

## Deficiency in p53 but not Retinoblastoma Induces the Transformation of Mesenchymal Stem Cells *In vitro* and Initiates Leiomyosarcoma *In vivo*

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### Abstract

Sarcomas have been modeled in mice by the expression of specific fusion genes in mesenchymal stem cells (MSC), supporting the concept that MSCs might be the target initiating cell in sarcoma. In this study, we evaluated the potential oncogenic effects of p53 and/or retinoblastoma (Rb) deficiency in MSC transformation and sarcomagenesis. We derived wild-type,  $p53^{-/-}$ ,  $Rb^{-/-}$ , and  $p53^{-/-}Rb^{-/-}$  MSC cultures and fully characterized their *in vitro* growth properties and *in vivo* tumorigenesis capabilities. In contrast with wild-type MSCs,  $Rb^{-/-}$ ,  $p53^{-/-}$ , and  $p53^{-/-}Rb^{-/-}$  MSCs underwent *in vitro* transformation and showed severe alterations in culture homeostasis. More importantly,  $p53^{-/-}$  and  $p53^{-/-}Rb^{-/-}$  MSCs, but not  $Rb^{-/-}$  MSCs, were capable of tumor development *in vivo* after injection into immunodeficient mice.  $p53^{-/-}$  or  $p53^{-/-}Rb^{-/-}$  MSCs originated leiomyosarcoma-like tumors, linking this type of smooth muscle sarcoma to p53 deficiency in fat tissue-derived MSCs. Sca1+ and Sca1 low/- cell populations isolated from *ex vivo*-established, transformed MSC lines from  $p53^{-/-}Rb^{-/-}$  tumors showed identical sarcomagenesis potential, with 100% tumor penetrance and identical latency, tumor weight, and histologic profile. Our findings define the differential roles of p53 and Rb in MSC transformation and offer proof-of-principle that MSCs could provide useful tools to dissect the sarcoma pathogenesis. *Cancer Res*; 70(10): 4185–94. ©2010 AACR.

### Introduction

Mesenchymal stem cells (MSC) are multipotent stem cells present in several tissues including bone marrow, cord blood, placenta, and fat tissue among others (1, 2). They differentiate *in vitro* and *in vivo* into multiple mesodermal tissues (2–4). Their multilineage differentiation potential coupled with

their immunoprivileged properties (5, 6) and their ability to home to sites of active tumorigenesis, metastasis processes, inflammation sites, and damaged tissues (2) is being exploited worldwide for both autologous and allogeneic cell replacement strategies, placing MSCs among the most promising adult stem cells for potential clinical use.

Increasing evidence suggests that a tumor resembles normal tissue development and retains a hierarchical organization (7, 8). According to the hierarchical model of cancer, a rare subset of cells display or regain the ability to divide asymmetrically, resulting in the generation of an identical daughter cell and more differentiated cells through which multiple subsequent divisions eventually generate the bulk of the tumor (9). These rare cells are thought to be responsible for initiating and/or sustaining the growth of the tumor and, if not completely eradicated, they may eventually drive tumor relapse (2, 9, 10). These so-called cancer-initiating cells have been identified both in hematologic malignancies and in an increasing number of solid tumors (11–13).

MSCs might constitute a target cell for some transforming mutations which may arise in a MSC or mesodermal precursor, giving rise to sarcomas including mixoid liposarcoma, rhabdomyosarcoma, and Ewing's sarcoma (14–18). In fact, cancer-initiating cells displaying MSC properties have been recently identified in Ewing's sarcoma (19). Together, these data support the idea that MSCs could play a relevant role and become an instrumental tool in studies aimed at dissecting the pathogenesis and origin of sarcomas (2). In addition

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**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

**Authorship:** R. Rubio designed and performed experiments, analyzed the data, and wrote the manuscript; J. García-Castro conceived the study and performed research; I. Gutiérrez-Aranda, P. Leone, and P. Catalina performed research; M. Santos and J. Paramio provided important feedback and contributed key reagents; P. Menendez analyzed the data and interpreted the results, supervised the study, and wrote the paper; R. Rodríguez conceived and supervised the study, wrote the paper, analyzed the data, and interpreted the results. P. Menendez and R. Rodríguez financially supported the study.

