E2F-1 Functions in Mice to Promote Apoptosis and Suppress Proliferation

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Summary

Members of the E2F transcription factor family (E2F-1–E2F-5) are believed to be critical positive regulators of cell cycle progression in eukaryotes although the in vivo functions of the individual E2Fs have not been elucidated. Mice were generated that lack E2F-1 and, surprisingly, these mice develop and reproduce normally. However, E2F-1−/− mice exhibit a defect in T lymphocyte development leading to an excess of mature T cells due to a maturation stage-specific defect in thymocyte apoptosis. As E2F-1−/− mice age they exhibit a second phenotype marked by aberrant cell proliferation. These findings suggest that while certain members of the E2F family may positively regulate cell cycle progression, E2F-1 functions to regulate apoptosis and to suppress cell proliferation.

Introduction

Normal growth and development involve competing processes of cell proliferation and apoptotic cell death. Transcription factors of the E2F family, comprised of E2F-1–E2F-5, have been suggested to play a key role in the regulation of cell proliferation (La Thangue, 1994; Adams and Kaelin, 1995; Weinberg, 1995). First, E2F binding sites are crucial for transcriptional activation of genes that regulate S phase entry—such as c-myc (Hiebert et al., 1989; Huang and Hearing, 1989; Thalmeier et al., 1989), cyclin D (Motokura and Arnold, 1993; Sala et al., 1994), and cyclin E (DeGregori et al., 1995; Duronio and O’Farrell, 1995)—and genes that function to engage DNA synthesis—such as dihydrofolate reductase (Blake and Azizkhan, 1989; Mudryj et al., 1990), thymidine kinase (Dou et al., 1992; Ogris et al., 1993), and DNA polymerase α (Pearson et al., 1991; Sala et al., 1994; DeGregori et al., 1995). Second, the activity of E2Fs as transcription factors is regulated by their interaction with other components of the cell cycle machinery—

including Rb (Bagchi et al., 1991; Chellappan et al., 1991; Chittenden et al., 1991), Rb’s relatives p107 (Cao et al., 1992; Devoto et al., 1992; Lees et al., 1992; Shirodkar et al., 1992) and p130 (Cobrinik et al., 1993), and cyclin-dependent kinases (Devoto et al., 1992; Lees et al., 1992; Pagano et al., 1992a, 1992b; Shirodkar et al., 1992; Dyllacht et al., 1994; Krek et al., 1994; Xu et al., 1994).

The best characterized member of the E2F family is E2F-1 (Helin et al., 1992; Kaelin et al., 1992; Shan et al., 1992). During the S phase of the cell cycle, E2F-1 has been hypothesized to play a role in activating the expression of genes important for the execution of S phase. The positive influence of E2F-1 on transcription of S phase genes may be restrained during G0 and G1 by E2F-1’s interaction with the dephosphorylated form of Rb. Prior to entry into S phase, Rb becomes highly phosphorylated by cyclin-dependent kinases. This renders Rb incapable of binding to E2F-1 and may allow released E2F-1 to activate gene expression (Weinberg, 1995).

Further evidence that E2Fs promote entry into S phase derives from studies of Drosophila lacking E2F function in which cell proliferation was arrested in the G1 phase of the cell cycle after the seventeenth embryonic division (Duronio et al., 1995). In addition, overexpression of E2F-1 in mammalian cells can cause oncogenesis (Johnson et al., 1993; Qin et al., 1994; Xu et al., 1995; Yang and Sladek, 1995) raising the possibility that E2F-1 may regulate expression of genes important for cell death. Another possible function of E2F-1 might be to activate genes that promote the G1 to S phase transition during cell proliferation.

However, several recent studies indicate that E2F-1 may have functions in addition to promoting entry into S phase. One of these may involve regulation of apoptosis. The overexpression of E2F-1 or mutated forms of E2F-1 in serum-starved or actively growing fibroblasts can trigger apoptosis (Qin et al., 1994; Shan and Lee, 1994; Wu and Levine, 1994; Kowalki et al., 1995; Krek et al., 1995) raising the possibility that E2F-1 may regulate genes important for cell death. Another possible function is suggested by the observation that Rb-bound E2F-1 can still interact with its cognate DNA sequence and its release from E2F (Sellers et al., 1995). This finding has prompted the hypothesis that, by repressing transcription, the Rb/E2F-1 complex may play an active role in suppressing progression through G1 until an appropriate signal triggers Rb phosphorylation and its release from E2F (Sellers et al., 1995).

Despite considerable progress in elucidating the multiple potential functions of E2F-1 during cell proliferation, a potential limitation of the studies so far is their use of overexpression of wild-type or mutant versions of E2F-1 under conditions where wild-type E2F-1 was present and functional. Thus, it is uncertain whether the predominant function of E2F-1 is to promote entry into...
Figure 1. Mutation of the E2F-1 Gene

(A) Partial structure of the E2F-1 gene determined from restriction mapping, Southern hybridization, and DNA sequencing (data not shown). Exons are depicted as open boxes. The E2F-1 gene replacement construct is derived from a 7.4 kb fragment of the E2F-1 gene disrupted in exon 3 by the PGK-neo selectable marker. In addition, exon 4 is deleted. Adjacent to the E2F-1 DNA is the negative selectable marker (PGK HSV-TK). Restriction enzyme recognition sites are abbreviated as follows: E, EcoRV; S, SacI; Sm, SmaI; X, XcmI; H, HindIII; C, ClaI.

