Inactivation of E2f1 Enhances Tumorigenesis in a Myc Transgenic Model

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ABSTRACT

Previous studies have demonstrated both oncogenic and tumor suppressive properties for the E2f1 transcription factor. In this study, E2f1-null mice were crossed with transgenic mice expressing Myc under the control of an epithelial-specific keratin 5 promoter to determine whether the absence of E2f1 would modulate the oncogenic activity of Myc. Inactivation of E2f1 was found to significantly accelerate tumor development in keratin 5 Myc transgenic mice. Acceleration of tumorigenesis occurred despite the fact that apoptosis levels were increased in transgenic tissue and tumors null for E2f1, whereas Myc-induced proliferation was unaffected by the status of E2f1. These findings provide new insight into the tumor suppressive activity of E2f1 and identify for the first time a specific oncogenic alteration that cooperates with the loss of E2f1 in tumorigenesis.

INTRODUCTION

Members of the E2F family of transcription factors play a crucial role in cell proliferation control by regulating the expression of genes important for DNA synthesis and cell cycle progression (1–3). A functional E2F factor is composed of a heterodimer between an E2F polypeptide (E2F1–E2F6) and a DP polypeptide (DP1 and DP2). The transcriptional activities of E2F factors are regulated through association with the Rb family tumor suppressor protein and the other pocket proteins, p107 and p130. Binding of a pocket protein inhibits the transcriptional activation capacity of E2F factors and, in at least some cases, can convert E2F factors to repressors of transcription. The activity of Rb is regulated during the cell cycle by cdks, primarily by cdk4 and cdk6, in association with D-type cyclins and cyclin E in association with cdk2. Phosphorylation of Rb by these cdks results in the dissociation of Rb from E2F and the derepression/activation of E2F-regulated genes.

The importance of E2F activity in regulating proliferation is illustrated by the fact that deregulated expression of several E2F family members can stimulate quiescent cells to enter the S phase of the cell cycle (4, 5). In addition to promoting proliferation, at least one E2F family member, E2F1, can also potently induce apoptosis when it is overexpressed or deregulated through Rb inactivation (5–8). Depending on the experimental system, the ability of E2F1 to induce apoptosis can either require wild-type p53 function or be independent of p53. Several possible mechanisms have been identified for E2F1-induced apoptosis. One involves the transcriptional activation of p73, a relative of p53. The p73 gene is up-regulated in response to E2F1 and, like p53, p73 activation can lead to apoptosis (9). This E2F1-p73 apoptotic pathway appears to play a physiological role in receptor activation-induced cell death during T-cell development (10). An ability to block survival pathway signaling from death receptors may also contribute to E2F1’s capacity to promote apoptosis (11). It has also been suggested that the ARF tumor suppressor, which functions as a negative regulator of mdm2, participates in the induction of p53-dependent apoptosis by E2F1. This hypothesis is based on the finding that E2F1 can transcriptionally activate the ARF gene (5, 12). However, we have shown recently that ARF is dispensable for E2F1-induced apoptosis and that ARF appears to be a negative regulator of E2F1 activity (13).

The role of E2F1 in the development of cancer is complex and not understood completely. Most human tumors have genetic or epigenetic alterations that result in the deregulation and activation of E2F1, as well as other E2F family members. However, mutations that may contribute to cancer are rarely, if ever, observed in E2F1 or other genes encoding E2F activity in human tumors. Various mouse models have demonstrated either an oncogenic or tumor suppressive property for E2F1. Transgenic mice overexpressing E2F1 in squamous epithelial tissues or in the liver are predisposed to developing tumors (14–16). This demonstrates that E2F1 can function as an oncogene in vivo. On the other hand, mice lacking a functional E2F1 allele are also predisposed to tumor development, demonstrating that E2F1 functions as a tumor suppressor gene (17). Other experiments with E2F1 knockout and E2F1 transgenic mice have demonstrated that both positive and negative effects on tumorigenesis can be observed whether E2F1 is absent or overexpressed (15, 18, 19). It has been suggested that E2F1 inhibits tumorigenesis by promoting apoptosis, but this has not been shown directly, and several other possible mechanisms for E2F1’s tumor suppressive activity exist.