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to these MSC-based cell transformation models, MSCs may transform spontaneously *in vitro* at higher frequency than other primary human or mouse stem cells (20–23).

Transformation of primary cells into tumorigenic variants is a multistep process, whereby each genetic insult confers a proliferative/survival advantage. Despite the above evidence of MSC transformation, little is still known about the potential mechanistic basis. MSC transformation has often been linked to the accumulation of chromosome instability (23–27). These observations, together with the high resistance of MSCs to apoptosis (28), support a potential key role of mechanisms that control proliferation and cell cycle in the transformation process. We therefore envision that the tumor suppressors p53 and retinoblastoma (Rb) might be intriguing and prominent candidates to be studied among all the potential cell cycle regulators expected to be disrupted in sarcoma development. Furthermore, many, if not all, human sarcomas show alterations in the p53 and/or Rb pathways (29). In this cell line, patients suffering from Li-Fraumeni syndrome, which is caused by p53 mutations, display an increased incidence of several sarcomas (30). Moreover, inactivation of both p53 and Rb in the osteoblastic lineage induced osteosarcoma development in mouse models (31–33).

In the present work, we aimed at analyzing, *in vitro* and *in vivo*, the potential oncogenic effects of p53 gene and Rb gene deficiency, alone or in combination, in MSC transformation and sarcomagenesis. We have derived wild-type (Wt), p53<sup>-/-</sup>, Rb<sup>-/-</sup>, and p53<sup>-/-</sup>Rb<sup>-/-</sup> MSC cultures after the Cre-driven excision of loxP sites, and we fully characterized their *in vitro* growth properties and their *in vivo* tumorigenesis ability. We report that in contrast with Wt and Rb<sup>-/-</sup> MSCs, p53<sup>-/-</sup> and p53<sup>-/-</sup>Rb<sup>-/-</sup> MSC cultures underwent *in vitro* transformation and were capable of *in vivo* tumor development. In all cases, either p53<sup>-/-</sup> or p53<sup>-/-</sup>Rb<sup>-/-</sup> MSCs gave rise to leiomyosarcoma-like tumors, linking this type of smooth muscle sarcoma with p53 deficiency in fat tissue-derived MSCs.

## Materials and Methods

**Generation of mutant MSCs.** MSCs were obtained and cultured as previously described (26) from fat tissue derived from FVB background mice bearing alleles for either p53, Rb, or both genes flanked by LoxP sites (34). Four mouse strains were used (a) Wt, (b) p53<sup>loxP/loxP</sup>, (c) Rb<sup>loxP/loxP</sup>, and (d) p53<sup>loxP/loxP</sup>Rb<sup>loxP/loxP</sup>. Mutant MSCs were generated by excision of the LoxP-flanked sequences by infection of all MSC cultures with human type 5 adenoviral vectors expressing the *Cre-recombinase* gene under the control of the hCMV promoter (Ad-CMV-Cre). Briefly, MSCs at 50% to 70% of confluence were transduced with Ad-CMV-Cre in serum-free medium at a multiplicity of infection of 10. Two hours later, the cultures were washed away twice with PBS (Life Technologies). The successful gene knockdown and subsequent generation of p53<sup>-/-</sup>, Rb<sup>-/-</sup>, and p53<sup>-/-</sup>Rb<sup>-/-</sup> MSC cultures after the excision of loxP regions was confirmed by genomic PCR and Western blot. Only cells between passages 5 and 15 were used in downstream experiments.

**Genomic PCR.** Total DNA was extracted using the DNeasy kit (Qiagen). Two hundred nanograms of DNA were used for each PCR reaction. PCR conditions were as follows: predenaturation at 94°C for 5 minutes followed by 29 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C (for p53), 60°C (for Rb), or 67°C (for  $\beta$ -actin) for 30 seconds and extension at 72°C for 50 seconds. Primer sequences: p53 and Rb as previously reported (34);  $\beta$ -actin forward 5-GCCATC-CAGGCTGTGCTGTC-3 and  $\beta$ -actin reverse 5-TGAGG-TAGTCTGTCAGGTCC-3.

