Tumorigenesis and Neoplastic Progression

Influence of *Hoxa5* on *p53* Tumorigenic Outcome in Mice

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***Hox* genes encode transcription factors of crucial importance in the pattern formation of a large spectrum of species. Several studies have now proposed a role for these developmental genes in cancer biology. It has been suggested that HOXA5 possesses growth-suppressive properties through activation of *p53* expression in human breast tissue. To assess the genetic cooperation that may exist between *Hoxa5* and *p53* in tumorigenesis, we generated *Hoxa5/p53* compound mutant mice. The presence of *Hoxa5* null alleles increased the susceptibility of *p53*^{-/-} mice to develop tumors with a high prevalence for thymic lymphoma, suggesting that the loss of function of the two genes collaborate in tumor formation. To extend our analysis to mammary tumorigenesis, we performed *Hoxa5/p53* whole mammary gland transplantations into wild-type hosts. In the *p53*^{-/-} background, the presence of one *Hoxa5* mutant allele had no impact on mammary tumor formation. In contrast, the complete loss of *Hoxa5* function influenced the tumorigenic outcome of *p53*^{+/-} mammary glands. However, the collaborative nature of this interaction did not depend on the transcriptional regulation of *p53* by *Hoxa5*. Altogether, our data establish that *Hoxa5* and *p53* cooperate in mammary tumorigenesis *in vivo*. (Am J Pathol 2010; 176:995–1005; DOI: 10.2353/ajpath.2010.090499)**

Tumorigenesis is a multistep process that reflects successive genetic alterations, some of which result in the activation of oncogenes and the loss of function of tumor suppressor genes. Mutations and loss of the *p53* tumor suppressor gene are common genetic lesions in a large proportion of human cancers.¹ The role of *p53* in tumor susceptibility was further demonstrated by the character-

ization of *p53* mutant mice. *p53*-deficient mice are viable, but they are prone to develop a variety of spontaneous tumors that dramatically reduce their lifespan.^{2–4} Analyses of *p53* compound mutant mice have also revealed that other genetic alterations involving various types of molecules, such as transcription factors, can participate with the loss of *p53* function to tumor initiation and progression.^{5–8}

Genes responsible for the control of transcriptional programs during normal development significantly contribute to cancer.⁹ Both normal development and cancer rely on shifts in the delicate balance between cell growth and differentiation. Cancer can arise from the misappropriation of signaling pathways normally used to control cell fate decisions during development. *Hox* genes occupy a critical position in the developmental hierarchy. They encode transcription factors essential for regulating embryo pattern formation, morphogenesis, and organogenesis.¹⁰ Several studies have also enlightened the potential role of *Hox* genes in tumor development, invasion, and metastasis. In numerous types of tumor, expression of specific *Hox* genes is either increased or decreased, suggesting that they may be involved in tumor suppression or promotion.^{11–14} The exact role of *Hox* genes in tumorigenesis remains unclear, but a likely explanation can be that their misregulation alters expression of downstream effectors, causing improper activation of embryonic developmental cascade(s), thereby disrupting normal programs of growth and differentiation and leading to neoplasia.⁹ The ability of *Hox* genes to control morphogenesis implies their role in multiple cellular processes. Consequently, changes in *Hox* gene expression in various cancers have been associated to altered proliferation, angiogenesis, apoptosis, DNA

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repair, and metastatic behavior.^{15–19} Increased incidence of malignancies correlates also with ectopic cervical ribs in humans, a skeletal transformation associated with several loss-of-*Hox* gene function mutations, suggesting that *Hox* genes might be a molecular link between congenital anomalies and cancer.²⁰

p53 gene expression has been shown to be under the control of HOX proteins. In the breast cancer cell line BT-20, constitutive *HOXA10* expression up-regulates *p53* expression, reducing the oncogenic potential of the cells.²¹ Transactivation and electrophoretic mobility shift assays have also revealed a direct binding of the HOXA5 protein to a putative HOX-binding motif in the *p53* promoter region.²² In breast cancer cell lines and patient tumors, reduced HOXA5 protein levels correlate with the lack of *p53* expression, supporting the idea that HOXA5 may possess growth-suppressive properties through activation of *p53* expression.^{22,23}

We produced a *Hoxa5* null mutant mouse line and demonstrated the crucial role of *Hoxa5* in regulating morphogenesis and specifying regional identity along the embryonic axis. The phenotypic survey of the mutant mice has unveiled the crucial role of *Hoxa5* in the development of several organs including the lung, gut, thyroid, and mammary glands.^{24–28} In the latter, the *Hoxa5* mutation causes inappropriate precocious mammary epithelium development. Proliferation is augmented while accelerated differentiation occurs in aged nulliparous and pregnant females, preceding the abnormal secretory activity at parturition that underlies the incapacity of *Hoxa5*^{−/−} dams to properly feed their pups.²⁸ The accelerated lobuloalveolar epithelium development can be rescued by grafting of mutant mammary epithelium into wild-type fat pad. Conversely, reciprocal grafting experiments demonstrate that *Hoxa5*^{−/−} stroma cannot support normal proliferation of wild-type epithelium. These data unveil the importance of *Hoxa5* in the precise equilibrium between cell growth and differentiation and establish the essential contribution of *Hoxa5* to mammary epithelium instruction via mesenchyme-epithelium crosstalk.

Hoxa5 null mutant mice are not prone to spontaneous tumorigenesis, indicating that the mutation is not a genetic lesion sufficient to initiate oncogenesis. However, the direct involvement of *Hoxa5* in cancer was revealed by data showing that leukemogenesis induced by the CALM-AF10 fusion protein requires *Hoxa5* up-regulation through the methylation of lysine 79 on histone H3 at the *Hoxa5* locus by the methyltransferase hDOT1L. In bone marrow cells from *Hoxa5*^{−/−} mice, CALM-AF10 fusion protein cannot cause leukemic transformation.¹³ Moreover, *HOXA5* gene misexpression was reported in various human cancers.^{14,23,29,30} For instance, nearly 70% of human breast carcinomas have decreased HOXA5 protein levels compared with normal breast tissue. The loss of *HOXA5* gene expression in human breast cancer also correlates with progression to higher-grade lesions, suggesting that it may act as a tumor suppressor gene.^{22,23} Thus, depending on the cellular context, either a gain or a loss of *Hoxa5* gene expression may disrupt normal growth and differentiation programs causing neoplasia.

