The Retinoblastoma Gene Regulates Somatic Growth during Mouse Development

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ABSTRACT

Overexpression of the retinoblastoma gene (Rb) in mice leads to the dwarf phenotype. To explore the potential mechanism of Rb effects on the somatic growth, bitransgenic mice with tetracycline-regulated Rb expression were generated, and their phenotypes were compared with those of previously established Rb mouse models. By gestational day 12.5, embryos lacking Rb and those expressing twice the regular amount of Rb are 15% larger and 10–30% smaller, respectively, compared with their wild-type littermates. The dwarf phenotype is associated with increased plasma levels of insulin-like growth factor-I (IGF-I) but not with growth hormone and glucose concentrations. Down-regulation of the Rb transgene results in a reduction of the IGF-I plasma concentrations to normalcy and an increase of somatic growth prenatally and postnatally. Consistent with the in vivo results, cells overexpressing Rb require higher thresholds of IGF-I to stimulate proliferation. Thus, Rb plays an integral role for mouse somatic growth and maintenance during ontogenesis, and IGF-I pathway is likely to be a target for such regulation.

INTRODUCTION

The Rb6 susceptibility gene (Rb) encodes a nuclear phosphoprotein with DNA-binding properties. Defects in Rb-associated pathways are considered to be among the most common mechanisms contributing to cancer (1–4). Reconstitution of Rb results in suppression of the malignant phenotype both ex vivo and in vivo (4–8). A better understanding of how Rb functions in vivo may directly facilitate the rational design of Rb gene therapy and prevention, as well as forecast potential tissue-specific effects of such treatments.

Ex vivo studies indicate pleiotropic functions of Rb in restriction of cell cycle progression, modulation of terminal differentiation, and control of cell survival (7, 9–17). Consistently, these biological functions of Rb were also revealed in animal studies. The complete absence of Rb leads to mouse death by GD 14.5 (18–20). By this stage, Rb−/− mice exhibit ectopic deregulated proliferation, excessive apoptosis, aberrant differentiation in the neural tissues and ocular lenses, and defects in erythropoiesis (12, 18–23). Mice expressing <50% of the amount of Rb in wild-type littermates survive until birth but have defects in skeletal muscle development (24). Moreover, chimeric animals with an extensive contribution from Rb-deficient cells develop normally (25, 26). These findings indicate an essential role of microenvironment in the survival and growth of Rb-deficient chimeras. Compared with their wild-type littermates, Rb+/− mice are larger, and their tissues contain approximately 60% of Rb present in wild-type littermates (1, 27). Conversely, overexpression of Rb in transgenic mice results in dose-dependent reduction in weight (28, 29). The timing of the influence of Rb during development and its contribution to the general maintenance of somatic growth remain unclear. Because Rb has pleiotropic functions in various cell lineages, its influence on body growth and weight may result from either central or peripheral mechanisms. In one scenario, alterations of Rb expression in neurogenesis might result in specific defects in cells responsible for growth regulation, e.g., neurons producing growth hormone-releasing factor (30) or hepatocytes producing serum IGF-I. In this case, expression of pertinent growth stimulatory factors, GH or IGF-I, might be lower than normal. Alternatively, Rb may influence the differentiation of specific tissues or cell types, e.g., adipocytes (16), serve as a general negative regulator of cell proliferation, or perhaps perform both functions. Such peripheral Rb functions would be expected to increase the amount of growth/differentiation-promoting factors because of feedback mechanisms.

To address how Rb regulates somatic growth, mice with conditionally regulated Rb were prepared. Tetracycline-mediated transgene regulation offers advantages over constitutive, continuous expression of exogenous gene, for it allows the evaluation of dosage-specific effects of the gene expression during a defined window of the time (31–37). Thus, the complexities of the whole organism in growth regulation and maintenance of homeostasis can be alleviated considerably. Using this mouse model, the results described in this study suggest that Rb plays an integral role for mouse somatic growth and maintenance during ontogenesis, and IGF-I is likely to be an important mediator for this process.

