Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 3092–3097, March 1999
Medical Sciences

Cooperative action of germ-line mutations in decorin and p53 accelerates lymphoma tumorigenesis

RENATO V. IOZZO*†‡, FATIMA CHAKRANI*, DANILO PERROTTI†, DAVID J. MCQUILLAN§, TOMASZ SKORSKI†, BRUNO CALABRETTA†, AND INGE EICHSTETTER*

*Department of Pathology, Anatomy, and Cell Biology, and †Kimmel Cancer Center, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA 19107; and §Center for Extracellular Matrix Biology, Institute of Biosciences and Technology, Texas A&M University, Houston, TX 77030

Communicated by Carlo M. Croce, Thomas Jefferson University, Philadelphia, PA, January 5, 1999 (received for review December 9, 1998)

ABSTRACT **Ectopic expression of decorin in a wide variety of transformed cells results in growth arrest and the inability to generate tumors in nude mice. This process is caused by a decorin-mediated activation of the epidermal growth factor receptor, which leads to a sustained induction of endogenous $p21^{WAF1/CIP1}$ (the cyclin-dependent kinase inhibitor p21) and growth arrest. However, mice harboring a targeted disruption of the decorin gene do not develop spontaneous tumors. To test the role of decorin in tumorigenesis, we generated mice lacking both decorin and p53, an established tumor-suppressor gene. Mice lacking both genes showed a faster rate of tumor development and succumbed almost uniformly to thymic lymphomas within 6 months [mean survival age (T_{50}) ~4 months]. Mice harboring one decorin allele and no p53 gene developed the same spectrum of tumors as the double knockout animals, but had a survival rate similar to the p53 null animals (T_{50} ~ 6 months). Ectopic expression of decorin in thymic lymphoma cells isolated from double mutant animals markedly suppressed their colony-forming ability. When these lymphoma cells were cocultured with fibroblasts derived from either wild-type or decorin null embryos, the cells grew faster in the absence of decorin. Moreover, exogenous decorin proteoglycan or its protein core significantly retarded their growth *in vitro*. These results indicate that the lack of decorin is permissive for lymphoma tumorigenesis in a mouse model predisposed to cancer and suggest that germ-line mutations in decorin and p53 may cooperate in the transformation of lymphocytes and ultimately lead to a more aggressive phenotype by shortening the tumor latency.**

Recent advances in the chemistry and biology of extracellular matrices have strengthened the concept that an understanding of the mechanisms underlying tumor cell proliferation and invasion of host tissues requires a knowledge of the complex interactions between the tumor cells and the surrounding connective tissue. Proteoglycans are key bioactive molecules that directly affect neoplastic development. Decorin, a prototype member of an expanding family of small leucine-rich proteoglycans, regulates matrix assembly, growth factor activities, and tumor cell growth (1). The observation that the tumor stroma of human colon cancer contains increased levels of decorin suggested that the abnormal expression of this gene would favor growth and infiltration of malignant cells (2). However, a mounting body of evidence indicates that decorin has growth-suppressive properties. For instance, *de novo* decorin gene expression totally suppresses the malignant phenotype of human colon carcinoma cells: the cells show a decline in growth rate, do not grow in soft agar, and fail to generate tumors in immunocompromised mice (3). The cells are arrested in G_1 and can reenter the cell cycle when decorin expression is abrogated by antisense oligodeoxynucleotides specific for decorin mRNA. This decorin-induced growth arrest is linked to a marked induction of p21 (4), a gene that is a potent cyclin-dependent kinase inhibitor (5) and that is transcriptionally induced by p53 (6). These cytostatic effects of decorin so far appear to be global because ectopic expression of decorin in transformed cell lines of various histogenetic origins leads to a marked growth suppression (7). In all stably transfected clones, growth arrest is associated with induction of p21 and its translocation into the nuclei (7), and p21 gene is required because cells harboring a disrupted p21 gene fail to be growth-suppressed by decorin (7). We recently discovered that decorin proteoglycan or protein core causes a rapid phosphorylation of the epidermal growth factor (EGF) receptor and a concurrent activation of mitogen-activated protein kinase signal pathway, which causes a protracted induction of endogenous p21 and, ultimately, cell cycle arrest (8). Moreover, decorin induces mobilization of cytosolic calcium, and this effect is blocked by AG1478, a specific inhibitor of EGF receptor activity (9).

To establish the role of decorin in development, we generated mice harboring a targeted mutation of both decorin alleles. These mutant mice express a skin fragility phenotype with abnormal tensile strength and deregulated collagen fibrillogenesis (10). However, the mutant animals do not develop spontaneous tumors, indicating that loss of decorin alone is not sufficient for tumorigenesis. We hypothesized that if decorin suppresses the growth of tumor cells, then its absence could favor cancer growth and invasion, given a suitable genetic background in which tumorigenesis is favored. To test this hypothesis, we generated double knockout mice deficient in both decorin and p53, a well established tumor-suppressor gene that, when genetically disrupted, produces an excellent animal model of tumorigenesis (11–13). Notably, mice deficient for both genes showed a faster rate of tumor emergence and developed almost uniformly thymic lymphomas positive for CD4 and CD8 markers. Mice harboring one decorin allele and no p53 gene developed the same spectrum of tumors as the double knockout animals, but had a survival rate similar to that of the singly p53 null animals. The growth of thymic lymphoma cells was markedly retarded when decorin was present as either exogenously supplied or naturally secreted by cocultured fibroblasts. Also, transient transfection with decorin markedly (>75%) reduced the colony-forming ability of the lymphoma cells. These results indicate that lack of decorin is permissive for lymphoma tumorigenesis and suggest that germ-line mutations of decorin and p53 may cooperate to decrease the time of overall tumor development. A functional synergism between a secreted ("extracellular tumor-repressor") and an intracellular ("tumor-suppressor") gene is proposed to play a role in lymphomagenesis.

