High Incidence of Thymic Epithelial Tumors in E2F2 Transgenic Mice*

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In virtually all human tumors, genetic and epigenetic alterations have been found which affect the INK4/Cyclin D/Rb pathway, which regulates cell cycle entry and exit in normal cells. E2F transcription factors are important downstream components of this pathway, which act by controlling the expression of genes involved in DNA replication and cell cycle progression. To determine whether E2F2 deregulation promotes proliferation and tumorigenesis in vivo, we generated E2F2 transgenic mice, in which the Eμ and murine pim1 promoter (pp) direct high expression of E2F2 in thymic epithelial cells. Eμ-pp-E2F2 mice start to develop cytokeratin- and ER-TR4-positive cortical thymomas from the age of 20 weeks, and within 1 year, nearly all mice succumb to gross thymic epithelial tumors. General thymic morphology is largely maintained, but T cell development is perturbed in thymomas, with proportionately less CD4+CD8+ double-positive thymocytes. In the first 3 months, E2F2 transgenic thymi exhibit only mild epithelial hyperplasia, and thereafter thymomas arise stochastically, probably following additional mutations. Interestingly, Eμ-pp-E2F2 mice do not display cortical thymomas. These data argue that E2F2 promotes unscheduled cell division and oncogenic transformation of thymic epithelial cells.

E2F transcription factors are key regulators of cell division and function by controlling the expression of genes that are critical for DNA synthesis, DNA repair, and mitosis (1–3). Six distinct genes encode seven different E2F proteins (E2F1, E2F2, E2F3a, E2F3b, and E2F4 to E2F6), which form heterodimers with DP1, or one of the four different splice variants (α, β, γ, and δ) of human DP2 (4) or mouse DP3 (5, 6). However, E2F7, which binds to consensus E2F DNA recognition sites and acts as a transcriptional repressor, lacks the residues necessary for DP dimerization (7, 8). E2F1, E2F2, and E2F3a are potent activators of E2F-responsive genes, but their transcriptional activity is inhibited by binding to the retinoblastoma protein (pRb).1 pRb is functionally inactivated at the G1-S transition by cyclin D-cyclin dependent kinase 4 (Cdk4)/Cdk6 and cyclin E-Cdk2-mediated phosphorylation (9, 10), thus enabling E2Fs to activate their target genes. Besides promoting cell division, E2F1, and to a lesser extent E2F2 and E2F3a, also induce programmed cell death through both p53-dependent and p53-independent mechanisms.

Studies performed on E2F gene-targeted mice have demonstrated both unique and redundant functions of the different E2Fs (11). E2f1−/− and E2f2−/− mice are fully viable, and each mutant shows distinct cell lineage-specific phenotypes: E2f2−/− thymocytes are defective in T cell receptor-mediated apoptosis (12–14), and T lymphocytes proliferate less upon antigen stimulation in the absence of E2F1 (15, 16). In contrast, E2F2-deficient thymocytes show normal apoptosis characteristics, whereas E2f2−/− splenic T cells display accelerated G1-S phase progression (15, 16). On the other hand, erythropoiesis and pre-B cell differentiation are defective in E2f2−/− mice, whereas there is increased B cell maturation in E2f1−/− animals (17). E2F3 deficiency dramatically impairs cell proliferation of mouse embryonic fibroblasts and results in reduced neonatal viability (18). E2F1 and E2F2 share redundant roles with E2F3 during embryonic development because deficiency for either E2F1 or E2F2 exacerbates the lethal phenotype observed in E2f3−/− embryos (19, 20). Although E2f1−/−; E2f2−/− mouse embryonic fibroblasts proliferate efficiently, the combined loss of E2F1, E2F2, and E2F3 completely abolishes entry into S phase and further cell division (20).

