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Absence of the *CD44* Gene Prevents Sarcoma Metastasis[1]

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ABSTRACT

To test the role of the *CD44* gene in tumorigenesis, mice with the min mutation of the *APC* gene or with the tm1 mutation of the *p53* gene were crossed with CD44 knockout mice. The absence of *CD44* gene products did not affect tumor incidence or survival; however, mice with disruption of the *CD44* gene showed virtually aborted metastasis formation of osteosarcomas. This is in agreement with the role attributed to CD44 variants in the spread of cancer. Therefore, *CD44* gene products are not essential for tumor incidence and growth but are important in regulating metastasis formation.

INTRODUCTION

The transmembrane glycoprotein CD44 is expressed on lymphocytes and macrophages. It serves as a homing receptor that mediates binding to high endothelial venules and has also been implicated in lymphoid development. Lymphocyte activation results in the expression of multiple, alternatively spliced products of the *CD44* gene, which are generated by the insertion of ≤10 variant exons into the extracellular domain. In pathophysiology, aberrant expression of certain CD44 splice variants has been connected causally to the spread of diverse malignant cells (1, 2) and may distinguish metastasizing from nonmetastasizing tumors. This function is mediated by the cytokine ligand osteopontin (3). Additional roles in carcinogenesis have been attributed to various forms of CD44. Expression of this receptor on tumor cells may support tumor growth (4, 5), possibly after adhesion to hyaluronate, and signal transduction through CD44 can induce oncogenes, such as *ras* (6). In contrast, the standard form that lacks variant exons may exert suppression of tumor growth and dissemination (7). The contributions of these diverse CD44 functions to carcinogenesis are not fully elucidated.

Despite ample experimental evidence for a role of some forms of CD44 in malignancy, clinical studies relating expression of CD44 or its variants to prognosis in diverse cancers have remained controversial (8). Nonconclusive results in patients may have been obtained because of insufficient sensitivity or specificity of the analyses. Thus, the measurements of mRNA for specific CD44 variants expressed by tumors do not detect posttranslational modifications that may affect function (9). Furthermore, the CD44 receptor expressed on tumors represents one component of a functional pair. Specific ligands may bind to selective splice variants, so that the availability of these ligands contributes to determining the phenotype. Similarly, many conventional rodent models of malignancies are compromised because they rely on the injection of preformed tumor cells, often in nonphysiological locations, rather than on the generation of endogenous tumors. We set out to analyze the role of CD44 in a genetically defined and homogeneous system, which most closely resembles the pathophysiology of human cancers. Mouse models, where the role of individual genes is tested by breeding the relevant gene-targeted mice with mice that have high susceptibility to tumors because of mutations in tumor suppressor genes, have provided substantial insights. We used two endogenous tumor models using mice with point mutations in tumor suppressor genes with or without targeted deletion of the *CD44* gene: Mice with the $APC^{+/min}$ genotype display a predisposition to multiple intestinal neoplasia. The murine min mutation is a nonsense mutation, which is analogous to mutations found in human autosomal dominantly inherited familial adenomatous polyposis, as well as in sporadic colorectal cancers (10). $APC^{+/min}$ mice develop multiple benign intestinal tumors, whose growth reflects early steps of transformation. Mutations of the *p53* gene contribute to the pathogenesis of a large percentage of human cancers. Similarly, mice with one mutant allele of the *p53* gene are susceptible to a larger spectrum of tumors, predominantly sarcomas and lymphomas. These mice allow the investigation of malignant dissemination.

MATERIALS AND METHODS

Mice. Mice with point mutations in tumor suppressor genes, $APC^{+/min}$ bred on C57Bl/6 background or $trp53^{+/tm1}$ on C57Bl/6 background, were obtained from The Jackson Laboratory. Either $APC^{+/min}$ mice or $trp53^{+/tm1}$ mice were mated with $CD44^{-/-}$ mice that had been back-crossed from 129 to C57BL/6 for four generations (10). The genotypes were assessed using PCR from genomic DNA (10–12), and CD44 expression was confirmed by flow cytometry from blood samples using the pan-CD44 antibody IM7 (PharMingen). Siblings were housed in groups of one to four per cage at the Redstone Animal Facility (DFCI) in alternate 12-h light and dark cycles. A diet of pelleted chow (Agway, Prolab 3000) and bottled water was administered *ad libitum*, and room temperature was kept at 25°C. The colony was tested frequently for endoparasitic and ectoparasitic infections, as well as for bacterial and viral infections by the Charles River Labs (Wilmington, MA). No infection was detected during the course of this study. Permission to exceed a tumor diameter of 2 cm was granted by the institutional animal care and use committee, and the mice were seen frequently by a veterinarian.