EXPERIMENTAL PROCEDURES

Materials. Fetal bovine serum (FBS) and fetal calf serum (FCS), DMEM, Medium 199, Dulbecco's PBS, and glutamine

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PNAS is available online at www.pnas.org.

Abbreviations: FBS, fetal bovine serum; MEF, mouse embryonic fibroblast.

‡To whom reprint requests should be addressed at: Department of Pathology, Anatomy, and Cell Biology, Room 249, Jefferson Alumni Hall, Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107. e-mail: iozzo@lac.jci.tju.edu.

were purchased from Mediatech (Herndon, VA). Gentamycin, penicillin/streptomycin, and Fungizone were obtained from GIBCO. LeukoStat Stain Kit was purchased from Fisher Scientific. Antibodies against mouse CD4 and CD8 antigens were from Southern Biotechnology Associates. Anti-mouse decorin antiserum was a gift from L. Fisher, National Institutes of Health. Recombinant decorin proteoglycan and protein core were purified from HT-1080 fibrosarcoma cells as described before (14).

Generation of *p53*−/− Decorin−/− Animals and Histology. To investigate possible cooperative effects on tumorigenesis of germ-line mutations in *p53* and *Dcn* genes, we mated mice homozygous for p53 mutation (11), purchased from Taconic Farms (Germantown, NY), with animals homozygous for *Dcn* mutation (10) to generate a series of animals heterozygous for both mutations (*p53*+/− *Dcn*+/−). The genotype of the animals was determined by PCR analysis using specific primers for *p53* (15) and *Dcn* (11) genes. The nature of the amplified bands was determined by DNA sequencing using the same primers. Double mutant animals were generated by intercrossing the double heterozygous according to standard protocols (16). Essentially, three groups of animals were compared, namely p53+/+ *Dcn*−/− ($n = 68$), *p53*−/− *Dcn* −/− ($n = 58$), and *p53*−/− *Dcn*+/− ($n = 28$). These sets of animals were monitored carefully, and animals exhibiting ill health were sacrificed and subjected to detailed autopsy. Tissues were fixed immediately in 10% neutral buffered formalin, embedded in paraffin, sectioned at 8 μm, and stained with hematoxylin and eosin (17). Cytospin preparations of thymic lymphoma cells were fixed in methanol for 5 min and subjected to immunohistochemical analysis using anti-CD4 and anti-CD8 antibodies and a detection system as described before (18).

Establishment of Tumor Cell Lines from Thymic Lymphomas of Double Mutant Animals. Three independent thymic lymphoma cell lines (designated PD85, PD99, and PD100) were isolated from spontaneously arising thymic lymphomas in *p53*−/− *Dcn*−/− mice. The tumors were finely minced and the fragments were placed in a flask with a small amount of medium to allow attachment of the tissue explants. After 6 days a rapidly growing adherent population of cells was observed. These cells could be subcultured readily without trypsin by using Hanks' balanced salt solution (HBSS) containing 100 μg/ml EDTA. After several subcultures a population of nonadherent lymphoma cells was isolated and expanded. The cells were grown routinely to high density in DMEM/10% FBS or Iscove's modified DMEM (IDMEM)/10% FBS and passaged at a 1:20 ratio in the same medium.

Coculture Experiments. Primary cultures of mouse embryo fibroblasts were prepared as described previously (10). Briefly, 10- to 12-day-old embryos from a timed pregnant mouse were removed aseptically. The head of the embryo was discarded and the body was minced finely with scalpels into 1-mm^3 fragments. After thorough rinsing with DMEM, the fragments were placed in a 25-cm^2 flask with a small volume (≈1–2 ml) of growth medium (DMEM/10% FBS) so that the surface tension allowed the fragments to adhere to the flask. Adherence and initiation of outgrowth of embryonic fibroblasts usually occurred within 24–36 hr. The cultures reached confluence within a week, after which they could be subcultured. Verification of the genotype was done by PCR or Southern blot analysis as described previously (10). The two established cell lines mouse embryonic fibroblast (MEF) *Dcn*+/+ and MEF *Dcn*−/− were used as a feeder layer to test the decorin effect on the growth of PD100 lymphoma cells. MEF *Dcn*−/− and MEF *Dcn*+/+ cells were grown in DMEM supplemented with 10% FBS in six-well dishes for 24 hr and then grown overnight in IDMEM supplemented with 5% FBS. After reaching confluence the cells were treated with mitomycin (10 μg/ml) for 3 hr to block cell division and washed extensively to remove any residual mitomycin. PD100 lymphoma cells were seeded at ≈10^5 per dish on the top of mitomycin-treated fibroblasts, which acted as a feeder layer. The number of the PD100 cells that grew in suspension was determined daily for the following 5 days. PD100 parental cells were also grown in the presence of decorin proteoglycan or protein core purified from human HT1080 as described before (14).

Retroviral Infection and Colony Formation Assay. Because the double knockout tumor cell lines were resistant to neomycin, we first transfected by electroporation the PD100 clone with the full-length mouse decorin using a pCDNA3.1/Zeo(+) vector (CLONTECH). However, several transfection experiments proved unsuccessful. For this reason, we utilized a retrovirus approach. The full-length mouse decorin cDNA was subcloned into the *Eco*RI restriction site of LXSP retroviral vector (a kind gift from A. Sacchi, Regina Elena Cancer Institute, Rome), which carries the puromycin-resistance gene. T cell lymphoma cells (PD100) obtained from the *p53*−/− *Dcn*−/− double knockout mice were kept in culture in IMDM supplemented with 10% FBS. Infection of 2.5×10^6 PD100 cells with the retrovirus carrying the full-length decorin (LXSP-Decorin) or with the empty virus LXSP (19) was carried out by cocultivation (20) of the PD100 cells with 293-derived BOSC 23 packaging cells transiently transfected by the calcium phosphate precipitation with the retroviral constructs mentioned above. Three days postinfection, 5×10^4 viable cells were plated in semisolid medium [0.9% methyl-cellulose (Methocult H4100; Stem Cell Technologies) in IMDM with 10% FCS] in the presence of puromycin (2 μg/ml). Colonies (>125 μm) were scored 12 days later.