MATERIALS AND METHODS

Constructs. To construct RBp-tTA, the SV40 late polyadenylation signal BamHI-HindIII fragment of pUHD15–1, containing tetR-VP-16 (tTA) chimeric gene (31), was substituted with the 300-bp bovine growth hormone polyadenylation site (38) derived from pGKneo (18) resulting in construct pGK6. The 1.6-kb EcoRI-HindIII RB promoter derived from plasmid pRB-CAT (39) was cloned to pBluescript II SK+/−, resulting in construct pBSK-RBp. The SalI-EcoRI 0.6-kb fragment containing the rabbit β-globin intron from pCMV-Neo Bam (40) and the 1.3-kb EcoRI-XhoI tTA-bpA from pGK6 were ligated to the SalI-digested pBSK-RBp, resulting in RBp-tTA. To generate hCMV+1-1p-RB, the 2.8-kb BamHI fragment of pRB44–2 containing full length RB CDNA (28, 41) and the 1.6-kb BamHI-HindIII β-globin polyadenylation site (28) were ligated into BamHI-HindIII restricted pUHD 10-3 (31).

Experimental Animals. To prepare transgenic mice, either the 4.9-kb XhoI-HindIII fragment of ICMV+1-1p-RB or 3.6-kb PspI-XhoI fragment of RBp-tTA (Fig. 1, A and B) were isolated with GELase (Epicentre Technologies, Madison, WI) and injected into fertilized eggs of superovulated C57BL mice at a concentration of 2 ng/μl. One-cell embryos were transferred to the oviducts of pseudopregnant CBA females according to Hogan et al. (42). Bitransgenic mice with conditionally regulated RB transgene were obtained as a result of a cross between transgenic hCMV+1-1p-RB and RBp-tTA animals. All of the animals were screened for transgene integration by FCR analyses of DNA from digits (Fig. 1C and below). Copy number, structure, and orientation of the transgenes were analyzed by Southern hybridization of genomic DNA after endonuclease restriction.

Preparation and characterization of mice with a mutant copy of the RB gene (18) and mice containing the human RB transgene under control of the RB
promoter (28) have been described previously. To exclude possible inadvertent effects of a mixed genetic background, all of the mice were intercrossed for more than 10 generations. All of the mice were maintained identically, following the recommendations of the Institutional Laboratory Animal Use and Care Committee. Animals were housed under minimum stress conditions and a circadian pattern that included light from 5:00 a.m. to 7:00 p.m. GD 1 and P

**Genotyping.** 

Both wild-type (Lanes 1 and 4), Tg Rbp-tTA (Lanes 5), Tg hCMV+1p-RB (Lanes 6 and 8), and bitransgenic RBp-tTA/hCMV+1p-RB (Lanes 2, 3, and 7) mice are identified. D, luciferase activity in primary mouse tail fibroblasts transfected with luciferase reporter plasmid pUHC-13.3. Average luminescence in transfected wild-type cell lysates (n = 10) was defined as one RU. All of the measurements were repeated twice in triplicates.

**Hormone and Glucose Measurements.** Repeated 100-μl blood samples were collected from the retroorbital venous plexus in heparinized tubes, whereas animals were anesthetized with chloral hydrate (400 μg/g body weight) at either 9:00 a.m. or 4:00 p.m. After centrifugation at 4°C, plasma samples were stored at −80°C until assayed. Growth hormone was quantified with a rat growth hormone (tgH pA) ELISA (Peninsula Laboratories, San Carlos, CA). Blood glucose concentrations were determined with One Touch quantitative test based on a colorimetric assay (LifeScan, Milpitas, CA).

**Body Weight and Skeleton Analyses.** Fetuses, placentas, and internal organs of adult animals were fixed in 4% paraformaldehyde and washed in PBS. Upon the elimination of excess of liquid, the mass of samples was estimated with precision to four significant figures. Weighing of living animals was performed at 4 p.m.