Inheritance. Mice with disrupted *CD44* genes were mated with heterozygotes for point mutations of the relevant tumor suppressor gene, yielding mice that were hemizygous for CD44 and either wild type or heterozygous for the tumor suppressor gene. Those two genotypes were interbred, which is expected to result in equal 12.5% representation of the genotypes of interest according to Mendelian inheritance (the remaining $2 \times 25\%$ are $CD44^{+/-}$). The litters from this second generation mating were screened. In the p53-related part of the study, 292 mice were analyzed, of which 16 were $trp53^{+/+}CD44^{+/+}$, 36 mice were $trp53^{+/+}CD44^{-/-}$, 24 mice had the genotype $trp53^{+/tm1}CD44^{+/+}$, and 26 mice had the genotype $trp53^{+/tm1}CD44^{-/-}$. In the APC-related part of the study, 217 mice were screened with the distribution of $APC^{+/+}CD44^{+/+}$ 21 mice, $APC^{+/+}CD44^{-/-}$ 20 mice, $APC^{+/min}CD44^{+/+}$ 10 mice, and $APC^{+/min}CD44^{-/-}$ 15 mice.

Because of the incomplete back-crossing from 129 to C57Bl/6, it is formally possible that a polymorphic modifier, linked to the CD44 locus, segregates with it and supersedes the influence of CD44 on tumor development. The tumor susceptibility locus Scc1 might be a candidate (13). This is unlikely, because quantitative trait loci, including Scc-1, depend strongly on interlocus

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interactions for influencing tumorigenesis (14). Cosegregation of one modifier could not affect the phenotype. It is generally improbable that genomic heterogeneity would account for the observations described here, because 97% of the genome are derived form the C57Bl/6 strain.

Necropsy. The animals were checked at least every 12 h, and total necropsy was performed on mice found moribund. Organs were fixed in formalin, and H&E slides were prepared for histological analysis. Histological evaluation was performed by an investigator blinded to the CD44 status of the samples.

Osteosarcoma metastases were observed in livers, lungs, and occasionally spleens. Enumeration of osteosarcoma metastases was performed on step sections from livers and lungs. For this purpose, every 10th microtome cut corresponding to a step thickness of 60 μm was analyzed.

Immunohistochemistry. CD44 expression on osteosarcomas was assessed by immunohistochemistry with antibody KM114 after antigen retrieval with citrate. Staining was done with 3,3′-diaminobenzidine. Staining of all tumor samples with secondary antibody served as negative control, and a normal mouse spleen was used as a positive control (data not shown).

Enumeration of Intestinal Polyps. Entire intestines from stomach to rectum were extracted, washed in PBS, and fixed in 10% buffered formalin, and the number of polyps was counted under a dissection microscope. As controls, intestines from 3 APC$^{+/+}$CD44$^{+/+}$ mice at the ages of 420–442 days and 3 APC$^{+/+}$CD44$^{-/-}$ mice at the ages of 433–442 days were examined for spontaneous polyps.

Statistical Evaluation. The data sets were analyzed for statistically significant differences at 95% confidence by *t* test (after confirmation of normal distribution and equal variance) and by Wilcoxon Mann-Whitney test (after testing for equal distribution). The prerequisites for applicability of either test were not fulfilled for the metastasis data. They were analyzed for equal variance by the Cochran test.

RESULTS

Absence of CD44 Prevents Tumor Metastasis. Because aberrant expression of CD44 splice variants may confer a malignant phenotype to tumor cells, we asked whether the targeted deletion of the *CD44* gene was sufficient to suppress the dissemination of solid tumors. Osteosarcomas developed mostly on the lower back. One trp53$^{+/tm1}$CD44$^{-/-}$ mouse had an osteosarcoma of the skull. Metastases were detected in the lungs and livers from trp53$^{+/tm1}$ mice with osteosarcoma. Step sections from livers and lungs identified 28 metastases in 6 CD44$^{+/+}$ mice and 1 metastasis in 4 CD44$^{-/-}$ mice (Fig. 1). One CD44$^{+/+}$ mouse also displayed a macroscopically visible metastasis in the spleen. All 6 CD44$^{+/+}$ mice had multiple osteosarcoma metastases, whereas in 4 CD44$^{-/-}$ mice, only one individual lung metastasis was detected. Consistently, CD44 expression was prominent in the osteosarcomas of CD44$^{+/+}$ mice (Fig. 2).

