BRIEF COMMUNICATION

E2F1 in Melanoma Progression and Metastasis

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Metastases are responsible for cancer deaths, but the molecular alterations leading to tumor progression are unclear. Overexpression of the E2F1 transcription factor is common in high-grade tumors that are associated with poor patient survival. To investigate the association of enhanced E2F1 activity with aggressive phenotype, we performed a gene-specific silencing approach in a metastatic melanoma model. Knockdown of endogenous E2F1 via E2F1 small hairpin RNA (shRNA) expression increased E-cadherin expression of metastatic SK-Mel-147 melanoma cells and reduced their invasive potential but not their proliferative activity. Although growth rates of SK-Mel-147 and SK-Mel-103 xenograft tumors expressing E2F1 shRNA or control shRNA were similar, mice implanted with cells expressing E2F1 shRNA had a smaller area of metastases per lung than control mice (n = 3 mice per group; 5% vs 46%, difference=41%, 95% confidence interval=15% to 67%; P = .01; one-way analysis of variance). We identified epidermal growth factor receptor as a direct target of E2F1 and demonstrated that inhibition of receptor signaling abrogates E2F1-induced invasiveness, emphasizing the importance of the E2F1–epidermal growth factor receptor interaction as a driving force in melanoma progression that may serve as a paradigm for E2F1-induced metastasis in other human cancers.


Tumor metastasis continues to be the most important problem in the field of cancer. Patients who present with metastatic disease or those who develop metastases after successful management of the primary tumor carry a universally grave prognosis. To improve treatment outcomes for these patients, a broader understanding of the determinants of this process is necessary.

The E2F1 transcription factor is considered to be the “final frontier” of the G1-to-S phase boundary that is tightly regulated through the retinoblastoma protein (Rb). Inactivation of Rb liberates E2F1 from the suppressive complex, which, in turn, induces continuous expression of target genes whose products promote cell cycle progression (1,2). Moreover, E2F1 can suppress differentiation of a variety of cell types, including keratinocytes and chondrocytes (3,4). Ectopic expression of E2F1 results in neoplastic transformation of rodent cells (5,6), and findings from transgenic models indicate that increased E2F1 activity is associated with tumor development in several tissues (7–9). In addition, deregulation of E2F1, either by overexpression or Rb inactivation, induces DNA damage and thus could contribute to cancer by causing mutations (10,11).

Abnormalities in E2F1 gene expression and/or E2F1 gene amplification have been described in various cancer cell lines and tumor types, including malignant melanoma (12–25). Of note, overexpression of E2F1 is frequently associated with high-grade tumors and poor patient survival prognosis (17,18,20,23,26,27), suggesting that its oncogenic properties extend beyond the ability to stimulate aberrant growth of neoplastic cells. Consistent with earlier reports indicating that Rb is constitutively hyperphosphorylated and inactive in melanoma cells from metastatic lesions (28), E2F1 is expressed at considerably higher levels in metastases than in primary tumors of melanoma patients (13,29). Based on these findings, we have investigated the role of E2F1 in cancer progression and characterized its molecular action in different human melanoma cell lines.

To determine the effect of endogenously high E2F1 levels (Figure 1, A, left) on invasiveness, highly metastatic melanoma cell lines SK-Mel-103 and SK-Mel-147 (a gift from Dr M. Soengas, Department of Dermatology, University of Michigan, Comprehensive Cancer Center) as verified by xenograft experiments (30,31) were infected with an adenoviral vector expressing gene-specific small hairpin RNA (shRNA), shown to efficiently knock down E2F1 (Figure 1, A, right), and tested for migration behavior using Boyden chamber and scratch assays. In both cell lines, cells expressing shE2F1 had reduced cell invasion and motility compared with cells expressing control shRNA (Supplementary Figure 1, available online). Growth rates of SK-Mel-147 cells expressing E2F1 shRNA or control shRNA and nontransfected cells were similar, as measured by viability assay. Loss of E2F1 expression had no inhibitory effect on G1-to-S phase progression, indicating that E2F1 is not required for the proliferation of metastatic melanoma cells. Because epithelial-to-mesenchymal transition is considered a key step for progression to metastatic stage (32), and loss of E-cadherin is frequently found in metastatic tumors (33), we examined whether suppression of E2F1 is associated with alterations of E-cadherin. We observed an increase of membranous E-cadherin expression in SK-Mel-147 cells when E2F1 activity was blocked, suggesting its involvement in E2F1-mediated invasion (Supplementary Figure 1, available online).