RESULTS

Accelerated Tumorigenesis and Mortality Rate in *p53*−/− *Dcn*−/− Animals. To investigate possible cooperative effects on tumorigenesis of germ-line mutations in *Dcn* and *p53* we mated animals homozygous for *Dcn* mutation with mice homozygous for *p53* mutation to obtain double heterozygous. These animals subsequently were intercrossed to yield double mutant mice. Mice lacking p53 are viable and fertile, but exhibit an increased rate of tumor development and are also prone to genome instability (21). In contrast, animals lacking the *Dcn* gene show no overt tumor formation but exhibit a skin-fragility phenotype because of the abnormal lateral fusion of collagen fibers (10). We investigated the survival rate, tumor formation, and histopathological spectrum in three groups of animals: *p53*+/+ *Dcn*−/− ($n = 68$), *p53*−/− *Dcn*−/− ($n = 58$), and *p53*−/− *Dcn*+/− ($n = 28$). The genotype of the animals was determined by PCR analysis using specific primers for p53 and decorin genes and their targeted alleles, respectively (Fig. 1). The animals were of mixed genetic background with an average of 50% C57BL/6, 25% 129/Sv, and 25% Bl/Swiss. Notably, the animals carrying a wild-type p53 and a null decorin gene did not develop any tumor for the 9-month period of observation (Fig. 2). In addition, we did not observe any preferential tumor development in the *p53*+/+ *Dcn*−/− animals after nearly 2 years of observation. We noticed the appearance of salivary gland hyperplasia in about 5% and a few sporadic cases of hyperplasia of the gastric mucosa in older animals (unpublished observation). Thus, lack of decorin expression alone does not predispose to tumor formation. In contrast, by ≈4 months of age, ≈50% of the double knockout animals died or were required to be sacrificed because of ill health (Fig. 2). By 5 months of age, ≈90% of the double knockout succumbed to tumor growth. No difference between male and female occurrence was noted. As expected, the *p53*−/− *Dcn*+/− animals survived longer with 50% mean survival rate of ≈6 months, similar to that observed in the *p53*−/− animals studied before (16, 21–23). In contrast to a previous report (12), we did not observe any increased incidence of infections, and this was confirmed by careful autopsy of all the animals and histopathology of several parenchymal organs.

Our data indicate that the combination of p53 and decorin deficiency predisposes the animals to an accelerated mortality because of enhanced tumorigenesis (see below). In addition, the presence of a single decorin allele is sufficient

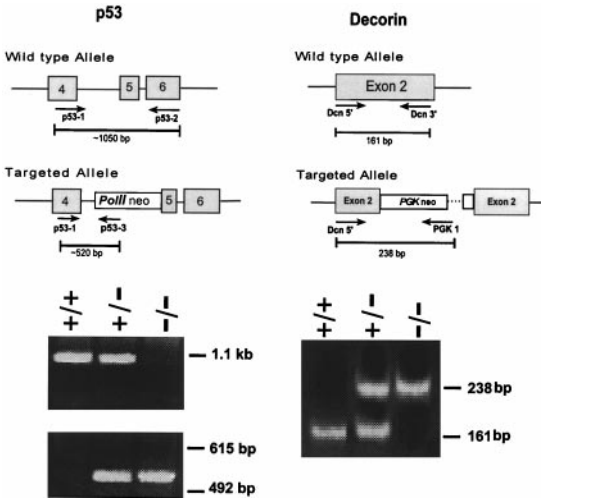

FIG. 1. Genotyping strategy of mice harboring germ-line mutations in p53 and/or decorin. (*Upper*) Schematic representations of the wild-type and targeted alleles and the primer position for the p53 and decorin genes, respectively. (*Lower*) Representative PCR products used to genotype animals derived from the mating of *p53*−/− and *Dcn*−/− mutant mice. The germ-line mutation of *p53* and *Dcn* consists of an insertion of *Pol*II-neo or *PGK-neo* in the fifth and second exon of their respective genes. Three separate sets of PCR amplifications were performed for each individual offspring and confirmed by DNA sequencing. The genotype is indicated at the top of the ethidium bromide-stained agarose gels, and the molecular weight in bp is indicated in the right margins.

to maintain a survival rate similar to that obtained when both wild-type decorin alleles are present in the context of a p53 null genetic background (22).

The Predominant Tumor of Double Knockout Animals Is Thymic Lymphoma. The spectrum of spontaneous tumors that generally are observed in *p53*−/− animals includes T cell lymphomas (>60%), soft tissues sarcoma (≈30%), and a variety of neoplasms encompassing teratomas, brain tumors, and rare carcinomas (12, 13, 21). In contrast, the spectrum of tumors in the *p53*+/− animals is different with osteosarcomas, soft tissue sarcomas, and lymphomas in roughly equal proportions (21). Moreover, *p53*+/− animals can develop classical carcinomas of the lung and colon as well as tumors of the pituitary gland (21). Another feature that is characteristic of both *p53*+/− and *p53*−/− background is the occurrence of multiple histogenetically distinct tumors in the same mutant animal (11). In contrast, the animals carrying both p53 and decorin null alleles showed a remarkable homogeneity in tumor spectrum and exhibited essentially no multiple tumors. More than 95% of the animals developed lymphomas in the thymic region and thus involving primarily the anterior mediastinum. Often the tumors encased the heart, pericardium, and lungs and extended into the soft tissues of the neck and chest wall. Because of the massive mediastinal involvement, the mice died as a direct consequence of pericardial compression or respiratory distress, in agreement with previous studies (16). The spleen was rarely affected, possibly because of the rapid appearance of the thymic lymphomas and the relatively shorter survival rate. In only one animal, a high-grade hemangiosarcoma was identified in the submandibular region. In a few (≈5%) animals, no neoplasm was grossly identified; however, histological evaluation showed marked enlargement of the thymus and foci of atypical lymphocytes often infiltrating the mediastinal soft tissues with signs of early-invading malignant lymphomas harboring a similar phenotype (not shown). The overall tumor spectrum in the *p53*−/− *Dcn*+/− animals essentially was identical to that observed in the double knockout animals.