Absence of CD44 Does Not Alter the Phenotype of Benign Tumors. The intestinal polyps caused by mutation in the *APC* gene grow noninvasively but express various splice variants of CD44. This occurs at the earliest stages of transformation diagnosed as aberrant crypt foci with dysplasia (15). Histology was performed on the largest intestinal polyps from each APC$^{+/min}$ mouse to assess malignancy. Consistent with previous reports, these tumors are noninvasive as judged by intact basement membranes in all cases (Fig. 3). No metastases were observed in other organs (three histological sections per organ). These results were not affected by the presence or absence of CD44. Histological findings included ectopic hepatic hematopoiesis and bone marrow

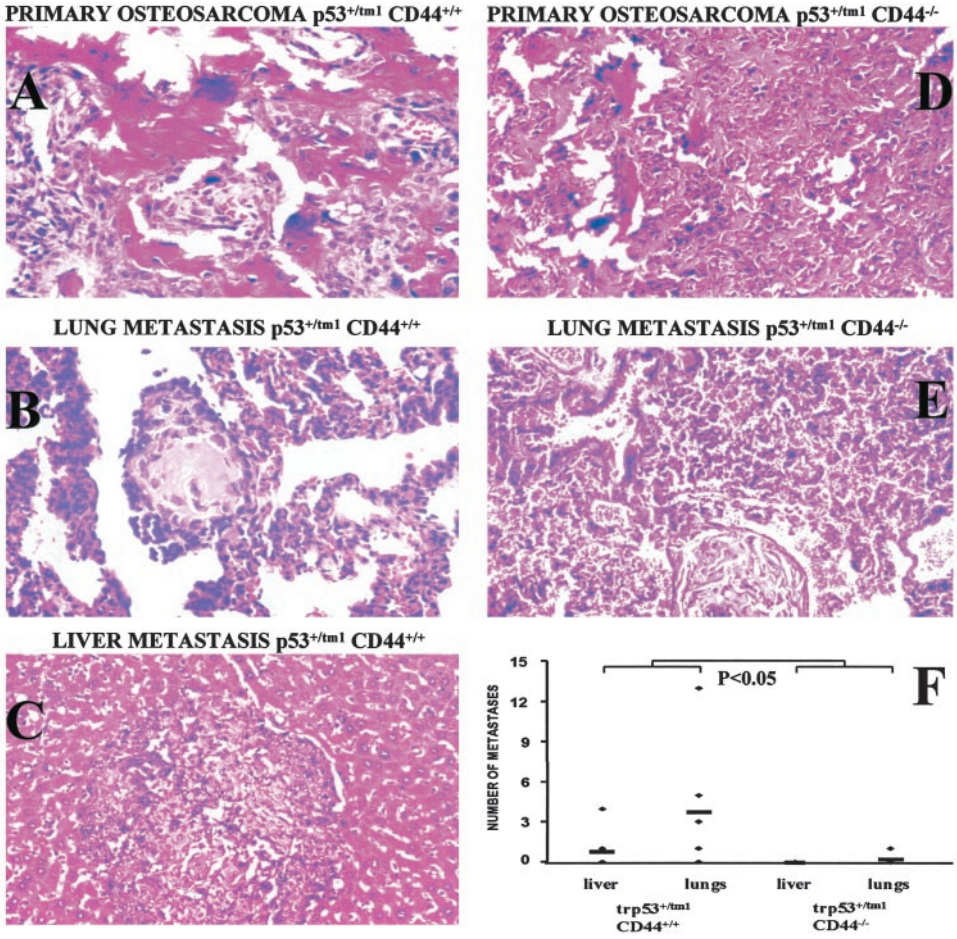

Fig. 1. Tumor invasiveness. *A–F*, osteosarcomas in trp53$^{+/tm1}$ mice and their metastases in lungs and liver. *A*, primary tumor in CD44$^{+/+}$ background; *B*, representative lung metastasis in CD44$^{+/+}$ background; *C*, representative liver metastasis in CD44$^{+/+}$ background; *D*, primary tumor in CD44$^{-/-}$ background; *E*, only metastasis detected in CD44$^{-/-}$ background (H&E); *F*, incidence of metastases in livers and lungs from osteosarcoma bearing mice.