Overexpression of the c-myc oncogene contributes to the development of many human cancers, including 11% of all primary tumors of epithelial origin (20). Myc is a transcription factor that, when heterodimerized with the Max polypeptide, can specifically bind to a DNA sequence (the E-box) and activate the expression of genes critical for cell cycle progression and metabolism. Like E2F1, deregulated expression of Myc induces quiescent cells to enter the S phase of the cell cycle in the absence of other mitogenic signals (21, 22). In addition, like E2F1, Myc is a potent inducer of apoptosis, and this activity is often, but not always, dependent on wild-type p53 function (23, 24). Unlike E2F1, however, a tumor suppressive activity for Myc has not been described, despite Myc’s ability to efficiently induce apoptosis.

Although Myc and E2F1 have several growth regulatory properties in common, the functional relationship between Myc and E2F1 is obscure. Different reports have suggested that Myc is either downstream of E2F, upstream of E2F, or functions in a parallel pathway with E2F to regulate cell proliferation. E2F DNA-binding sites are found in the c-myc gene promoter, and, under at least some conditions, these sites are responsive to E2F1 and other regulators of E2F-dependent transcription (25–28). However, the endogenous c-myc gene is not significantly up-regulated in response to E2F1 overexpression, either in rat embryo fibroblast cultures or in transgenic epidermis (29, 30). It has also been reported that overexpression of Myc can stimulate the expression of E2F1, as well as E2F2 and E2F3 (31–33). This suggests that E2F family members could participate in one or more of the growth regulatory pathways regulated by Myc. Using a Myc transgenic model and null mice for E2f1, we now dem-
onstrate that E2F1 is not required for proliferation, apoptosis, or tumorigenesis induced by Myc. Rather, inactivation of E2F1 enhances both Myc-induced tumorigenesis and apoptosis in this model system.

MATERIALS AND METHODS

Mice. K5 Myc transgenic mice (34), E2f1 knockout mice (17), and p53 knockout mice (35) have been described. K5 Myc transgenic mice (line MM5 and MM3) were bred to mice null for E2f1 to generate K5 Myc transgenic mice heterozygous for E2f1. These mice were then bred to E2f1 heterozygous mice to generate K5 Myc transgenic and nontransgenic mice that were either homozygous, heterozygous, or nullizygous for E2f1. K5 Myc transgenic mice (line MM5) heterozygous for both E2f1 and p53 were also generated and bred to double E2f1, p53 heterozygous mice. This breeding generated K5 Myc transgenic and nontransgenic sibling mice that were homozygous for E2f1 and p53, null for either E2f1 or p53, or null for both E2f1 and p53. The genetic background of these mice was a mixture of SSIn and 129/Sv strains. Sibling mice were used for comparisons in all experimental procedures.

Brdu Incorporation. Mice were injected with 170 μl of 20 mM Brdu 20 min before sacrifice. Skin samples were fixed in formalin, paraffin-embedded, and immunohistochemically stained using an antibody to Brdu (Becton Dickinson; 1:500 dilution). Interfollicular basal cells were examined microscopically to determine the number of unstained and stained cells. At least 1000 cells were counted per section.

TUNEL Assay. TUNEL assays were performed in formalin-fixed, paraffin-embedded skin sections using the ApopTag in situ apoptosis detection kit (Oncor). TUNEL-positive epidermal keratinocytes were visualized microscopically by peroxidase-diaminobenzidine staining, and the average number of positive epidermal keratinocytes per 10 mm of linear skin was determined.

Activated Caspase 3 Immunohistochemistry. Formalin-fixed, paraffin-embedded skin sections were immunohistochemically stained with an antibody specific for the activated form of caspase 3 (R&D Systems, Inc.; 1:2000 dilution) using the Histostain-Plus kit (Zymed). The stained slides were examined microscopically to determine the average number of positive epidermal keratinocytes per 10 mm of linear skin.

Western Blot Analysis of p53 in Epidermal Lysates. Epidermal protein was collected by scraping dorsal skin and resuspending epidermal tissue in lysis buffer (50 mM HEPES (pH 7.9), 250 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% IGEPA, 0.4 mM NaF, 0.4 mM Na3VO4, 10% glycerol, and 1 mM DTT] containing 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin. Protein extract was prepared by freeze thawing, followed by supernatant collection after high-speed centrifugation. Protein samples (100 μg) were separated on a 10% SDS-PAGE and transferred to nitrocellulose membrane. Western blot analysis was performed using anti-rabbit p53 antibody (Cell Signaling; 1:500 dilution) in 1× Tris-buffered saline containing 5% blocking reagent and 0.5% Tween 20 (Bio-Rad). Visualization of p53 bands was accomplished using enhanced chemiluminescence reagent (Amersham) and autoradiography. Even loading of samples was confirmed using Ponceau staining.