**Morphological Analyses and Immunostainings.** All of the mouse tissues were either immersed in Bouin’s fluid or fixed in phosphate-buffered 4% paraformaldehyde by perfusion at 90 mm of Hg in mice anesthetized with avertin. After evaluation and dissection under a stereo operation microscope (Nikon), all of the samples were embedded in paraffin (Paraffin X-TRA; Fisher), sectioned at 4 μm, and stained with Mayer’s hematoxylin and eosin. Immunohistochemical detections of human and mouse Rb on paraffin sections of paraformaldehyde-fixed material were performed essentially as described earlier (7, 43).

**Protein Analyses.** Immunoprecipitations were performed as in Bignon et al. (28). Briefly, subconfluent cells or snap-frozen tissues were washed three times with ice-cold PBS and lysed in 250 buffer [50 mM Tris-HCl (pH 7.4); 250 mM NaCl; 5 mM EDTA; 0.1% NP40; 50 mM NaF; 1 mM phenylmethylsulfonyl fluoride; 1 mM DTT; 50 μg/ml aprotinin; 1 μg/ml pepstatin A; and 1 μg/ml leupeptin]. After a freeze-thaw cycle, precleared supernatants were immunoprecipitated with either rabbit polyclonal immunoglobulin fraction C-15 (Santa Cruz Biotechnology; Santa Cruz, CA) or Mab 11D7 (45), followed by protein Sepharose CL-4B (Pharmacia Biotech; Piscataway, NJ). Western immunoblotting was performed with Mab 245 (46).

**Primary Cell Culture.** Tail fragments of 2 cm in length were collected from adult mice, minced, and incubated in 10 ml of cDMEM containing 1 mg/ml dispase for 1 h at 37°C. Dispersed cells were then plated on two 10-cm dishes with fresh cDMEM. Medium was changed daily, and outgrown cells were passed on the seventh day of culture.

**Cell Transfection.** Forty eight h after transfection with pUHC-13.3 (31), cells were collected and processed for luciferase detection assay according to the manufacturer’s protocol (Promega, Madison, WI).

**Analysis of IGF Effects on Cells in Culture.** Primary tail fibroblasts were continuously kept in cDMEM with 1 μg/ml of tetracycline hydrochloride. For analyses, cells were split onto 12-well plates (0.22 mm; Corning). In the test group, medium was changed for cDMEM containing 1 μg/ml dispase for 4 h after incubation. Twenty h later, medium was changed for serum-free DMEM (Life Technologies, Inc.) containing 50 μg/ml transferrin (Sigma Chemical Co.) and 0.1% BSA Fraction V (Sigma Chemical Co.) with and without tetracycline. After 48 h, cells were exposed to growth factors and 10 μg/ml BrdUrd (Sigma Chemical Co.) as described in Fig. 6. After incubation for 16 h, cells were washed with PBS, fixed for 4% paraformaldehyde for 30 min at 4°C, treated with 4 N HCl for 10 min to denature the DNA, and stained according to the regular immunohistochemical protocol (43). For estimation of BrdUrd index, at least 200 cells were counted in triplicate, and all of the experiments were repeated twice.

**Statistical Analyses.** All of the statistical analyses were performed with programs InStat, InPlot, and Prism (GraphPad, San Diego, CA). The life spans of animals were determined as the intervals from birth until natural death. Survival curves were compared by log rank Mantel-Haenszel test. Two-tailed
RESULTS

Generation of Mice with Conditional Regulation of RB Expression. To establish an animal model in which human RB gene expression could be regulated, two groups of transgenic mice were prepared. In the first group, mice harbor a transgene consisting of the 1.6-kb human RB promoter driving expression of the chimeric transcription transactivator tetrVP16 (Tg tetRPV16; Fig. 1, A and C). Tissue-specific and developmentally adequate patterns of expression provided by this RB regulatory element have been demonstrated earlier (28, 39). In the second group, mice harbor a transgene consisting of a minimal CMV promoter-tet operator fused to the wild-type human RB cDNA (Tg hCMV*-1p-RB; Fig. 1, B and C). The potential for leakage of the minimal CMV promoter was reduced by designing the hCMV*-1p/RB junction portion devoid of additional translation initiation sites. Because the minimal CMV promoter-tet operator becomes activated only upon binding of the tetR/VP16, expression of this transgene will be detected only in bitransgenic mice derived from the cross between Tg tetRPV16 and Tg hCMV*-1p-RB. Tetracycline or its analogues inactivate the chimeric transcription transactivator tetRPV16; therefore, their administration allows for conditional regulation of RB expression in the bitransgenic mice.