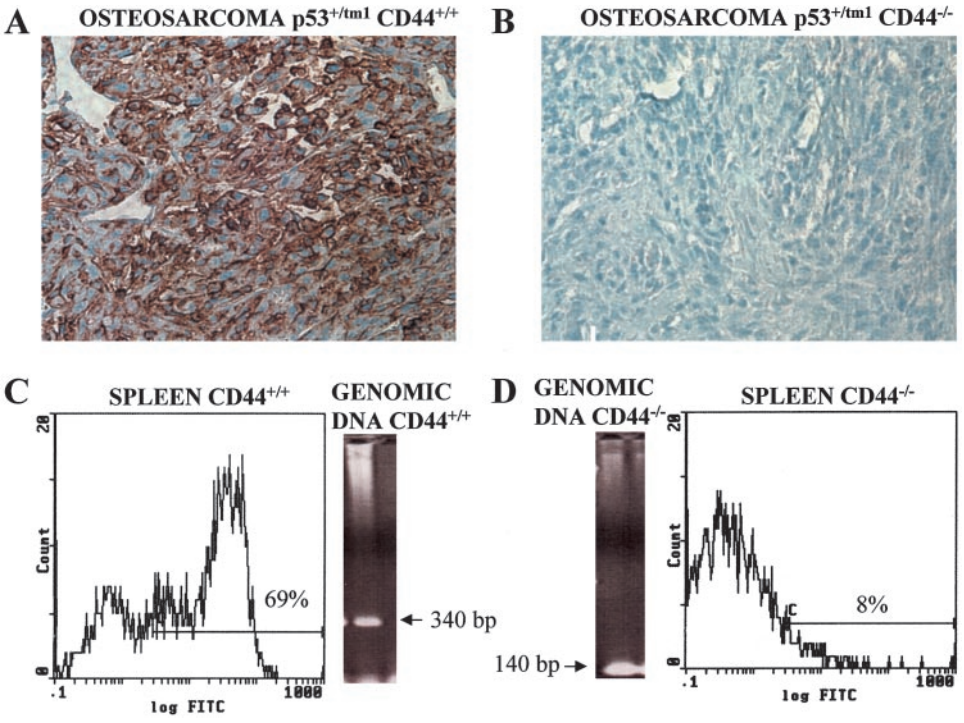

Fig. 2. Expression of CD44. *A* and *B*, immunohistochemistry for CD44 expression in osteosarcomas from a trp53$^{+/tm1}$ CD44$^{+/+}$ (*A*) and a trp53$^{+/tm1}$ CD44$^{-/-}$ mouse (*B*). In *C* and *D*, typing of the mice was performed by flow cytometry with FITC-anti-CD44 on spleen cells and lymph node cells (data not shown) and by PCR on genomic DNA with published primers (11); *C*, CD44$^{+/+}$; *D*, CD44$^{-/-}$.

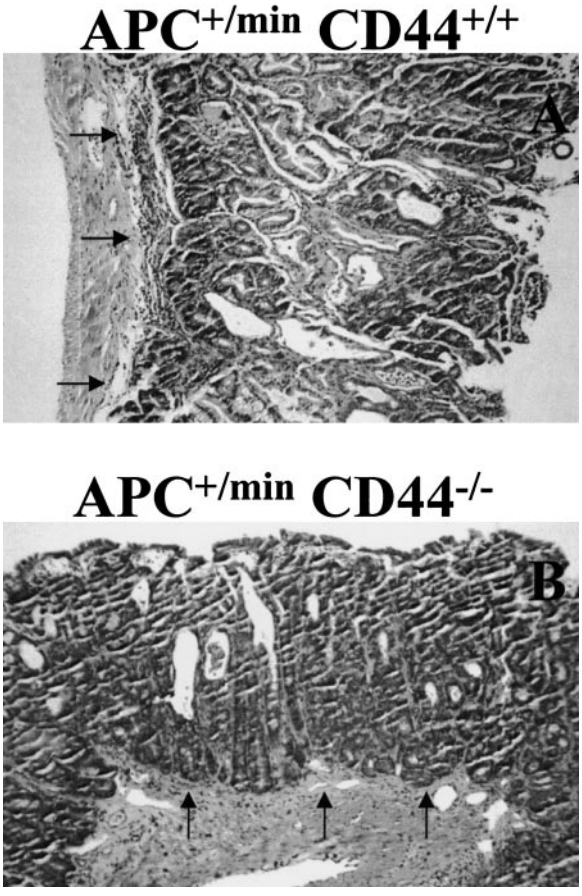

Fig. 3. Intestinal polyps caused by the min mutation of the *APC* gene are not invasive. Histology of representative intestinal polyps from APC$^{+/min}$ mice. The largest polyp from each intestine was sectioned in the middle and stained with H&E for histological assessment of signs for malignancy. Regardless of the presence of the *CD44* gene, the basement membrane remains intact (*arrows*). *A*, CD44$^{+/+}$; *B*, CD44$^{-/-}$.