(B) Southern hybridization demonstrates the presence of the targeted E2F-1 mutation in mice. Mouse genomic DNA was digested with the restriction enzyme EcoRV, and analyzed by Southern blotting using an E2F-1 genomic probe (as depicted in [A]). The wild-type E2F-1 gene yields a band of 3.0 kb, while the mutant gene yields a band of 2.3 kb. Southern hybridization using other restriction enzymes and both 5' and 3' E2F-1 genomic probes demonstrates that the structure of the mutant gene is as expected in mice derived from all three ES cell lines (data not shown). Further, these data and the use of a probe for neo sequences indicate that additional copies of the gene targeting construct do not exist elsewhere in the genome (data not shown).

(C) Northern hybridization demonstrates that the homozygous E2F-1 mutation abolishes E2F-1 mRNA expression. RNA was harvested from cells arrested in S phase from two wild-type and two E2F-1 \(^{2/2}\) mice and used for Northern hybridization with a probe derived from the 3' coding region of the human E2F-1 cDNA (see Experimental Procedures). As shown, wild-type fibroblasts express an \(\sim 3.0\) kb message corresponding to the E2F-1 mRNA. This mRNA is not present in the E2F-1 homozygous mutant fibroblasts. The integrity and amount of RNA in each lane was demonstrated by stripping and rehybridizing the same blot with a probe specific for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

S phase, suppress G1 progression, and/or regulate apoptosis. To address this issue, we generated and studied E2F-1 deficient mice. Although these mice were found to develop and reproduce normally, they exhibited a number of defects in T cell development indicating that E2F-1 functions to positively regulate apoptosis and suppress cell proliferation. E2F-1 appears not to be critical for entry into S phase. Rather, this function may be served by other members of the E2F family. Taken together with previous findings demonstrating that E2F-1 can cause oncogenesis when overexpressed, the observations described here reveal that a single gene, depending on its level of expression or cellular environment, can function as either an oncogene, or paradoxically, as a suppressor of cell proliferation.

Results

Targeted Mutation of the E2F-1 Gene

A 7.4 kb fragment of the E2F-1 gene was used to generate a targeting vector in which the DNA binding and dimerization domains of the E2F-1 gene were disrupted by the insertion of a PGK-neo cassette (Figure 1A). The site of insertion lies within a region of E2F-1 in which mutations abolish the ability of E2F-1 to bind DNA, activate transcription, drive cell cycle progression, or trigger apoptosis (Helen et al., 1992; Kaelin et al., 1992; Cress et al., 1992; Johnson et al., 1993; Shan and Lee, 1994).

The E2F-1 targeting construct also deletes E2F-1 exon 4, encoding the leucine zipper and part of a "marked box" protein–protein interaction domain (Kaelin et al., 1992; Lees et al., 1993). Therefore, any RNA transcripts expressed from the mutated E2F-1 locus should not encode a functional protein. Gene-targeted J1 embryonic stem (ES) cell lines were used to produce mice heterozygous for the E2F-1 mutation. Heterozygotes (+/-) were intercrossed to generate homozygous mutant mice (-/-), as demonstrated by Southern hybridization (Figure 1B). Homozygotes produced from three independent ES cell clones were bred into both 129/Sv and 129/Sv × C57BL/6 backgrounds and gave similar results in all experiments described here.

RNA expression of primary embryonic fibroblasts isolated from wild-type and E2F-1-/- mouse embryos was examined by Northern analysis. Fibroblasts were synchronized at the start of S phase by exposure to hydroxyurea. Under these conditions, E2F-1 mRNA expression is maximal (Kaelin et al., 1992; Li et al., 1994),...
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and wild-type fibroblasts express a 3.0 kb E2F-1 transcript (Figure 1C). This transcript was not detected in the E2F-1+/− fibroblasts indicating that the engineered mutation successfully disrupted E2F-1 mRNA expression and therefore E2F-1 function.

**E2F-1 is Not Essential for Overall Development or Survival**

E2F-1+/− mice are born and appear overtly normal. Of 351 mice born to crosses of E2F-1+/− mice, 97 (28%) were of the +/+ , 172 (49%) of the +/− , and 82 (23%) of the −/− genotype, indicating that E2F-1 +/+ , +/− , and −/− mice were equally viable. Moreover, crosses between E2F-1+/− mice also produced overtly normal offspring. Although E2F-1−/− mice weighed 17% less than wild-type littermates for at least the first 8 months of life (p < 0.01, n = 56 wild type, n = 43 E2F-1+/−), no obvious gross or microscopic abnormalities were noted in the liver, brain, gut, cardiac or skeletal muscle, bone, spleen, kidneys, or pancreas at four to six weeks of age. Thus, E2F-1 is not required for overall development, survival, or reproduction of mice.