In the preliminary screen for tetRPV16 expression, transgenic mouse tail fibroblasts were transfected with luciferase reporter plasmid pUHC-13-3 (31). In cells from four of six transgenic lines, luciferase activity was more than 4-fold higher compared with transfected cells derived from nontransgenic fibroblasts (Fig. 1D). To evaluate possible adverse biological side effects of tetRPV16, growth, body weight, fertility, and feeding habits of Tg RBp-tTA mice were evaluated (Table 1 and data not shown). In five of six Tg RBp-tTA lines, no obvious toxic effects of tetRPV16 on development were observed.

Because of the significant transactivating activity observed in primary tail fibroblast cell culture transfection assays (Fig. 1D) and the lack of detectable influence on the body weight (Table 1), the Tg RBp-tTA L14 founder line (Tg L14) was selected for subsequent crosses with Tg hCMV*-1p-RB lines. Compared with nontransgenic and single-transgenic littermates, bitransgenic mice in two of eight Tg hCMV*-1-RB lines crossed with Tg L14 were significantly smaller (Table 1). The Tg hCMV*-1-RB L9 line (Tg L9) was used further to screen the remaining Tg RBp-tTA mice. Bitransgenic mice with either Tg hCMV*-1p-RB L9 or L10, and either Tg RBp-tTA L14 or L15 were chosen for subsequent analyses based on their obvious dwarf phenotypes and abundant expression of exogenous RB (Table 1; Fig. 2). Mice with these transgene combinations are almost identical in their phenotype and hereafter will be described together as mice with regulated expression of RB transgene (RTg). In pilot experiments, similar results were observed both in females and in males. To reduce the number of animals required for study, the majority of the subsequent experiments were performed on females.

Exogenous RB Gene Is Biologically Effective and Can Be Tightly Regulated. RB expression was demonstrated by IP-WB analyses in all of the tissues of bitransgenic mice but not Tg hCMV*-1p-RB mice (Fig. 2). Similar to a line of mice (RB3) expressing the human RB transgene constitutively (Table 1; Refs. 27, 28), bitransgenic mice with 15–25% body weight reduction expressed exogenous RB at 80–100% of endogenous levels (Fig. 2; Table 1). In concert with endogenous RB, transgenic RB is hypo- and hyper-phosphorylated in brain and spleen, respectively (Fig. 2). At the cellular level, expression patterns of exogenous and endogenous RB/Rb were very similar; e.g., in the brain, both Rb and RB were located in the nuclei of neuronal and glial cells (Fig. 3, B–D and F). Endothelial cells contained undetectable amounts of both proteins (Fig. 3, B–D and F), as observed previously (43).

As an additional important test for exogenous, transgenic RB function, carcinogenesis in transgenic mice carrying a single copy of endogenous RB was studied. Nontransgenic mice carrying a single allele of the Rb gene develop multiple neuroendocrine tumors and die prematurely at a mean of 346 ± 5 days (SE; median, 346 days; 7, 8, 43, 44). The mean survivals ± SE of Rb+/− mice with either Tg hCMV*-1p-RB (n = 11) or Tg RBp-tTA (n = 12) were 345 ± 15 days (median, 355 days) or 345 ± 25 days (median, 354 days), respectively. In contrast, all of the 12 bitransgenic Rb+/− mice sur-

