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*Advances in Brief*

# Development of Rhabdomyosarcoma in *HER-2/neu* Transgenic *p53* Mutant Mice[1]

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## Abstract

**Rhabdomyosarcomas derive from the skeletal muscle lineage and harbor a variety of genetic and molecular lesions. However, it is not clear which molecular alterations have a pathogenetic role. We show that activation of the *HER-2/neu* oncogene coupled with inactivation of the oncosuppressor gene *p53* causes rhabdomyosarcoma in mice. At the age of 11–21 weeks, all male mice carrying both genetic lesions developed embryonal rhabdomyosarcomas expressing desmin, myosin, and insulin-like growth factor-II, in the genitourinary tract. Our findings led to the hypothesis that the interaction between HER family genes and the p53 pathway might be involved in the origin of human rhabdomyosarcoma.**

## Introduction

Rhabdomyosarcoma, the most common soft-tissue sarcoma of childhood, results from the neoplastic transformation of cells of the skeletal muscle lineage (1). Rhabdomyosarcomas harbor a variety of genetic and molecular lesions that frequently include autocrine growth factor circuits along with alterations of oncosuppressor genes. However the actual causes of rhabdomyosarcoma are still unknown, nor is it clear which molecular alterations have a role in its etiology and progression. Genetically modified mice carrying individual genes derived from human studies either were not tumor-prone (2) or showed a stochastic development of rhabdomyosarcoma at low incidence (1), suggesting that additional hits are required to generate such tumors. Involvement of the oncosuppressor gene *p53* in rhabdomyosarcoma is suggested by the presence of *p53* mutations in a proportion of human tumors and by the occurrence of rhabdomyosarcomas in human families and in knockout mice carrying a germ-line mutation in one *p53* allele (1). The low incidence of rhabdomyosarcoma in individuals carrying *p53* alterations indicates that additional genetic lesions are required to cause this tumor. Human rhabdomyosarcoma can express receptor tyrosine kinases of the HER/ErbB family, which can play different roles in the malignant phenotype: HER-1/EGF-R sustains rhabdomyosarcoma cell growth whereas HER-3 induces myogenic differentiation *in vitro* (3); and both HER-1 and HER-3 heterodimerize with HER-2. Activation of *HER-2* can lead to transformation *in vitro* and *in vivo* in many cell types and is required for myoblast cell survival (4). HER-2 is expressed in approximately one-half of human rhabdomyosarcomas[3]; however, its role in the genesis of rhabdomyosarcoma is unknown. Here we show that p53 inactivation coupled with HER-2/neu activation produces rhabdomyosarcomas in mice.

## Materials and Methods

**Mice.** BALB/c $p53^{+/-}$ mice (BALB/cJ-*Trp53*$^{tm1Tyj}$) were purchased from The Jackson Laboratory, Bar Harbor, MI. BALB/c *HER-2/neu* transgenic mice (referred to as BALB-NeuT) carrying a mutant rat *neu* oncogene under control of a MMTV-LTR[4] were bred in our animal facilities as described previously (5). In BALB-NeuT mice, HER-2/neu is expressed in several tissues including skeletal muscle[3] because MMTV-LTR promoter is active in many organs and tissues apart from mammary gland (6). BALB/c $p53^{+/-}$ mice were crossed with BALB-NeuT and mice bearing the $p53^{+/-}/neu^{+/-}$ genotype were selected by PCR analysis.

**PCR.** All of the mice used in this study were genotyped by PCR both for the presence of rat *HER-2/neu* transgene and for the status of *p53* by a multiplex PCR (The Jackson Laboratory). To study gene expression, we performed total RNA extraction, retrotranscription, and semiquantitative RT-PCR as described previously (3) with specific primer pairs for GAPDH (Clontech, Palo Alto, CA), rat HER-2/neu (5′-AGGGCAACTTGGAGCTTACCTACG-3′ and 5′-GGGTTCTGCCTGGGGTGGA-3′), IGF-II (7) and IGF-I-R (5′-AATACGGGTCGCAAGTCGAG-3′ and 5′-TCTGTCCATGACCCATTCCC-3′), and IGF-II-R (5′-TCAGAGCGGAGTCAGGCTGT-3′ and 5′-ACACGCCCGAGCTTCTTCT-3′), GR, and AR (8). The mouse mammary carcinoma cell line TS/A was used as a control.