siderosis in several mice, which are likely attributable to blood loss through the intestinal polyps.

CD44 Does Not Affect Tumor Incidence. We tested whether deletion of the *CD44* gene alters tumor incidence as judged by the number of intestinal polyps in mice with one mutated *APC* allele. All APC$^{+/min}$ mice succumbed to intestinal polyposis. At the time of death, APC$^{+/min}$ mice had developed around a mean of 66 polyps in CD44$^{+/+}$ background and 58 polyps in CD44$^{-/-}$ background (Fig. 4), suggesting that the tumor development in this model does not depend on the presence of *CD44* gene products.

Mice with the trp53$^{+/tm1}$ genotype developed, predominantly, sarcomas and lymphomas. The mesenchymal tumors were diagnosed as fibrosarcomas, osteosarcomas, hemangiosarcomas, and histiocytic sarcomas. Their incidence, associated life span, and tumor weight on death were not affected by the presence or absence of the *CD44* gene (Table 1). Like the osteosarcomas, the fibrosarcomas were located mostly on the lower back.

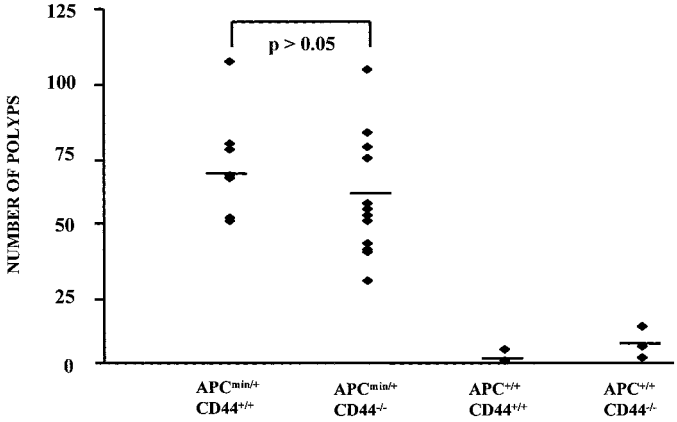

Fig. 4. Tumor incidence. The numbers of intestinal polyps were counted in APC$^{+/min}$ mice at the end of their life span (average 209 days for 10 APC$^{+/min}$CD44$^{+/+}$ mice and 236 days for 15 APC$^{+/min}$CD44$^{-/-}$ mice). The incidence of spontaneous polyps was assessed at an average of 433 days for 3 APC$^{+/+}$CD44$^{+/+}$ mice and 437 days for 3 APC$^{+/+}$CD44$^{-/-}$ mice. *Symbols*, individual data points; mean values are presented as *horizontal lines*.

Table 1 *Characterization of solid tumors in trp53$^{+/tm1}$ mice. Incidence, associated life span, and tumor weight at the time of death are specified for each histologic type of sarcoma for CD44$^{+/+}$ and CD44$^{-/-}$ genetic background. Life span and tumor weight are indicated as mean ± standard error.*

Tumor	Incidence	Life span		Weight	
Osteosarcoma					
trp53$^{tm1/+}$CD44$^{+/+}$	6 (25%) 5 f, 1 m[a]	532 ± 30 days	$P > 0.05$	7.7 ± 2.4 grams	$P > 0.05$
trp53$^{tm1/+}$CD44$^{-/-}$	4 (15%) 3 f, 1 m	467 ± 56 days		6.1 ± 1.7 grams	
Fibrosarcoma					
trp53$^{tm1/+}$CD44$^{+/+}$	7 (29%) 4 f, 3 m	410 ± 23 days	$P > 0.05$	21.9 ± 5.0 grams	$P > 0.05$
trp53$^{tm1/+}$CD44$^{-/-}$	7 (27%) 5 f, 2 m	403 ± 30 days		12.3 ± 2.9 grams	
Hemangiosarcoma					
trp53$^{tm1/+}$CD44$^{+/+}$	3 (12%) 2 f, 1 m	304 ± 51 days		10.7 ± 8.1 grams	
trp53$^{tm1/+}$CD44$^{-/-}$	0 (0%)				
trp53$^{+/+}$CD44$^{-/-}$	1 (3%) 1 m	595 days		2.9 grams	
Histiocytic sarcoma					
trp53$^{tm1/+}$CD44$^{+/+}$	2 (8%) 2 m	589/590 days			
trp53$^{tm1/+}$CD44$^{-/-}$	1 (4%) 1 f	420 days			
trp53$^{+/+}$CD44$^{+/+}$	1 (6%) 1 m	600 days			