Table 1  Body weight of transgenic female mice

<table>
<thead>
<tr>
<th>Transgenes</th>
<th>None</th>
<th>L14</th>
<th>L15</th>
<th>L16</th>
<th>L18</th>
<th>L19</th>
<th>L20</th>
</tr>
</thead>
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<tr>
<td>hCMV*-1p-RB</td>
<td>100 ± 1 (104)</td>
<td>99 ± 3 (28)</td>
<td>102 ± 5 (6)</td>
<td>96 ± 4 (12)</td>
<td>98 ± 5 (9)</td>
<td>99 ± 5 (3)</td>
<td>77 ± 5 (8)</td>
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<tr>
<td>L6</td>
<td>103 ± 3 (14)</td>
<td>93 ± 8 (5)</td>
<td>ND</td>
<td>102 ± 1 (2)</td>
<td>81 ± 2 (3)</td>
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<td>ND</td>
</tr>
<tr>
<td>L7</td>
<td>95 ± 5 (6)</td>
<td>88 ± 2 (1)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L8</td>
<td>98 ± 7 (10)</td>
<td>106 (1)</td>
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<td>ND</td>
</tr>
<tr>
<td>L9</td>
<td>99 ± 2 (14)</td>
<td>85 ± 3 (26)*</td>
<td>84 ± 3* (11)</td>
<td>84 ± 3* (7)</td>
<td>97 ± 4 (13)</td>
<td>92 ± 7 (5)</td>
<td>86 (1)</td>
</tr>
<tr>
<td>L10</td>
<td>102 ± 4 (14)</td>
<td>88 ± 4 (10)*</td>
<td>70 ± 3* (7)</td>
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<td>ND</td>
<td>90 ± 5 (5)</td>
<td>ND</td>
</tr>
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<td>ND</td>
</tr>
<tr>
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<td>89 ± 8 (13)*</td>
<td>93 ± 4 (3)</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L13</td>
<td>101 ± 3 (11)</td>
<td>89 ± 3 (9)</td>
<td>ND</td>
<td>80 (1)</td>
<td>ND</td>
<td>ND</td>
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</table>

* Student’s P < 0.05, as compared with wild-type mice. Weight of transgenic mice was determined between P 24 and P 28. It was estimated as percentage (± SE) of mean weight of wild-type littersmates; (n), number of animals. At least three litters were tested for each, but L6L16 (2 litters), L7L14 (2 litters), L8L14 (1 litter), L9L20 (1 litter), L11L14 (1 litter), and L13L16 (1 litter). ND, not determined.

Fisher’s and Student’s t tests were used to compare mean values when appropriate. Survival fractions were calculated using the Kaplan-Meier method.
vived over 440 days without developing tumors. Thus, the 1.6-kb fragment of RB promoter of Tg RBp-tTA is sufficient to direct a pattern of expression similar to that of endogenous Rb and to rescue the malignant phenotype in Rb+/− mice, as described previously (27, 28). Because a 10% increase in the amount of exogenous Rb is sufficient for prevention of tumor formation (27), the above results indicate no significant leakage of the RB transgene under the control of the CMV minimal promoter. Furthermore, they argue against inadvertent effects on carcinogenesis by the chimeric transactivator Tg RBp-tTA itself. The expected patterns of expression, nuclear localization, appropriate phosphorylation, and efficient rescue of malignant phenotypes all indicate the biological competence of the exogenous RB in the established mouse bitransgenic model.

To test whether RB expression could be conditionally regulated, tetracycline was administered to mice, either in drinking water or by drug pellets (see “Materials and Methods”). In agreement with earlier observations on the tetracycline-mediated regulation of transgene expression (33, 35, 36, 47), tetracycline administration for 3 days was sufficient for complete repression of transgene expression, as determined by both IP-WB and immunohistochemistry (Fig. 2; Fig. 3, A–F; and Fig. 6A). A period of 7 days was required to observe an increase in the amount of RB after tetracycline withdrawal (data not shown). Thus, RB expression can be tightly controlled by treatment with tetracycline.