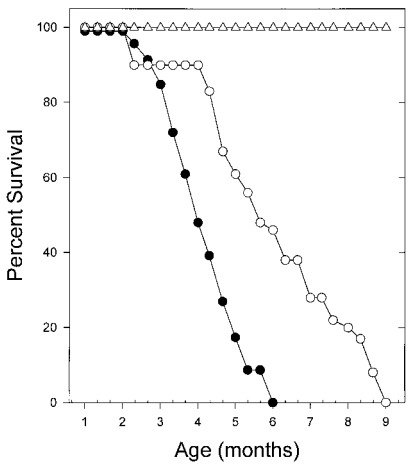

FIG. 2. Survival curve of mice harboring germ-line mutations in p53 and/or decorin. The graph summarizes the survival of *p53*−/− *Dcn*−/− (●, $n = 58$) relative to *p53*−/− *Dcn*+/− (○, $n = 28$), and *p53*+/+ *Dcn*−/− (△, $n = 68$). The mean survival age (T_{50}) was ≈4 and ≈6 months for the *p53*−/− *Dcn*−/− and *p53*−/− *Dcn*+/− genotype, respectively. None of the *p53*+/+ *Dcn*−/− animals developed tumors or needed to be sacrificed for ill health during the 9-month period of observation.

Histopathological examination of the tumors revealed high-grade lymphomas (Fig. 3*A*) with a starry-sky appearance. The tumor cells infiltrated the soft tissues of mediastinum (Fig. 3 *D* and *J*), the salivary glands (Fig. 3 *E* and *F*), the periaortic spaces (Fig. 3*G*), the pericardium (Fig. 3*H*), and the bronchial wall (Fig. 3*I*). The microscopic features of these lymphomas were identical in the *p53*−/− *Dcn*+/− animals (Fig. 3*L*). The only nonlymphomatous tumor, a high-grade hemangiosarcoma, is shown in Fig. 3*K*. Primary cultures of three independent thymic lymphomas reveled that the vast majority of the neoplastic cells reacted with antibodies against murine CD4 and CD8 (Fig. 3 *B* and *C*), indicating that they arose from the transformation of immature thymocytes (13). Thus, the absence of decorin in a p53-deficient genetic background accelerates lymphoma tumorigenesis. These data suggest that decorin cooperates with p53 in allowing a rapid appearance of the most common tumor in *p53*−/− animals. In fact, the incidence of thymic lymphomas has been reported to vary from 71 to 82% (11–13), depending on the genetic background (Table 1). The genetic background of our animals is similar to that described by others (11, 13, 16). This supports the notion that the shortened latency of lymphoma tumorigenesis is linked directly to the absence of decorin rather than to a modifier gene associated with a specific genetic background.

Absence of Decorin Is Permissive for the Growth of Lymphoma Cells. To investigate the role of decorin in lymphoma tumorigenesis we isolated several clones from freshly minced thymic lymphomas carrying both p53 and decorin null alleles. After establishing the cultures and testing them for CD4 and CD8 reactivity (Fig. 3 *B* and *C*), we cultured one clone (designated PD100) over a confluent monolayer of MEFs derived from either wild-type or *Dcn*−/− animals. Cell division of MEFs was arrested by mitomycin C. Thus, the only difference between the two sets of feeder layers was the synthesis and release of decorin proteoglycan in the medium as shown by Western immunoblotting using an antibody directed against the N terminus of mouse decorin (24) (Fig. 4*A*). Notably, PD100 lymphoma cells grew faster in the absence of decorin, and after 6 days of continuous culture there were approximately four times more tumor cells than control (Fig. 4*B*). These experiments were repeated twice with similar results. Cell cycle analysis at each day of coculture using fluorescence-activated cell sorting revealed no cell death or significant block in G_1 (not shown). The latter differs from our previous results using attached tumor cells in which we found an overall increase in the proportion of cells in the G_1 phase of the cell cycle in all the decorin-transfected clones (7). Thus, it appears that decorin causes growth retardation in unattached lymphoma