Our *in vivo* observations that *Hoxa5*^{−/−} mice display hyperplasia and inappropriate differentiation of the mammary epithelium suggest a role for *Hoxa5* in mammary tumorigenesis.²⁸ This is further strengthened by the coordinated loss of *p53* and *HOXA5* expression in human breast tumors, which indicates that both genes can cooperate in tumorigenesis.²² To directly address this question, we generated *Hoxa5/p53* compound mutant mice. Aside from the perinatal lethality associated with the *Hoxa5* homozygous mutation, *Hoxa5/p53* mutants mainly died from lymphomas similarly to *p53* mutants. However, the loss of *Hoxa5* function in *p53* mutant mice increased the susceptibility to thymic lymphomas. The high predisposition of *Hoxa5/p53* mutants to lymphoma limited their lifespan and precluded studies of tumors with longer latencies such as mammary tumors. To overcome this problem, we performed grafts of whole mammary glands from females carrying *Hoxa5/p53* allelic combinations into wild-type hosts. We report here a cooperative action of *Hoxa5* and *p53* in mammary gland tumorigenesis.

Materials and Methods

Mice, Genotyping, and Tissue Collection

The establishment of the *Hoxa5* mutant mouse line was previously reported.²⁴ The *p53*-deficient mouse line was obtained from the Jackson Laboratory (Bar Harbor, ME). Both mutant lines were maintained in the 129/Sv inbred genetic background. Experimental animals were genotyped by Southern blot analysis as described.^{3,24}

Hoxa5 mutant mice were interbred with *p53* mutant mice to obtain double heterozygous animals (*Hoxa5*^{+/-}/*p53*^{+/-}). The latter were intercrossed to generate mice of all possible allelic combinations. A total of 11 *Hoxa5*^{+/+}/*p53*^{+/+}, 9 *Hoxa5*^{+/-}/*p53*^{+/+}, 18 *Hoxa5*^{-/-}/*p53*^{+/+}, 18 *Hoxa5*^{+/+}/*p53*^{+/-}, 22 *Hoxa5*^{+/-}/*p53*^{+/-}, 46 *Hoxa5*^{-/-}/*p53*^{+/-}, 26 *Hoxa5*^{+/+}/*p53*^{-/-}, 69 *Hoxa5*^{+/-}/*p53*^{-/-}, and 15 *Hoxa5*^{-/-}/*p53*^{-/-} mice were monitored daily during the first week, then weekly for 80 weeks, and sacrificed when overt tumor development was detected or when signs of morbidity were evident. Pathological and control tissues were excised, fixed overnight in cold 4% paraformaldehyde prepared in phosphate-buffered sulfate (PBS), processed, paraffin-embedded, and sectioned (6 μm). In some cases, thymus, lymph node, and spleen specimens were processed for flow cytometric analyses (see below). All experiments were performed according to the guidelines of the Canadian Council on Animal Care and approved by the institutional animal care committee.

Whole Mammary Gland Transplantation Studies

Whole mammary gland transplantation experiments were performed as described.³¹ Entire inguinal glands containing both epithelium and stroma were removed from 4-week-old (on average) wild-type and *Hoxa5/p53* compound mutant mice and transplanted in the place of the excised right inguinal gland of two 4-week-old wild-type

female recipients. The left inguinal gland of the female recipients was also excised and reinserted as control to monitor the efficiency of the graft (autograft). Recipients were monitored daily during the first week, then weekly, and sacrificed when overt tumor development was detected or when signs of morbidity were evident. The transplanted glands (experimental and autograft) and the right endogenous thoracic gland were excised and processed for histological and immunohistological analyses.

Histological, Immunohistochemical, and in Situ Hybridization Analyses

Sections from tumors and control tissues were stained with hematoxylin eosin (H&E) for evaluation by light microscopy. Further characterization of the tumors was assessed by immunohistochemistry with different cell markers: a rat monoclonal antibody against cytokeratin 8 (CK8, 1:20 dilution; gift from Dr. Normand Marceau, Université Laval) for luminal epithelial cells; a rabbit polyclonal antibody against cytokeratin 14 (CK14, 1:150; gift from Dr. Marceau) for myoepithelial cells; a mouse monoclonal antibody against α -smooth muscle actin (1:500; clone 1A4; Sigma, Oakville, Ontario, Canada) for myoepithelial cells and pericytes; a rat monoclonal antibody against CD31 (1:200; MEC13.3; Pharmingen, Mississauga, Ontario, Canada) for endothelial cells; a rabbit polyclonal antibody against vimentin (1:500; gift from Dr. Marceau) for fibroblasts; a rat monoclonal antibody against CD3 (1:100; Clone CD3-12; Serotec, Raleigh, NC) for T lymphocytes; and a rabbit monoclonal antibody against CD20 (1:20; Clone EP459Y; Abcam, Cambridge, MA) for B lymphocytes.

For all antibodies, antigen retrieval was performed by heat treatment in 10 mmol/L sodium citrate buffer pH6, except for CD31, which required an enzymatic digestion with trypsin-EDTA (0.025%) at room temperature for 8 minutes. Endogenous peroxidases were inactivated in a 3% H₂O₂ solution for 30 minutes. Nonspecific binding was then blocked with 3% normal goat serum at room temperature for 1 hour. Slides were incubated with the primary antibodies at the appropriate dilution overnight at 4°C. Secondary antibody incubation with biotinylated goat anti-rabbit (Vector Laboratories, Burlington, Ontario, Canada), anti-rat, or anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA) antibodies was performed for 30 minutes at a dilution of 1:200 or 1:500, followed by avidin-biotin complex reagent revelation (Vector Laboratories). Horseradish peroxidase activity was detected with the diaminobenzidine reagent kit (Zymed Laboratories, South San Francisco, CA). Slides were counterstained with Mayer's hematoxylin.