Since the E2Fs are believed to play a crucial role in regulating the G1/S transition, we examined the requirement for E2F-1 in cell proliferation and cell cycle progression. Surprisingly, in E2F-1+/− primary embryonic fibroblasts, the doubling time, cell cycle distribution, and timing of reentry into the cell cycle from G0 were indistinguishable from that of wild-type fibroblasts (Figures 2A and 2B). Likewise, when lymph node cells from four- to six-week-old E2F-1+/− mice and wild-type littermates were compared, they were found to respond to mitogenic signals equally well (Figure 2C). Cell proliferation in vivo was examined by injecting mice with 5-bromo-2′-deoxyuridine (BrdU) to label cells in S phase. As shown in Figures 2D and 2E (also Figure 5B and data not shown), proliferation in gut epithelium, spleen, lymph nodes, and thymus was indistinguishable between wild-type and E2F-1−/− mice at four to six weeks of age. By contrast, in older mice differences in proliferation between wild-type and E2F-1−/− mice were detected (see below). Nevertheless, on the basis of the findings in younger animals, we can conclude that under many circumstances E2F-1 is not required for cell proliferation or cell cycle progression. This may reflect the ability of other E2F family members to compensate for the absence of E2F-1.

**Thymus Abnormalities in E2F-1−/− Mice**

E2F-1 was found to play a critical role in normal thymic development. Upon dissection, the thymuses of four- to six-week-old E2F-1−/− mice were noticeably enlarged compared with those of +/+ littermates, even though other organs were consistently smaller. The ratio of thymus:body weight in E2F-1−/− mice is increased by 25% relative to wild-type mice (Figure 3A; p < 0.03, Student’s t test, n = 14 wild-type, n = 12 E2F-1−/−). The increased thymus size reflects a consistent increase in thymic cellularity demonstrated by a 55% increase in the number of thymocytes per thymus (Figure 3B; p < 0.02, Student’s t test, n = 10 wild-type, n = 10 E2F-1−/−). Several mechanisms might explain the increased number of thymocytes. These include increased seeding of the thymus with progenitors from the bone marrow, increased proliferation of thymocytes within the thymus, failure of mature thymocytes to leave the thymus, or a decreased rate of thymocyte death.

To investigate these possibilities, the developmental profiles of thymocytes from E2F-1−/− and wild-type mice were compared. By monitoring the expression of the CD4 and CD8 cell surface markers, the extent of thymocyte maturation was assessed. As thymocytes mature they progress sequentially through double-negative (CD4−/CD8−), double-positive (CD4+/CD8+), and single-positive (CD4+/CD8− or CD4−/CD8+) stages (Scollay et al., 1988). Upon staining with anti-CD4 and anti-CD8 antibodies, thymuses of four- to six-week-old E2F-1−/− mice were found consistently to contain a higher fraction of mature thymocytes (CD4+ or CD8+) than wild-type littermates (Figure 3C). To establish further the identity of the population of thymocytes expanded in the E2F-1−/− mice, thymocytes were stained with an antibody to CD3e, a component of the T cell receptor expressed at high levels in mature cells. The percent of CD3e+ thymocytes was significantly increased in E2F-1−/− compared with wild-type mice (44% ± 12% versus 18% ± 3% in a typical experiment), supporting the conclusion that in the absence of E2F-1 the fraction of mature thymocytes is increased. Taking into account the total number of thymocytes per thymus, the absolute number of cells in all thymocyte populations is increased in E2F-1−/− mice (Figure 3D). The E2F-1−/− mice exhibit a small increase in the absolute number of immature (CD4−/CD8− double-negative and double-positive) thymocytes. However, E2F-1−/− mice exhibit a larger, selective increase in the number of mature (CD4+CD8− single-positive) thymocytes. Although the smaller increase in the number of immature thymocytes could reflect increased seeding of the thymus with precursor cells from the bone marrow, the larger selective increase in the number of mature thymocytes could not. In subsequent experiments, we focused our attention on elucidating the mechanism by which a mutation in E2F-1 leads to a selective increase in the number of mature thymocytes.

A failure of thymocytes to exit the thymus and colonize the periphery does not account for the increase in the number of mature thymocytes in E2F-1−/− mice. First, flow cytometric analysis of lymph node cells using antibodies to CD3e or Thy1.2 (markers for T cells) and B220 (a marker for B cells) revealed that, as in wild-type mice, the lymph nodes of E2F-1−/− mice are composed primarily (70% to 80%) of T cells. In addition, E2F-1−/− mice have visibly enlarged, hypercellular lymph nodes (Figure 4; p < 0.01; Student’s t test, n = 6 wild-type, n = 6 E2F-1−/−), indicating that E2F-1−/− mice also have an increase in the number of mature T peripheral cells. Thus, mature T cells effectively exit the thymus of E2F-1−/− mice.