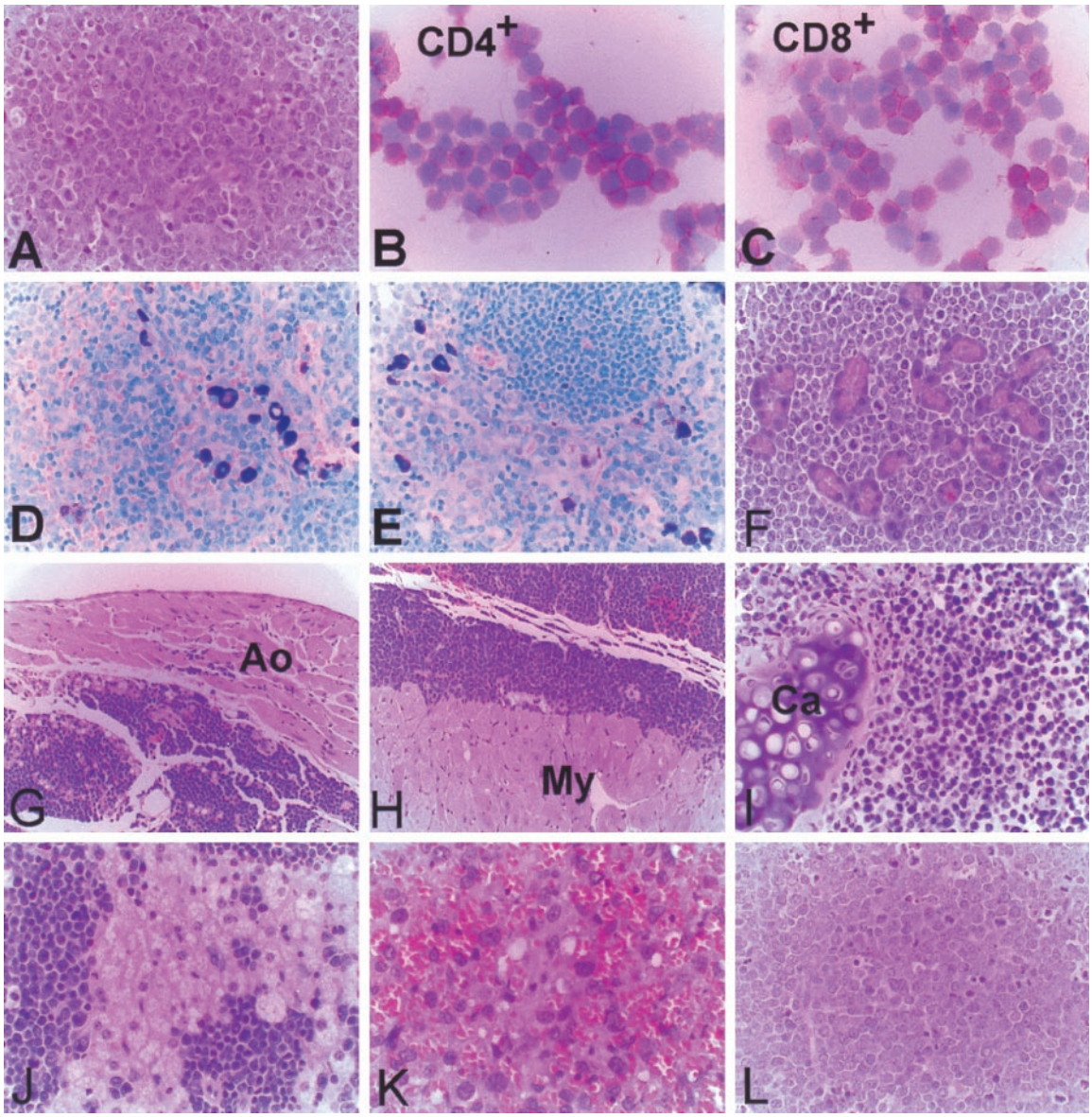

FIG. 3. Gallery of digitized images illustrating the morphology of tumors in *p53/Dcn* double mutant animals. Histopathological examination of the tumors revealed high-grade lymphoma (*A*). *B* and *C* represent cytospin preparations of the cultured PD100 lymphoma shown in *A* after immunostaining with anti-CD4 and anti-CD8 antibodies. The tumor cells are highly invasive and infiltrate the soft tissues of mediastinum (*D* and *J*), the salivary glands (*E* and *F*), the periaortic spaces (*G*), the pericardium (*H*), and the bronchial wall (*I*). *K* shows a section of the only nonlymphoid tumor, a high-grade hemangiosarcoma, found in a double mutant animal. *L* is a section of a thymic lymphoma detected in the *p53−/− Dcn+/−* animals showing microscopic features identical to those observed in the double mutant animals. The images were digitized with a Pixera digital camera at resolution of $\approx 10^6$ pixels/inch. [×150 (*A*, *F*, *I*, *J*, *L*); ×300 (*B*, *C*, *K*); ×100 (*D*, *E*, *G*, *H*).]

cells without causing a block of cell cycle progression but, rather, with a slowing down of the cell cycle.

To further investigate these effects, we cultured PD100 lymphoma cells in the presence of 1 μM recombinant human decorin or its protein core. The results showed a significant inhibition of growth (Fig. 4*C*), and the core protein exhibited a greater cytostatic effect than the proteoglycan (Fig. 4*D*). These data, thus, demonstrate that the inhibitory activity resides in the protein moiety rather than the dermatan sulfate chains and indicate further that these cytostatic properties of decorin transcend

Table 1. Frequency of lymphomas in mice lacking p53 or decorin alleles vis-à-vis those lacking p53 alone in various genetic backgrounds

Genotype	Genetic background (estimated proportion)	Incidence of thymic lymphomas (n = total number)	Reference
p53−/− Dcn−/−	C57Bl/6, 129/Sv, Bl/Swiss (50%, 25%, 25%)	95% (n = 58)	This study
p53−/− Dcn+/−	C57Bl/6, 129/Sv, Bl/Swiss (50%, 25%, 25%)	94% (n = 28)	This study
p53−/− Dcn+/+	C57Bl/6, 129/Sv (75%, 25%)	77% (n = 26)	Donehower *et al.* (11)
p53−/− Dcn+/+	129/Sv (100%)	65% (n = 26)	Harvey *et al.* (22)
p53−/− Dcn+/+	C57BL/6, 129/Sv (75%, 25%)	70% (n = 44)	Harvey *et al.* (22)
p53−/− Dcn+/+	129/Sv (100%)	47% (n = 43)	Donehower *et al.* (16)
p53−/− Dcn+/+	C57Bl/6, 129/Sv (≈50%, 50%)	71% (n = 55)	Jacks *et al.* (13)
p53−/− Dcn+/+	129/Ola (≈50%, 50%)	82% (n = 17)	Purdie *et al.* (12)