**Western blot.** Whole cell extracts were prepared as previously described (35). Proteins were resolved on 10% SDS-PAGE gels and blotted onto a nitrocellulose membrane (Bio-Rad). Proteins were detected using a chemiluminescence detection system (Bio-Rad) according to the recommendations of the manufacturer, using anti-p53 (1:500 dilution; Santa Cruz Biotechnology), anti-Rb (1:750 dilution; BD-PharMingen), and anti- $\beta$ -actin (1:20,000 dilution; Sigma).

**Flow cytometry and cell sorting.** The immunophenotype of cultured MSCs was determined by flow cytometry using monoclonal antibodies for Sca-1, CD11b, CD14, CD29, CD44, and CD45 (Becton Dickinson) as previously described (26).

In sorting experiments, MSCs were incubated with Sca1 monoclonal antibodies and the Sca1<sup>+</sup> and Sca1<sup>low/-</sup> cell fractions were purified using a FACSARIA cell sorter (Becton Dickinson). Sorted cells ( $2 \times 10^5$ ) were immediately inoculated into nonobese diabetic/severe combined immunodeficient (NOD/SCID) IL2R $\gamma$ <sup>-/-</sup> mice as described below. For cell cycle analysis, cells were stained with propidium iodide as described previously (36, 37). Stained nuclei were analyzed on a FACSCanto II flow cytometer using FACSDiva (Becton Dickinson) and ModFit LT (Verity) software.

**In vitro culture homeostasis and differentiation analysis.** Phase contrast morphology of the different MSC cultures was observed daily. As for the growth kinetics of the different MSC cultures, cells were counted every 4 days and re-plated at a density of  $3 \times 10^3$  cells/cm<sup>2</sup>. Cumulative population doublings were calculated at each passage according to  $X_i \times 2^n = X_f$ , where  $X_i$  is the initial number of cells seeded,  $n$  is the number of population doublings, and  $X_f$  is the final number of cells counted.

G-banding karyotype analysis was performed as previously described (38). Fifty metaphases were analyzed for each MSC genotype. *In vitro* differentiation studies of MSCs into the osteogenic and adipogenic lineages were performed as previously described in detail (26).

**Anchorage-independent cell growth.** Soft agar colony formation assay was carried out using the CytoSelect 96-Well Cell Transformation Assay Kit (Cell Biolabs, Inc.) following the instructions of the manufacturer. Briefly,  $1.5 \times 10^4$  MSCs from each genotype were suspended in DMEM containing 0.4% low-melting agarose and 10% FCS and seeded onto a coating composed of DMEM containing 1% of low-melting agarose and 10% FCS. In this assay, the number of colonies showing more than 50 cells was scored 4 weeks later. For each genotype, two independent experiments were performed in triplicate. The HeLa cell line was used as a positive control.

**In vivo tumorigenesis assays.** NOD/SCID *Cg-Prkdc<sup>scid</sup> IL2Rg<sup>tmWjl/sj</sup>* (NOD/SCID *IL2Rγ<sup>-/-</sup>*) mice were obtained from The Jackson Laboratory (39). All mice were housed under specific pathogen-free conditions, fed *ad libitum* according to animal facilities guidelines, and used at 8 to 12 weeks old. NOD/SCID *IL2Rγ<sup>-/-</sup>* mice were inoculated s.c. with  $5 \times 10^6$  MSCs or  $2 \times 10^5$  tumor-derived cells, according to the United Kingdom Coordinating Committee for Cancer Research guidelines for the welfare of animals in experimental cancer research. Animals were killed when tumors reached ~10 mm or 4 months after infusion. Upon tumor removal, half the tumor was mechanically disaggregated to establish *ex vivo* MSC-transformed cell lines as previously described (26). The remaining portion of the tumor was used for immunohistopathology analysis.