**RB Is Involved in Regulation of Somatic Growth.** The present work on RTg mice and previous work (27, 28) on mice carrying an RB minitransgene (RB3) demonstrated dwarf phenotypes in mice...
Animals were presented. All of the animals were evaluated with matched wild-type and mice without (Rb2C(Rb perinatal tetracycline administration. Animals were either not exposed to tetracycline). Representative examples from each group of at least 12 applied starting at P24 (Rt21, Rt3, and RtTet) and with (RTg Tet21, RTg Tet-14, or exposed beginning at GD 14.5. All of the values (mean ± SE) represent normalized RUs, 6 and placental (A Fig. 4. Conditional regulation of growth by Rb. Body (A) and placental (B) weights of mice without (Rb+/−, Rb+/+, RB3, and RTg Tet−) and with (RTg Tet+) exposure to tetracycline from the time of fertilization. Lethality prevented collection of Rb−/− embryos from GD 13.5. Because of normally large individual deviations (64), placental weights were not determined after GD 16.5. C, body weights after temporary perinatal tetracycline administration. Animals were either not exposed to tetracycline (Rb+/+, RB3, and RTg Tet−), exposed from fertilization until the time of body weight measurement (RTg Tet+), exposed until GD 14.5 (RTg Tet-14), or exposed beginning at GD 14.5 (RTg Tet+14). All of the values (mean ± SE) represent normalized RUs, defined as a percentage of values for wild-type littermates subjected to identical exposure to tetracycline. D, transgenic/nontransgenic weight ratio (percentage) of L9L14 (RTg) mice without (RB3, RTg Tet−) and with (RTg Tet+) administration of tetracycline applied starting at P24 (arrow). Representative examples from each group of at least 12 animals are presented. All of the animals were evaluated with matched wild-type and single transgenic littermates as controls.

Fig. 4. Conditional regulation of growth by Rb on mouse growth. To test whether Rb expression influences body weight during postnatal development, animals were exposed to tetracycline from P24. The removal of tetracycline resulted in an increased growth rate for the few subsequent days (Fig. 4D). On average, animals regained up to 10–15% of their body weights (Fig. 4D; Table 2). To characterize potential tissue-specific effects of Rb-mediated dwarfism and its reversion by RB down-regulation, organs and body dimensions were carefully monitored. The weights of carcasses and major organs and the parameters of longitudinal growth (lengths of nose to anus distance, tail, and humerus) were measured in RTg mice before and after tetracycline application (Table 2). Continuous RB overexpression results in relatively proportional reductions in the weight of

8 A. Y. Nikitin, unpublished observations.

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Rb ROLE IN MOUSE GROWTH

Table 2: Effects of Rb on postnatal weight and length

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Genotype</th>
<th>RB3</th>
<th>RTgtet−</th>
<th>RTgtet+</th>
<th>Gain (tet−−)−(tet+)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n = 4</td>
<td>n = 6</td>
<td>n = 6</td>
<td></td>
</tr>
<tr>
<td>Weight</td>
<td>n = 11</td>
<td>83 ± 0.4a</td>
<td>93 ± 3.3a</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Head</td>
<td>100 ± 1.9</td>
<td>73 ± 3.1a</td>
<td>88 ± 5.8a</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Carcass</td>
<td>100 ± 1.6</td>
<td>82 ± 0.9a</td>
<td>89 ± 4.3</td>
<td>7</td>
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<tr>
<td>Skin</td>
<td>100 ± 2</td>
<td>71 ± 0.9a</td>
<td>93 ± 3.6a</td>
<td>22</td>
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<tr>
<td>Brain</td>
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<td>76 ± 1.1a</td>
<td>89 ± 4.6a</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Pituitary</td>
<td>100 ± 3.8</td>
<td>74 ± 3.5a</td>
<td>90 ± 3.2a</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td>100 ± 4.6</td>
<td>87 ± 4.5</td>
<td>103 ± 2.5a</td>
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<tr>
<td>Lungs</td>
<td>100 ± 2.1</td>
<td>84 ± 3.8a</td>
<td>94 ± 2</td>
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<tr>
<td>Heart</td>
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<td>89 ± 3.7a</td>
<td>94 ± 5.2</td>
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<td>Spleen</td>
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<td>54 ± 2.3a</td>
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<td>Kidneys</td>
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<td>83 ± 3.1a</td>
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<td>Adrenal glands</td>
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<td>Ovaries</td>
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<td>71 ± 3.2a</td>
<td>103 ± 13.8a</td>
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<tr>
<td>Para-ovarian fat</td>
<td>100 ± 7.2</td>
<td>43 ± 8.7a</td>
<td>48 ± 13.6</td>
<td>5</td>
<td></td>
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<tr>
<td>Total</td>
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<td>94 ± 0.6b</td>
<td>96 ± 0.9</td>
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<tr>
<td>Tail</td>
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<td>93 ± 2.8b</td>
<td>95 ± 1.6</td>
<td>2</td>
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<tr>
<td>Nose to anus</td>
<td>100 ± 0.5</td>
<td>94 ± 1.6b</td>
<td>96 ± 0.9</td>
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*All values are percentage (±SE) of mean in wild-type littermates. n, number of animals; tet− and tet+ are RTg animals with and without tetracycline administration, respectively.

