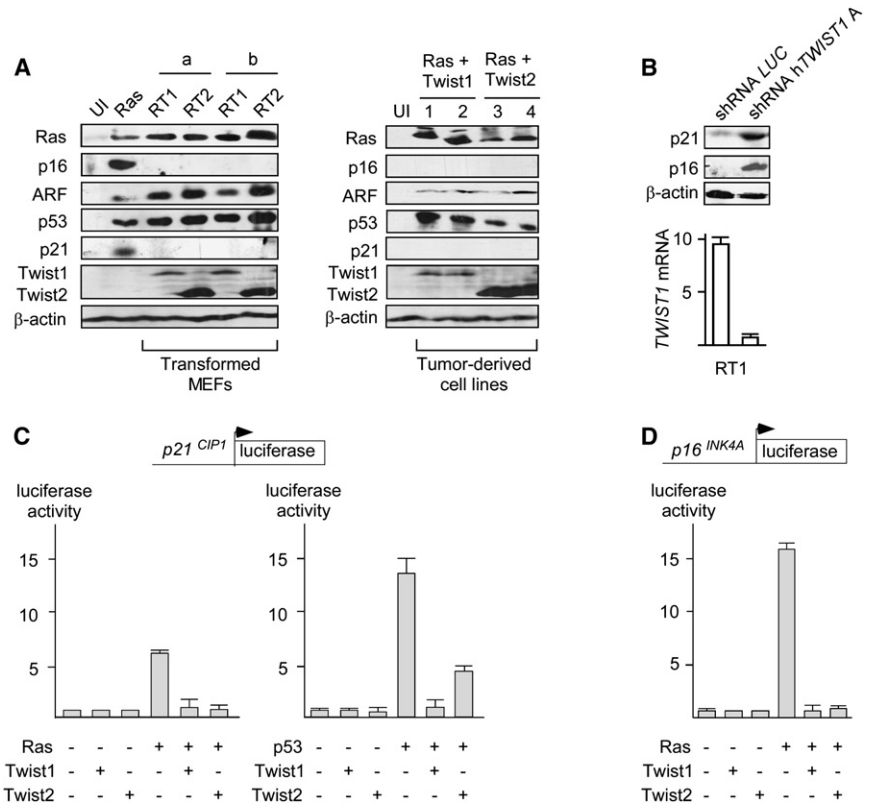

Figure 6. Effects of Twist1 and Twist2 Overexpression on Key Regulators of the p53 and Rb Signaling Pathways in Murine and Human Cells

(A) Western blots of cell lysates. Ras, $p16^{Ink4a}$, ARF, p53, $p21^{Cip1}$, Twist1, and Twist2 protein levels are shown as indicated. Left panel: cell lysates from uninfected (UI) MEFs, MEFs transiently infected with a H-Ras^{V12} (Ras) retroviral expression construct, and transformation assay-derived cell lines coexpressing H-Ras^{V12} and Twist1 (RT1) or H-Ras^{V12} and Twist2 (RT2). "a" and "b" represent two independent infection experiments. Right panel: cell lysates from uninfected MEFs or from murine tumor-derived cell lines coexpressing H-Ras^{V12} and Twist1 or H-Ras^{V12} and Twist2 as indicated.

(B) Cell lysates from cell lines coexpressing H-Ras^{V12} and Twist1 infected with a shRNA directed against the *TWIST1* gene (shRNA *TWIST1* A) or the *luciferase* gene (shRNA *LUC*). Inhibition of *TWIST1*, as assessed by real-time qPCR (relative mRNA levels are shown), is associated with the restoration of $p16^{Ink4a}$ and $p21^{Cip1}$ protein expression.

(C and D) Twist1 and Twist2 act as transcriptional repressors of both $p16^{INK4A}$ and $p21^{CIP1}$ genes. E1A-immortalized MEFs were transiently cotransfected with H-Ras^{V12}, p53, Twist1, and Twist2 expression constructs as indicated, along with the human $p21^{CIP1}$ (C) and $p16^{INK4A}$ (D) promoters. Relative luciferase activity is shown.

Error bars represent mean ± SD of triplicate experiments.

shown in earlier studies (Yang et al., 2004), ectopic expression of Twist1 alone in HMECs triggered several features of EMT (Figure 7). However, Twist1-mediated EMT was only partial as demonstrated by the observed cellular morphology, the significant but incomplete decrease of the E-cadherin epithelial marker, and the modest increase of the vimentin mesenchymal marker (Figures 7A and 7B). Of note, a similar effect was also observed with Twist2. In contrast, expression of both Twist1 (or Twist2) and H-Ras^{V12} (or ErbB2) triggered a total loss of E-cadherin, an increase in vimentin expression (Figure 7 and data not shown), and a concomitant acquisition of invasive properties (Figure 7C). A cooperative effect between Twist and mitogenic oncoproteins on the acquisition of mesenchymal features and invasive properties was also observed in Ras-transformed mammary epithelial MCF10A cells (MCF10A cells retrovirally infected by Ras^{V12}, MCF10A pLX Ras^{V12}) (Figure S5) (Konishi et al., 2007).