E2f1−/− as well as E2f2−/− mice develop a similar spectrum of tumor types after a latency of 16 months, including lymphomas, hemangiosarcomas, and histiocytic sarcomas, arguing that E2f1 acts as a tumor-suppressor gene (21). The tumor-inhibitory function of E2F1 is illustrated further by the finding that overexpression of E2F1 inhibits glioma tumor growth (22), induces apoptosis in breast and ovarian carcinoma cell lines (23), and increases chemosensitivity of melanoma and pancreatic carcinoma cells (24, 25). Dominant negative mutants of E2F2/DP inhibit apoptosis and induce tumor growth of a non-malignant breast epithelial cell line (26). In addition, E2F1 suppresses tumorigenesis in mice expressing a c-Myc transgene under control of an epithelial-specific keratin (K5) promoter (27). E2f2−/− mice display aberrant peripheral immune tolerance and acquire autoimmune disease (15), but on an E2f1−/− background, E2f2−/− mice largely succumb to additional hematological malignancies (16), suggesting that E2F2 may also act as a tumor suppressor. Importantly, loss of E2F3-dependent kinase; DP, double positive; Eμ, immunoglobulin heavy chain enhancer; HA, hemagglutinin; K5, keratin 5; LTR, long terminal repeat; pp, pim1 promoter; TEC, thymic epithelial cell; DAB, 3,3′-diaminobenzidine tetrahydrochloride.

1 The abbreviations used are: pRb, retinoblastoma protein; Cdk, cyclin-dependent kinase; DP, double positive; Eμ, immunoglobulin heavy chain enhancer; HA, hemagglutinin; K5, keratin 5; LTR, long terminal repeat; pp, pim1 promoter; TEC, thymic epithelial cell; DAB, 3,3′-diaminobenzidine tetrahydrochloride.

Published, JBC Papers in Press, December 18, 2003, DOI 10.1074/jbc.M313682200
expression harbors no increased susceptibility for the development of tumors, both in wild type and E2F1 mutant mice.

On the other hand, enforced expression of E2F1 in the liver results in hepatocellular adenomas as well as large cell dysplasia (28). Transgenic K5-E2F1 expression induces spontaneous tumors that originate from epithelial basal cells (29), accelerates formation of benign skin papillomas in collaboration with a v-Ha-ras transgene (30), and enhances skin tumorigenesis in p53+/− mice (31). Furthermore, E2F1 deficiency reduces thymid and pituitary tumorigenesis in Rh+/− mice (32), delays the onset of Eµ-Myc-induced pre-B cell lymphomas (33), and enhances keratinocyte apoptosis after UVB exposure and γ-radiation (34). Interestingly, the absence of E2F1 reverses the UVB-induced apoptosis defect in primary fibroblasts and early onset thymic lymphomas seen in p53+/− mice, arguing that E2F1 acts functionally downstream of p53 (34).

These data demonstrate that in vivo E2F1 has both tumour-suppressive as well as tumor-promoting activity depending on cell lineage, stage of differentiation, status of the p53/ARF/MDM2 pathway, and expression level of Rb. However, there is much less information about the potential diverse functions of E2F2 in different cell lineages. E2F2 has an unexpected negative role in controlling antigen-stimulated T cell proliferation (17). In postmitotic lens fibers overexpression of E2F2 contributes to promote cell division (20), and in hematopoietic progenitors E2F2 is required for efficient S phase progression (17). In postmitotic lens fibers overexpression of E2F2 induces cell cycle entry and subsequent apoptosis (35). In this study, we provide evidence that E2F2 has a distinct function in promoting oncogenic transformation of nonlymphoid thymic epithelial cells (TECs).