RESULTS

E2F1 Suppresses Tumorigenesis in K5 Myc Transgenic Mice. E2f1 knockout mice are predisposed to developing spontaneous tumors late in life, suggesting that E2F1 functions as a tumor suppressor (17). However, the loss of E2F1 has not been demonstrated to promote tumorigenesis in any defined animal model of cancer predisposition. To determine whether E2F1 could modulate the tumorigenic activity of Myc, transgenic mice expressing Myc under the control of a K5 promoter (34) were crossed with mice null for E2f1 (17). K5 Myc transgenic mice (line MM5) heterozygous for E2f1 were then crossed to E2f1 heterozygous mice to generate transgenic and nontransgenic siblings that were either wild type, heterozygous, or null for E2f1. These mice were then maintained in colony for >1 year without treatment.

Almost all (92%) K5 Myc transgenic mice lacking a functional E2f1 allele had developed tumors by 1 year of age (Fig. 1 and Table 1). The K5 Myc transgenic mice heterozygous for E2f1 had a tumor incidence of 56%, whereas the transgenic mice wild type for E2f1 had a tumor incidence of only 28% at 1 year of age. The difference in incidence at 1 year is even more dramatic when only the head and neck tumors are considered. The overall incidence of skin papillomas was not significantly different between E2F1 genotypes. However, the E2f1+/− allele had developed tumors by 1 year of age (Fig. 1 and Table 1).

The head and neck tumors appeared to be squamous odontogenic tumors by their morphological similarities with these rare human tumors (Fig. 2, C and D). Some areas acquired characteristics that were difficult to distinguish from a squamous cell carcinoma, but the presence of cells in the tumors with characteristics of clear cells, as well as their association with simple keratocysts and the molar alveoli, indicates that these tumors were derived from the odontogenic epithelium. The apparent evolution of these tumors based on the obser-
Inactivation of a number of mice appears to start with a keratin cyst in close proximity with the odontogenic epithelium. Larger tumors presented a clear pattern of invasion affecting surrounding tissues, including bone. Again, no obvious pathological differences were observed between squamous odontogenic tumors from $E2f1^{+/+}$ and $E2f1^{-/-}$ mice.

Inactivation of $E2f1$ Enhances Myc-induced Apoptosis. To address the mechanism by which $E2f1$ inactivation cooperates with Myc overexpression in tumorigenesis, the skin of young K5 Myc transgenic mice with and without $E2f1$ was analyzed. Eight-week-old mice were injected with BrdUrd and sacrificed, and skin samples were taken for analysis. Consistent with previous results (34), K5 Myc transgenic mice exhibit hyperproliferative epidermis as measured by the percentage of interfollicular keratinocytes incorporating BrdUrd (Fig. 3A). Proliferation in the epidermis of transgenic mice was unaffected by the absence of $E2f1$. This was true for both the lower-expressing line (MM5) used in the tumor study and a higher-expressing K5 Myc transgenic line (MM3). Thus, the absence of $E2f1$ does not affect the ability of Myc to induce proliferation in primary epidermal tissue.

To examine the effect of $E2f1$ status on Myc-induced apoptosis, skin sections from K5 Myc transgenic mice, with and without functional $E2f1$, were subjected to an in situ TUNEL assay. As shown previously (34), deregulated expression of Myc in the basal cell layer of the epidermis results in increased levels of apoptosis in both K5 Myc transgenic lines (Fig. 3B). Surprisingly, apoptosis was further increased in the epidermis of K5 Myc transgenic mice when $E2f1$ was inactivated, compared with when $E2f1$ was wild type (Fig. 3B). To confirm this unexpected result, a second assay for apoptosis was performed that detects the activated form of caspase 3 by immunohistochemistry. Once again, there was an increase in the apoptotic index in K5 Myc transgenic epidermis when $E2f1$ was absent, compared with when $E2f1$ was present (Figs. 3C). In both the TUNEL and caspase 3 assays, differences in the level of apoptosis between Myc transgenic mice with $E2f1$ and Myc transgenic mice without $E2f1$ were statistically significant ($t$ test, $P < 0.05$). These results demonstrate that $E2f1$ is not required for Myc to induce apoptosis in mouse epidermal keratinocytes in vivo. Instead, inactivation of $E2f1$ increases the level of apoptosis in transgenic epidermis overexpressing Myc.