SK-Mel-103 and SK-Mel-147 cells expressing either E2F1 shRNA or control shRNA were implanted subcutaneously to generate localized xenografts or intravenously to induce disseminated tumors. Although both cell lines, irrespective of whether they expressed control shRNA or E2F1 shRNA, formed primary tumors that grew at similar rates (Figure 1, B), E2F1-knockout cells...
Prior knowledge
E2F1, a key regulator of the cell cycle, is often overexpressed in aggressive tumors.

Study design
Effects of E2F1 knockdown on proliferation, tumor growth, and invasive and metastatic potential of metastatic melanoma cells and xenografts derived from them were examined. The role of epidermal growth factor receptor was also examined in vitro.

Contributions
Knockdown of E2F1 expression reduced epidermal growth factor receptor expression and reduced the invasive potential but not proliferation of metastatic melanoma cells in vitro. Knockdown of E2F1 expression did not alter growth of xenograft tumors but reduced their metastatic potential in vivo. Blocking epidermal growth factor receptor activity in cells that overexpressed E2F1 led to reduced invasive potential.

Implications
Epidermal growth factor receptor was identified as a direct target of E2F1-mediated metastasis.

Limitations
Because the study was based on specific in vitro and in vivo models, it is still unclear whether these mechanisms are useful targets in human cancer.

From the Editors

Figure 1. Inhibition of pulmonary metastasis using E2F1 shRNA. A) Immunoblot analysis of high endogenous E2F1 levels in metastatic SK-Mel-103 and SK-Mel-147 vs nonmetastatic SK-Mel-19 and SK-Mel-29 cells expressing low amounts of E2F1 (left) and after infection of SK-Mel-147 cells with equal multiplicities of infection (50 moi) of control- or shE2F1-expressing adenovirus (right panel). Melanocytes and normal fibroblasts are shown as negative control. Equal amounts of total protein (50 µg) were loaded in each lane. Blots were probed with mouse monoclonal E2F1 antibody (1:2000; BD Pharmingen, Heidelberg, Germany). Actin expression (Sigma-Aldrich, München, Germany) is shown as a control for loading and transfer. B) The effect of E2F1 inhibition on localized tumor growth was analyzed by subcutaneous injection of 5 x 10^4 metastatic melanoma cells expressing control shRNA or shRNA against E2F1 in the flank of athymic nude mice (n = 10). Volumes of subcutaneous xenografts were estimated as V = 0.52 x L x W^2, where L and W stand for tumor length and width, respectively. Data represent the mean from each SK-Mel-147 group (n = 10 tumors per experimental condition). Error bars = upper and lower 95% confidence intervals. C) As surrogate for metastatic dissemination of melanoma cells (30), 2 x 10^6 melanoma cells were injected intravenously into the mouse tail vein (n = 10). Representative photos and hematoxylin and eosin sections on the right of lungs are shown. Slides of 4 µm were scanned by a Mirax Scanner (Carl Zeiss, Göttingen, Germany), and areas of metastatic melanoma and total lung tissue were measured using the Mirax Viewer (Carl Zeiss, Göttingen, Germany). The relative area (in %) of metastases per lung area from three individual mice carrying control shRNA-expressing cells (I–III) and E2F1 shRNA (IV–VI) expressing cells is indicated in bottom bar graphs (light green tumor fraction). Metastatic nodules, 31% (II); 56% (II); 52% (III); 5.8% (IV); 6% (V); 10% (VI). Scale bar = 2 mm. Statistical significance was determined by one-way analysis of variance for two groups. P values of .05 or less were deemed statistically significant (P < .05). All mouse procedures were conducted in adherence to ethical standards and with approval of Rostock Animal Care Committee. D) SK-Mel-103 and SK-Mel-147 melanoma cells were harvested 48 hours after infection with Ad.control shRNA or Ad.shE2F1 for RNA extraction. Equal amounts of RNA were analyzed using Affymetrix GeneChip Human Genome U133 Plus 2.0 Arrays (Affymetrix Inc., Santa Clara, CA). Expression profiles of both cell lines expressing either control shRNA or shE2F1 were grouped and statistically analyzed using CA. Expression profiles of both cell lines expressing either control shRNA or shE2F1 were grouped and statistically analyzed using t test and multiple testing correction (Benjamini and Hochberg False Discovery Rate). Only targets displaying a minimum twofold induction or reduction (P < .05) by E2F1 knockdown were included for clustering. Green shading represents low gene expression, whereas red shading indicates higher expression. Sets of coregulated genes were analyzed using Web-based Gene Set Analysis Toolkit at bioinfo.vanderbilt.edu/webgestalt. Gene expression levels for subsets of genes that are E2F1 target genes or related to tumor progression (adhesion/migration, green; metastasis, blue; aggressiveness/poor prognosis, orange) are indicated. The relative mRNA levels were measured in SK-Mel-147 melanoma cells by quantitative real-time polymerase chain reaction analysis. Fold expression was calculated after normalization with 36B4 (S3) and beta-2-microglobulin (B2M) housekeeping genes. Bar graphs represent the mean of three separate samples measured in triplicate. Error bars show upper 95% confidence intervals, p.i., post injection.
Figure 2. Effect of epidermal growth factor receptor (EGFR) expression on E2F1-mediated cell motility and invasiveness. A) Endogenous EGFR protein expression was verified by immunoblot analysis of whole cell lysates in metastatic melanoma cells with control shRNA or E2F1 shRNA using mouse monoclonal EGFR antibody (1:2000; Sigma-Aldrich, München, Germany). Parental cells were used as positive control. Blots were probed with mouse monoclonal actin antibody (1:4000; Sigma-Aldrich) as a control for equal loading and transfer. B) Reverse transcription–polymerase chain reaction (RT-PCR) of epidermal growth factor receptor, cyclin E, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) expression in serum-starved nonmetastatic SK-Mel-19.ER-E2F1 cells [generated from parental cells by transfection with pBabe-HAERE2F1 as described (36)] grown for 24 hours in 4-hydroxytamoxifen (4-OHT) and/or cycloheximide (CHX) was performed as described (37).
revealed four putative motifs of E2F1, which cluster in the region from −998 to −583 of the full-length EGFR promoter. Its relevance to E2F1-mediated direct EGFR regulation was confirmed by luciferase assays using full-length pER1-Luc fragment and a 5′deletion of the E2F-binding sites spanning region [pER10-Luc, provided by Dr. A. C. Johnson, Center for Cancer Research, National Cancer Institute (40)] cotransfected with E2F1 in SK-Mel-29 cells, whereas DNA-binding or transactivation-defective E2F1 mutants E132 and dTA had no effect (Figure 2, C, lower). Chromatin immunoprecipitation assays in stable SK-Mel-29.ER-E2F1 cells demonstrated that direct binding of E2F1 occurs via the motif at position −998 (Figure 2, D). Implication of E2F1 in the increased expression of EGFR observed was further supported by the observation that its enhanced activity in nonmetastatic SK-Mel-29.ER-E2F1 cells led to strong EGFR staining, whereas exposure of SK-Mel-147 cells to infection with Ad. shE2F1 substantially abolished EGFR expression (Figure 2, E, top). E2F1 and EGFR cotranslocated in the nucleus (Figure 2, E, bottom), directly interacted (data not shown), and resulted in increased expression of B-Myb (Figure 2, E). Importantly, blockade of EGFR tyrosine kinase activity in 4-hydroxytamoxifen (4-OHT)–induced SK-Mel-29.ER-E2F1 cells, generated from parental cells after stable transfection of ER-E2F1, severely reduced the number of invading cells (Figure 2, F, top). Consistent with cytoplasmic receptor signaling (41), activation of E2F1 in these cells was associated with increased phosphorylation of extracellular signal-regulated kinase (ERK) and Akt compared with uninduced SK-Mel-29.ER-E2F1 cells (Figure 2, F, bottom). In support of the established E2F1–EGFR link, we found coordinated increases in E2F1 and EGFR RNA and protein levels in patient melanoma metastases (Supplementary Figure 2, available online).