Because the role of Twist1 in the induction of EMT was originally demonstrated in polarized Madin-Darby canine kidney (MDCK) epithelial cells (Yang et al., 2004), we next examined the effects of Twist1 or Twist2 in the presence or absence of H-Ras^{V12} in these cells. In line with previous observations, overexpression of Twist1 or Twist2 alone was sufficient to trigger some morphological features of EMT, including a fibroblastic morphology, associated with a significant decrease of epithelial markers and increase of mesenchymal protein levels. Nevertheless, as demonstrated by the additional reduction of epithelial markers and further increase of mesenchymal protein expression, complete EMT required the presence of H-Ras^{V12}, confirming the cooperative effect (Figure 8). The mechanisms underlying this observation remain unknown. EMT is regulated by an elaborate interplay of signaling pathways. However, it is noteworthy that oncogenic Ras has been shown to cooperate with TGF-β to induce EMT (Oft et al., 1996) and that TGF-β is believed to act through the induction of several transcription repressors, including Twist1 (Kang and Massague, 2004).

Taken together, our results highlight an unexpected double-barreled oncogenic cooperation between Twist proteins and mitogenic oncoproteins such as Ras and ErbB2. On one hand, Twist1 or Twist2 allows cancer cells to override premature senescence, and on the other hand, concomitant activation of Twist and mitogenic signaling promotes complete EMT, thereby facilitating invasion and metastasis.

DISCUSSION

Aberrant cell proliferation and malignant transformation induced by some oncogenes are restrained by cellular senescence (Serrano et al., 1997). First considered as a cell culture artifact, premature senescence was recently confirmed in vivo as an early barrier against tumor progression (Bartkova et al., 2005; Chen et al., 2005; Gorgoulis et al., 2005; Collado et al., 2005). Indeed, it occurs in many different precancerous tissues in both humans and mice but is absent in malignant tumors, suggesting that its inactivation is a general requirement for full-blown cancer progression. However, the mechanisms allowing premalignant cells to bypass this primary failsafe program remain

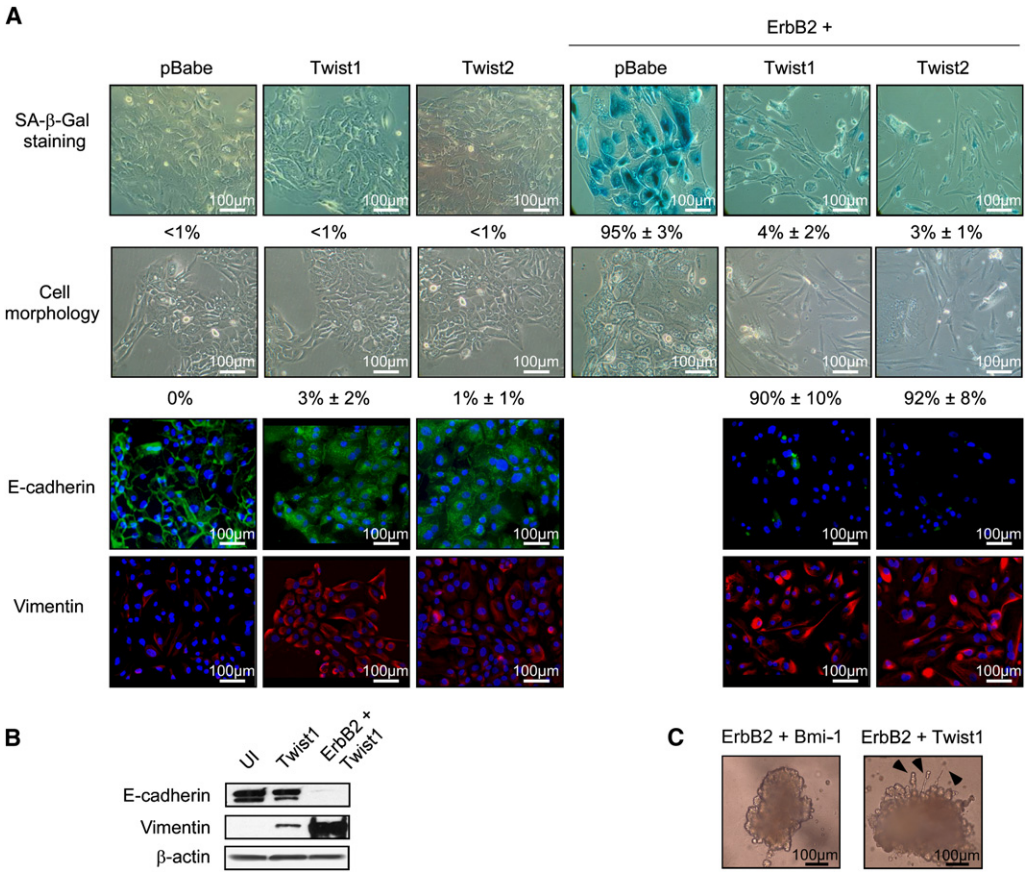

Figure 7. Twist-Dependent Failsafe Program Escape in Human Mammary Epithelial Cells Is Associated with Complete EMT and Acquisition of Invasive Properties

(A) Human mammary epithelial cells (HMECs) were infected with Twist1 or Twist2 retroviral expression constructs (left three columns) or sequentially infected with ErbB2 and Twist1 or Twist2 retroviral expression constructs (right three columns) as indicated. First row: representative photomicrographs of SA-β-Gal staining. Percentages of SA-β-Gal-positive cells (±SD of triplicate experiments) are indicated. Second row: phase-contrast microscopy showing cell morphology. Percentages of fibroblastic cells (±SD of triplicate experiments) are indicated. Third and fourth rows: E-cadherin (epithelial marker) and vimentin (mesenchymal marker) expression analysis by immunofluorescence. Nuclei were counterstained using Hoechst.