[a] f = female; m = male.

Sporadic carcinomas also occurred in trp53$^{+/tm1}$ mice independently of their CD44 status with one case of squamous cell carcinoma in a trp53$^{+/tm1}$CD44$^{+/+}$ mouse and one incident of lung carcinoma among the trp53$^{+/tm1}$CD44$^{-/-}$ mice (Fig. 5).

There were four cases (17%) of lymphoma, typical of those observed in p53$^{+/tm1}$ mice (12, 16), in trp53$^{+/tm1}$CD44$^{+/+}$ mice with an associated mean life span of 445 days. In comparison, there were six cases (23%) of lymphomas, resembling anaplastic large cell lymphomas (17), in trp53$^{+/tm1}$CD44$^{-/-}$ mice with an associated life span of 503 days (data not shown). The morphology of the lymphoid malignancies in CD44$^{-/-}$ mice appeared unusual but requires further characterization.

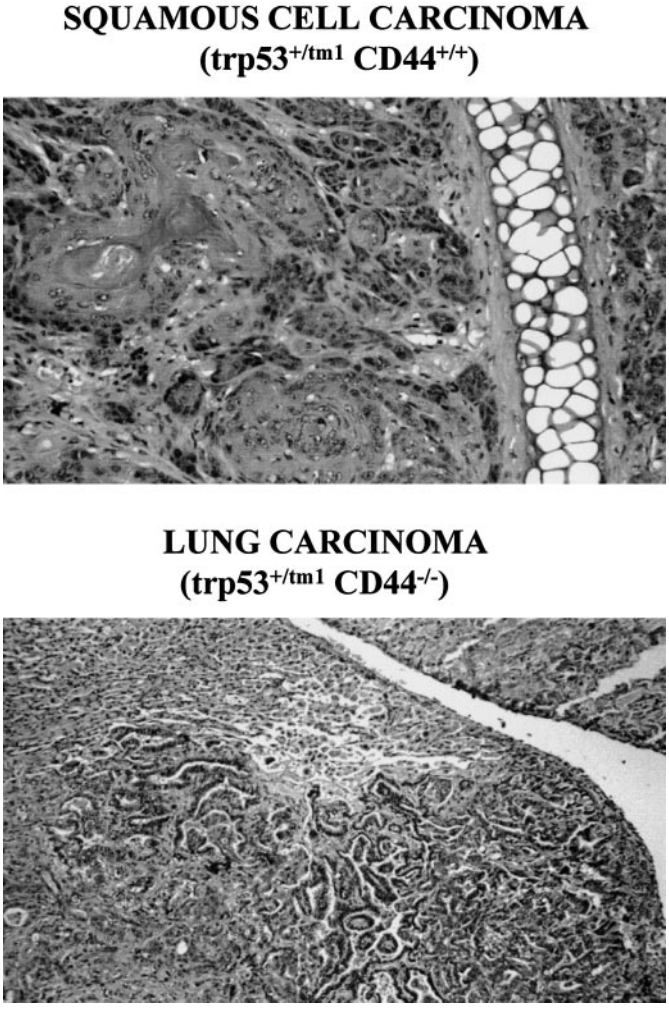

Fig. 5. Carcinomas in mice with one mutant *p53* allele. Histology of sporadic carcinomas in trp53$^{+/tm1}$ mice, including a squamous cell carcinoma (CD44$^{+/+}$; *top*) and a lung carcinoma (CD44$^{-/-}$; *bottom*). The slides are stained with H&E.