**Histologic analysis.** Tumor samples were fixed in formol, embedded in paraffin, cut into 4- $\mu$ m sections, and stained with H&E. The histologic diagnosis was performed following WHO criteria as described by Ernst and colleagues (40). Multiple tumor sections were stained with specific antibodies against caldesmon (1:500 dilution; Upstate), smooth muscle actin (1:50 dilution; Dako), myosin (1:100 dilution; Dako), and myogenin (1:50 dilution; Dako).

## Results

### Generation, phenotypic, and functional characterization of Wt, *p53<sup>-/-</sup>*, *Rb<sup>-/-</sup>*, and *p53<sup>-/-</sup>Rb<sup>-/-</sup>* mouse MSCs.

Adipose tissue-derived MSC cultures were successfully established from Wt, *p53<sup>loxP/loxP</sup>*, *Rb<sup>loxP/loxP</sup>*, and *p53<sup>loxP/loxP</sup>Rb<sup>loxP/loxP</sup>* mice. Wt, *p53<sup>-/-</sup>*, *Rb<sup>-/-</sup>*, and *p53<sup>-/-</sup>Rb<sup>-/-</sup>* MSCs were generated by excision of the LoxP-flanked sequences by transduction of Wt, *p53<sup>loxP/loxP</sup>*, *Rb<sup>loxP/loxP</sup>*, and *p53<sup>loxP/loxP</sup>Rb<sup>loxP/loxP</sup>* cultures with adenoviral vectors expressing the *Cre-recombinase* gene under the control of the hCMV promoter (Ad-CMV-Cre). Depletion of either *p53*, *Rb*, or both was confirmed by genomic PCR (Fig. 1A) and Western blot (Fig. 1B). Of note, p53 protein was upregulated in *Rb<sup>-/-</sup>* MSCs as compared with Wt MSCs, both in unstressed conditions and after the induction of DNA damage by treatment with the topoisomerase I inhibitor camptotecin. Conversely, Rb protein is heavily downregulated in *p53<sup>-/-</sup>* MSCs as compared with Wt MSCs.

Upon generation of the different MSC mutants, we analyzed their phenotypic, morphologic, and functional properties *in vitro*. All MSC cultures showed typical MSC phenotypes regardless of the genotype: lack of hematopoietic markers including CD45, CD14, and CD11b and high expression levels of CD44 and CD29. Interestingly, *Sca1* expression differed between genotypes: Wt MSCs were *Sca1*<sup>+</sup> whereas *p53<sup>-/-</sup>Rb<sup>-/-</sup>* MSCs showed a reduced *Sca1* expression and both *p53<sup>-/-</sup>* and *Rb<sup>-/-</sup>* MSCs were almost negative for this marker (Fig. 1C). From a morphological point of view, Wt and *Rb<sup>-/-</sup>* MSCs had a fibroblast-like morphology whereas *p53<sup>-/-</sup>* and *p53<sup>-/-</sup>Rb<sup>-/-</sup>* MSCs displayed slight morphologic changes showing smaller round morphology (Fig. 1D). Most importantly, a skew in the differentiation potential was observed among the distinct MSC genotypes (Fig. 1D). As