(B) Expression of E-cadherin and vimentin proteins in the same cells was analyzed by western blotting.

(C) Comparison by Matrigel invasion assay of the invasive properties of HMEC cells sequentially infected with ErbB2 and either Bmi-1 or Twist1 retroviral expression constructs as indicated. Bmi-1 was used as a control due to its ability to override oncogene-induced senescence.

largely unknown. Our results show that Twist1 and Twist2, two important regulators of embryogenesis, are frequently activated in human cancer cells and that their induction is sufficient to override oncogene-induced senescence in both murine and human cells by inhibiting both the p53 and Rb tumor suppressor pathways. In line with these observations, Twist proteins cooperate with Ras to transform MEFs, further demonstrating their oncogenic properties. At least in vitro, the Twist1 and Twist2 oncogenic activities appear to rely largely upon the inhibition of premature senescence. Indeed, in conditions where Ras activation does not trigger senescence, e.g. targeted knockin of a mutant *K-Ras* gene in immortalized MCF10A breast cells, no Twist-dependent transforming activity as assessed by soft agar assay (Figure S6) could be observed. Of note, MCF10A is a nontumorigenic but immortal HMEC cell line displaying a rearrangement and a deletion affecting the *INK4/ARF* locus that lead to a homozygous loss of the $p16^{INK4A}$, *ARF*, and $p15^{INK4B}$ genes (Cowell et al., 2005).

The role of Twist proteins in the inhibition of premature senescence is concordant with previous studies that reported the induction of Twist1 expression during the progression toward malignant stages in several cancer types including prostate, bladder, and pancreatic lesions (Kwok et al., 2005; Zhang et al., 2007; Ohuchida et al., 2007). The inverse correlation between $p16^{Ink4a}$ and Twist1 protein levels in nevi and melanoma samples also supports the role of Twist proteins as negative modulators of $p16^{INK4A}$ and as crucial inhibitors of oncogene-induced senescence in human cancer cells. As we have previously demonstrated that Twist1 and Twist2 are able to inhibit Myc-induced apoptosis in vitro and in vivo (Maestro et al., 1999; Valsesia-Wittmann et al., 2004), Twist proteins appear to act as general inhibitors of oncogene-induced safeguard programs. Noticeably, in response to activated Ras, Twist1 and Twist2 appear to act by downregulating the cyclin-dependent kinase inhibitors $p16^{Ink4a}$ and $p21^{Cip1}$ without affecting ARF levels, whereas ARF is a crucial Twist target in response to aberrant