**Student’s P < 0.05 for values of RB3 or RTgtet− compared with those of wild-type animals.

***Student’s P < 0.05 for values of RTgtet− compared with those of RTgtet+ animals.

major organs and carcass. As compared with other organs, the weights of pituitary gland and spleen were somewhat lower, both in RB3 and in RTg mice. Interestingly, only minor changes were observed in the longitudinal growth parameters of either group of mice. Down-regulation of Rb expression postnatally resulted in a 10–18% gain of the weight of parenchymal organs. The most dramatic effect was observed in the weight increase of ovaries (32%), spleen (31%), and skin (22%). Only very slight changes were observed in the weight of heart and para-ovarian fat in body and tail lengths. Morphological analyses of brain, cerebellum, pituitary gland, thyroid gland, lungs, thymus, stomach, small and large intestine, liver, kidney, adrenal gland, gonads, spleen, pancreas, salivary glands, prostate/uterus, mammary glands, trigeminal nerve, eyes, heart, and striated muscles of the thigh demonstrated proportional changes in the size of particular tissue compartments. No significant hypoplasia, hyperplasia, edema, and/or adipose accumulation were observed in any of the tissues analyzed. However, we cannot exclude subtle changes in cellular size and/or proliferation beyond the limits of direct microscopic evaluation. Taken together, our results demonstrate continuous involvement of Rb in weight regulation of the internal organs.

Rb Expression Correlates with Levels of Plasma IGF-I. To determine which major pathways regulating somatic growth during organogenesis were subject to Rb influence, amounts of plasma IGF-I, GH, and glucose (as an indicator for insulin) were measured in RTg mice before and after tetracycline application. No significant deviations in the concentration of GH and glucose were observed (Fig. 5, A and C). In contrast, IGF-I levels were 194% ± 15% (mean ± SE; n = 16; P < 0.0001) and 180% ± 13% (n = 9; P < 0.0001) higher in RTg and RB3 mice when compared with sex-matched wild-type littermates (100 ± 8; n = 14; Fig. 5B). Application of tetracycline to RTg mice for 3 days resulted in decreased plasma IGF-I concentrations to normal values, coincident with Rb down-regulation. The normal IGF-I concentrations were maintained after tetracycline administration for 14 days. In a reverse experiment, IGF-I, GH, and glucose concentrations were measured in RTg animals receiving tetracycline in utero since fertilization, both before and after tetracycline withdrawal. Three days after tetracycline withdrawal, increased RB expression coincided with an elevation of plasma IGF-I concentrations but not with changes in either GH or glucose concentrations. IGF-I concentrations increased further when animals were maintained for 14 days in tetracycline-free conditions. This gradual increase probably reflected a requirement for plasma clearance of tetracycline. Thus, a tight correlation exists between amounts of RB expressed and plasma IGF-I concentration, but neither GH nor glucose concentrations were significantly affected by RB.

High Concentrations of IGF-I Are Required to Stimulate Proliferation of Fibroblasts Overexpressing RB. To test directly the existence of IGF-mediated signal transduction via Rb, primary fibroblast cultures derived from tails of either RTg or nontransgenic mice were exposed to different concentrations of IGF-I either in the presence or in the absence of tetracycline. The addition of 5 ng/μl IGF-I was sufficient for stimulation of DNA synthesis in G0-arrested RTg fibroblasts with repressed expression of RB to levels similar with those of wild-type fibroblasts (Fig. 6, A and B). In contrast, RTg fibroblasts with RB expression required the addition of 100 ng/μl of IGF-I for 70% stimulation of BrdUrd incorporation. Thus, RB expression can effectively block cell proliferation induced by IGF-I. This block can be partially overcome by increased amounts of IGF-I in a dose-dependent manner. These results further suggest that RB may serve as a general negative regulator of cell proliferation stimulated by IGF-I.