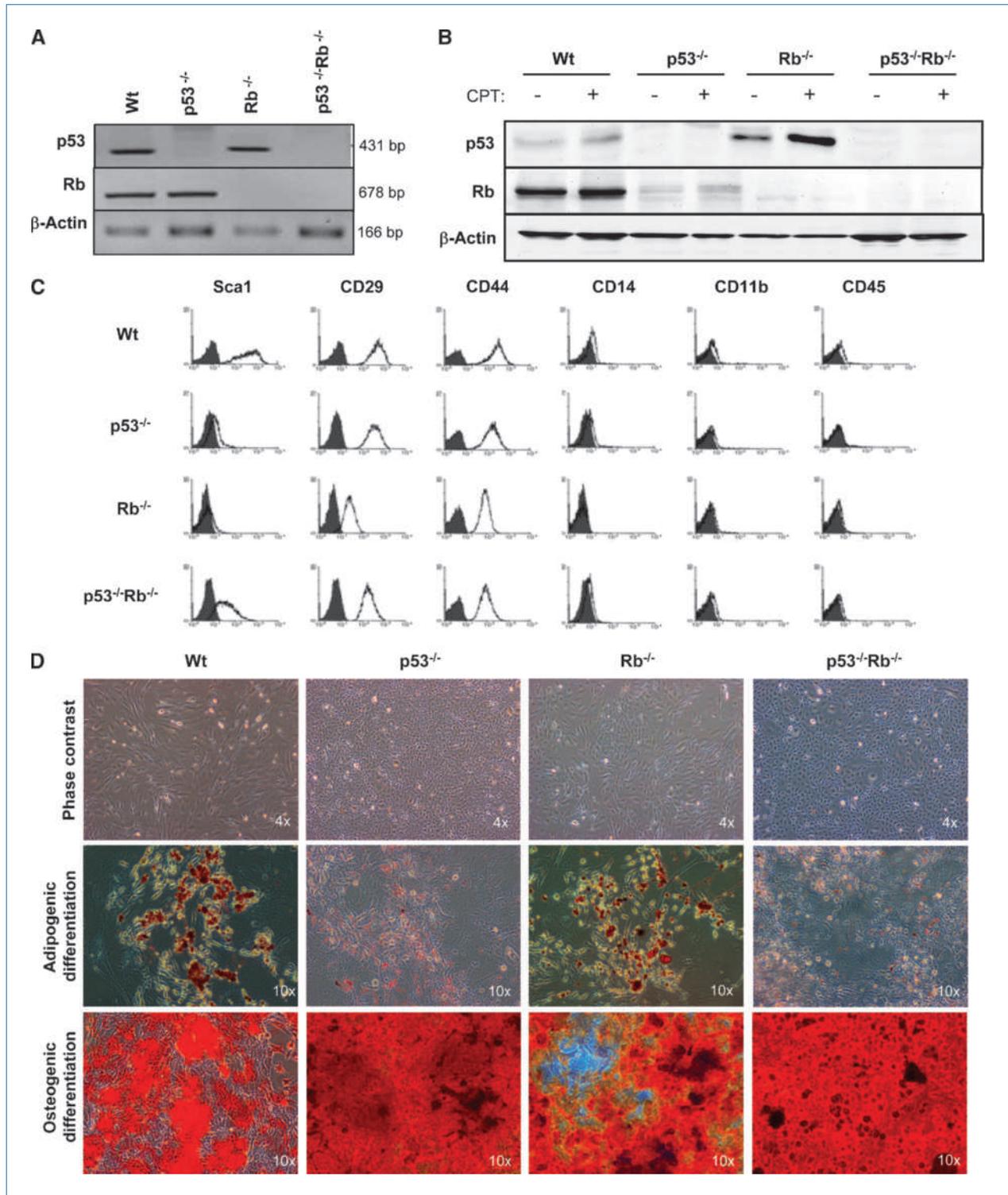
shown in Fig. 1D, *p53<sup>-/-</sup>* and *p53<sup>-/-</sup>Rb<sup>-/-</sup>* MSCs displayed a robust impairment in adipogenic differentiation (almost absent), whereas osteogenic differentiation was strongly enhanced. These *in vitro* functional differences observed between *p53<sup>-/-</sup>* and *p53<sup>-/-</sup>Rb<sup>-/-</sup>* MSCs versus Wt and *Rb<sup>-/-</sup>* MSCs suggest a potential role for p53 in the maintenance of MSC culture homeostasis.

**Either *p53* or *Rb* deficiency alters MSC culture homeostasis but only *p53* deficiency induces *in vitro* transformation of MSCs.** We initially analyzed the *in vitro* culture homeostasis of Wt, *p53<sup>-/-</sup>*, *Rb<sup>-/-</sup>*, and *p53<sup>-/-</sup>Rb<sup>-/-</sup>* mouse MSCs. When p53 and/or Rb were depleted, MSC primary cultures grew faster and could be maintained significantly longer than Wt MSCs (Fig. 2A). Cell cycle analysis based on either propidium iodide or propidium iodide/bromodeoxyuridine staining confirmed that mutant MSC cultures contain a higher proportion of cycling (S/G<sub>2</sub>/M) cells than Wt MSC cultures (Fig. 2B; Supplementary Fig. S1).