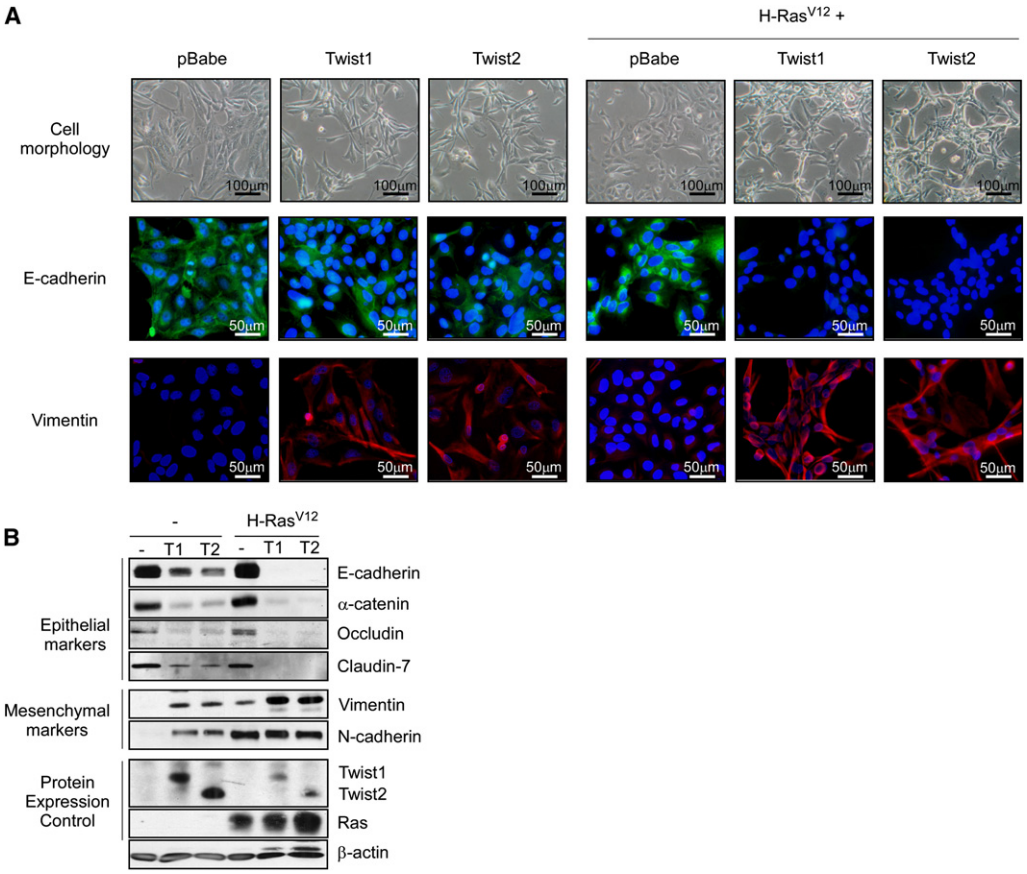

Figure 8. Twist Proteins Cooperate with Ras to Induce Complete EMT in MDCK Cells

Madin-Darby canine kidney (MDCK) cells were infected with Twist1 or Twist2 retroviral expression constructs (left three columns) or sequentially infected with H-Ras^{V12} and Twist1 or Twist2 retroviral expression constructs (right three columns) as indicated.

(A) First row: representative photomicrographs of cells obtained by phase-contrast microscopy. Second and third rows: E-cadherin and vimentin expression analysis by immunofluorescence. Nuclei were counterstained with Hoechst.

(B) Expression analysis of epithelial and mesenchymal markers, Twist1, Twist2, and Ras proteins by western blotting.

Myc activation (Maestro et al., 1999; Valsesia-Wittmann et al., 2004). Although the underlying mechanisms remain to be determined, the differential effect on *ARF* gene expression has been confirmed in reporter assays (data not shown).

Escape from oncogene-induced senescence and apoptosis being a general prerequisite for malignant conversion, our observations suggest that Twist1 and Twist2 are early drivers of tumor progression and provide a rationale for the high frequency of *TWIST1* and *TWIST2* overexpression in a large variety of human tumors. Furthermore, these findings shed new light on the role of Twist proteins in EMT and metastasis. Several groups have previously reported that high Twist1 expression promotes EMT and correlates with tumor invasion and metastasis in a variety of human cancers (Yang et al., 2004; Mironchik et al., 2005; Kang and Massague, 2004). Overall, our data show that in cells with aberrant mitogenic signaling, Twist1 or Twist2 reactivation overrides primary safeguard mechanisms to trigger cell transformation and, as a side effect, promotes EMT in cooperation with mitogenic oncoproteins, favoring invasiveness. In conditions where the mitogenic activation does not trigger senescence (due either to low levels of oncoprotein or to alternative mechanisms leading to a deregulation of safeguard programs), Twist1 and Twist2 do not display major transforming capabilities but still cooperate with activated oncoproteins in promoting EMT (Figures S5 and S6).

A prevailing model proposes that transcription factors involved in EMT promotion, such as Twist1, Snail, and Slug, are induced in rare cancer cells residing at the invasive edge of advanced carcinomas in response to signals released by the activated stroma (Scheel et al., 2007). According to this model, *TWIST1* induction will not provide any growth advantage within the primary tumor and will specifically favor cell dissemination, particularly the steps of motility and invasiveness. Based on our observations, we propose an alternative model in which, in the presence of aberrant mitogenic signaling, reactivation of the developmental proteins Twist1 and Twist2 promotes the transition from a premalignant to a malignant stage by inactivation of innate failsafe programs. Due to the cooperative effect of Twist1 or Twist2 and mitogenic factors on EMT promotion, this model predicts that most of the cancer cells within the primary tumor will be responsive to EMT-inducing signals from

the microenvironment, highly increasing the risk of metastatic dissemination.

Challenging the classical view of metastasis as the final step of tumor progression, recent observations further support the notion that early transformed cells are capable of dissemination. Specifically, Klein and colleagues have reported that disseminated cancer cells in the bone marrow of breast cancer patients exhibit different and fewer alterations than their matched primary tumors (Schmidt-Kittler et al., 2003; Schardt et al., 2005). By using BALB/c mice transgenic for the activated rat *ErbB2*/*Neu* gene, the same group recently demonstrated that dissemination of epithelial cells begins shortly after expression of the oncogenic transgene, as early as the stage of atypical hyperplastic lesion (Husemann et al., 2008). Strikingly, unlike both normal mammary glands and the invasion front of advanced tumors, these early lesions are characterized by a significant overexpression of *TWIST1* (of note, *TWIST2* expression was not examined by these authors). Although further work is warranted to confirm these observations in human cancers, these and our findings support the hypothesis that reactivation of Twist proteins in vivo promotes malignant conversion concomitantly with metastatic dissemination.

EXPERIMENTAL PROCEDURES

Constructs

N-terminally FLAG-tagged human Twist1, Twist2, and H-Ras^{V12} proteins were expressed from the pBabe, pCI-neo, or pcDNA3 expression vectors (Promega). The human *ERBB2* cDNA was subcloned into the pBabe retroviral expression vector.