The RNA *in situ* hybridization protocol was essentially based on that of Ref. 32. A 850-bp *Bgl*II-*Hind*III genomic fragment containing the 3'-untranslated region of the second exon of the murine *Hoxa5* gene was used as a template for synthesizing a [³⁵S] UTP-labeled riboprobe. A minimum of three wild-type adult specimens was used for the analysis of *Hoxa5* expression in the thymus and the spleen.

Microscopic analyses were performed with a Leica DMR microscope or a Wild M8 binocular (Leica Microsystems, Richmond Hill, Ontario, Canada) coupled to a QImaging camera. Image acquisition was done by using the QCapture software (QImaging, Surrey, British Columbia, Canada).

Preparation and Flow Cytometric Staining of Cell Suspensions

Cells from thymus, spleen, and lymph nodes (axillary, brachial, and inguinal) were released by mincing the tissues and filtrating the resulting suspensions through nylon sieves. Before filtration, cell suspensions were treated with 150 mmol/L NH₄Cl, 10 mmol/L KHCO₃, and 0.1 mmol/L EDTA to lyse erythrocytes and washed twice in RPMI 1640 medium containing 5% fetal bovine serum. Cells were counted by using a hemacytometer, suspended in PBS containing 1% fetal bovine serum (FACS medium), and stained with anti-CD4-fluorescein isothiocyanate (clone RM4-5; Pharmingen), anti-CD8-phosphatidylethanolamine (clone 53-6.7; Pharmingen), anti-B220-fluorescein isothiocyanate (clone RA3-6B2; Pharmingen), and anti-TcR β -phosphatidylethanolamine (clone H57-597; Pharmingen) antibodies on ice for 25 minutes. Cells were washed twice in FACS medium before flow cytometric analysis on a EPICS XL cytometer (Beckman Coulter, Mississauga, Ontario, Canada). Listmode data were collected with live gating on 10⁴ relevant cellular events and analyzed by using Expo32 ADC software (Beckman Coulter).

Quantitative RT-PCR Experiments

Total RNA was isolated from paraffin-embedded whole mammary grafts by using the High Pure formalin-fixed, paraffin-embedded (FFPE) tissue section RNA Micro kit (Roche Diagnostics, Laval, Québec, Canada) and cDNA was synthesized with Superscript II Reverse Transcriptase (Invitrogen, Burlington, Ontario, Canada) by using random primers. Real-time PCR was performed by using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and a thermal cycler ABI PRISM 7000. Samples were analyzed in triplicate. *p53* and *Mlh1* transcripts were detected with the following primers overlapping exons 1 to 3 and exons 3 to 4, respectively: *p53* forward 5'-TCTGAGCCAGGAGACATTTTCA-3'; *p53* reverse 5'-AGCAACAGATCGTCCATGCA-3'; *Mlh1* forward 5'-GGATATTGTGTGTGAGAGGTTCACTAC-3'; and *Mlh1* reverse 5'-ACATGGGCCACATGGCTTAT-3'. The *Rpl19* gene was used as control by using the primers *Rpl19* forward 5'-GCTCGGATGCCTGAGAAGGT-3' and *Rpl19* reverse 5'-TCCATGAGGATGGCGCTTGTT-3'.

p53 Loss-of-Heterozygosity Analysis

Genomic DNA was isolated from paraffin-embedded whole mammary grafts by using the QIAamp DNA FFPE Tissue kit (Qiagen, Mississauga, Ontario, Canada). Due to the small amount of DNA per sample, *p53* loss of

heterozygosity (LOH) was determined by semiquantitative PCR analysis to specifically amplify the wild-type and mutant alleles with primers used for genotyping.^{33,34} The *p53* wild-type allele was detected with exon 6 (*p53* forward 5'-CCCAGATATCTGGAAGACAG-3') and exon 7 (*p53* reverse 5'-ATAGGTCGGCGGTCAT-3') specific primers giving a band of 548 bp. The *p53* mutant allele was identified by using the primers *NEOP53* forward 5'-CTTGGGTGGAGAGGCTATTC-3' and *NEOP53* reverse 5'-AGGTGAGATGACAGGAGATC-3', for a 280 bp band. The intensity of *p53* wild-type and mutant PCR products was quantified by using ImageJ software (NIH Image). LOH was considered when the ratio between the intensity of the wild-type band versus the mutant band was <0.5 .³⁵

Statistical Analyses

Mouse survival duration was defined as the time from birth until the animals died or became moribund. Kaplan-Meier survival curves were constructed and compared by using the log-rank test. For categorical variables, between-group differences were assessed by using Pearson's χ^2 test or Fisher's exact test when appropriate. *p53* and *Mlh1* expression levels in mammary grafts were compared among independent groups by using the nonparametric method Kruskal-Wallis one-way analysis of variance by rank test with Monte Carlo estimation of the exact *P* value. If the results from a comparison of multiple groups by using the Kruskal-Wallis test yielded a statistical significance, the permutation method (SAS Proc Multest) was applied to obtain the adjusted *P* value for each group in pair-wise comparisons to the control group. Statistical analyses were performed by using the SAS 9.1.3 statistical software (SAS Institute, Cary, NC). A significance level of $<5\%$ ($P < 0.05$) was considered statistically significant.