Importantly, cell cycle analysis revealed differences in the DNA content among the distinct MSC genotypes (Fig. 2B). Although the majority (79%) of Wt MSCs were diploid, a very high proportion of mutant MSCs were aneuploid: 77%, 97%, and 99% for *p53<sup>-/-</sup>*, *Rb<sup>-/-</sup>*, and *p53<sup>-/-</sup>Rb<sup>-/-</sup>*, respectively (Fig. 2B). To further confirm this increased DNA content indicative of genomic instability, G-banding karyotype assays were carried out. As shown in Fig. 2C and Supplementary Fig. S2, a minor degree of aneuploidy commonly detected in culture-expanded murine cells was observed in Wt MSCs (median, 50 chromosomes; range, 40–65). However, the remaining MSC genotypes displayed a near-tetraploid DNA content: *p53<sup>-/-</sup>* MSCs (median, 65 chromosomes; range, 41–95), *Rb<sup>-/-</sup>* MSCs (median, 71 chromosomes; range, 49–78), and *p53<sup>-/-</sup>Rb<sup>-/-</sup>* MSCs (median, 74 chromosomes; range, 52–87; Fig. 2C). These data not only support the faster cell growth kinetics of mutant MSCs and their cell cycle profile but also indicate that the absence of these key cell cycle regulators make the MSCs more prone to overall genomic instability.

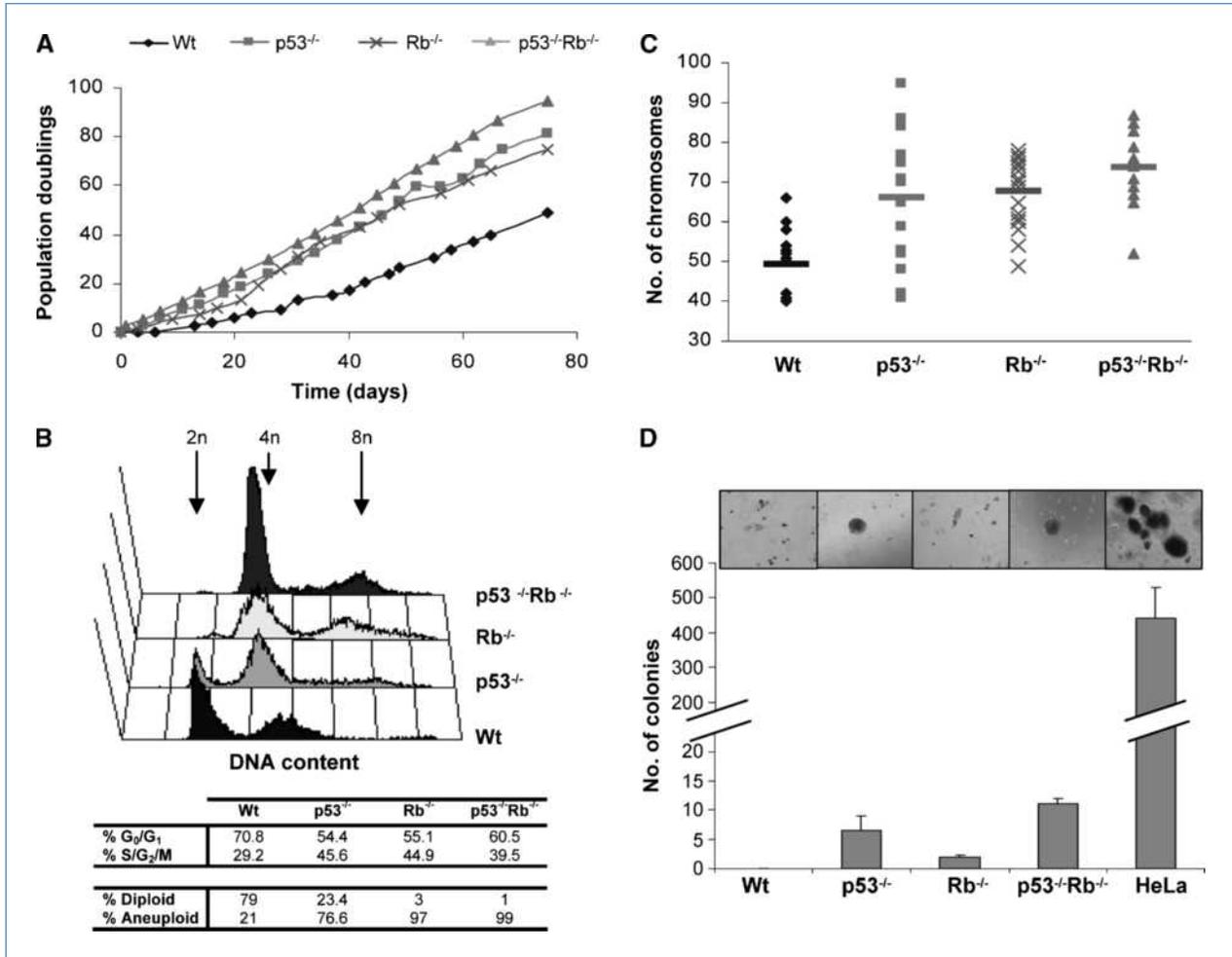
We next wondered whether these mutant MSC genotypes were transformed. A relevant *in vitro* feature of transformed cells is their ability to grow as clonogenic colonies in semi-solid medium. We thus performed anchorage-independent growth-based *in vitro* transformation assays with Wt, *p53<sup>-/-</sup>*, *Rb<sup>-/-</sup>*, and *p53<sup>-/-</sup>Rb<sup>-/-</sup>* MSCs. As shown in Fig. 2D, Wt MSCs formed no clonogenic colonies. Similarly, *Rb<sup>-/-</sup>* MSCs barely formed clonogenic colonies, whereas *p53<sup>-/-</sup>* and *p53<sup>-/-</sup>Rb<sup>-/-</sup>* MSCs gave rise to ~9 to 12 clonogenic colonies per  $1.5 \times 10^4$  plated MSCs. The HeLa cell line was used as a positive control. This data indicates that either p53 or Rb deficiency might alter MSC culture homeostasis but only p53 deficiency seems to induce *in vitro* transformation of MSCs.