EXPERIMENTAL PROCEDURES

E2F2 Transgenic Mice—For the generation of Eµ-pp-E2F2 mice, a 1.3-kb HA-tagged human E2F2 cDNA was inserted into the SacI-KpnI site of pJ31. The Nol-Hpal fragment containing HA-E2F2, followed by splice donor and acceptor elements derived from SV40 small t antigen intervening sequences present in pJ31, was cloned in EagI-Hpal site of Eµ-pim1 promoter-Moloney murine leukemia virus LTR transgenic vector. The assembled transgene was liberated with HindIII from the vector backbone, microinjected into pronuclei of FVB zygotes, and transferred to (B6 × DBA/F1)F1, founder mice. Eµ-pp-E2F2 founder mice and subsequent transgenic progeny were identified with PCR analysis on genomic DNA, and each of the founders was transmitted the E2F2 transgene to their progeny, except founder TEF2-6. To analyze expression of the different Eµ-pp-E2F transgenes in the thymus and compare transgene-driven E2F mRNA levels, Northern blot hybridization was performed using the Moloney murine leukemia virus U3LTR probe. Both Eµ-pp-E2F1 (TEF1-5) and Eµ-pp-E2F2 (TEF2-35 and TEF2-37) transgene were identified by PCR analysis on genomic DNA, and each of the founders was transmitted the E2F2 transgene to their progeny, except founder TEF2-6. To analyze expression of the different Eµ-pp-E2F transgenes in the thymus and compare transgene-driven E2F mRNA levels, Northern blot hybridization was performed using the Moloney murine leukemia virus U3LTR probe. Both Eµ-pp-E2F1 (TEF1-5) and Eµ-pp-E2F2 (TEF2-35 and TEF2-37) transgene were identified by PCR analysis on genomic DNA, and each of the founders was transmitted the E2F2 transgene to their progeny, except founder TEF2-6. To analyze expression of the different Eµ-pp-E2F transgenes, relative levels of transgene-driven HA-tagged E2F1 and E2F2 transgenic mRNA levels (Fig. 1B). Immuno blotting confirmed protein expression of transgene-derived HA-tagged E2F1 and E2F2 (data not shown). Western blotting for cyclin E and cyclin A protein levels on thymic cell extracts indicated that transgene-driven E2F2 expression, but not E2F1, resulted in enhanced cyclin E (Fig. 1C). Both cyclin E (Ccn1) and cyclin A (Ccn2) gene transcriptions are controlled by the pRb/E2F pathway (3), suggesting that overexpression of E2F2 in murine thymus activates cyclin E expression.

Eµ-pp-E2F1 and Eµ-pp-E2F2 founder lines were mated with FVB mice to generate larger cohorts of E2F transgenic mice. Interestingly, we observed a remarkable difference in phenotypes between E2F1 and E2F2 transgenic mice. Eµ-pp-E2F1 mice displayed a strong delay in endochondral ossification with concomitant dwarfism and a relatively high perinatal lethality among Eµ-pp-E2F2 animals (60% of transgenic offspring) (39). Thus with great difficulty, only one Eµ-pp-E2F1 line (TEF1-5) could be maintained. In contrast, progeny of all three Eµ-pp-E2F2 founder lines (TEF2-33, -35, and -37) were born alive with normal Mendelian ratios and showed no evidence of skeletal abnormalities. Therefore, subsequent functional studies were largely restricted to E2F2 transgenic mice.

Eµ-pp-E2F2 Transgene Is Highly Expressed in TECs—We decided to examine in more detail the expression pattern of the Eµ-pp-E2F2 transgene in the two major cell types present in thymus, thymocytes and TECs. Thymic epithelium forms a structurally and functionally important component of the thymus microenvironment and is required for selection of the
Cortical Thymomas in E2F2 Transgenic Mice

High Incidence of Spontaneous Thymic Tumors in Eμ-pp-E2F2 Transgenic Mice—The predominant phenotype observed in aging Eμ-pp-E2F2 animals of each transgenic line, which became apparent between the age of 5 and 11 months, was respiratory distress and dyspnea. Shortly thereafter, these E2F2 transgenic mice became cachectic and had to be sacrificed (Fig. 3). At autopsy, diseased Eμ-pp-E2F2 animals displayed severe hyperplastic thymi, often occupying the entire thoracic cavity (Fig. 4A). There was no evidence of enlarged secondary lymphoid tissues (spleen and lymph nodes) or macroscopic invasion of other organs. In most cases, the normal thymic bilateral structure was preserved in the solid hyperplastic thymi.