**Immunohistochemistry and Immunofluorescence.** Histological and immunohistochemical evaluations were performed as reported previously (5). Anti-desmin monoclonal antibody DE-B-5 (Boehringer Mannheim, Milan, Italy) and anti-embryonic myosin monoclonal antibody BF-G6 were used (3). Membrane expression of $p185^{neu}$ was studied by flow cytometry with monoclonal antibodies recognizing rat Her-2/neu (7.16.4; Oncogene Research Products, Cambridge, MA) or human HER-2 (MGR-3) as reported previously (3).

## Results

**Development of Rhabdomyosarcoma in $p53^{+/-}/neu^{+/-}$ Mice.** To investigate whether *p53* and *HER-2/neu* genetic defects might cooperate in the genesis of rhabdomyosarcoma, we crossed *p53* knockout mice (9) with mice carrying an activated *HER-2/neu* transgene (5) to obtain $p53^{+/-}/neu^{+/-}$ mice on BALB/c inbred background, indicated here as BALB-p53neu. A very high spontaneous incidence of rhabdomyosarcoma was observed in male BALB-p53neu mice, but not in parental mice carrying one of the genetic lesions (Fig. 1*A*), which, as expected, developed a variety of other tumor histotypes (10) several months later (data not shown). Male and female BALB-p53neu mice also developed salivary gland tumors, as reported for another line of $p53^{+/-}/neu^{+/-}$ mice (11).

All of the rhabdomyosarcomas arose around 11–21 weeks of age in the genitourinary tract (Fig. 1*B*) from the sphincter of small striated muscle fibers present from the base of the verumontanum to the prostate apex (Fig. 1*C*). Tumors were composed of undifferentiated cells with scant cytoplasm and round-to-spindled centrally placed nuclei, and differentiating cells with tapering bipolar cytoplasm showing cross-striations and multiple nuclei (Fig. 1*D*). The morphology

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[3] Our unpublished observations.

[4] The abbreviations used are: MMTV-LTR, mouse mammary tumor virus 3′ long terminal repeat; AR, androgen receptor; GR, glucocorticoid receptor; IGF, insulin-like growth factor(s); GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; IGF-I-R, IGF-I receptor; IGF-II-R, IGF-II receptor.