Results

Survival of *Hoxa5/p53* Compound Mutant Mice

To determine whether mutations in *Hoxa5* and *p53* cooperate during tumorigenesis, *Hoxa5* homozygous mutant mice were interbred with *p53* homozygous mutant mice to generate double heterozygous mutants (*Hoxa5*^{+/-}/*p53*^{+/-}). These animals were subsequently intercrossed to yield compound mutants of all possible *Hoxa5/p53* allelic combinations. In the 129/Sv genetic background, the survival of *Hoxa5* homozygous single mutants (*Hoxa5*^{-/-}/*p53*^{+/+}) was less than a day for the majority of animals (Figure 1A) as for *Hoxa5*^{-/-}/*p53*^{+/-} (Figure 1B) and *Hoxa5*^{-/-}/*p53*^{-/-} mutant mice (Figure 1C). This corroborates the high rate of perinatal lethality associated with the *Hoxa5* mutation previously reported.^{24,36} The survival curve of *p53* homozygous single mutants (*Hoxa5*^{+/+}/*p53*^{-/-}) superimposed that of *Hoxa5*^{+/-}/*p53*^{-/-} mice ($P = 0.6309$; Figure 1C). The median latency to morbidity and/or mortality of *Hoxa5*^{+/+}/*p53*^{-/-} mice corresponded to 19 weeks while it was 17 weeks for *Hoxa5*^{+/-}/*p53*^{-/-} mice. As well, the survival curve of the

Figure 1. Kaplan-Meier curves showing survival of *Hoxa5/p53* compound mutant mice monitored for 80 weeks. The *p53* genotype is indicated above each panel. Vertical dashed lines represent the median time at which 50% of the animals died. The median time of survival for *Hoxa5*^{-/-} is less than a day, regardless of the *p53* genotype (A–C). The median time of survival for *Hoxa5*^{+/+}/*p53*^{-/-} and *Hoxa5*^{+/-}/*p53*^{-/-} mice is 19 and 17 weeks, respectively (C). The *P* value was calculated by using the log-rank test; *n*: number of mice analyzed per genotype.

Hoxa5^{+/+}/*p53*^{+/-} animals was not significantly different from that of the double heterozygous mutants ($P = 0.5414$; Figure 1B). The median latency to morbidity and/or mortality corresponded to 69 weeks for *Hoxa5*^{+/+}/*p53*^{+/-} mice and to 80 weeks for *Hoxa5*^{+/-}/*p53*^{+/-} mice. Thus, the presence of one *Hoxa5* mutant allele did not significantly impact on the survival of the animals regardless of the *p53* genotype, indicating a lack of synergistic effect between *p53* and *Hoxa5* on survival. These data

Table 1. Tumor Spectrum of *Hoxa5/p53* Compound Mutant Mice

Genotype	n	Animals developing tumors*	Tissues developing tumors [†]			
			Lymphoid tissues	Testis	Mammary glands	Others [‡]
<i>p53</i> ^{+/+}						
<i>Hoxa5</i> ^{+/+}	11	0				
<i>Hoxa5</i> ^{+/-}	9	0				
<i>Hoxa5</i> ^{-/-}	8	0				
<i>p53</i> ^{+/-}						
<i>Hoxa5</i> ^{+/+}	17	3 (18)	1 (33)			2 (67)
<i>Hoxa5</i> ^{+/-}	21	3 (14)	2 (67)			1 (33)
<i>Hoxa5</i> ^{-/-}	16	2 (13)	1 (50)			1 (50)
<i>p53</i> ^{-/-}						
<i>Hoxa5</i> ^{+/+}	22	18 (82)	17 (94)	4 (22)	2 (11)	1 (6)
<i>Hoxa5</i> ^{+/-}	62	61 (98)	57 (93)	7 (11)	4 (7)	1 (2)
<i>Hoxa5</i> ^{-/-}	4	4 (100)	3 (75)	2 (50)		

Data presented as n (%) unless indicated otherwise. n, number of mice analyzed per genotype.

*Percentage relative to the number of animals analyzed.

[†]Percentage relative to the number of animals developing tumors.

[‡]Sarcomas affecting various tissues.

also demonstrated the predominant role of *p53* in long-term survival of *Hoxa5/p53* compound mutants.

Tumor Spectrum of *Hoxa5/p53* Compound Mutant Mice

The surviving *Hoxa5*^{-/-}/*p53*^{+/+} mutant mice did not develop neoplastic lesion, showing that the loss of *Hoxa5* function was not sufficient to initiate tumor formation (Table 1). These data agreed with our observations made over the years concerning the absence of tumor formation in *Hoxa5*^{-/-} mice. The presence of either one or two *Hoxa5* mutant alleles did not significantly impact the tumor susceptibility of *p53*^{+/-} mice. In contrast, in *p53*^{-/-} animals, the introduction of one *Hoxa5* mutant allele increased the number of mice developing tumors (82% *Hoxa5*^{+/+}/*p53*^{-/-} versus 98% *Hoxa5*^{+/-}/*p53*^{-/-} mice; *P* = 0.0155). In all four *Hoxa5*^{-/-}/*p53*^{-/-} mice obtained, tumor formation occurred. These results suggest that the

loss of *Hoxa5* function increases tumor susceptibility of our *p53*^{-/-} cohort.

It is known that *p53* mutant mice mainly develop lymphoid-derived tumors.^{2,3,37} Similarly, lymphomas were the most frequent tumors encountered in our *p53*^{-/-} cohort regardless of the *Hoxa5* genotype (Table 1). In *p53*^{-/-} mutants, we observed testicular tumors and few mammary gland tumors, independently of the *Hoxa5* genotype. Some sarcomas were also observed in *p53*^{+/-} and *p53*^{-/-} cohorts. Other pathologies, such as kidney cysts and soft-tissue derived lesions, were detected. In *p53*^{-/-} mice, lymphoid tumors were mostly found in the thymus and the spleen with few cases in the lymph nodes. However, when *Hoxa5* mutant alleles were present, thymic tumors were more prevalent (*Hoxa5*^{+/+}/*p53*^{-/-}, 12 of 17 mice [71%]; *Hoxa5*^{+/-}/*p53*^{-/-}, 54 of 57 mice [95%]; and *Hoxa5*^{-/-}/*p53*^{-/-}, 3 of 3 mice [100%]; *P* = 0.0281), suggesting an increased susceptibility to thymus-derived tumors (Supplemental Table S1, see

Table 2. Tumor Development in Whole Mammary Grafts from *Hoxa5/p53* Compound Mutants