Together, we have shown that deregulated E2F1 enhances invasion and metastasis of malignant melanoma cells independent from its proliferative activity. This observation is consistent with the notion that E2F3 is critical for cellular proliferation of tumor cells, but E2F1 is not (42). Apart from identifying multiple E2F1 target genes that are good candidates for further examination of their possible involvement in melanoma aggressive behavior, E2F1-induced tumor progression was found to be mediated through increased expression and activation of EGFR. Because overexpression of EGFR in tumors is often accompanied by expression of EGF-family ligands (43), autocrine regulation through EGFR by such ligands could provide the mechanism for activation of downstream cascades, such as the Ras/Mapk/ERK and PI3K/Akt dependent pathway (44), and metastasis. In particular, expression of nuclear EGFR, which has been linked to aggressiveness and poor clinical outcome (45), was clearly increased following E2F1 activation, and nuclear cotranslocation of EGFR and E2F1 was associated with enhanced expression of B-Myb. Consistently, B-Myb expression was severely impaired after inhibition of E2F1 in microarray analyses of metastatic melanoma cell lines. However, irrespective of previous reports suggesting that cyclin D1 expression contributes to EGFR-driven tumorigenesis and its suppression leads to E2F1 inhibition (46,47), EGFR signaling that is initiated through E2F1 in melanoma cells appears to be independent from cyclin D1. Considering that oncogenic signaling by EGFR (48) and PI3K/Akt (49) has been implicated in restraining E2F1 apoptotic activity during normal growth, such a “feedback loop” could facilitate E2F1-mediated metastasis by blocking its proapoptotic function. Melanoma cells from advanced lesions, however, exhibit severe defects in the apoptotic pathways that are normally activated by E2F1 (28,50,51). These defects may, a priori, select against apoptotic consequences of deregulated E2F1 activity (52). This notion is supported by the observed lack of apoptosis in metastatic cell lines in which E2F1 was inactivated or in nonmetastatic cells in which E2F1 was overexpressed. This observation also suggests that EGFR may not inhibit E2F1-mediated apoptosis in melanoma cells and that this mechanism may not be required for E2F1-related metastasis.