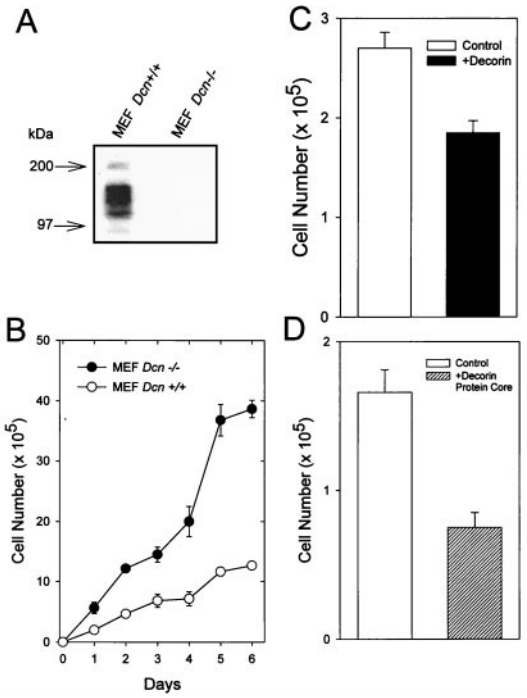

FIG. 4. Decorin inhibits the growth of PD100 thymic lymphoma cells derived from *p53*−/− *Dcn*−/− animals. (*A*) Western immunoblotting of serum-free medium conditioned by embryonic fibroblasts derived from *Dcn*+/+ and *Dcn*−/− mouse embryos (MEF) using LF-113 polyclonal antibody directed against a synthetic peptide in the N terminus of decorin spanning residues 36–49 of the mouse protein core (24). The migration of the molecular mass markers is indicated in the left margin. Under such conditions, decorin migrates as a smear centering around 100 kDa (10). (*B*) Growth of PD100 thymic lymphoma cells in the presence or absence of a feeder layer composed by mitomycin-arrested MEFs. The genotype is indicated in the top margin. (*C* and *D*) Growth of PD100 thymic lymphoma cells cultured in the absence or presence of recombinant human decorin or its protein core (1 μM each). Cells were counted after 60 hr of culture in 5% FBS. The values represent the mean of quadruplicate determinations ±SEM.

species because human decorin can suppress the growth of murine thymic lymphoma cells.

Ectopic Expression of Decorin Reduces Colony Formation in the Thymic Lymphoma Cells. Because the double knockout tumor cell lines were resistant to neomycin, we first electroporated the PD100 clone with the full-length mouse decorin using a pCDNA3.1/Zeo(+) vector. However, several transfection experiments proved unsuccessful. For this reason, we used a retrovirus-based approach. The full-length mouse decorin cDNA was subcloned into the *Eco*RI restriction site of LXSP retroviral vector, which carries the puromycin resistance gene driven by the simian virus 40 early promoter. The expression of decorin is under the control of the long terminal repeat of the mouse Maloney virus (20). About 2.5×10^6 PD100 T cell lymphoma cells obtained from the *p53*−/− *Dcn*−/− double knockout mice were infected with the retrovirus carrying the full-length decorin (LXSP-Decorin) or with the empty LXSP retroviral vector as described before (20, 25). Proper expression of decorin was tested by immunoblotting of medium conditioned for 2–3 days by the total cells before plating (not shown). Three days postinfection, 5×10^4 viable cells were plated in semisolid medium in the presence of 2 μg/ml puromycin. After 12 days of selection in puromycin, the number of puromycin-resistant colonies arising from the decorin-infected cells was markedly diminished (>75% inhibition) as compared with that of cells infected with the vector alone (Fig. 5). Thus, ectopic expression of decorin reduces colony-forming ability of lymphoma cells. Collectively, the data presented above point to a primary role of decorin in malignancy and establish a functional synergism in lymphoma tumorigenesis

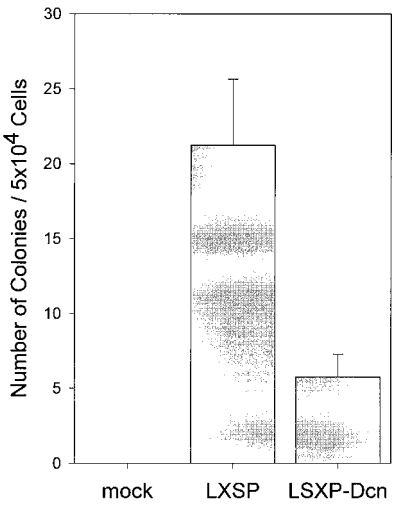

FIG. 5. Inhibition of PD100 colony formation by ectopic decorin expression. Colonies from 5×10^4 freshly infected PD100 thymic lymphoma cells were grown in methyl cellulose in the presence of puromycin (2 μg/ml) and scored 12 days later. Mock, noninfected cells; LXSP, cells infected with the empty vector LXSP; LSXP-Dcn, cells infected with the retrovirus carrying the full-length mouse decorin cDNA. Error bars indicate ±SD of the mean of four independent experiments performed in duplicate. Notice the marked suppression of colony-forming ability in the decorin-expressing clones.

dependent on the effects of a natural inhibitor of tumor cell growth (that we call "extracellular tumor repressor") in a genetic background deficient of intracellular p53 ("tumor-suppressor") gene.

DISCUSSION

This study tests the function of a secreted proteoglycan and a tumor-suppressor gene in the process of spontaneous *in vivo* tumorigenesis. Our findings indicate that lack of decorin in an otherwise wild-type background is not sufficient by itself to induce tumorigenesis. However, the lack of decorin in a *p53* null background accelerates the appearance of tumors, essentially all T cell-derived lymphomas. We do not know whether the selective appearance of lymphomas is due solely to an acceleration of the "usual" tumorigenic process observed in the *p53* null animals because these mutant animals spontaneously develop thymic lymphomas at high frequency (21). An interesting hypothesis is related to the existence of various temporal windows of opportunity for tissue-specific tumor development (16). During neonatal stages, for example, the highest rate of cell division and potential genetic rearrangement is the lymphoid compartment. In the thymus, decorin is localized primarily in the capsule and the fibrovascular septa (26). Thus, the absence of functional p53 and decorin may allow a faster accumulation of genetic abnormalities and also may favor tumor progression in the lymphoid system. The observation of thymic hyperplasia and foci of lymphoid atypia in cases in which no overt tumor was found further supports the concept that absence of decorin in a p53 null genetic background favors lymphomagenesis. Reactive lymphoid hyperplasia also has been observed in mice carrying a large deletion of the *p53* gene (12) and at a lower frequency in exon 5 mutant mice (11).