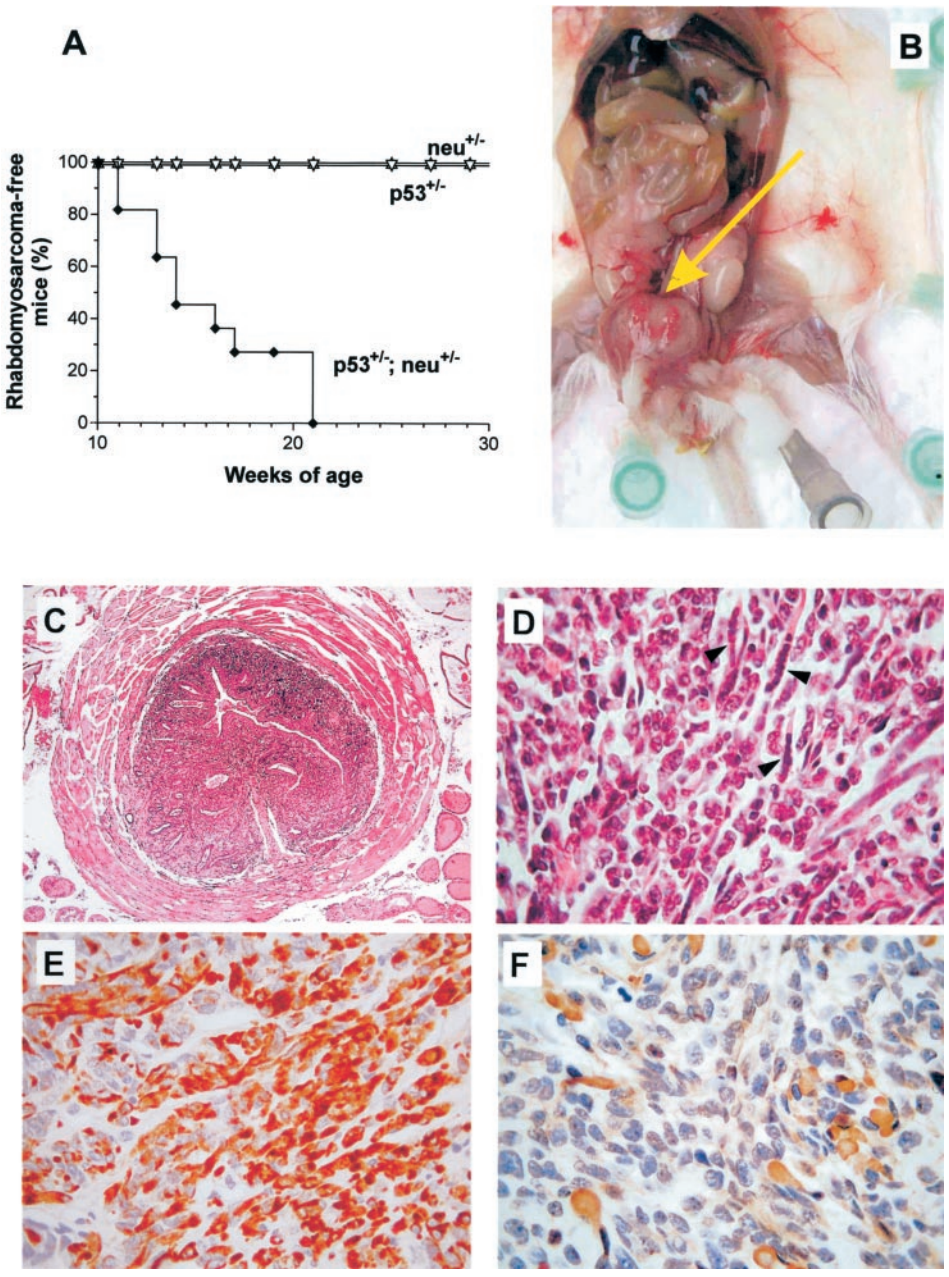


Fig. 1. Development of rhabdomyosarcoma in BALB-p53neu mice (*A* and *B*); morphological analysis (*C* and *D*), and immunohistochemical detection of myogenic markers (*E* and *F*) in BALB-p53neu rhabdomyosarcomas. *A*, rhabdomyosarcoma incidence (Kaplan-Meier curves) in groups of 10 male mice of the indicated genotype. *B*, at necropsy all of the rhabdomyosarcomas were found as pelvic masses lying behind the urinary bladder. Seminal vesicles and bladder were extroflected to better expose the tumor mass. *C*, coronal section of the prostatic urethra showing that the tumor surrounded the urethra and involved peri-urethral soft tissues and the prostate apex (H&E; ×50). *D*, typical embryonal rhabdomyosarcoma containing differentiated rhabdomyoblasts (*arrowheads*) with multiple nuclei and eosinophilic cytoplasm (H&E; ×400). *E*, expression of desmin throughout the tumor, in particular by spindle cells with cytoplasmic cross-striations. *F*, expression of embryonic myosin.

was highly reminiscent of human embryonal rhabdomyosarcoma. Morphological diagnosis was confirmed by the expression of desmin in all of the tumor cells and of striated muscle myosin in a much lower proportion of elements (Fig. 1, *E* and *F*). This indicates a defect of myogenic differentiation at the level of myosin, similar to the arrest of differentiation of most human rhabdomyosarcomas (1).

**Establishment of Rhabdomyosarcoma Cell Cultures.** To obtain pure populations of tumor cells for additional studies, we cultured *in vitro* cells disaggregated from primary rhabdomyosarcomas of BALB-p53neu mice. *In vitro* cultures were established quickly and grew rapidly. Like human rhabdomyosarcoma cells lines, mouse cultures contained small mononuclear proliferating rhabdomyoblasts along with differentiated, extremely elongated multinuclear rhabdomyotubes (Fig. 2*A*). All of the cells expressed desmin (Fig. 2*B*), thus confirming their myogenic nature, whereas only a fraction of elements, including all rhabdomyotubes, expressed the embryonic myosin isoform (Fig. 2*C*). Cultured cells were injected s.c. into syngeneic immunocompetent male and female mice giving rise to tumors that grew early and rapidly only in males, as opposed to female mice, in which tumors grew slowly and with a longer latency (Fig. 2*D*).

**Molecular Analysis of *p53* and *HER-2/neu* in Rhabdomyosarcomas.** Both BALB-p53neu rhabdomyosarcomas and cultured cells were consistently $p53^{null}$ because of the loss of the remaining *p53* allele (Fig. 3*A*). Expression of *HER-2/neu* mRNA was found in all of the tumors examined and in the cultured cells (Fig. 3*B*). Preneoplastic retrovesical tissue of 7-week-old male mice already showed HER-2 expression. $p185^{neu}$, the product of the *HER-2/neu* gene, was expressed on the surface of murine rhabdomyosarcoma cells (Fig. 3, *C* and *D*) at a level similar to the levels found in human rhabdomyosarcoma cells (Fig. 3, *E* and *F*). The intensity of p185 in murine and human rhabdomyosarcomas is about 10 times lower than that of murine and human mammary carcinomas (5, 12), thus suggesting a tissue-specific regulation of HER-2/neu oncogenesis in this mesenchymal tumor.