***p53<sup>-/-</sup>* and *p53<sup>-/-</sup>Rb<sup>-/-</sup>* MSCs give rise to leiomyosarcoma *in vivo*.** To assay the *in vivo* tumorigenic potential of the different MSC genotypes, NOD/SCID *IL2Rγ<sup>-/-</sup>* mice were inoculated s.c. with either Wt, *p53<sup>-/-</sup>*, *Rb<sup>-/-</sup>*, and *p53<sup>-/-</sup>Rb<sup>-/-</sup>* MSCs. Animals were killed when tumors reached ~10 mm or 4 months after infusion. In line with the *in vitro* data,



**Figure 1.** Characterization of Wt, p53<sup>-/-</sup>, Rb<sup>-/-</sup>, and p53<sup>-/-</sup>Rb<sup>-/-</sup> mouse MSCs. A, genomic PCR confirming depletion of either p53 or Rb in the indicated MSC genotypes. β-Actin was used as a housekeeping control. B, Western blot performed in the presence or absence of camptothecin (CPT; 0.5 μmol/L for 24 hours) confirming lack of expression of either p53 (top) or Rb (middle) protein in the indicated MSC genotypes. β-Actin (bottom) was used as a loading control. C, immunophenotypic profile of the indicated MSC genotypes analyzed by flow cytometry. Representative dot plots are shown for Sca-1, CD29, CD44, CD14, CD11b, and CD45. Filled lines represent the irrelevant isotypes. Empty lines display antibody-specific staining. D, phase contrast morphology (top), adipogenic (Oil red staining; middle), and osteogenic (Alizarin red staining; bottom) differentiation potential of MSCs with the distinct genotypes indicated. Original magnification is indicated.