Table 1. Increased tumorigenesis in K5 Myc mice deficient for $E2f1$

<table>
<thead>
<tr>
<th>$E2f1$ genotype</th>
<th>No. of mice with tumors</th>
<th>Tumor type</th>
</tr>
</thead>
<tbody>
<tr>
<td>$+/+$</td>
<td>5/18 (27.8%)</td>
<td>3 Skin papillomas</td>
</tr>
<tr>
<td>$+/+$</td>
<td>14/25 (56.0%)</td>
<td>2 Head and neck tumors</td>
</tr>
<tr>
<td>$-/+$</td>
<td>11/12 (91.7%)</td>
<td>3 Skin papillomas</td>
</tr>
</tbody>
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* By 1 year of age.

$E2f1^{-/-}$ does not affect the ability of Myc to induce proliferation in primary epidermal tissue.

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Myc Activation of p53 Is Unaffected by E2F1 Status. The p53 tumor suppressor plays a key role in mediating apoptosis and suppressing tumorigenesis in response to Myc overexpression. To determine whether the absence of E2F1 affected p53 induction by Myc in transgenic epidermis, Western blot analysis was performed on epidermal lysates from K5 Myc transgenic and nontransgenic mice either wild type or null for E2F1 (Fig. 4A). p53 protein was barely detectable in nontransgenic epidermis but was clearly induced in K5 Myc transgenic epidermis. The absence of E2F1 did not affect the level of p53 induced by Myc.

To determine whether Myc-induced apoptosis in transgenic epidermis lacking E2F1 still required functional p53, K5 Myc transgenic mice null for both E2F1 and p53 were generated. This was done by crossing K5 Myc transgenic mice (line MM5) that were heterozygous for both E2F1 and p53 with double E2F1/p53 heterozygous mice. In this way, siblings that were wild type for E2F1 and p53, null for either E2F1 or p53, or null for both E2F1 and p53 could be compared. As observed previously (34), K5 Myc transgenic mice null for p53 had a significant reduction in levels of epidermal apoptosis, whereas transgenic mice null for E2F1 had increased levels of epidermal apoptosis (Fig. 4B). Apoptosis in K5 Myc transgenic epidermis lacking E2F1 was still dependent on functional p53, as evidenced by the suppression of apoptosis in transgenic epidermis null for both E2F1 and p53 (Fig. 4B).

Increased Levels of Apoptosis in Skin Tumors Lacking E2F1. Previous experiments have demonstrated that although E2F1 is not required for proliferation and development in normal tissues, the loss of E2F1 can affect proliferation and apoptosis in tumors induced by Rb inactivation (7, 18). To examine the effect of E2F1 inactivation on proliferation and apoptosis in tumors induced by Myc overexpression, skin papillomas from K5 Myc transgenic mice, either wild type or null for E2F1, were analyzed. Consistent with the acceleration of tumorigenesis in the absence of E2F1, the three papillomas from the transgenic mice null for E2F1 arose at an average of 14 weeks earlier than the three papillomas from the transgenic mice wild type for E2F1. Genetic analysis of mouse skin papillomas has found that these tumors usually have wild-type p53 and contain an activating mutation in a ras gene (37, 38).

Proliferation in the papillomas was increased compared with nontumorigenic transgenic epidermis (Fig. 5A, compare with Fig. 3A). As observed in the epidermis, the proliferative index in these papillomas was unaffected by the status of E2F1 (Fig. 5A). The apoptotic index in the skin papillomas was also increased compared with nontumorigenic transgenic epidermis (Fig. 5B, compare with Fig. 3B). As was the case in transgenic epidermis, the apoptotic index in skin papillomas was higher when E2F1 was absent compared with when E2F1 was present (Fig. 5, B and C). Thus, inactivation of E2F1 enhances apoptosis but does not effect proliferation in skin papillomas, as well as in epidermis, from K5 Myc transgenic mice.

These findings that inactivation of E2F1 increases apoptosis in tumors while at the same time accelerating tumor development may at first appear paradoxical. However, many other factors besides alterations in proliferation and apoptosis affect the complex process of tumorigenesis, e.g., in many tissues, such as the epidermis, tumor growth is significantly affected by cell loss through differentiation. Moreover, the growth of tumors over time was not measured in this study. It is quite possible that although tumors appear earlier in an E2F1-null background, the tumors lacking E2F1 may grow in size more slowly than tumors containing E2F1 because of the increased levels of apoptosis.