Human *ARF*, *p16^{INK4A}*, and *p21^{CIP1}* luciferase reporter constructs have been described previously (Linggi et al., 2002; Hara et al., 1996; el-Deiry et al., 1993). The H-Ras^{V12} pBabe and the Bmi-1 pBabe expression constructs were generous gifts from the laboratories of Robert A. Weinberg and Goberdhan P. Dimri.

shRNAs directed against the human *TWIST1* gene (shRNA h*TWIST1* A, 5′-GCTGAGCAAGATTCAGACC-3′ [Yang et al., 2004]; shRNA h*TWIST1* B, 5′-GTACATCGACTTCCTCTAC-3′), the human *TWIST2* gene (shRNA h*TWIST2* A, 5′-GCAAGAAGTCGAGCGAAGA-3′; shRNA h*TWIST2* B, 5′-GCTGAGCAAGATCCAGACG-3′), and the murine *TWIST2* gene (shRNA m*TWIST2* A, 5′-GCAAGAAATCGAGCGAAGA-3′; shRNA m*TWIST2* B, 5′-GCTCAGCAAGATCCAGACG-3′) and control shRNAs directed against the *luciferase* GL3 gene (shRNA *LUC*, 5′-CTTACGCTGAGTACTTCGA-3′) and the prion pRpC protein (shRNA *PRPC*, 5′-TGAGCAGGCCCATCATACA-3′) were subcloned into the pRETRO-SUPER and the lentiviral pLVTHM expression vectors.

Cell Culture and Retroviral Infection

Primary MEFs (derived from C57BL/6 × DBA2 F1 mice), E1A-immortalized MEFs, transformed MEF cell lines, and tumor-derived cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1 mM HEPES (GIBCO), and 0.1 mM nonessential amino acids. RPMI 7951, T47D, MDCK, MCF10A, and HMEC cell lines were grown as recommended by the American Type Culture Collection (ATCC).

Plt-E and Plt-A producer cells were used to generate retroviral stocks. 3 × 10^6 Plt-E (-A) cells were transfected by calcium phosphate precipitation with 10 μg of retroviral vector and then placed after 24 hr in 5 ml of fresh medium for viral production. Forty-eight hours after transfection, the supernatant was collected, filtered (0.45 μm, Millipore), and supplemented with 4 μg/ml of polybrene (Sigma). The viral stock was placed in contact with 1 × 10^6 MEFs (or 2.5 × 10^5 human cells) for 3 hr, and infected cells were selected after 24 hr with appropriate antibiotics. Cooperation assays were performed by sequential infections using Ras- or ErbB2-expressing constructs and Twist1, Twist2, or empty (pBabe) expression constructs, spaced by a 48 hr period of time. Foci formation and growth on agar were assessed as described in Petrenko et al. (2003). *TWIST1* and/or *TWIST2* depletion experiments were performed by a double 48 hr-spaced infection of cells with retroviral or lentiviral shRNA expression virus using Plt-E or 293T cells as packaging cell lines.

Real-Time qRT-PCR

SYBR green quantitative PCR (Figure 1, Figure 2, Figure 3, and Figure 6) was performed using *36B4* as an internal control gene. Trans-species primer pairs were 5′-GCAGGACGTGTCCAGCTC-3′ and 5′-CTGGCTCTTCCTCGCTGTT-3′ for *TWIST1*, 5′-GCAAGAAGTCGAGCGAAGAT-3′ and 5′-GCTCTGCAGCTCCTCGAA-3′ for *TWIST2*, 5′-GGCGGGGAATAAAACGGAGCGA-3′ and 5′-CCTCACGGGCACAAGTCTGGAA-3′ for *DEC1* (Collado et al., 2005), and 5′-GCTGATGGGCAAGAACACCA-3′ and 5′-CCGGATGTGAGGCAGCAGTT-3′ for *36B4*.

The TaqMan quantitative PCR method used to analyze *TWIST* expression in human primary tumors and tumor-derived cell lines is described in detail in the Supplemental Experimental Procedures.

Human Tumor Samples

Human tissue samples were obtained from the Centre de Ressources Biologiques (Centre Léon Bérard, French agreement number DC-2008-99) after approval by the Comité de Protection des Personnes Lyon Est and by the institutional review board and ethics committee of Centre Léon Bérard, with fully informed patient consent.

Reporter Assays

3 × 10^5 E1A-immortalized MEFs were cotransfected with 1.5 μg of reporter construct, 0.5 μg of expression vector, and 0.3 μg of internal pRL-TK-luc construct (Promega) using jetPEI transfectant (Q-Biogen). Twenty-four hours after transfection, luciferase activity was monitored using the Dual-Luciferase Reporter Assay System (Promega) and a luminometer (Luminoskan Ascent, Labsystem).

Mouse Xenografts

Animal maintenance and experiments were carried out in accordance with the animal care guidelines of the European Union and with French laws and were validated by the Comité Régional d'Ethique Animale CNRS Rhône-Alpes. Six-week-old female athymic Swiss nude mice (Charles River Laboratories) were subcutaneously grafted in the left flank with 1 × 10^5 Ras + Twist1 or Twist2 retrovirally infected MEFs. Tumor growth was monitored twice a week with calipers at the site of injection. Animals were allowed to form tumors up to 1.5 cm in diameter, at which point they were euthanized. Each tumor was dissected and either fixed in formaldehyde and processed for histopathologic examination or used for protein or RNA extraction.