An increase in the rate of cell proliferation within the thymus of young E2F-1−/− mice was also ruled out as the explanation for the thymic hypertrophy observed in these animals. Thymocyte proliferation in vitro in response to stimulation with mitogens was found to be indistinguishable when thymocytes isolated from four- to six-week-old wild-type and E2F-1−/− mice were compared. When the cell cycle distribution of freshly harvested thymocytes was analyzed, thymocytes from four- to six-week-old wild-type and E2F-1−/− mice were
found to have similar cell cycle distributions (Figure 5A). To examine in vivo the fraction of cells in S phase in the thymuses of wild-type and E2F-1-/- mice, four- to six-week-old mice were injected with BrdU. Wild-type thymuses, as expected, demonstrated labeling predominantly of cells of the thymic cortex (Figure 5B), indicating proliferation of immature thymocytes. E2F-1-/- thymuses demonstrated a similar distribution and extent of labeling. Together, these experiments indicate that thymocyte proliferation both in vitro and in vivo for six-week-old mice is not affected by the absence of E2F-1.

On the other hand, our analysis revealed a clear difference in the extent of apoptotic cell death when thymocytes from wild-type and E2F-1-/- mice were compared. Apoptosis was initially examined by monitoring the percent viability of in vitro cultured wild-type and E2F-1-/- mouse embryos and cultured in vitro. Doubling time was measured by following the increase in cell number over time as described in Experimental Procedures. Wild-type and E2F-1-/- primary embryonic fibroblasts had similar doubling times of 36.5 ± 3.2 hr and 34.7 ± 2.9 hr, respectively (mean ± SEM). Error bars in this and subsequent figures indicate the SEM.

(B) The cell cycle distributions of exponentially growing wild-type and E2F-1-/- primary embryonic fibroblasts were found to be similar. To assay cell cycle distribution, 75% confluent dishes of fibroblasts were pulse labeled with BrdU for 1 hr, harvested, and fixed. DNA content was assessed by staining with propidium iodide, and DNA synthesis assessed by staining with an antibody to BrdU, as described in Experimental Procedures.

(C) Lymphocytes isolated from the lymph nodes of four- to six-week-old wild-type and E2F-1-/- mice were stimulated in vitro with 1.5 μg/ml concanavalin A and 100 U/ml interleukin-2 to assess cell cycle progression to S phase from G0. At various times after stimulation, cell cycle distribution was analyzed, as described in Experimental Procedures. As shown, lymphocytes from wild-type and E2F-1-/- mice responded equally well to concanavalin A and interleukin-2. In other experiments, other stimuli including phorbol myristate acetate and ionomycin or staphylococcal enterotoxin B and interleukin-2 were also shown to produce similar responses from wild-type and E2F-1-/- lymphocytes.

(D and E) In vivo BrdU labeling (as described in Experimental Procedures) identifies cells in S phase (appearing red) in organs from four- to six-week-old mice. As shown, gut epithelium (D) from four- to six-week-old wild-type (+/+, on the left) and E2F-1-/- (on the right) mice have similar numbers of cells in S phase. Similarly, the spleens (E) from four- to six-week-old wild-type (+/+, on the left) and E2F-1-/- (on the right) mice also have similar numbers of cells in S phase.
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Figure 3. E2F-1−/− Mice Have an Excess Number of Mature Thymocytes and T Cells

(A) The weights of the thymuses from 14 wild-type and 12 E2F-1−/− mice four to six weeks of age are expressed as fraction of total body weight. As shown, relative thymus size of E2F-1−/− mice is 25% greater than their wild-type littermates. For comparison, liver weight as a fraction of total body weight is 7% less for E2F-1−/− mice than their wild-type littermates (data not shown). The increase in size of the thymuses in E2F-1−/− mice compared with wild-type is statistically significant, with p < 0.03 (Student’s t test).

(B) Thymocytes were harvested from the thymuses of 10 wild-type and 10 E2F-1−/− mice. The number of thymocytes per thymus was counted and is shown in the graph. E2F-1−/− mice show a 55% increase in the number of thymocytes per thymus compared with wild-type littermates (mean ± SEM for wild type = 99 ± 11 × 10^6 cells per thymus, for E2F-1−/− = 154 ± 18 × 10^6 cells per thymus). The effect of genotype on number of thymocytes per thymus is statistically significant, with p < 0.02 (Student’s t test).

(C) Thymocyte expression of the CD4 and CD8 cell surface antigens was examined by flow cytometry. Thymocytes from wild-type and E2F-1−/− mice were labeled with a fluorescein conjugated antibody to CD8 and an R-phycoerythrin conjugated antibody to CD4. CD4 and CD8 expression per cell were analyzed by flow cytometry. In the graphs shown, each dot indicates the CD4 and CD8 expression levels for a single cell. The cell populations are divided into quadrants representing CD4/CD8 double-negative (lower left), CD4/CD8 double-positive (upper right), CD4 single-positive (upper left), and CD8 single-positive (lower right) populations. The numbers in each quadrant represent the percentage of cells in that quadrant (mean ± SEM). As shown, the more mature CD4 and CD8 single-positive populations are expanded in the E2F-1−/− mice. The experiment shown represents duplicate measurements from one pair of mice and is representative of seven experiments comparing in total 13 wild-type to 13 E2F-1−/− mice.