DISCUSSION

Rb plays a significant role in the prenatal development of neural, hematopoietic, and skeletal muscle tissues. However, its functions in postnatal development have been less understood. Our results demonstrate that Rb is an important component in integrative regulation of body growth during the most of organogenesis. Quantitative changes in the Rb amount and phosphorylation status can be detected by GD 10.5 (12). At this time, the amount of Rb increases, and the hypophosphorylated, functionally active fraction becomes apparent. By GD 14.5, phosphorylated, inactive forms of Rb are nearly undetectable. Interestingly, this period coincides with a general decrease in embryonic growth rate (49). As shown in the present study, the absence of Rb results in an increase in total embryo weight by GD 12.5. This weight increase can be measured only for a short period, for the Rb−/− embryos die by GD 14.5. At the same time, the overexpression of RB decreases embryonic weight in a dose-dependent fashion (the present study and Refs. 27, 28). Thus, RB modulates body growth from GD 12.5 forwards.

Rb-mediated dwarfism begins during prenatal development and is
maintained through adult life. Continuous expression of Rb during postnatal development (43, 48) indicates a persistent function for Rb in the majority of organs and tissues. The preparation of mice with tightly regulated Rb expression allowed us to determine that maintenance of the mass of organs and tissues is continuously responsive to Rb. Indeed, lowering Rb levels results in compensatory growth during both prenatal and postnatal development. Because such regulation of growth was observed in all of the organs tested, this novel function of Rb should be biologically relevant for the majority of cell types. It should be noted that although we were unable to observe any specific alterations in cell differentiation and survival upon modulation of Rb quantity, additional functions of Rb in postnatal cells could not be excluded by these studies. Indeed, the spectrum of Rb effects and their thresholds undoubtedly vary in different cell types. It was noted that developmental and carcinogenic effects associated with Rb deficiency are observed only in some of tissues with the highest expression of the protein (43, 48, 50). In some cells, biological effects of Rb may not be evident because of compensatory modulation of the related proteins, p107 and p130 (51, 52). Additional studies are warranted to address the issue regarding the extent of Rb contribution to the maintenance of differentiation and the survival of specific cell types in vivo.

Phosphorylation leading to the inactivation of Rb growth inhibitory functions is achieved by cyclin-dependent protein kinases and cyclins (for review, see Refs. 3, 53). On the other hand, these kinase activities are modulated by cyclin-dependent protein kinase inhibitors of the INK and Cip/Kip families, p15, p16, p18, p19 and p21, p27, p57, respectively. As expected from these functions established ex vivo, cyclin D and p27 are indeed involved in growth regulation. Mice without functional cyclin D are smaller than their littermates (54, 55). In contrast, mice deficient for p27 are larger (56–58). Intriguingly, other genes such as p21, p16, and p57 implicated in the regulation of Rb function either do not have an effect on somatic growth (59, 60) or affect development of only some compartments, such as limbs (61). In the latter case, the phenotype of shortened extremities is quite similar to that observed in mice with simultaneous inactivation of the p107 and p130 genes (62). Thus, genetic studies of animal models pinpoint interactions between Rb, cyclin D, and p27 as most biologically critical and nonredundant. Some phenotypes in mice with cyclin D and p27 disruption differ from those in Rb-transgenic mice; e.g., defects in mammary gland development and female sterility are observed only in cyclin D- and p27-deficient mice, respectively. The relevance of these phenotypes to Rb-mediated pathways remains to be evaluated. Future analyses of mice with conditionally regulated expression of Rb should enable us to evaluate the possible contribution of other Rb-upstream regulators as temporarily and spatially active effectors of the somatic growth.
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