Specific genetic backgrounds play a role in the manifestation of tumors affecting both the rate and spectrum of neoplastic development in the mutant animals (23). Normal C57BL/6 mice have a relatively high incidence of lymphomas, but the average age of onset is about 27 months (23). Because ≈75% of the C57BL/6, 129/Sv *p53* −/− animals developed lymphomas, it was hypothesized initially that the high incidence of this type of malignancy might be a result of a specific genetic background of the inbred animals (11). In a subsequent study, however, it was discovered that in a pure 129/Sv strain, loss of p53 causes a high incidence (65%) of lymphomas (23), although the frequency and type of

other tumors were markedly different from the mixed-background mice (summarized in Table 1). Thus, lymphoma development appears to be directly linked to p53 loss and is not a strain-specific effect. It has been proposed that p53 plays a key role in the surveillance of gene amplification, abnormal recombination events, and the control of ploidy (27). The development of thymic lymphomas in the p53 nullizygous mice (11) and the role p53 plays in controlling T cell recombination events (28) support these concepts.

Our findings also indicate that although the survival rate of the *p53*−/− *Dcn*+/− was not significantly different from that of *p53*−/− *Dcn*+/+ animals, the mice lacking one decorin allele showed the same type of lymphomas in 94% of the cases. We have not investigated the loss of heterozygosity in the tumors because decorin is not expressed by the neoplastic cells but, rather, by the stromal elements. We have noticed previously, however, that the levels of decorin were relatively low in the heterozygous animals although with some degree of variability (10). In addition, we observed that there was no compensatory mechanism and that the homologous proteoglycan biglycan was not increased in its expression. Thus, reduced expression of decorin also may help to accelerate the appearance of malignant lymphoma. Alternatively, decorin expression may be directly related to lymphoma tumorigenesis. To address this question more directly, we isolated lymphoma cells from tumors of double knockout animals and infected them with a retrovirus expression vector containing the full-length mouse decorin. The data clearly showed a marked inhibition of colony formation in methylcellulose when decorin was expressed. Moreover, the growth of wild-type lymphoma cells from the double knockout animals was markedly reduced when the cells were cultured on top of a feeder layer composed of *Dcn*+/+ embryonic fibroblasts vis-à-vis *Dcn*−/− fibroblasts. Because the only difference between the two fibroblast feeder layers was the presence of secreted decorin, we conclude that absence of decorin is permissive for the *in vitro* growth of thymic lymphoma cells. If decorin does not affect the cell cycle in these thymic lymphoma cells (as our data indicate) but does retard the overall growth of the tumor cells, then it is possible that *in vivo* the reduced rate of mitosis may attenuate or delay the rate of mutations in a *p53*-deficient genetic background.

The cytostatic effects of murine decorin in coculture settings were corroborated by experiments in which recombinant human decorin proteoglycan or its protein core caused a marked inhibition of growth in thymic lymphoma cells isolated from double mutant animals. Interestingly, the protein core exhibited a greater cytostatic effect than the proteoglycan itself, indicating that the inhibitory activity resides in the protein moiety rather than the dermatan sulfate side chains. Our findings indicate further that the cytostatic properties of decorin transcend species because human decorin can suppress the growth of murine thymic lymphoma cells. This is in agreement with our previous data in which we found growth suppression of mouse M2 melanoma cells upon stable transfection with a human decorin cDNA (7).

To assess cooperativity between p53 and other tumor-associated genes, several investigators have crossed p53-deficient mice to other strains of tumor-susceptible transgenic or knockout mice (21). Among these studies, two reports have shown an accelerated lymphomagenesis. First, double mutant animals lacking both p53 and the catalytic subunit of DNA protein kinase at the *scid* locus (*p53 scid*) resulted in the development of lymphoma with strikingly early onset (29). Second, mice lacking *p53* and either *Rag1* or *Rag2* genes, which are needed for V(D)J recombination, developed thymic lymphomas at high frequency (70 and 86% of total, respectively), and the tumor arose with a short latency (30). In both sets of bitransgenic animals, the mortality rate and the spectrum of tumors were nearly identical to those observed in the *p53*−/− *Dcn*−/− mice. In contrast, mice deficient in both *p53* and *Rb* genes show an accelerated tumorigenesis but also a different spectrum of tumors not observed in the parental singly deficient animals, including pinealoblastomas, islet cell tumors, and other neuroendocrine neoplasms of the thyroid and pituitary glands (22, 31). Thus, it is possible that germ-line mutations of both *p53* and *Dcn* genes may cooperate in the transformation of thymic lymphocytes.

Another possibility to be considered is that decorin may be involved in modulating the immune response. A recent study has shown in an animal model of brain tumor that ectopic expression of decorin in C6 rat glioma cells results in a strong inhibition of tumor formation *in vivo* (32). Notably, the decorin-expressing glioma cells show a marked increase in activated T lymphocytes infiltrating the tumors, and these effects can be abrogated by steroid-mediated immune suppression. Thus, ectopic expression of decorin significantly enhances antiglioma immune response *in vivo*.

In conclusion, this study provides genetic evidence that decorin, a natural inhibitor of tumor growth, plays an important role in restraining thymic lymphoma cells from rapidly infiltrating the mediastinal soft tissues. The potential cooperation of two genes, one acting on the transcriptional machinery within the nucleus (an established "tumor-suppressor gene") and one acting at the cell/matrix boundary (which we term "extracellular tumor-repressor gene"), opens new avenues of research and novel, potential therapeutic approaches.