Genotype	n	Onset of tumor formation (wk ± SD)*	Sacrifice after transplantation (wk ± SD)	Tumor type
<i>p53</i> ^{+/+}				
<i>Hoxa5</i> ^{+/+}	9		73 ± 5	No tumor
<i>Hoxa5</i> ^{+/-}	10		72 ± 10	No tumor
<i>Hoxa5</i> ^{-/-}	16		64 ± 10	No tumor
<i>p53</i> ^{+/-}				
<i>Hoxa5</i> ^{+/+}	10		74 ± 6	No tumor
<i>Hoxa5</i> ^{+/-}	9		68 ± 16	No tumor
<i>Hoxa5</i> ^{-/-} †	9	46	67 ± 18	Lymphoma (1/9); mammary intraepithelial neoplasia (1/9); carcinosarcoma (1/9)
<i>p53</i> ^{-/-}				
<i>Hoxa5</i> ^{+/+} ‡§	10	45 ± 10	53 ± 8	Angiosarcoma (9/10); carcinoma (1/10); adenocarcinoma (1/10); pseudosarcomatous carcinoma (1/10)
<i>Hoxa5</i> ^{+/-} §	10	45 ± 9	52 ± 13	Angiosarcoma (9/10); carcinoma (1/10); carcinosarcoma (1/10); pseudosarcomatous carcinoma (1/10)

n, number of successful mammary gland transplants analyzed per genotype.

*As determined by palpation; not applicable for lymphoma and mammary intraepithelial neoplasia.

†One case of atypical lymphoid infiltration was observed.

‡One case of concomitant angiosarcoma and pseudosarcomatous carcinoma.

§One case of concomitant angiosarcoma and carcinoma.

Figure 2. *In situ* hybridization of *Hoxa5* expression in thymus (A–B) and spleen (C–D) of wild-type adult mice. Bright-field views are shown in A and C. B: In the thymus, *Hoxa5* transcripts are detected in a punctate manner (arrows), while in the spleen (D) *Hoxa5* expression is found in the red pulp. r, red pulp; w, white pulp. Scale bars = 50 μ m (A); 500 μ m (C).

<http://ajp.amjpathol.org>). In contrast, spleen tumors were less abundant in the presence of *Hoxa5* mutant alleles (*Hoxa5*^{+/+}/*p53*^{-/-}, 10 of 17 mice [59%]; *Hoxa5*^{+/-}/*p53*^{-/-}, 11 of 57 mice [19%]; and *Hoxa5*^{-/-}/*p53*^{-/-}, 0 of 3 mice [0%]; *P* = 0.0056). No statistically significant difference was observed in the proportion of lymph node-derived tumors regardless of the *Hoxa5* genotype in the *p53*^{-/-} cohort. To further characterize the lymphoid tumors, immunophenotyping performed by using B220, TcR β , CD4, and CD8 antibodies was done by flow cytometry. No anomalies were observed in the B and T cell populations in thymus, spleen, and lymph nodes of *Hoxa5*^{-/-} mice, as previously reported (not shown).³⁸ Independently of the *Hoxa5* genotype, all spleen- and lymph node-derived tumors from the *Hoxa5/p53* compound mutants were mainly from B-cell origin as they mostly expressed the B220 marker. In thymus-derived tumors, mature lymphoid T-cell populations (TcR β ⁺ CD4⁻ CD8⁺ or TcR β ⁺ CD4⁺ CD8⁻) were detected in all four *Hoxa5*^{+/+}/*p53*^{-/-} specimens analyzed, whereas more immature T-cell populations (TcR β ⁺ CD4⁺ CD8⁺) were found in two of six *Hoxa5*^{+/-}/*p53*^{-/-} specimens. Thus, the loss of *Hoxa5* function in a *p53* mutant con-

text may affect the susceptibility to develop thymic lymphoma.

As *Hoxa5* appears to influence the frequency of the *p53* lymphoid tumors, we assessed *Hoxa5* expression in the thymus and the spleen by *in situ* hybridization. In adult thymus, *Hoxa5* was expressed in a punctate manner (Figure 2, A and B), while in the spleen, *Hoxa5* transcripts were mainly detected throughout the red pulp (Figure 2, C and D). Thus, *Hoxa5* expression in spleen and thymus is compatible with a role, yet to be defined, for *Hoxa5* in these tissues.

In summary, although the *Hoxa5* mutation did not affect the tumor latency and tumor spectrum in *Hoxa5/p53* mutants, it increased the susceptibility of *p53*^{-/-} mice to develop lymphoid tumors with a higher prevalence for thymic lymphoma. Altogether, these observations suggest that a genetic interaction may occur between *Hoxa5* and *p53* in tumorigenesis.

Development of Mammary Tumors in *Hoxa5/p53* Whole-Gland Grafts

Despite evidence suggesting a crucial role for *p53* in human breast cancer, *p53* mutant mice rarely develop mammary tumors.^{2,3} In our *p53*^{-/-} cohort, only 7% of the *p53*^{-/-} animals developing tumors presented mammary neoplasias, regardless of the *Hoxa5* genotype (Table 1). The phenotype was also sex-independent. Out of the six cases, three angiosarcomas, one hemangioma, one mammary intraepithelial neoplasia (MIN), and one lymphoma were diagnosed.

The high predisposition of *Hoxa5/p53* mutants to develop lymphoma limited their lifespan and precluded studies of tumors with longer latencies like mammary tumors. To directly address the genetic interaction between *Hoxa5* and *p53* in mammary tumor formation *in vivo*, we performed grafts of whole mammary glands from females of all possible *Hoxa5/p53* allelic combinations into wild-type hosts, with the exception of *Hoxa5*^{-/-}/*p53*^{-/-} specimens, which were never obtained. More than 95% of the grafts were accepted by the recipients. No tumor developed in the whole-graft transplants derived from mice of the following genotypes: *Hoxa5*^{+/+}/*p53*^{+/+}

Figure 3. Histology of the mammary grafts from *Hoxa5/p53* allelic combinations that did not develop tumors. H&E stained sections of representative mammary glands are shown. A: Control mammary graft displayed large adipocytes and ducts surrounded by a thin stroma. In B and D, no obvious alterations were detected in *Hoxa5*^{+/+}/*p53*^{+/+} (B) and *Hoxa5*^{+/+}/*p53*^{+/-} (D) grafts. C: Precocious lobuloalveolar development and epithelial hyperplasia were observed in *Hoxa5*^{-/-}/*p53*^{+/+} specimens as previously shown in *Hoxa5*^{-/-} females.²⁸ E: Adipocytes with microvesicular fat droplets (arrows) and intense reactive stroma were often seen in *Hoxa5*^{+/-}/*p53*^{+/-} grafts. Scale bar = 100 μ m.