(D) Calculation of the absolute number of thymocytes in each subpopulation based on the percentages measured in (C) and the total number of cells per thymus for each animal. E2F-1−/− mice have a small increase in the absolute number of CD4/CD8 double-negative (lower left), CD4/CD8 double-positive (upper right), and CD8 single-positive (lower right) populations. The numbers in each quadrant represent the percentage of cells in that quadrant (mean ± SEM). As shown, the more mature CD4 and CD8 single-positive populations are expanded in the E2F-1−/− mice. The experiment shown represents duplicate measurements from one pair of mice and is representative of seven experiments comparing in total 13 wild-type to 13 E2F-1−/− mice.

Since the thymocyte population in E2F-1−/− mice differed from that of wild-type mice in that it includes a higher proportion of mature cells, a clear demonstration of a defect in apoptosis in the E2F-1−/− thymocytes required a comparison of the survival of developmentally homogeneous subpopulations of wild-type and E2F-1−/− thymocytes. CD4/CD8 double-positive or single-positive thymocytes from wild-type or E2F-1−/− mice were purified by cell sorting, and their survival in culture was assayed. Purified CD4+/CD8+ thymocytes from E2F-1−/− mice displayed a clearly enhanced level of survival in culture when compared with their wild-type counterparts (Figure 6B), confirming a defect in apoptosis in the E2F-1 deficient thymocytes. By contrast, the survival
E2F-1-deficient mice are not as susceptible to apoptosis as wild-type thymocytes. The lymph node cells from E2F-1−/− mice have similar numbers of cells in S phase in the thymus in vivo. Moreover, E2F-1−/− thymocytes demonstrate a defect in T cell receptor-stimulated apoptosis in vivo, as might be expected if there is a defect in negative selection in these mice. The defect in CD4+/CD8+ double-positive thymocyte apoptosis in E2F-1−/− mice is likely the cause of the increased production of single-positive thymocytes and T cells in these animals and probably accounts for the thymic and lymph node hypertrophy observed in the E2F-1−/− mice. We conclude that a primary function of E2F-1 in vivo is to regulate apoptosis in immature thymocytes.

Effects of E2F-1 Loss in Older Mice

We next considered the possibility that the decreased apoptosis in the CD4+/CD8+ thymocytes of young E2F-1−/− mice might explain the observed increase in thymocyte apoptosis in E2F-1−/− mice. The injection of anti-CD3 antibodies into wild-type mice led to a significant increase in thymocyte apoptosis. By contrast, thymocytes from E2F-1−/− mice exhibited significantly less (50%) anti-CD3-induced thymocyte apoptosis (Figure 7; p < 0.002, paired t test, n = 5 wild-type, n = 5 E2F-1−/−). Thus, E2F-1−/− mice display a defect in CD4+/CD8+ thymocyte apoptosis both in vitro and in vivo. Moreover, E2F-1−/− thymocytes demonstrate a defect in T cell receptor-stimulated apoptosis in vivo, as might be expected if there is a defect in negative selection in these mice. The defect in CD4+/CD8+ double-positive thymocyte apoptosis in E2F-1−/− mice is likely the cause of the increased production of single-positive thymocytes and T cells in these animals and probably accounts for the thymic and lymph node hypertrophy observed in the E2F-1−/− mice. We conclude that a primary function of E2F-1 in vivo is to regulate apoptosis in immature thymocytes.
morphology of liver, gut, brain, cardiac and skeletal muscle, spleen, bone, and kidney were found to be similar in wild-type and E2F-1−/− mice. However, older E2F-1−/− mice displayed moderate testicular atrophy (~60% reduction in weight, data not shown). The reduction in testicular weight seems to result from a decrease in the number of sperm found in many of the seminiferous tubules. Furthermore, these same tubules are observed to contain an overgrowth of interstitial Leydig cells. While the mechanism of testicular atrophy is uncertain, it is possible that an overgrowth of Leydig cells is the cause of decreased sperm production, and thus the decrease in testicular weight.

The most dramatic finding in the older E2F-1−/− mice was again in the thymus. Examination of cell proliferation in 10 wild-type and 11 E2F-1−/− mice by in vivo labeling with BrdU revealed a striking increase in proliferation in the thymus of older E2F-1−/− mice compared with wild-type littermates (Figure 8A). The proliferating cells in most of these E2F-1−/− mice maintained normal morphology and were localized to the thymic cortex but not the medulla. The localization of these proliferating thymocytes within the cortex suggests that they are likely to be immature thymocytes. The large increase in the number of proliferating cells in the thymic cortex of older E2F-1−/− mice was seen in all 11 E2F-1−/− thy- muses examined, indicating a role for E2F-1 in the control of thymocyte proliferation in older mice. Other tissues, including gut epithelium, spleen, lymph nodes, and liver in the E2F-1−/− mice displayed BrdU labeling indices that were comparable to those seen in wild-type mice (Figure 8B). One of the 11 older E2F-1−/− animals, in addition to displaying increased thymic proliferation,
also contained a region of the thymus with altered morphology indicative of a lymphoblastic lymphoma (Figure 8C). In vivo labeling of this animal with BrdU demonstrated that cells within the tumor were rapidly proliferating. None of the 10 wild-type littermates that were examined contained detectable tumors. More extensive analysis of older mice by Yasuyuki et al. (1996 [this issue of Cell]) has revealed sporadic tumorigenesis in a wide variety of tissues in E2F-1−/− animals, suggesting that our detection of a tumor in one of the 11 older animals that we have sacrificed may reflect a real increase in the rate of tumorigenesis in these mice. Consistent with this possibility, we have found that as our E2F-1−/− mice age they die at a significantly higher rate than their wild-type littermates. Although for many the cause of death remains to be elucidated, preliminary evidence suggests that a number of these mice had tumors. What we can conclude from our experiments is that as E2F-1−/− mice age they exhibit a consistent phenotype of thymic hyperproliferation that at least in one case resulted in the formation of a lymphoblastic lymphoma. Together, these findings indicate that E2F-1 functions in vivo as a suppressor of cell proliferation.