DISCUSSION

The functional relationship between Myc and E2F1 appears to be complex and can vary dependent on the context. A recent report by
Leone et al. (39) examined the requirement of individual E2F family members for Myc-induced proliferation and apoptosis in primary MEF cultures. As in transgenic epidermis, the absence of E2F1 had no effect on Myc-induced proliferation in MEF cultures. In sharp contrast to our findings, however, inactivation of E2F1 impaired Myc’s ability to induce apoptosis in MEF cultures (39). This difference in results is likely attributable to the very different experimental systems used in the two studies. At present, it is unclear whether the increase in apoptosis observed in K5 Myc epidermis and tumors when in an E2F1-null background is cell autonomous. It is quite possible that inactivation of E2F1 in the whole animal causes an indirect effect that results in increased levels of apoptosis in Myc-overexpressing tissues. Nonetheless, these results do demonstrate that E2F1 is dispensable for Myc-induced apoptosis under at least some conditions.

E2F1 appears to have the unique ability to function as both onco- gene and tumor suppressor gene. There is much indirect evidence to suggest that the activation of E2F1 and other E2F family members, via perturbation of the p16-cyclin D-Rb pathway, is a key event in the development of tumors. This hypothesis is supported by the finding that deregulated expression of E2F1 can oncogenically transform cells in vitro and contribute to tumor development in transgenic mice (14–16, 40–42). On the other hand, inactivation of E2F1 predisposes mice to a variety of tumors late in life, including reproductive tract sarcomas and lymphomas (17). A tumor suppressor function for E2F1 is further demonstrated in this study by the identification of Myc overexpression as a specific oncogenic alteration that cooperates with E2F1 inactivation to promote tumorigenesis.

When E2F1 knockout mice were used in other mouse models of cancer, the absence of E2F1 either had no effect or inhibited tumor development. Rb heterozygous mice develop pituitary and thyroid tumors that invariably lose the wild-type Rb allele (43). When mice heterozygous for Rb were crossed into an E2F1-null background, the development of pituitary and thyroid tumors was actually suppressed by the absence of E2F1 (18). This reduction in tumor development may be attributable to decreased tumor cell proliferation because the absence of E2F1 in Rb+/− embryos suppresses inappropriate proliferation, as well as p53-dependent apoptosis (8). Inactivation of E2F1 also did not accelerate tumorigenesis in a transgenic model in which SV40 T antigen is used to inactivate Rb family members in the choroid plexus (7). In this T antigen transgenic model, the absence of E2F1 reduced apoptosis in the tumors, but it also reduced proliferation. This resulted in no change in the timing of tumor development. Hepatocarcinogenesis after treatment with diethylnitrosamine was also unaffected by the status E2F1 (44).

The effects of E2F1 inactivation on tumorigenesis in these different model systems provide some insight into the mechanism of tumor suppression by E2F1. A leading theory for the mechanism underlying E2F1’s tumor suppressive activity has been that E2F1 participates in a protective apoptotic pathway that is induced in response to cell cycle deregulation. In this model, the functional inactivation of Rb, through deregulation of cdk activity or mutation of the Rb gene, leads to the constitutive activation of E2F1. This in turn leads to the activation of p53 and the promotion of apoptosis. In this way, cells that might otherwise go on to form a tumor are eliminated. In the absence of E2F1, this apoptotic pathway would be impaired, and the cell could continue to proliferate and accumulate additional oncogenic alterations.

If this protective apoptotic pathway model for tumor suppression by E2F1 is correct, it might have been expected that the absence of E2F1 would have accelerated tumor development in Rb heterozygous mice because of impaired apoptosis. Instead, Rb+/− mice null for E2F1 have an increased lifespan and develop fewer pituitary and thyroid tumors than Rb+/− mice with wild-type E2F1 (18). Our finding that inactivation of E2F1 accelerates tumorigenesis in K5 Myc mice while at the same time increasing apoptosis in transgenic epidermis and tumors also does not support the model in which E2F1 suppresses tumor development by inducing apoptosis. It is possible, however, that E2F1 has multiple tumor suppressive activities and that under some conditions, the induction of apoptosis by E2F1 does function to suppress tumorigenesis.

The observation that E2F1 has different roles during tumorigenesis in these different mouse models of cancer has implications for the use of potential therapies designed to target E2F1. Given the result from the Rb knockout model, the inhibition of E2F1 in tumors lacking Rb might suppress tumor growth by reducing tumor cell proliferation. On the other hand, in lesions in which Myc is overexpressed, inhibiting E2F1 might actually promote tumorigenesis. A more complete understanding of the role of E2F1 in cancer development is clearly required before rationale therapies targeting E2F1 can be designed.

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