Hematoxylin and eosin-stained sections indicated that the functional organization of cortex and medulla was still present in all E2F2 thymic tumors examined, but darker stained lymphocyte-rich cortical areas were largely increased in size compared with their normal counterparts (Fig. 4, B and C). Pale stained medulla-like areas with Hassall corpuscle-like structures were present as isolated islets in enlarged thymus instead of one continuous area within a normal thymic lobe. These macroscopic and histological features recapitulated the characteristics of epithelial thymomas. A few Eμ-pp-E2F2 tumors showed small necrotic areas, but overall there was no evidence for high grade malignancy. None of the 18 Eμ-pp-E2F1 mice that survived into late adulthood developed thymomas or showed an enlarged thymus upon necropsy. These findings indicate that the induction of thymomas in Eμ-pp-E2F2 transgenic animals results as a consequence of deregulated E2F2 expression, but not E2F1. This different ability to induce tumors in thymic epithelium may well be related to the differential ability of E2F1 and E2F2 to induce apoptosis.

Phenotypic Analyses of Thymocytes in Eμ-pp-E2F2 Thymomas—To investigate whether thymocytes present in hyperplastic thymus of Eμ-pp-E2F2 transgenic mice showed evidence of transformation, T cell receptor rearrangements were assessed by Southern blotting. T cell lymphomas are normally mono- or oligoclonal in origin, which can be demonstrated by a specific and unique rearrangement pattern at the joining regions of the T cell receptor β locus (Fig. 4D, lane 2). However, Eμ-pp-E2F2 tumors contained either a regular germ line configuration, as seen in wild-type thymus (Fig. 4D, lanes 1, 3, 5, 6, and 8), or polyclonal rearrangements (Fig. 4D, lanes 4 and 7), arguing against the presence of T lymphoid tumor cells.

These findings were corroborated by flow cytometric analyses, which also showed no indications for clonal outgrowth of specific thymocyte subsets in Eμ-pp-E2F2 thymic tumors as defined by CD4 and CD8 coreceptor expression (Fig. 4E). Instead, we found altered ratios of thymocyte subsets in Eμ-pp-E2F2 thymomas, with relatively more immature CD4–CD8– double negative pre-T cells as well as a higher proportion of postselected CD4+ CD8– double positive thymocytes (Fig. 4E and Table 1). Young Eμ-pp-E2F2 mice without thymomas showed normal CD4/CD8 thymocyte distributions (data not shown). These analyses indicate that enforced expression of E2F2 in TECs results in cortical thymomas with concomitant perturbation of T cell development.

Thymomas Express Increased Levels of Transgenic E2F2—We wanted to assess whether the induction of thymic tumors in E2F2 transgenic mice was associated with altered E2F2 expression levels. Northern blot analysis demonstrated that the occurrence of thymomas correlated with more abundant Eμ-pp-E2F2 transgene expression. All tumor samples showed enhanced levels of transgenic E2F2-LTR transcripts compared with nontumorigenic counterparts (Fig. 5A). Similar results were observed when E2F2 protein levels were analyzed by immunoblotting. Human HA-E2F2, which migrates slower...

Fig. 1. Eμ-pp-E2F1 and Eμ-pp-E2F2 transgene construction and expression. A, schematic representation of the Eμ-pp-E2F transgenes, where HA-tagged human E2F1 or E2F2 cDNAs, in conjunction with small t antigen splice donor and splice acceptor sequences, were cloned into the EagI-HpaI enzyme restriction sites of the Eμ-pim1 promoter transgenic vector. The Moloney murine leukemia virus LTR provides a polyadenylation signal. B, Northern blot analysis on total RNA isolated from thy-mi of 5–8-week-old wild type (WT), Eμ-pp-E2F1 (TEF1-5), and Eμ-pp-E2F2 (TEF2-35 and TEF2-37) transgenic mice. Blots were hybridized with U3LTR probe to detect transgene expression and β-actin as loading control. C, immunoblotting demonstrates cyclin E and cyclin A protein levels in total cell extracts of wild-type, E2F1 (TEF1-5 and TEF1-62), and E2F2 (TEF2-35 and TEF2-37) transgenic thymi, with actin as loading control.