Figure 4. Neoplasias observed in *Hoxa5*^{+/+}/*p53*^{-/-} and *Hoxa5*^{+/-}/*p53*^{-/-} mammary grafts. Hematoxylin & eosin stained sections are shown on the left panel for each tumor type (A–D). **A:** The angiosarcoma, characterized by the presence of red blood cells within the sarcoma (inset), was positive for CD31, a marker of endothelial cells, and vimentin (Vim). **B:** The carcinoma was confirmed by its positivity for CK8 while being negative for CK14, a myoepithelial marker. **C:** The adenocarcinoma was composed of clusters of neoplastic epithelial cells positive for CK8 that are organized around small lumens (arrows) and surrounded by Vim-positive fibroblasts. **D:** The pseudosarcomatous carcinoma with typical fusiform epithelial cells co-stained for CK8 and Vim. Scale bar = 100 μ m.

p53^{+/+}; *Hoxa5*^{+/+}/*p53*^{+/+}; *Hoxa5*^{-/-}/*p53*^{+/+}; *Hoxa5*^{+/+}/*p53*^{+/-}; and *Hoxa5*^{+/-}/*p53*^{+/-} after 70 weeks on average (Table 2; Figure 3). In contrast, all *p53*^{-/-} mammary grafts developed tumors detectable approximately 45 weeks after transplantation, regardless of the *Hoxa5* genotype (Table 2; Figure 4). The histology of the tumors revealed that angiosarcoma was the most prevalent tumor type observed in *Hoxa5*^{+/+}/*p53*^{-/-} (90%) and *Hoxa5*^{+/-}/*p53*^{-/-} (90%) grafts, as confirmed by CD31, vimentin, and α -smooth muscle actin immunostaining assays (Figure 4A; not shown). Few mammary grafts from the *Hoxa5*^{+/+}/*p53*^{-/-} and *Hoxa5*^{+/-}/*p53*^{-/-} genotypes displaying angiosarcoma also developed either a carcinoma, as detected by CK8 immunostaining (Figure 4B), or a pseudosarcomatous carcinoma (Table 2; Figure 4D). In addition, an adenocarcinoma and a carcinosarcoma were detected in *Hoxa5*^{+/+}/*p53*^{-/-} and *Hoxa5*^{+/-}/*p53*^{-/-} grafts, respectively (Table 2; Figure 4C; not shown). Thus, the presence of one *Hoxa5* mutant allele has no major impact on mammary tumor latency and type in the *p53*^{-/-} background, indicating a predominant role for *p53* in mammary tumorigenesis.

All *Hoxa5*^{+/+}/*p53*^{+/-} and *Hoxa5*^{+/-}/*p53*^{+/-} mammary transplants remained tumor-free. In contrast, the homozygous loss of *Hoxa5* function in a *p53*^{+/-} context was

Figure 5. Characterization of *Hoxa5*^{-/-}/*p53*^{+/-} mammary tumors. Hematoxylin & eosin stained sections are shown on the left panel for each tumor type (A–C). **A:** The carcinosarcoma with its sarcomatous and carcinomatous (asterisks) components is shown. Each component was revealed by CK8 (carcinoma) and by vimentin (Vim; sarcoma) immunostaining. **B:** The lymphoma was confirmed by immunostaining with the CD3 and CD20 lymphoid markers, labeling T- and B-cells, respectively. **C:** The mammary intraepithelial neoplasia was characterized by a CK8-positive epithelial hyperplasia within the mammary duct (arrows), the presence of CK14-positive myoepithelial cells (arrowhead), and the atypical nuclear cytology (not shown), suggesting a high-grade lesion. Scale bar = 100 μ m.

associated with the development of lymphoma (1 of 9), MIN (1 of 9), and carcinosarcoma (1 of 9; Table 2; Figure 5, A–C). The lymphoma was validated by immunostaining with lymphoid specific antibodies (Figure 5B). The MIN was characterized by an epithelial hyperplasia positive for the CK8 marker, the presence of CK14⁺ myoepithelial cells within the mammary duct, and the atypical nuclear cytology suggesting a high-grade lesion (Figure 5C).³⁹ Thus, the loss of both *Hoxa5* alleles may trigger mammary tumor development in a *p53*^{+/-} background.

HOXA5 was reported to be a potential transcriptional regulator of *p53* expression in human breast cancer.²² We thus monitored *p53* expression levels in our mammary graft specimens by quantitative RT-PCR (Figure 6A). No significant change of *p53* expression was detected in *Hoxa5*^{-/-}/*p53*^{+/+} grafts compared with wild-type controls. The residual *p53* expression in *Hoxa5*^{+/+}/*p53*^{-/-} tumor tissues was most likely due to the vascular contribution of the wild-type host to the tumor. *Hoxa5*^{+/+}/*p53*^{+/-} and *Hoxa5*^{+/-}/*p53*^{+/-} mammary grafts showed a statistically significant reduction of 46% and 33%, respectively, when compared with wild-type specimens, probably reflecting the haplo-insufficiency at the *p53* locus. Likewise, a 43% decrease of *p53* expression was detected in *Hoxa5*^{-/-}/*p53*^{+/-} specimens that did not develop tumors. The *Hoxa5*^{-/-}/*p53*^{+/-} lymphoma and preneoplastic lesion MIN showed a similar reduction than the tumor-free specimens, whereas the *Hoxa5*^{-/-}/*p53*^{+/-} carcinosarcoma presented a pronounced decrease of *p53* expression. These results suggest that the loss of *Hoxa5* function did not alter *p53* expression as shown in *Hoxa5*^{-/-}/*p53*^{+/+} and *Hoxa5*^{-/-}/*p53*^{+/-} grafts. Since we