**Molecular Features of BALB-*p53neu* Rhabdomyosarcomas.** A hallmark of human embryonal rhabdomyosarcoma is the presence of autocrine loops involving IGF-II and the IGF-I-R, which contribute to

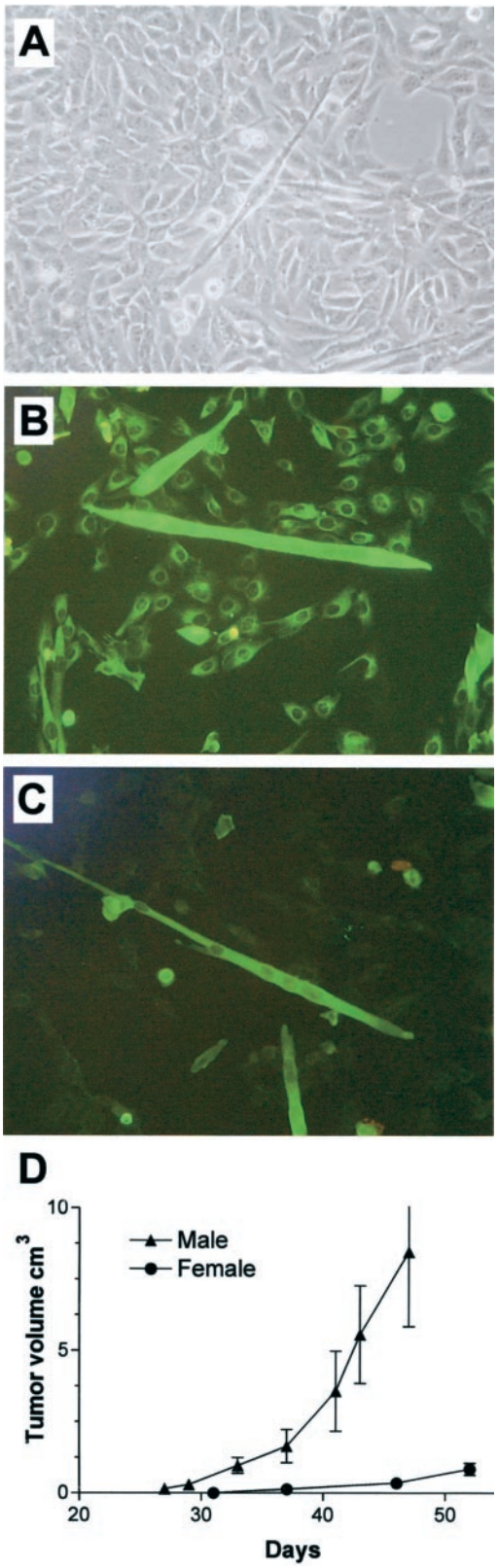


Fig. 2. *In vitro* (*A–C*) and *in vivo* (*D*) properties of cell lines derived from BALB-p53neu rhabdomyosarcomas. Cell culture morphology (*A*), expression of desmin (*B*) and embryonic myosin (*C*), and tumorigenic ability (*D*) in syngeneic immunocompetent male and female mice after the s.c. injection of $10^5$ cells.