Immunoblot Analysis

Cell lysates were prepared in RIPA buffer. Protein expression was examined by western blotting using rabbit anti-mouse p16^{Ink4a} M-156 and anti-human p16^{Ink4a} H-156 (Tebu-bio), anti-mouse p21^{Cip1} C-19 (Tebu-bio), anti-Ras C-20 (Tebu-bio), anti-mouse ARF Ab80 (Abcam), anti-human ARF Ab1 (Neo-Markers), anti-mouse p53 CM5 (Novocastra), and anti-Claudin-7 (Zymed) polyclonal antibodies and mouse anti-human p21^{Cip1} SX118 (Dako), anti-β-actin AC15 (Sigma), anti-FLAG M2 (Stratagene), anti-E-cadherin, anti-N-cadherin, anti-α-catenin, anti-ZO-1 (BD Biosciences), anti-vimentin V9 (Dako), and anti-occludin (Zymed) monoclonal antibodies for primary detection. Horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse antibodies (Amersham) were used as secondary antibodies. Western blots were revealed using an ECL Detection Kit (Amersham).

Characterization of Senescent Cells

SA-β-Galactosidase Staining

Cells were fixed in 3% formaldehyde, washed three times with PBS, and incubated at 37°C for 12 hr in buffer containing 40 mM sodium phosphate (pH 6), 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 150 mM NaCl, 2 mM $MgCl_2$, and 1 mg/ml X-Gal.

Flow Cytometry Analysis

Trypsinized cells were washed in PBS, fixed in cold 70% ethanol/PBS for 30 min, washed, and incubated for 1 hr in 1 ml PBS containing 0.1 mg/ml RNase A and 20 mg/ml propidium iodide. Cell-cycle profiles were then

determined using a FACSCalibur flow cytometer (BD Biosciences), and data were analyzed using CellQuest software.

Immunofluorescence Analysis

Cells were seeded onto glass coverslips 24 hr prior to treatments. Cells were fixed with 4% formaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 20 min at room temperature, saturated for 30 min with 10% FCS in PBS, washed in PBS, incubated overnight at 4°C with monoclonal anti-E-cadherin (BD Biosciences) or anti-vimentin V9 (Dako) antibodies, and then incubated for 1 hr with FITC- or TRITC-conjugated rabbit anti-mouse antibodies (Dako). Cells were counterstained with 2 μg/ml Hoechst (33258, Sigma). Coverslips were then mounted with Fluoromount-G (Southern Biotech).

Immunohistochemistry

Routine formalin-fixed and paraffin-embedded excision biopsies of a series of melanocytic neoplasms (including 12 nevi and 10 melanomas) were analyzed. Immunohistochemistry was performed on 5 μm tissue sections. All specimens were immunostained with a non-biotin detection system (Bond Polymer Refine, Leica Microsystems), with diaminobenzidine development. Heat-induced antigen retrieval was performed using Tris-EDTA buffer (pH 9.0) in a water bath at 95°C for 30 min. Monoclonal anti-$p16^{Ink4a}$ (clone JC8, NeoMarkers) and anti-Twist1 (clone Twist2C1a, Biomatrix) antibodies were used. Stainings were performed with an automatic immunostainer (Bond System, Leica Microsystems).

Matrigel Invasion Assays

Matrigel (BD Biosciences) was added to the wells of an eight-well Labtek chamber in a volume of 300 μl/well. A Matrigel plug of about 1 mm diameter was removed. The hole was successively filed with 1×10^5 cells and 100 μl of Matrigel. Appropriate growth medium was added on top. Cultures were analyzed for up to 4 days. Areas of migration were visualized using an Olympus IX50 (NA 0.075). Samples were performed in duplicate.

ACCESSION NUMBERS

Microarray data have been entered into the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE11756.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, Supplemental References, six figures, and one table and can be found with this article online at http://www.cancercell.org/cgi/content/full/14/1/79/DC1/.

ACKNOWLEDGMENTS

The authors thank Isabelle Treilleux and Jean-Philippe Michot (Anatomy-Cytopathology Unit, Centre Léon Bérard); Joël Lachuer (ProfileExpert, Bron, France); and Gaël Grelier, Carole Audoynaud, Claire Ciancia, Fanny Courbis-Poncet, and Isabelle Durand for technical assistance. We also thank Eric Tabone (Centre de Ressources Biologiques, Centre Léon Bérard), Elisabeth Brambilla, and Jean Tanitakis for providing tumor samples; Gordon Peters and Takahiro Maeda for providing the luciferase reporter constructs; Patrizia Casalini for providing the *ErbB2* expression vector; R.A. Weinberg for providing the $H\text{-}Ras^{V12}$ retroviral expression construct; G.P. Dimri for providing the Bmi-1 retroviral expression construct; and Ben Ho Park for providing the MCF10A-derived cell lines. The authors extend special thanks to Patrick Mehlen and Raphaël Rousseau for scientific discussions.

This work was supported by grants from the Institut National du Cancer, the Association pour la Recherche contre le Cancer (ARC), the Ligue Nationale contre le Cancer, the Comités de l'Ain et du Rhône, and the Breast Cancer Research Foundation. R.M. is supported by the Italian Association for Cancer Research (AIRC).

Received: October 10, 2007
Revised: May 9, 2008
Accepted: June 11, 2008
Published: July 7, 2008

REFERENCES

Bartkova, J., Horejsi, Z., Koed, K., Kramer, A., Tort, F., Zieger, K., Guldberg, P., Sehested, M., Nesland, J.M., Lukas, C., et al. (2005). DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature *434*, 864–870.