We thank A. Kovatich for help with the immunohistochemistry of cytospin preparations, L. Fisher and A. Sacchi for gifts of valuable reagents, and P. Wlodarski and K. G. Danielson for help in the initial stages of this study. This work was supported in part by National Institutes of Health Grants RO1 CA39481 and RO1 CA47282 (R.V.I.) and CA 46782 (B.C.). D.P. was supported by a fellowship from the American-Italian Foundation for Cancer Research, New York.

1. Iozzo, R. V. (1998) *Annu. Rev. Biochem.* **67,** 609–652.
2. Adany, R., Heimer, R., Caterson, B., Sorrell, J. M. & Iozzo, R. V. (1990) *J. Biol. Chem.* **265,** 11389–11396.
3. Santra, M., Skorski, T., Calabretta, B., Lattime, E. C. & Iozzo, R. V. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7016–7020.
4. De Luca, A., Santra, M., Baldi, A., Giordano, A. & Iozzo, R. V. (1996) *J. Biol. Chem.* **271,** 18961–18965.
5. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K. & Elledge, S. J. (1993) *Cell* **75,** 805–816.
6. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W. & Vogelstein, B. (1993) *Cell* **75,** 817–825.
7. Santra, M., Mann, D. M., Mercer, E. W., Skorski, T., Calabretta, B. & Iozzo, R. V. (1997) *J. Clin. Invest.* **100,** 149–157.
8. Moscatello, D. K., Santra, M., Mann, D. M., McQuillan, D. J., Wong, A. J. & Iozzo, R. V. (1998). *J. Clin. Invest.* **101**, 406–412.
9. Patel, S., Santra, M., McQuillan, D. J., Iozzo, R. V. & Thomas, A. P. (1998) *J. Biol. Chem.* **273,** 3121–3124.
10. Danielson, K. G., Baribault, H., Holmes, D. F., Graham, H., Kadler, K. E. & Iozzo, R. V. (1997) *J. Cell Biol.* **136,** 729–743.
11. Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S. & Bradley, A. (1992) *Nature (London)* **356,** 215–221.
12. Purdie, C. A., Harrison, D. J., Peter, A., Dobbie, L., White, S., Howie, S. E. M., Salter, D. M., Bird, C. C., Wyllie, A. H., Hooper, M. L. & Clarke, A. R. (1994) *Oncogene* **9,** 603–609.
13. Jacks, T., Remington, L., Williams, B. O., Schmitt, E. M., Halachmi, S., Bronson, R. T. & Weinberg, R. A. (1994) *Curr. Biol.* **4,** 1–7.
14. Ramamurthy, P., Hocking, A. M. & McQuillan, D. J. (1996) *J. Biol. Chem.* **271,** 19578–19584.
15. Wlodarski, P., Wasik, M., Ratajczak, M. Z., Sevignani, C., Hoser, G., Kawiak, J., Gewirtz, A. M., Calabretta, B. & Skorski, T. (1998) *Blood* **91,** 2998–3006.
16. Donehower, L. A., Harvey, M., Vogel, H., McArthur, M. J., Montgomery, C. A., Jr., Park, S. H., Thompson, T., Ford, R. J. & Bradley, A. (1995) *Mol. Carcinogen.* **14,** 16–22.
17. Stanley, K. K. (1988) *Curr. Topics Microbiol. Immunol.* **140,** 49–65.
18. Handler, M., Yurchenco, P. D. & Iozzo, R. V. (1997) *Dev. Dyn.* **210,** 130–145.
19. Sevignani, C., Wlodarski, P., Mercer, E., Danielson, K. G., Iozzo, R. V. & Calabretta, B. (1998) *J. Clin. Invest.* **101**, 1572–1580.
20. Pear, W. S., Nolan, G. P., Scott, M. L. & Baltimore, D. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8392–8396.
21. Donehower, L. A. (1996) *Semin. Cancer Biol.* **7,** 269–278.
22. Harvey, M., Vogel, H., Lee, E. Y. H. P., Bradley, A. & Donehower, L. A. (1995) *Cancer Res.* **55,** 1146–1151.
23. Harvey, M., McArthur, M. J., Montgomery, C. A., Jr., Bradley, A. & Donehower, L. A. (1993) *FASEB J.* **7,** 938–943.
24. Fisher, L. W., Stubbs, J. T., III, & Young, M. F. (1995) *Acta Orthop. Scand.* **66,** 61–65.
25. Perrotti, D., Bonatti, S., Trotta, R., Martinez, R., Skorski, T., Salomoni, P., Grassilli, E., Iozzo, R. V., Cooper, D. R. & Calabretta, B. (1998) *EMBO J.* **17,** 4442–4455.
26. Scholzen, T., Solursh, M., Suzuki, S., Reiter, R., Morgan, J. L., Buchberg, A. M., Siracusa, L. D. & Iozzo, R. V. (1994) *J. Biol. Chem.* **269,** 28270–28281.
27. Levine, A. J. (1997) *Cell* **88,** 323–331.
28. Guidos, C. J., Williams, C. J., Grandal, I., Knowles, G., Huang, M. T. F. & Danska, J. S. (1996) *Genes Dev.* **10,** 2038–2054.
29. Nacht, M., Strasser, A., Chan, Y. R., Harris, A. W., Schlissel, M., Bronson, R. T. & Jacks, T. (1996) *Genes Dev.* **10,** 2055–2066.
30. Nacht, M. & Jacks, T. (1998) *Cell Growth Differ.* **9,** 131–138.
31. Williams, B. O., Remington, L., Albert, D. M., Mukai, S., Bronson, R. T. & Jacks, T. (1994) *Nat. Genet.* **7,** 480–484.
32. Ständer, M., Naumann, U., Dumitrescu, L., Heneka, M., Löschmann, P., Gulbins, E., Dichgans, J. & Weller, M. (1998) *Gene Therapy* **5,** 1187–1194.