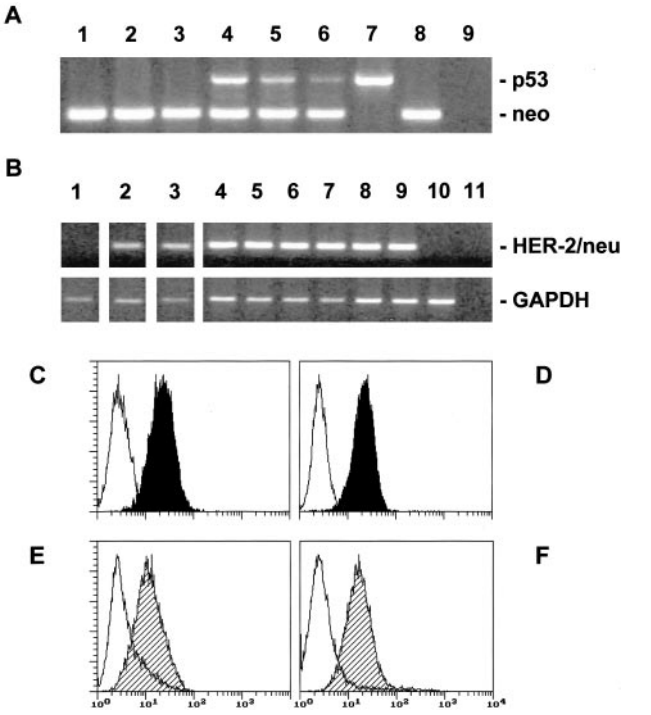


Fig. 3. HER-2/neu and p53 in tumors and cell lines. *A*, loss of *p53* allele in rhabdomyosarcoma detected by multiplex PCR with primers for *p53* and for the knockout allele (*neo* cassette). *Lanes 1–3*, rhabdomyosarcoma DNA from two *in vitro* cultures and one tumor mass; *Lanes 4–6*, somatic (tail) DNA from BALB-p53neu mice in which tumors shown in *Lanes 1–3* arose; *Lane 7*, $p53^{+/+}$ mouse tail DNA; *Lane 8*, $p53^{-/-}$ mouse tail DNA; *Lane 9*, negative control (water). *B*, expression of the *HER-2/neu* transgene (30 cycles of amplification) in BALB-p53neu rhabdomyosarcomas by RT-PCR. *Lanes 1–3*, preneoplastic retrovesical tissue from 7-week-old mice (*Lane 1*, $p53^{+/-}$; *Lane 2*, BALB-neuT; *Lane 3*, BALB-p53neu); *Lanes 4–7*, rhabdomyosarcoma tumor samples deriving from four different mice; *Lanes 8–9*, two *in vitro* cultured rhabdomyosarcoma cell lines; *Lane 10:* HER-2/neu-negative mouse mammary adenocarcinoma TS/A; *Lane 11*, negative control (water). *GAPDH* housekeeping gene expression at 20 cycles of amplification is shown for comparison. *C-D*, membrane expression of HER-2/neu in mouse rhabdomyosarcoma cell lines derived from independent tumors analyzed by flow cytometry. *E* and *F*, expression of HER-2 in two human embryonal rhabdomyosarcoma cell lines is shown for comparison (3).

the unrestricted growth of tumor cells (13). We found a copious expression of IGF-II, IGF-I-R, and IGF-II-R both in tumors and in cultured cells (Fig. 4*A*), revealing a further parallel with human rhabdomyosarcomas.

Both *p53* and *HER-2/neu* are known to cause multiple tumors of different histological origin (11). Cooperation of the two genetic lesions was required to produce rhabdomyosarcomas, which were not found either in $p53^{+/-}$ mice or in *HER-2/neu*$^{+/-}$ transgenic mice (Fig. 1*A*). Male and female BALB-p53neu mice developed salivary gland carcinomas, but rhabdomyosarcomas arose only in the genitourinary tract of male mice, suggesting the involvement of additional sex-related causative factor(s). In BALB-p53neu mice *HER-2/neu* expression was driven by MMTV-LTR, which is known to be responsive to steroid hormones, particularly androgens and glucocorticoids (14). All of the BALB-p53neu rhabdomyosarcomas expressed AR as well as GR (Fig. 4*B*), which could explain the specific origin from the genitourinary tract of male mice.

## Discussion

Our results demonstrate for the first time that the combination of p53 inactivation and HER-2/neu activation could lead to the onset of rhabdomyosarcoma and raises the possibility that similar genetic lesions could be involved also in the genesis of human rhabdomyosarcoma. Starting from the results shown here, the analysis of genes