Bialek, P., Kern, B., Yang, X., Schrock, M., Sosic, D., Hong, N., Wu, H., Yu, K., Ornitz, D.M., Olson, E.N., et al. (2004). A twist code determines the onset of osteoblast differentiation. Dev. Cell *6*, 423–435.

Chen, Z., Trotman, L.C., Shaffer, D., Lin, H.K., Dotan, Z.A., Niki, M., Koutcher, J.A., Scher, H.I., Ludwig, T., Gerald, W., et al. (2005). Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. Nature *436*, 725–730.

Chen, Z.F., and Behringer, R.R. (1995). twist is required in head mesenchyme for cranial neural tube morphogenesis. Genes Dev. *9*, 686–699.

Collado, M., Gil, J., Efeyan, A., Guerra, C., Schuhmacher, A.J., Barradas, M., Benguria, A., Zaballos, A., Flores, J.M., Barbacid, M., et al. (2005). Tumour biology: senescence in premalignant tumours. Nature *436*, 642.

Cowell, J.K., Laduca, J., Rossi, M.R., Burkhardt, T., Nowak, N.J., and Matsui, S. (2005). Molecular characterization of the t(3;9) associated with immortalization in the MCF10A cell line. Cancer Genet. Cytogenet. *163*, 23–29.

el-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W., and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell *75*, 817–825.

Elias, M.C., Tozer, K.R., Silber, J.R., Mikheeva, S., Deng, M., Morrison, R.S., Manning, T.C., Silbergeld, D.L., Glackin, C.A., Reh, T.A., and Rostomily, R.C. (2005). TWIST is expressed in human gliomas and promotes invasion. Neoplasia *7*, 824–837.

Entz-Werle, N., Stoetzel, C., Berard-Marec, P., Kalifa, C., Brugiere, L., Pacquement, H., Schmitt, C., Tabone, M.D., Gentet, J.C., Quillet, R., et al. (2005). Frequent genomic abnormalities at TWIST in human pediatric osteosarcomas. Int. J. Cancer *117*, 349–355.

Gorgoulis, V.G., Vassiliou, L.V., Karakaidos, P., Zacharatos, P., Kotsinas, A., Liloglou, T., Venere, M., DiTullio, R.A., Jr., Kastrinakis, N.G., Levy, B., et al. (2005). Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature *434*, 907–913.

Guy, C.T., Webster, M.A., Schaller, M., Parsons, T.J., Cardiff, R.D., and Muller, W.J. (1992). Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc. Natl. Acad. Sci. USA *89*, 10578–10582.

Hara, E., Smith, R., Parry, D., Tahara, H., Stone, S., and Peters, G. (1996). Regulation of p16CDKN2 expression and its implications for cell immortalization and senescence. Mol. Cell. Biol. *16*, 859–867.

Hay, E.D. (1995). An overview of epithelio-mesenchymal transformation. Acta Anat. (Basel) *154*, 8–20.

Hoek, K., Rimm, D.L., Williams, K.R., Zhao, H., Ariyan, S., Lin, A., Kluger, H.M., Berger, A.J., Cheng, E., Trombetta, E.S., et al. (2004). Expression profiling reveals novel pathways in the transformation of melanocytes to melanomas. Cancer Res. *64*, 5270–5282.

Husemann, Y., Geigl, J.B., Schubert, F., Musiani, P., Meyer, M., Burghart, E., Forni, G., Eils, R., Fehm, T., Riethmuller, G., and Klein, C.A. (2008). Systemic spread is an early step in breast cancer. Cancer Cell *13*, 58–68.

Kang, Y., and Massague, J. (2004). Epithelial-mesenchymal transitions: twist in development and metastasis. Cell *118*, 277–279.

Konishi, H., Karakas, B., Abukhdeir, A.M., Lauring, J., Gustin, J.P., Garay, J.P., Konishi, Y., Gallmeier, E., Bachman, K.E., and Park, B.H. (2007). Knock-in of mutant K-ras in nontumorigenic human epithelial cells as a new model for studying K-ras mediated transformation. Cancer Res. *67*, 8460–8467.

Kwok, W.K., Ling, M.T., Lee, T.W., Lau, T.C., Zhou, C., Zhang, X., Chua, C.W., Chan, K.W., Chan, F.L., Glackin, C., et al. (2005). Up-regulation of TWIST in prostate cancer and its implication as a therapeutic target. Cancer Res. *65*, 5153–5162.

Li, L., Cserjesi, P., and Olson, E.N. (1995). Dermo-1: a novel twist-related bHLH protein expressed in the developing dermis. Dev. Biol. *172*, 280–292.

Linggi, B., Muller-Tidow, C., van de Locht, L., Hu, M., Nip, J., Serve, H., Berdel, W.E., van der Reijden, B., Quelle, D.E., Rowley, J.D., et al. (2002). The t(8;21) fusion protein, AML1 ETO, specifically represses the transcription of the p14(ARF) tumor suppressor in acute myeloid leukemia. Nat. Med. *8*, 743–750.

Maestro, R., Dei Tos, A.P., Hamamori, Y., Krasnokutsky, S., Sartorelli, V., Kedes, L., Doglioni, C., Beach, D.H., and Hannon, G.J. (1999). Twist is a potential oncogene that inhibits apoptosis. Genes Dev. *13*, 2207–2217.