Five mice with one mutant *p53* allele had multiple tumors. In trp53$^{+/tm1}$CD44$^{+/+}$ mice, one osteosarcoma occurred together with a histiocytic sarcoma. Frequently, lymphomas were diagnosed in conjunction with solid tumors. One trp53$^{+/tm1}$CD44$^{+/+}$ mouse had lymphoma and osteosarcoma. In trp53$^{+/tm1}$CD44$^{-/-}$ mice, lymphoma was seen in conjunction with osteosarcoma, fibrosarcoma, and histiocytic sarcoma in one case each.

CD44 Does Not Affect Survival. The mice with one mutant allele of the *APC* gene developed symptoms of ruffled fur, bloated abdomen, and black stools followed by lethargy and succumbed around a mean of 209 days (range 133–350 days) of age, for CD44$^{+/+}$ background, or 236 days (range 96–326 days) of age, for CD44$^{-/-}$ background (Fig. 6*A*). At the end of their life span, 2 of the CD44$^{-/-}$ mice also suffered from rectal prolapse. None of the APC$^{+/+}$ mice died during the 420-day period of observation, regardless of their CD44 status.

Mice with one mutant *p53* allele developed various tumors with a predominance of sarcomas and lymphomas and had a 50% survival of ~470 days of age regardless of their CD44 genotype (Fig. 6*B*). The period of observation was limited to 600 days, at which point 1 of 24 trp53$^{+/tm1}$CD44$^{+/+}$ mice (4%) was still alive and 6 of 26 trp53$^{+/tm1}$CD44$^{-/-}$ mice (23%) survived. In the control groups, 14 of 16 trp53$^{+/+}$CD44$^{+/+}$ mice (87%) and 30 of 36 trp53$^{+/+}$CD44$^{-/-}$ mice (83%) were alive. Conversely, 1 trp53$^{+/tm1}$CD44$^{+/+}$ mouse (4%), 3 trp53$^{+/tm1}$CD44$^{-/-}$ mice (11%), 1 trp53$^{+/+}$CD44$^{+/+}$ mouse (6%), and 5 trp53$^{+/+}$CD44$^{-/-}$ mice (14%) died without detectable signs of malignancies. Although five of them were diagnosed with histological signs of inflammation (abscess, periarteriitis nodosa, glomerulonephritis, dermatitis, and pneumonia), the contribution of these conditions to the death of the mice is unknown. The higher incidence of deaths unrelated to neoplasms (8 CD44$^{-/-}$ mice of 50 when disregarding the trp53 status, compared with 2 CD44$^{+/+}$ mice of 40) implies that the lack of the *CD44* gene may increase the susceptibility to other pathogenic influences.

DISCUSSION

Diverse roles in cancer have been ascribed to various *CD44* gene products, but their contributions to endogenous tumors have not been studied. Here, we have tested the consequences of targeted deletion of the *CD44* gene in the development of endogenous tumors caused by mutations in two distinct tumor suppressor genes, which are also mutated frequently in human cancers. We show that the absence of the