Figure 6. Quantitative RT-PCR analysis of *p53* and *Mlh1* expression in *Hoxa5/p53* mammary grafts. The genotypes of the mammary grafts and the number of specimens (*n*) analyzed are indicated below each bar. **A:** Relative *p53* expression levels are represented. No significant change of *p53* expression was detected in *Hoxa5*^{-/-}/*p53*^{+/+} grafts compared with wild-type controls, while statistically significant reductions occurred in *Hoxa5*^{+/+}/*p53*^{-/-}, *Hoxa5*^{+/+}/*p53*^{+/-}, *Hoxa5*^{-/-}/*p53*^{+/-}, and *Hoxa5*^{-/-}/*p53*^{-/-} specimens. The *Hoxa5*^{-/-}/*p53*^{+/-} carcinosarcoma presented a pronounced decrease of *p53* expression. **B:** Relative *Mlh1* expression levels are represented. No significant change of *Mlh1* expression was detected in all specimens analyzed regardless of the genotype. CS, carcinosarcoma; L, lymphoma; **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

could not rule out the possibility of a vascular and stromal contribution of the wild-type host to the grafts, we examined *p53* expression in whole mammary glands from 4-month-old *Hoxa5*^{-/-} nulliparous females (Supplemental Figure S1, see <http://ajp.amjpathol.org>). No statistically significant difference was observed supporting the notion that *Hoxa5* is not a major regulator of *p53* expression in the murine mammary gland.

The decreased *p53* expression in the *Hoxa5*^{-/-}/*p53*^{+/-} carcinosarcoma suggests that *p53* LOH has occurred. To examine the status of the *p53* wild-type allele in the *Hoxa5*^{-/-}/*p53*^{+/-} carcinosarcoma, we performed a semiquantitative PCR analysis (Supplemental Figure S2, see <http://ajp.amjpathol.org>).^{33,34} The intensity ratio of the band corresponding to the wild-type allele versus that of the mutant allele was superior to 0.5 in the *Hoxa5*^{-/-}/*p53*^{+/-} tumor-free specimens, while that of the *Hoxa5*^{-/-}/*p53*^{+/-} carcinosarcoma was inferior to 0.5, supporting the notion of LOH in the *Hoxa5*^{-/-}/*p53*^{+/-} carcinosarcoma.³⁵

As *Hoxa5* may participate to the integrity of the genome through the regulation of the expression of the mismatch repair gene *Mlh1*, the loss of *Hoxa5* function could indirectly render *p53*^{+/-} mammary glands more susceptible to a second hit mutation.¹⁷ Therefore, we examined *Mlh1* expression in our mouse mammary grafts. *Mlh1* expression was reduced by 52% in *Hoxa5*^{+/+}/*p53*^{+/-} grafts and by 57% in *Hoxa5*^{-/-}/*p53*^{+/-} grafts, regardless of tumor formation (Figure 6B). However,

these variations were not statistically significant. As well, no changes in *Mlh1* expression were observed in all other genotypes analyzed.

In summary, whole mammary graft experiments clearly demonstrate the major role of the *p53* gene in mammary tumorigenesis. They also establish that the loss of *Hoxa5* function, despite no direct impact on *p53* expression, can significantly contribute to mammary tumor formation in a *p53*^{+/-} context, validating our hypothesis that *Hoxa5* can cooperate with *p53* in mammary neoplasia *in vivo*.

Discussion

In this report, we aimed to directly assess the genetic cooperation of *Hoxa5* and *p53* in tumorigenesis by generating *Hoxa5/p53* compound mutant mice. In mice, the susceptibility to tumorigenesis and the spectrum of tumors that develop depend on the genetic background.^{2,37,40} The average lifespan of our 129/Sv *p53*^{-/-} cohort was about 18 weeks, which is comparable with the ~15 to 17 weeks previously reported for the same genetic background.^{37,41} *p53* has a predominant function in long-term survival of *Hoxa5/p53* compound mutants. In contrast, the *Hoxa5* mutation causes perinatal mortality with no major impact on *p53*-dependent survival of the compound mutants. The number of *Hoxa5*^{-/-} surviving animals decreases in presence of *p53* mutant alleles. This may reflect an additive action of both mutations on embryonic development as it is known that a small fraction of *p53*^{-/-} female embryos fail to develop normally.^{42,43} This consequence may account for the paucity of *Hoxa5*^{-/-}/*p53*^{-/-} female mice obtained.

The tumor spectrum of our cohort of *p53*^{-/-} single mutant mice corresponds to that previously described for *p53*^{-/-} mice in the 129/Sv genetic background with lymphoma being the most frequent tumor occurring in 94% of the affected mice.³⁷ In the presence of *Hoxa5* mutant alleles, the tumor susceptibility of *p53*^{-/-} mice increases. Furthermore, the incidence of thymic lymphoma raises with the number of *Hoxa5* mutant alleles in the *p53*^{-/-} background, suggesting some collaborative role between *Hoxa5* and *p53* in lymphomagenesis. *Hoxa5* is expressed in restricted areas of the thymus that could correspond to regions of T-cell maturation, which may be consistent with our observation that some *Hoxa5*^{+/+}/*p53*^{-/-} thymic lymphomas seem inclined to display immature T-cell populations. Altogether, these data indicate that the loss of *Hoxa5* function could influence the susceptibility to develop thymic lymphoma. Inactivation of *HOXA5* expression by hypermethylation was previously reported in myeloid and lymphoid malignancies in humans and strongly correlates with progression to blast crisis.³⁰ Also, overexpression of *HOXA5* in human patients suffering from T acute lymphoblastic leukemia is associated with the production of the CALM-AF10 fusion protein.⁴⁴ This was confirmed by genetic analysis showing that *Hoxa5* up-regulation is required for inducing leukemia transformation mediated by CALM-AF10 fusion protein in mice.¹³ Thus, deregulated expression of *Hoxa5* may contribute to malignant hemopathies.