Michaloglou, C., Vredeveld, L.C., Soengas, M.S., Denoyelle, C., Kuilman, T., van der Horst, C.M., Majoor, D.M., Shay, J.W., Mooi, W.J., and Peeper, D.S. (2005). BRAFE600-associated senescence-like cell cycle arrest of human naevi. Nature *436*, 720–724.

Mironchik, Y., Winnard, P.T., Jr., Vesuna, F., Kato, Y., Wildes, F., Pathak, A.P., Kominsky, S., Artemov, D., Bhujwalla, Z., Van, D.P., et al. (2005). Twist overexpression induces in vivo angiogenesis and correlates with chromosomal instability in breast cancer. Cancer Res. *65*, 10801–10809.

Oft, M., Peli, J., Rudaz, C., Schwarz, H., Beug, H., and Reichmann, E. (1996). TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. Genes Dev. *10*, 2462–2477.

Ohuchida, K., Mizumoto, K., Ohhashi, S., Yamaguchi, H., Konomi, H., Nagai, E., Yamaguchi, K., Tsuneyoshi, M., and Tanaka, M. (2007). Twist, a novel oncogene, is upregulated in pancreatic cancer: clinical implication of Twist expression in pancreatic juice. Int. J. Cancer *120*, 1634–1640.

Petrenko, O., Zaika, A., and Moll, U.M. (2003). deltaNp73 facilitates cell immortalization and cooperates with oncogenic Ras in cellular transformation in vivo. Mol. Cell. Biol. *23*, 5540–5555.

Qian, Y., Zhang, J., Yan, B., and Chen, X. (2008). DEC1, a basic helix-loop-helix transcription factor and a novel target gene of the p53 family, mediates p53-dependent premature senescence. J. Biol. Chem. *283*, 2896–2905.

Schardt, J.A., Meyer, M., Hartmann, C.H., Schubert, F., Schmidt-Kittler, O., Fuhrmann, C., Polzer, B., Petronio, M., Eils, R., and Klein, C.A. (2005). Genomic analysis of single cytokeratin-positive cells from bone marrow reveals early mutational events in breast cancer. Cancer Cell *8*, 227–239.

Scheel, C., Onder, T., Karnoub, A., and Weinberg, R.A. (2007). Adaptation versus selection: the origins of metastatic behavior. Cancer Res. *67*, 11476–11479.

Schmidt-Kittler, O., Ragg, T., Daskalakis, A., Granzow, M., Ahr, A., Blankenstein, T.J., Kaufmann, M., Diebold, J., Arnholdt, H., Muller, P., et al. (2003). From latent disseminated cells to overt metastasis: genetic analysis of systemic breast cancer progression. Proc. Natl. Acad. Sci. USA *100*, 7737–7742.

Serrano, M., Lee, H., Chin, L., Cordon-Cardo, C., Beach, D., and DePinho, R.A. (1996). Role of the INK4a locus in tumor suppression and cell mortality. Cell *85*, 27–37.

Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., and Lowe, S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell *88*, 593–602.

Simpson, P. (1983). Maternal-zygotic gene interactions during formation of the dorsoventral pattern in Drosophila embryos. Genetics *105*, 615–632.

Sosic, D., Richardson, J.A., Yu, K., Ornitz, D.M., and Olson, E.N. (2003). Twist regulates cytokine gene expression through a negative feedback loop that represses NF-kappaB activity. Cell *112*, 169–180.

Thisse, B., el Messal, M., and Perrin-Schmitt, F. (1987). The twist gene: isolation of a Drosophila zygotic gene necessary for the establishment of dorsoventral pattern. Nucleic Acids Res. *15*, 3439–3453.

Trost, T.M., Lausch, E.U., Fees, S.A., Schmitt, S., Enklaar, T., Reutzel, D., Brixel, L.R., Schmidtke, P., Maringer, M., Schiffer, I.B., et al. (2005). Premature senescence is a primary fail-safe mechanism of ERBB2-driven tumorigenesis in breast carcinoma cells. Cancer Res. *65*, 840–849.

Valsesia-Wittmann, S., Magdeleine, M., Dupasquier, S., Garin, E., Jallas, A.C., Combaret, V., Krause, A., Leissner, P., and Puisieux, A. (2004). Oncogenic cooperation between H-Twist and N-Myc overrides failsafe programs in cancer cells. Cancer Cell *6*, 625–630.

Wolf, C., Thisse, C., Stoetzel, C., Thisse, B., Gerlinger, P., and Perrin-Schmitt, F. (1991). The M-twist gene of Mus is expressed in subsets of mesodermal cells and is closely related to the Xenopus X-twi and the Drosophila twist genes. Dev. Biol. *143*, 363–373.

Yang, J., Mani, S.A., Donaher, J.L., Ramaswamy, S., Itzykson, R.A., Come, C., Savagner, P., Gitelman, I., Richardson, A., and Weinberg, R.A. (2004). Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell *117*, 927–939.

Zhang, Z., Xie, D., Li, X., Wong, Y.C., Xin, D., Guan, X.Y., Chua, C.W., Leung, S.C., Na, Y., and Wang, X. (2007). Significance of TWIST expression and its association with E-cadherin in bladder cancer. Hum. Pathol. *38*, 598–606.