C) Luciferase activity of the human EGFR promoter in SK-Mel-29 and SK-Mel-147 cells according to different E2F1 protein levels is shown in Figure 1, A (top). Schematic diagram of the full-length (pER1) and ER10 deletion mutant (bottom). Positions of putative E2F1-binding sites are indicated relative to the transcriptional start site (+1). The TP73 reporter (36) has been used as a positive control. SK-Mel-29 cells were cotransfected with 0.5 µg pER1, pER10, or pGL3basic and increasing amounts of expression plasmid encoding E2F1 (0.5, 0.75) or the E2F1 mutants E132 and dTA (38). Luciferase activity was measured 36 hours after transfection and normalized to total protein concentration in the cell extract. Promoter activity of pGL3basic was normalized to 1.0 and shown as relative light units (RLU). Error bars = upper 95% confidence intervals. Protein expression levels of E2F1 and EGFR in E2F1 transfected cells are as indicated. Blots were probed with actin as a control for equal loading and transfer. D) Chromatin immunoprecipitation (ChIP) assays were performed essentially as described (39). Cross-linked chromatin was prepared from serum-starved SK-Mel-29.ER-E2F1 cells grown with and without 4-OHT for 24 hours and immunoprecipitated with an anti-E2F1 antibody or control IgG (BD Pharmingen, Heidelberg, Germany). Binding of E2F1 to EGFR promoter regions as outlined in (C) (E2F site 1 [−998], 2 and 3 [−875 to −834], 4 [−593]) was detected by PCR amplification using specific primer pairs (Supplementary Table 2, available online). The Apaf-1 promoter was used as positive control. E) EGFR expression in response to E2F1 activation by 4-OHT (top left panel) and after E2F1 knockdown (top right panel) was detected by using mouse monoclonal anti-EGFR (1:2000; Sigma-Aldrich). Nuclear cotranslocation of E2F1 and EGFR using rabbit polyclonal E2F1 antibody (1:2000; Santa Cruz Biotechnology, Heidelberg, Germany) (left) and activation of B-Myb expression (MYBL2, right) in SK-Mel-29.ER-E2F1 cells is shown (bottom). Increased expression of cyclin D1 (CCND1) and iNOS (NOS2) was not observed. Relative mRNA levels were measured by quantitative RT-PCR analysis. Fold expression was calculated after normalization with B2M. Bar graphs represent the mean of three independent experiments. Error bars show upper 95% confidence intervals. Alexa Fluor 488 and 633 and DAPI were used for visualization by laser scanning microscopy. Scale bars = 10 µm. F) Number of invading ER-E2F1 expressing SK-Mel-29 cells at 36 hours after 4-OHT induction and/or treatment with EGFR inhibitor AG 1478 (top). Values of invasion were obtained from five fields per membrane. Data are means of three separate experiments with error bars showing 95% confidence intervals. Mann–Whitney pair wise tests (two-sided) were preceded by an overall test indicating statistically significant differences between groups (⁎ P = .008). Immunoblot of total and phosphorylated ERK1/2 and Akt levels in inducible SK-Mel-29.ER-E2F1 cells 36 hours after E2F1 activation by 4-OHT (bottom). Extracts were probed with anti-ERK1/2 (1:2000; BD Pharmingen), mouse monoclonal AKT (pan 11E7), Phospho-Akt (Ser473), p44/42 MAP Kinase (3A7), or Phospho-p44/42 MAPK (Thr202/Tyr204) antibodies from Cell Signaling Technology (Beverly, MA) at 1:2000 dilution. Blots were probed with actin as a control for loading and transfer.
A potential limitation of this study is the small number of patient samples. Overall, we provide evidence that E2F1 is crucial for malignant melanoma cells to exhibit aggressive behavior. Because elevated expression of E2F1 and EGFR has been observed in other tumor types (19,38), the established mechanistic link may also be important in other human cancers. This association should be explored in future studies.

References


**Notes**

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