Angiosarcoma was the prevalent tumor type affecting the mammary glands of *Hoxa5/p53* compound mutant mice (Table 1). In our whole graft experiments of *Hoxa5*^{+/+}/*p53*^{-/-} and *Hoxa5*^{+/-}/*p53*^{-/-} mammary glands, all specimens develop tumors and most lesions consist of angiosarcomas (Table 2). Thus, the presence of one *Hoxa5* mutant allele in the *p53*^{-/-} background has no major consequence on mammary tumor latency and type, showing the predominant role of the *p53* homozygous mutation in the development of spontaneous mammary tumors. However, one limitation of our analysis was the lack of *Hoxa5*^{-/-}/*p53*^{-/-} whole mammary grafts due to the extremely low viability of *Hoxa5*^{-/-}/*p53*^{-/-} female mice that precluded the study of the impact of the homozygous loss of *Hoxa5* function on mammary tumorigenesis in absence of *p53* function. The use of the *Hoxa5* conditional mutant mouse line should provide a means to circumvent this hurdle by producing double homozygous null *Hoxa5/p53* female donors.⁴⁵

Breast cancer is a heterogeneous disease, which includes a wide range of histological subtypes and a diversity of clinical behaviors and patient's outcome.⁴⁶ In humans, primary breast angiosarcomas account for only 0.04% of all malignant breast tumors.⁴⁷ They mainly arise in younger women. Secondary angiosarcomas occur most frequently after breast conservation therapy with radiation therapy (0.15%).⁴⁸ However, the risk is expected to raise as more women with breast cancer are treated with breast conserving therapy.⁴⁹ Moreover, secondary angiosarcomas tend to have a poor prognosis.⁴⁷ In the BALB/c background, 75% of *p53*^{-/-} whole mouse mammary gland transplants develop carcinomas or adenocarcinomas without report of occurrence of angiosarcoma.⁵⁰ Thus, our mouse model in the 129/Sv genetic environment may provide an insightful system to study the genetic events responsible for this aggressive neoplastic lesion.

Adenocarcinoma and carcinoma were rarely observed in our *Hoxa5*^{+/+}/*p53*^{-/-} mammary grafts (Table 2). One likely explanation for this difference in the mammary tumor spectrum may be the genetic background. The 129/Sv and C57Bl/6 mouse strains are more resistant to mammary tumor development than the BALB/c mouse strain.^{40,50} Moreover, except for lymphomas and teratomas, soft-tissue sarcomas are the most prevalent tumors in 129/Sv *p53*^{-/-} mice.⁴¹ The development of sarcomatous lesions, either angiosarcomas or carcinosarcomas, in our *Hoxa5*^{+/+}/*p53*^{-/-} and *Hoxa5*^{+/-}/*p53*^{-/-} mammary grafts may reflect the predisposition of the mice from the 129/Sv strain to this type of tumors. It would also explain why only one case of adenocarcinoma occurred in the *Hoxa5*^{+/+}/*p53*^{-/-} grafts, a tumor type rarely detected in 129/Sv *p53*^{-/-} mice.⁴¹

The impact of the loss of *Hoxa5* function in *p53*^{+/-} mutant glands clearly demonstrates that *Hoxa5* collaborates with *p53* in mammary tumor formation. Our whole graft experiments show that the lack of both *Hoxa5* functional alleles in *p53*^{+/-} mammary glands causes neoplastic or preneoplastic lesions in 30% of the specimens, while all *Hoxa5*^{+/+}/*p53*^{+/-} and *Hoxa5*^{+/-}/*p53*^{+/-} grafts remain tumor-free. The tumor types detected correspond

to mammary lymphoma, carcinosarcoma, and MIN. In humans, primary breast lymphoma is a rare entity, accounting for 0.04 to 1.1% of all breast tumors and for 1.7 to 2% of all non-Hodgkin's lymphomas.⁵¹ It has a fair outcome.⁵² Human breast carcinosarcomas are also rare with an incidence estimated to 0.1 to 0.2% of breast carcinomas.⁵³ However, they are very aggressive with a poor prognosis. True carcinosarcoma is strictly defined as a mixed tumor with both epithelial and mesenchymal components without a transition zone between the two elements. This type of tumor is often confounded with other metaplastic carcinomas, suggesting that they may be more abundant than reported. MINs are preneoplastic lesions that possess characteristics of both benign and malignant cells. In humans, these lesions affect epithelial tissues before or accompanying invasive carcinomas.³⁹

While in human breast tumors and cell lines, HOXA5 is proposed to activate *p53* expression, our *p53* expression analysis for the *Hoxa5*^{-/-}/*p53*^{+/+} and *Hoxa5*^{-/-}/*p53*^{+/-} mouse mammary grafts does not support the notion that *Hoxa5* significantly contributes to *p53* transcriptional regulation (Figure 6A; Supplemental Figure S1, see <http://ajp.amjpathol.org>).²² This may reflect that in mice, *Hoxa5* is not a key regulator of *p53* expression since *Hoxa5*^{-/-} mice and mammary grafts never develop tumors. Moreover, the decreased *p53* expression in the *Hoxa5*^{-/-}/*p53*^{+/-} carcinosarcoma was associated to *p53* LOH (Supplemental Figure S2, see <http://ajp.amjpathol.org>). Incidence of *p53* LOH is frequent in human primary breast cancers and it was also reported in mouse mammary tumors.^{50,54}

It was shown that HOXA5 may participate to the integrity of the genome of human breast cells through the transcriptional regulation of the mismatch repair gene *MLH1*.¹⁷ Our data rather indicate that the loss of *Hoxa5* function does not impact *Mlh1* expression in mammary grafts. In fact, *Mlh1* expression levels are not significantly perturbed in all grafts analyzed regardless of the genotype. Although *Mlh1* null mutant mice are prone to cancer, they mainly develop gastrointestinal tract and lymphoma tumors, with no reported occurrence of mammary tumors.⁵⁵ As well, *hMLH1* does not seem to play an essential role in human breast carcinoma.⁵⁶ Together, these data are in agreement with our observations that tumor formation in *Hoxa5/p53* mammary grafts does not involve *Mlh1* misregulation.

In summary, our study endorses the hypothesis that *Hoxa5* occupies a pivotal position in mammary gland homeostasis and tumorigenesis since its loss of function contributes with other genetic lesions to breast tumor formation. It now remains to define the mechanisms that underlie the genetic cooperation between *Hoxa5* and *p53* in mammary oncogenesis.

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