Discussion

To investigate the functions of E2F-1, we generated E2F-1 deficient mice. Surprisingly, mice deficient in E2F-1 are in many ways normal. E2F-1−/− mice undergo normal embryonic development, are fully viable at birth, and mature to become reproductively competent. The ability of E2F-1−/− mice to do so well may be indicative of the ability of other E2F family members to provide sufficient E2F function for most developmental pathways. Nevertheless, further examination of the E2F-1−/− mouse has led to the identification of important nonredundant functions of E2F-1.

Most notably, within one month of birth E2F-1−/− mice display visible enlargement of the thymus and lymph nodes despite a reduction in the size of other organs. These enlarged lymphoid organs reflect an expansion of the population of mature CD4/CD8 single-positive thymocytes and peripheral T cells. This expansion appears to be due to a defect in a normal pathway of apoptosis. The defect in CD4+CD8+ thymocyte apoptosis in E2F-1−/− mice is observed in every animal examined, indicating a consistent and important role for E2F-1 in thymocyte apoptosis.

Apoptosis has been previously shown to be an important event during normal thymocyte maturation. Notably, the elimination of thymocytes bearing self-reactive T cell receptors occurs during negative selection and is believed to be mediated by T cell receptor-stimulated apoptosis of CD4/CD8 double-positive thymocytes just prior to their conversion into mature thymocytes (Fowlkes et al., 1988; Kisielow and von Boehmer, 1991). These observations, taken together with our finding that E2F-1−/− mice have a defect in T cell receptor-stimulated apoptosis of CD4/CD8 double-positive thymocytes, raise the possibility that E2F-1 may play an important role in the process of negative selection.

As E2F-1−/− mice age, a second phenotype, characterized by a dramatic increase in thymocyte proliferation, is observed. This aberrant hyperproliferation occurs in the thymic cortex, but not thymic medulla or lymph nodes, suggesting that it is due to the presence of proliferating immature thymocytes. The finding that the disruption of E2F-1 function leads to increased thymocyte proliferation implies that E2F-1 normally functions as a suppressor of cell proliferation.

The fact that the decreased apoptosis detected in young mice and the increased cell proliferation seen in older mice both were observed in the thymus suggests that the two phenotypes may be mechanistically related. For example, inactivation of E2F-1's apoptotic function may lead indirectly to the hyperproliferation of immature thymocytes. Thus, the role proposed here for E2F-1 may be analogous to that of the p53 tumor suppressor gene.

p53 is believed to play a critical role in suppressing inappropriate cell proliferation. In cells that have undergone a mutation that leads to their aberrant proliferation, p53 somehow triggers cell death. In the absence of p53, for example in the cells of p53-deficient animals, these inappropriately proliferating cells do not die. Rather, they expand in number and can give rise to tumors. Similarly, the observed failure of immature thymocytes in E2F-1−/− mice to undergo normally scheduled apoptosis may result in the survival of an excessive number of proliferating thymocytes as is observed in older E2F-1−/− mice. Interestingly, this suggests that E2F-1 may play a direct role in regulating apoptosis independent
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of its function as a regulator of cell proliferation. Several recent findings provide support for this possibility. First, we find that the thymuses of young E2F-1−/− mice exhibit a defect in apoptosis in the absence of any measurable change in cell cycle distribution. Second, in a recent mutagenesis study E2F-1’s roles as a regulator of apoptosis and cell cycle progression were found to be separable (Krek et al., 1995).

Nevertheless, it remains a possibility that E2F-1 functions primarily as a suppressor of cell proliferation. Consistent with this possibility is the observation that E2F DNA binding sites in the promoters of several cell cycle genes function primarily to repress transcription of these genes during G0 and G1 (Weintraub et al., 1992, 1995; Neuman et al., 1995), presumably by binding E2F-Rb complexes. Recent data suggest that by repressing transcription E2F-1-Rb complexes suppress entry into S phase (Sellers et al., 1995). Thus, an E2F-1 deficient cell may be unable to tether Rb to E2F-1 DNA binding sites during G0 or G1. This might lead to aberrant expression of particular cell cycle regulatory genes and inappropriate cell proliferation. A role for E2F-1 in the suppression of thymocyte cell cycle progression might explain the aberrant hyperproliferation seen in the thymus of older E2F-1−/− mice.

