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Article

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

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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

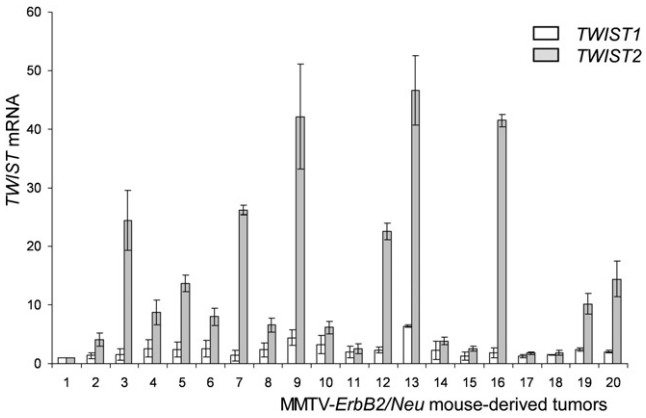

Figure 1. *TWIST2* Is Overexpressed in MMTV-*ErbB2/Neu*-Derived Mammary Tumors

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