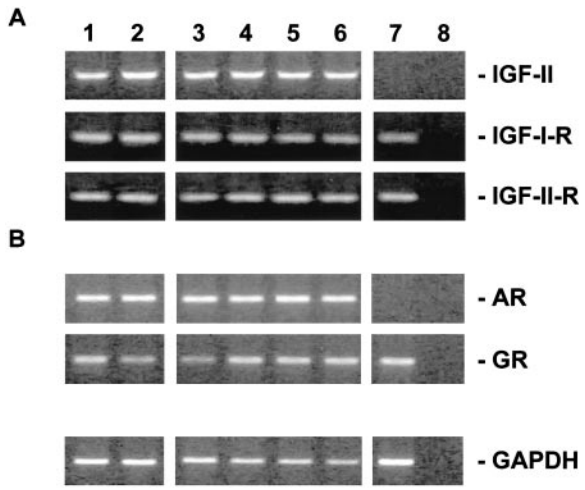


Fig. 4. IGF-system and steroid hormone receptors expression in BALB-p53neu tumors and cell lines by RT-PCR. *A*, expression of IGF-II (20 cycles of amplification) and its receptors IGF-I-R and IGF-II-R (30 cycles). *B*, expression of AR and GR (28 cycles). *GAPDH* housekeeping gene expression at 20 cycles of amplification is shown for comparison. *Lanes 1–2*, two rhabdomyosarcoma cell cultures; *Lanes 3–6*, tumor samples derived from different animals; *Lane 7*, mouse mammary adenocarcinoma TS/A; *Lane 8*, negative control (water).

involved in the genesis of the corresponding human tumors should be extended also to other members of the HER family of receptor tyrosine kinases, which are known to be expressed by human rhabdomyosarcomas (3), as well as to various genes of the p53 pathway. According to some studies the prevalence of p53 inactivation is lower in rhabdomyosarcomas as compared with other human neoplasms (15), but in human tumors, p53 function is hampered also by other mechanisms, for example by the inactivation of key regulators of its pathway like $p21^{WAF1}$, which is strongly methylated and consequently hypoexpressed in up to 50% of rhabdomyosarcoma tumor samples (16).

In the BALB-p53neu model, rhabdomyosarcomas arose as a consequence of the combined *HER-2/neu* activation and *p53* inactivation. The only other mouse model of rhabdomyosarcoma, recently described, shows that the concomitant inactivation of *INK4a/ARF* and the aberrant signaling through c-Met leads to rhabdomyosarcoma development (17). In both model systems, p53 function impairment was crucial for the development of tumors, because $p14^{ARF}$ acts via p53, but a second genetic lesion was necessary for rhabdomyosarcoma development. HER-2/neu and c-Met are implicated in different steps of normal myogenesis, and their abnormal activation could lead to rhabdomyosarcoma onset by alternative pathways.

Occurrence of rhabdomyosarcoma was not described for a different line of $p53^{+/-}/neu^{+/-}$ mice on FVB background (11) that develop salivary tumors like the mice described here. Parental HER-2/neu transgenic mice used in our study showed a more efficient and pregnancy-independent mammary carcinogenesis (5) than those used by Brodie *et al.* (11). Differences in expression of the *HER-2/neu* transgene or in the genetic background could account for the different tumor patterns.

A convergence of HER-2/neu and p53 in oncogenesis has been found in several murine models leading to the onset of tumors of diverse histological origin. It is likely that in diverse cell types, HER family and p53 pathway alterations converge in mediating carcinogenesis through tissue-specific mechanisms. In myoblasts, from which rhabdomyosarcoma derives, HER-2/neu is required for cell survival, and its absence in knockout mice leads to apoptotic cell death (4), whereas wild-type p53 triggers myogenic differentiation (18). Probably the combined genetic events involving *HER-2/neu* and *p53* in BALB-p53neu mice contribute to the survival and differentiation arrest in rhabdomyosarcoma cells.

A distinctive feature of embryonal rhabdomyosarcoma is the activation of IGF-II (13). Forced expression of wild-type p53 in human rhabdomyosarcoma inhibits IGF-II through the binding of specific promoter elements (19), thus contributing to the explanation for the overexpression of IGF-II in tumors.

In BALB-p53neu mice, rhabdomyosarcoma arose only in the genitourinary tract of male mice, suggesting the involvement of additional sex-related causative factors. BALB-p53neu also developed other tumor types, mainly salivary gland carcinomas, which were found in both males and females, thus excluding any global sex specificity of transgene expression. The best candidate for additional studies on this aspect seems to be HER-2/neu, which, in this system, was driven by MMTV-LTR. This sequence is known to be responsive to various classes of steroid hormones, including androgens (14). It has been recently reported that in transgenic rats harboring a MMTV-*HER-2/neu* construct similar to the one used here, mammary carcinomas developed exclusively in males (20), and that castration prevented carcinogenesis, thus confirming a role of male hormones. In mouse rhabdomyosarcoma cells, we found abundant expression of AR, which could be implicated in the sex-specificity of tumor onset.

The BALB-p53neu animal model, displaying a high incidence of rhabdomyosarcoma caused by genes involved in the corresponding human neoplasia, will be a useful tool to investigate the natural history of this malignant tumor, and to devise therapeutic approaches directed to specific molecular targets.

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