If E2F-1 functions primarily as a suppressor of cell cycle progression, then the defect in apoptosis seen in young E2F-1−/− mice could be an indirect consequence of a defect in cell cycle progression. Although we detected no change in the cell cycle distribution of thymocytes in young E2F-1−/− mice, we cannot exclude the possibility that there is a subtle increase in proliferation or progression through the cell cycle in the E2F-1−/− cells. If it is necessary that a thymocyte be in a particular phase of the cell cycle to engage apoptosis, a change in cell cycle distribution caused by the mutation of E2F-1 might lead indirectly to a decrease in apoptosis.

A direct role for E2F-1 in either apoptosis or as a
suppressor of cell cycle progression may explain additional phenotypes that we and Yamasaki et al. (1996) observe in the E2F-1−/− mice such as testicular atrophy, increased mortality, and sporadic tumor formation. For example, the overgrowth of interstitial Leydig cells in the testes may be due to a failure of these cells to undergo apoptosis or growth arrest. Similarly, sporadic tumors in the E2F-1−/− mice might be the result of a defect in apoptosis or growth suppression in these cells. Although in the thymus E2F-1 plays a consistent role in preventing abnormal cell proliferation, the sporadic nature of the abnormal proliferation seen in other cell types suggests that in these cell types random mutations may be required to unmask E2F-1’s role in growth suppression.

The phenotype of the E2F-1−/− mice is surprising given the widely held view that E2Fs function primarily as positive regulators of cell cycle progression and that E2F-1 overexpression can cause oncogenesis. Our data does not support a unique role for E2F-1 as a promoter of cell proliferation. Rather, we find that E2F-1 plays a critical role as a positive regulator of apoptosis and a suppressor of cell proliferation. How can these roles be reconciled with the observed ability of E2F-1 to drive cell cycle progression and oncogenesis when overexpressed? It is likely that the level of E2F-1 expression is important. Under normal circumstances, in G0 and G1 E2F-1 may be completely bound up by Rb so that it functions primarily as a repressor of transcription and a suppressor of cell proliferation. When E2F-1 is overexpressed, there may not be sufficient Rb present in the cell to bind up all the E2F-1. Under such a circumstance, free E2F would be present and might be capable of promoting inappropriate entry into S phase and ultimately oncogenesis. Since under normal circumstances E2F-1 may have a dual role as a suppressor of cell proliferation in G0/G1 and an activator of proliferation in G1/S, the effect of a mutation in E2F-1 might be difficult to predict. The fact that a deletion of E2F-1 leads to a decrease in apoptosis and enhanced proliferation suggests that in vivo the negative effects of E2F-1 on cell cycle progression may be more important than the positive effects. Nevertheless, the fact that E2F-1 is a member of a large family of related genes leaves open the possibility that E2F-1 also plays an important role in promoting S phase entry in many cell types but that in its absence this role is compensated for by other family members.

A more thorough understanding of the multiple overlapping functions of different E2F family members must await the production of mice containing mutations of other E2F family members, either singly or in combination. Furthermore, the generation of E2F-1 deficient mice is a start in this direction and has provided new insight into the function of the E2F family. Somewhat unexpectedly the analysis of this animal, taken together with previous studies, has revealed that a single gene, depending on the circumstance, can function both as an oncogene and as a growth suppressor gene.

Experimental Procedures

Cloning

A murine E2F-1 genomic clone was isolated from a 129/Sv genomic library in λgt11 (Stratagene) screened by hybridization using GeneScreen (NEN) according to the instructions of the manufacturer. The hybridization probe was prepared from a 0.9 kb Sall–EcoRI fragment of the human E2F-1 cDNA plasmid pSP72-RBAP-1 (Kaelin et al., 1992) and labeled by random priming. DNA was isolated from purified positives and a 7.4 kb fragment spanning the bulk of the E2F-1 gene was subcloned into pBluescript KS(+) from two independent λ clones. Further restriction mapping, Southern hybridization, and DNA sequencing suggested that these two fragments were identical and represented the bona fide E2F-1 gene. To generate a gene targeting vector, a fragment containing PGK-neo was inserted by blunt-end ligation into the XcmI site in exon 3 of E2F-1 and a 1.7 kb Small/EcoRV fragment containing E2F-1 exon 4 was excised. The disrupted E2F-1 genomic fragment was then inserted into a backbone containing a PGK-TK gene to provide a negative selectable marker (Tybulewicz et al., 1991).

Embryonic Stem Cell Culture and Embryo Manipulation

J1 ES cell culture, electroporation, and selection of subclones were performed essentially as described (Li et al., 1992). Injection of ES cells into host blastocysts and their surgical introduction into foster mothers were performed essentially as described (Robertson, 1987).