appropriate T cell repertoire on the cell surface of thymocytes (40, 41). Total mRNA was extracted from freshly isolated thymocytes or TECs that were cultured short term in vitro to obtain pure epithelial cells in sufficient quantity. Surprisingly, Northern blot analysis indicated that Eμ-pp-E2F2 was highly expressed in purified TECs and very low or nondetectable in primary thymocytes (Fig. 2A). Similarly, transgenic E2F2 expression was absent in mature peripheral T cells (data not shown). TECs isolated from Eμ-pp-E2F1 mice displayed reduced viability and were not able to grow in vitro, suggesting that E2F1 overexpression promotes apoptosis in these cells. Immunohistochemical analysis performed on thymic sections of Eμ-pp-E2F2 mice confirmed HA-E2F2 nuclear staining in TECs from E2F2 transgenic thymi but not wild-type controls (Fig. 2, B and C), and high E2F2 protein levels coincided with the network of cytokeratin-positive TECs interspersed between islets of thymocytes (Fig. 2, D and E). Thus, pim1 promoter in conjunction with Eμ sequences direct high transgene expression of E2F2 in TECs.

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than murine E2F2 during SDS-PAGE, was strongly up-regulated in total cell extracts of Eμ-pp-E2F2 thymomas compared with nontumorigenic thymi of young E2F2 transgenic animals (Fig. 5B). In contrast, endogenous levels of mouse E2F2 remained similar in normal and hyperplastic thymi from Eμ/H9262-pp-E2F2 mice of each transgenic line tested (Fig. 5B). Thus, thymomas that arise in Eμ-pp-E2F2 mice show an increase in transgenic E2F2 expression levels, which most likely results from the expansion of non-T lymphoid E2F2-expressing TECs.

E2F2-induced Thymomas Arise from ER-TR4/H11001 Thymic Cortical Epithelial Cells—To confirm that E2F2-induced thymic tumors indeed originated from cytokeratin-positive thymus epithelium, immunohistochemistry was performed on normal and Eμ/H9262-pp-E2F2 hyperplastic thymi. In wild-type thymus, cortical and medullary areas could easily be distinguished morphologically and by pan-cytokeratin staining, with thymic cortical epithelial cells oriented perpendicularly toward the capsule that envelops the thymus (Fig. 6A). The cortical areas in E2F2 thymomas, however, displayed a more disoriented architecture, notably at subcapsular areas and cortical-medullary junctions (Fig. 6B). Overall, epithelial cells were increased in numbers and formed small clusters, giving rise to more intense cytokeratin staining.

Three classes of epithelium can be phenotypically distinguished in thymus: subcapsule (ER-TR5\(^+\)), cortex (ER-TR4\(^+\)), and medulla (ER-TR5\(^-\)), even though many different subtypes of epithelial cells exist in each of these areas (41, 42). Immunohistochemical analysis indicated that E2F2-induced thymomas showed more intensive staining and larger areas of ER-TR4\(^+\) epithelial cells compared with normal thymus (Fig. 6, C).
**FIG. 4.** Characterization of thymomas in Eμ-pp-E2F2 transgenic animals. A, macroscopic representation of severe hyperplastic thymus filling thoracic cavity of TEF2-35 animal at age of 8 months. The lungs and heart are almost completely obscured by the thymic tumor. The scale bar represents 8 mm. B and C, hematoxylin and eosin-stained paraffin sections of wild-type (WT) thymus at age of 4 weeks and TEF2-33 thymoma at age of 7 months. Medulla (M) and cortical (C) areas are indicated. Scale bars represent 5 mm. D, Southern blot analysis on genomic DNA isolated from thymi of wild-type (WT), Moloney murine leukemia virus-induced T cell lymphoma, Eμ-pp-E2F2 thymomas of founder TEF2-6 (F2-6), and TEF2-33 (F2-33), and E2F2 transgenic animals 44, 771 (both TEF2-33), 691, and 410 (both TEF2-35). Each genomic DNA sample is digested with PvuII, blotted, and hybridized with a T cell receptor β2 probe to detect T cell receptor rearrangements. The arrow indicates the position of the germ line band. The asterisk indicates the monoclonal rearranged T cell receptor β allele. E, flow cytometry was performed on a single cell suspension of thymocytes isolated from wild-type thymus and Eμ-pp-E2F2 thymoma of founder TEF2-6, stained with anti-CD4 and anti-CD8 antibodies. The percentage of cells in each quadrant is indicated.