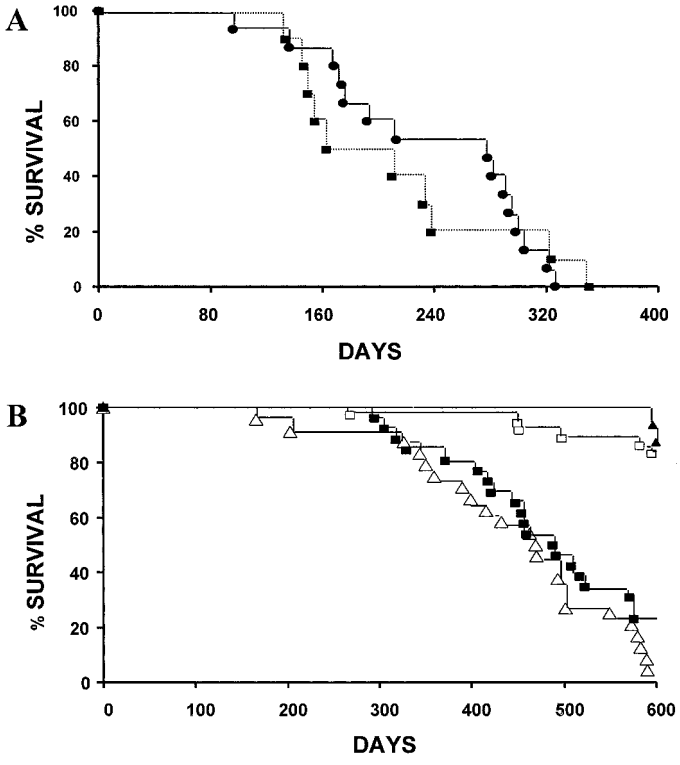

Fig. 6. Kaplan-Meier survival curves. *A*, survival of $APC^{+/min}$ mice with wild-type (■) or deleted (●) *CD44* gene. All $APC^{+/+}$ mice survived the 420-day period of observation. *B*, survival of $trp53^{+/tm1}$ mice. Within 600 days, mice with the $trp53^{+/tm1}$ genotype succumbed to various tumors independently of their CD44 status, leading to survival of 4% or 1 mouse ($CD44^{+/+}$, △) and 23% or 6 mice ($CD44^{-/-}$, ■). Control mice with the wild-type *trp53* gene had survival rates of 87% or 14 mice ($CD44^{+/+}$, ▲) and 83% or 30 mice ($CD44^{-/-}$, □).

CD44 gene products virtually abrogates osteosarcoma metastasis. In contrast, we did not find evidence for a role of CD44 in tumor growth or survival.

In various cancers, expression of CD44 splice variants is necessary and sufficient to cause metastasis formation (1, 2). In the present study, the role of CD44 in malignancy of nonhematopoietic origin was limited to inducing dissemination (Fig. 1) and corroborating the role of *CD44* as a metastasis gene in solid tumors. The expression of CD44 is sufficient to confer metastatic properties to primary osteosarcoma cells, but the expression of multiple CD44 variants on intestinal adenomatous polyps (15) is not associated with invasive growth. This may be accounted for by the dominance of metastasis suppressor gene products over gene products that induce dissemination (18). Intestinal cells frequently express the adhesion molecule DCC, which may prevent CD44-mediated invasion. Alternatively, metastasis formation by the intestinal polyps may be suppressed by elevated β-catenin, secondary to loss of APC protein function. This decreases enterocyte crypt villus migration (19) and may prevent invasive behavior. Osteosarcoma cells, in contrast, do not display any prominent expression of metastasis suppressor genes so that the expression of CD44 variants is sufficient to cause a malignant phenotype.

Various genetic influences can affect tumor multiplicity in $APC^{+/min}$ mice. They include genes for cell cycle control, DNA repair, and metalloproteinases. The genetic modifier Mom1 encodes a secretory phospholipase, Pla2g2a, expressed throughout the intestinal tract. The active allele of Pla2g2a leads to a reduction in the growth rate and multiplicity of intestinal adenomas (20). $APC^{+/min}$ mice homozygous for a null allele of p53 developed significantly more intestinal adenomas than those homozygous for the wild-type allele of p53. Similarly, the intact DNA mismatch repair gene *Pms2* reduces the number of intestinal tumors as compared with mice with a targeted deletion of this gene (21). In contrast, deletion of the gene for the metalloproteinase Matrilysin leads to substantial reduction in intestinal tumors, despite a lack of destruction of the basement membrane by these polyps (22). The intestinal polyps caused by the *APC* gene mutation express various splice variants of CD44 at the earliest stages of transformation, diagnosed as aberrant crypt foci with dysplasia (15); however, the contributions by *CD44* gene products to the pathogenesis of the intestinal polyps were unknown. In this study, the numbers of polyps and associated life spans were not influenced by the absence of *CD44* gene products. The size of the individual polyps did not appear to be compromised.

The expression of CD44 on tumors has been described to not only affect metastatic spread but also tumor growth (4, 5) and induction of oncogenes, such as *ras* (10). This opened the possibility that deletion of the *CD44* gene might influence disease progression. In $APC^{+/min}$ mice, the incidence of polyps and associated life spans were, however, not altered. Similarly, incidence, survival, and tumor weight of sarcomas in $trp53^{+/tm1}$ mice were not influenced by the absence of CD44, arguing against a prominent role for CD44 in early transformation or tumor growth. In contrast, the dissemination of osteosarcomas was virtually abrogated by the absence of *CD44* gene products (29 microscopically and macroscopically identified metastases in 6 $CD44^{+/+}$ mice, compared with 1 metastasis identified in 4 $CD44^{-/-}$ mice). We have found previously metastasis gene products to constitute a unique group of cancer-related biomolecules, which is distinct from growth controlling oncogene or tumor suppressor gene products. They are dysregulated in cancer at the levels of gene expression or mRNA splicing (18). The present results confirm the role of CD44 as a metastasis gene and refine our insights into the contributions of CD44 to cancer.

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