Southern Hybridization and Northern Hybridization

DNA isolated (Laird et al., 1991) from the offspring of chimeras and subsequent generations was digested with EcoRV (or other restriction enzymes) and used in standard Southern blots (Church and Gilbert, 1984) using a probe prepared from E2F-1 genomic sequences to determine the genotypes of the mice.

For Northern hybridization, embryonic fibroblasts were isolated by standard methods (Robertson, 1987), grown to 75% confluence, and arrested in early S phase by treatment with 100 μM hydroxyurea for 16 hr. RNA was isolated (Chomczynski and Sacchi, 1987) and used in standard Northern hybridizations (Church and Gilbert, 1984) using a probe prepared from a 0.9 kb SalI–EcoRI fragment of the human E2F-1 cDNA (Kaelin et al., 1992) or a 1.3 kb probe prepared from a PstI restriction fragment of the rat glyceraldehyde-3-phosphate dehydrogenase cDNA (Fort et al., 1985).

Isolation of Primary Embryonic Fibroblasts and Measurement of Growth

Primary embryonic fibroblasts were harvested from 13.5- or 14.5-day-old embryos using the protocol of Robertson (1987) with minor modifications. Doubling times were measured by plating fibroblasts on multiple dishes, trypsinizing, and counting cell numbers on paired dishes after one, two, or three days of culture. Doubling time was calculated from the slope of the best fit line of logarithm of cell number as a function of time. Data were pooled from multiple experiments comparing in total fibroblasts independently isolated from four wild-type and four E2F-1−/− mouse embryos.

Harvest of Thymocytes and Lymph Node Cells

Thymuses or lymph nodes were mechanically dissociated between two pieces of ground glass. Debris was allowed to settle, the cells washed in medium (Dulbecco’s modified Eagle’s medium), and contaminating erythrocytes removed by hypotonic lysis (155 mM NaCl, 10 mM KHCO3, 0.1 mM EDTA [pH 7.3]) on ice for 5 min. The cells were washed again in medium and then used in subsequent experiments.

Cell Cycle Analysis

Cells were pulse labeled with 10 μM BrdU for 1 hr, washed in ice-cold phosphate-buffered saline (PBS), and fixed in ice-cold 70% ethanol. One to ten days later, cells were processed for flow cytometric analysis of DNA content (staining with propidium iodide) and DNA synthesis (staining with fluorescein isothiocyanate-conjugated antibody to BrdU) according to a protocol from the supplier of the antibody (Becton-Dickinson).

In Vivo S Phase Labeling

Mice were injected intraperitoneally with BrdU in PBS at a dose of 50 μg per gram of body weight. Mice were sacrificed 4 hr later, and organs harvested and fixed in 10% formalin. Slides of tissue sections...
were stained with antibody to BrdU and processed using the Vecta-Stain ABC alkaline phosphatase immunostaining kit (Vector Laboratories) according to the instructions of the manufacturer. Sections were counterstained with hematoxylin.

**Flow Cytometry and Fluorescence-Activated Cell Sorting**

Cells were suspended in blocking buffer (3% fetal bovine serum, 1% bovine serum albumin in PBS) and mixed with a fluorescein isothiocyanate-conjugated antibody (either anti-CD8 [clone 53-6.7], anti-CD3ε [clone 145-2C11] or anti-Thy1.2 [clone 30-H12]) and an R-phycocerythrin-conjugated antibody (either anti-CD4 [clone H129.19], anti-CD8 [clone 53-6.7], or anti-B220 [clone RA3-6B2], all purchased from Pharmingen). After 15 min, labeled cells were washed twice in blocking buffer, once in PBS, and fixed in fresh 1% paraformaldehyde in PBS for flow cytometry or resuspended in medium for fluorescence-activated cell sorting. Appropriate single color controls were used to set flow cytometer compensation and quadrants.

**In Vitro Apoptosis**

Thymocytes or lymph node cells were plated in 96-well plates at a concentration of 5 × 10^4 cells per 100 μl in licoce's modified Dulbecco's medium supplemented with 10% heat-inactivated fetal bovine serum, 0.5 mM β-mercaptoethanol, and 1 mM HEPES (pH 7.4). Immediately after plating, or at various time intervals, aliquots from replicate wells were mixed with an equal volume of 0.4% trypan blue, and the concentrations of live and dead cells counted on a hemacytometer. In situ nick translation performed by the method of Gold et al. (1993) was used to verify that DNA fragmentation was an early event in cell death.

**In Vivo Apoptosis**

Antibody to CD3ε (clone 145-2C11) was injected intraperitoneally at a dose of 1.9 to 2.5 μg per gram body weight (in a given experiment all mice were injected at the same dose). Control mice were injected with a similar volume (0.2 ml) of PBS. Thymocytes were isolated 16 hr later and processed for TUNEL (Gavriel et al., 1992) according to the instructions for the fluorescein Apoptag kit (Oncon). Compensation and gates for flow cytometry were set using appropriate controls. For negative controls TUNEL was performed omitting the terminal deoxynucleotidyl transferase. As positive controls, thymocytes were treated with 1 μM dexamethasone for 4 hr in vitro, or fixed thymocytes were treated with DNase.

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