### Table I

**Latency and distribution thymocyte subsets of Eμ-pp-E2F2 thymomas**

The age at which moribund Eμ-pp-E2F2 animals were sacrificed is indicated together with individual mouse identification numbers and transgenic founder lines of origin. Fraction of CD4⁺/CD8⁺ double negative, CD4⁺/CD8⁺ double positive, CD4⁺/CD8⁻ single positive, and CD4⁺/CD8⁻ single positive thymocyte subsets are shown as determined by flow cytometric analysis on freshly isolated thymic tumor single cell suspensions. For reference, the normal distribution in wild-type (WT) FVB mice is indicated. For some thymomas, the distribution of thymocyte subsets was not determined (ND).

| Animal | Eμ-pp-E2F2 line | Age (weeks) | CD4⁺/CD8⁻ (WT, 4%) | CD4⁺/CD8⁺ (WT, 78%) | CD4⁺/CD8⁻ (WT, 14%) | CD4⁺/CD8⁺ (WT, 4%) |
|--------|----------------|-------------|---------------------|---------------------|---------------------|---------------------|
| 35     | TEF2-6 founder | 36          | 22%                 | 39%                 | 27%                 | 12%                 |
| 44     | TEF2-33 founder | 48          | 14%                 | 48%                 | 27%                 | 11%                 |
| 771    | TEF2-33 F₁      | 24          | 6%                  | 71%                 | 19%                 | 6%                  |
| 773    | TEF2-33 F₁      | 30          | ND                  | ND                  | ND                  | ND                  |
| 409    | TEF2-35 F₁      | 32          | 9%                  | 67%                 | 19%                 | 5%                  |
| 691    | TEF2-35 F₁      | 31          | 6%                  | 55%                 | 30%                 | 9%                  |
| 354    | TEF2-37 F₁      | 36          | ND                  | ND                  | ND                  | ND                  |
| 496    | TEF2-37 F₁      | 30          | 5%                  | 64%                 | 24%                 | 7%                  |
and D). In contrast, ER-TR5+ medullary and subcapsule epithelial cells in thymomas exhibited an architecture similar to and expression level equal to those in wild-type thymus (Fig. 6, E and D). Similarly, there was no enrichment of ER-TR7+ mesenchymal fibroblasts in thymomas (Fig. 6, G and H), implying that Eμ-pp-E2F2 thymomas are primarily related to ER-TR4+ cortical epithelial cells.

Secondary Mutations Are Required for Tumor Phenotype in E2F2 Mice—Overexpression of cyclin D1 and D2 in mouse thymic epithelium induces severe hyperplasia, which is already evident at the age of 8 weeks (43, 44). To investigate the extent of thymic hyperplasia in young Eμ-pp-E2F2 mice and study the development of E2F2-induced thymomas in time, thymi from transgenic animals and wild-type siblings from line TEF2-35 were harvested at ages of 8, 12, 16, and 20 weeks, and thymus wet weight was determined. In normal FVB mice, involution of thymic hyperplasia in young E2F2 Mice—Overexpression of cyclin D1 and D2 in mouse thymic epithelium induces severe hyperplasia, which is already evident at the age of 8 weeks (43, 44). To investigate the extent of thymic hyperplasia in young Eμ-pp-E2F2 mice and study the development of E2F2-induced thymomas in time, thymi from transgenic animals and wild-type siblings from line TEF2-35 were harvested at ages of 8, 12, 16, and 20 weeks, and thymus wet weight was determined. In normal FVB mice, involution of thymic hyperplasia in young E2F2 transgenic mice, the first significant hyperplastic thymi were harvested at ages of 8, 12, 16, and 20 weeks, and thymus size started to appear at the age of 20 weeks, displaying a 20-fold increase in thymus weight and histological evidence of malignant transformation. Thus, there was a clear lag phase before thymic tumors all show characteristics reminiscent of human type B1 thymoma (43, 44).

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**DISCUSSION**

E2F transcription factors have important functions in controlling cell cycle progression, apoptosis, differentiation, and replicative senescence (45, 46). Ectopic expression of E2F2, like E2F1, induces S phase entry in immortalized quiescent rodent fibroblasts (47, 48), primary adult rat sensory neurons (49), and postmitotic epithelial lens fiber cells (35). However, it has been suggested that in primary diploid fibroblasts, entry into S phase by E2F1 or E2F2 overexpression requires the additional inactivation of pRb- or p53-mediated G1 checkpoint control (50). Interestingly, E2F2 seems to have opposing roles in regulating cell division of hematopoietic cells because antigen-stimulated E2F2+/− splenic T cells show accelerated G1-S phase progression (15, 16), whereas S phase is stalled and prolonged in E2F2−/− hematopoietic progenitor cells (17).

In this study, we demonstrate that enforced expression of E2F2 results in mild hyperproliferation of the thymus in young Eμ-pp-E2F2 transgenic mice and the subsequent development of thymomas. The Eμ-pp-E2F2 thymic tumors all show characteristics reminiscent of human type B1 thymoma ("organoid" thymoma). The tumor mimics normal thymus with areas of cortical and medullary differentiation, however with architectural disorganization between the distinct compartments and within the cortical compartment. This is reflected by concentrated patches of ER-TR4+ cortical TECs, without an in-
isolated TECs cultured in vitro cells of the thymus, as shown by RNA expression analysis on SV40 small t antigen intervening sequences at the 3′ of artificial splice donor and acceptor sequences, consisting of (data not shown). Therefore, we speculate that the introduction pattern of E/H9262 transgenic cDNA inserts, may have modified the expression results from the expansion of cytokeratin-positive epithelial cells. It is not clear why the E/H9262 transgene does not drive detectable E2F2 mRNA expression in the T cell lineage, which is normally the case. Similarly, we have not been able to detect E/H9262-E2F1 mRNA expression in isolated thymocytes (data not shown). Therefore, we speculate that the introduction of artificial splice donor and acceptor sequences, consisting of SV40 small t antigen intervening sequences at the 3′-end of the transgenic cDNA inserts, may have modified the expression pattern of E/H11001 and pim1 promoter. Alternatively, thymocytes expressing high levels of human HA-E2F1/2 could have been negatively selected.

Some epithelial cell lineages, like skin epidermis or gut epithelium, have a rapid cell turnover and are characterized by the presence of stem cells that give rise to progenitor and terminally differentiated cells (51). It has been demonstrated that E2F expression and activity are tightly regulated during development and differentiation of these epithelial tissues (52, 53), suggesting a critical role of E2F transcription factors in controlling epithelial cell cycle kinetics. Thymic epithelium originates from the third pharyngeal pouch endoderm, and recently a common epithelial precursor cell has been identified in embryonic thymus (54, 55). However, it has not yet been established whether this embryonic thymic precursor cell harbors the capacity for self-renewal and whether it represents an adult thymic epithelial stem cell. A unique feature of the thymus is its involution quite early in life, which correlates with deterioration of thymic stromal structures, including epithelium. This observation may argue against the existence of true epithelial stem cells within the adult thymus and suggests the presence of progenitors with more restricted self-renewal capacity and high differentiation probability (transit-amplifying cells).

Our data suggest that enforced E2F2 expression prevents the onset of thymic involution because thymus size does not decline in E2F2 transgenic animals beyond the age of 12 weeks as normally occurs in wild-type mice. Between the age of 8 and 12 weeks, Eμ-pp-E2F2 mice display mild thymic hyperplasia with on average a 2-fold increase in thymus weight compared with control littermates. One likely explanation for the observed thymic hyperplasia is an increased proliferative capacity of E2F2 transgenic TECs, which correlates with our finding of up-regulated cyclin E expression in Eμ-pp-E2F2 thymi. Furthermore, E2F2 transgenic TECs demonstrate a higher plating efficiency when cultured in vitro compared with wild-type control cells (data not shown). Therefore, it is plausible that enforced E2F2 expression could also extend the replicative potential of these TECs. Additional studies will be performed in the near future to address these issues in more detail. In addition, it remains to be established whether cortical epithelial cells are more susceptible than medullary TECs for E2F2-induced hyperproliferation or if this is just the result of differential transgene expression. Remarkably, E2F1 transgenic TECs display reduced viability and cannot be expanded in vitro, showing earlier on hallmarks of senescence. Thus, E2F1 and E2F2 transgenic TECs display opposite phenotypes, arguing that different transcriptional targets are regulated by E2F1 and E2F2 in these TECs.

After the initial stage of mild thymic hyperplasia which is observed in all Eμ-pp-E2F2 mice of three independent founder lines (TEF2-33, TEF2-35, and TEF2-37), there is a significant delay of about 5 months before overt thymomas arise from which the mice eventually succumb. These findings suggest that additional genetic mutations need to accumulate to achieve complete immortalization of Eμ-pp-E2F2 cortical epithelial cells. The course of thymic hyperplasia is in this respect rather different from K5-cyclin D1 and D2 mice, where gross TEC hyperplasia is already evident at a much earlier age of 8–10 weeks (43, 44). Thus, deregulated E2F2 expression alone seems not to be sufficient for epithelial cell transformation. Disruption of both p16INK4A/pRb and p53 pathway is essential to bypass the senescence checkpoint in human epithelial cells (56), and inactivation of both pathways contributes to the onset of thymic epithelial tumors in SV40 large T and human papillomavirus 16 E6/E7 transgenic mice (57, 58). These data suggest that mutation of p53/MDM2/p19ARF pathway could be a rate-limiting step for the induction of cortical thymomas in Eμ-pp-E2F2 mice. Alternatively, activation of proto-oncogenes like c-myc (59) or c-fos (60) may contribute to transformation of TECs.

Our data show that Eμ-pp-E2F1 and Eμ-pp-E2F2 mice display completely distinct phenotypes. Overexpression of E2F1 inhibits chondrocyte differentiation and delays endochondral ossification, resulting in dwarf mice with severely reduced postnatal viability (61). On the other hand, enforced expression of E2F2 has no appreciable effect on endochondral bone formation but selectively induces TEC tumors. Although we have not been able to perform extensive analysis on Eμ-pp-E2F2 mice because of the small numbers of viable adult mice, it is clear that none of the 18 adult E2F1 transgenic animals, subjected to postmortem examination, displayed evidence of thymic hyperplasia. These findings are corroborated by the lack of thymic epithelial hyperplasia in K5-E2F1 transgenic mice (29, 30). Our results support an emerging theme that E2F1 and E2F2 have several different nonredundant roles in vivo (11). E2F1 is required for efficient negative selection of thymocytes (12, 14) and impairs pre-B cell development (17). E2F1 inhibits terminal differentiation of various mesenchymal cell lineages, including chondrocytes (61), and suppresses the formation of
lymphomas, hemangio- and histiocytic sarcomas (21). On the other hand, E2F2 is essential for proper erythropoiesis and S phase progression of hematopoietic progenitor cells (17) and regulates peripheral immune tolerance (15).

In conclusion, the presented data show that E2F2 acts as a positive regulator of cell proliferation in TECs and induces thymic epithelial tumors (cortical thymomas), thereby establishing for the first time oncogenic activity of E2F2 in vivo.

Acknowledgments—We thank Dr. Willem van Ewijk for antibodies, Animal Pathology for histological analysis, and the Transgene Core facility for generating and maintaining mice.

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Correlation of Thymoma in E2F2 Transgenic Mice

10483

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