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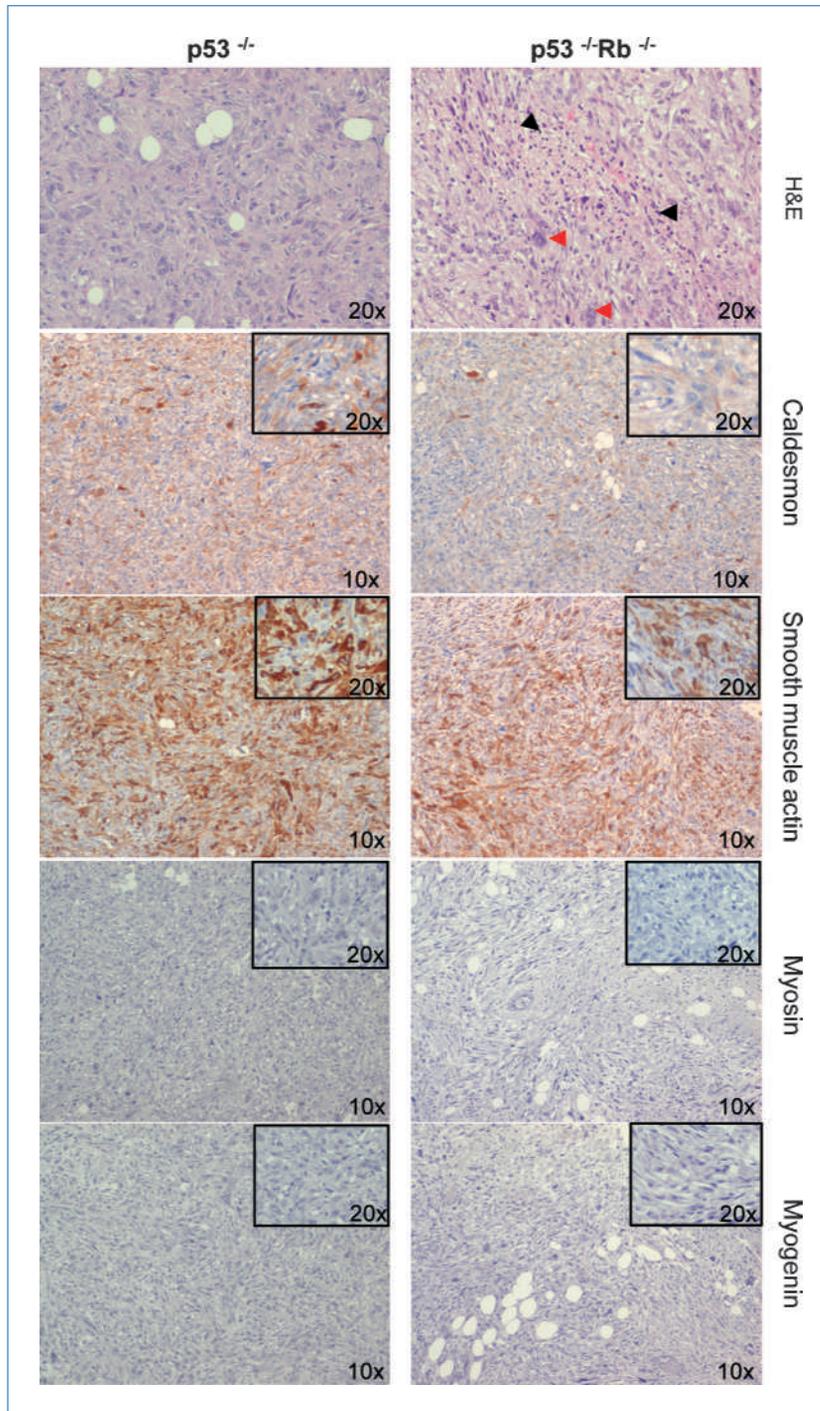


**Figure 2.** *In vitro* cell growth properties of Wt,  $p53^{-/-}$ ,  $Rb^{-/-}$ , and  $p53^{-/-}Rb^{-/-}$  mouse MSCs. A, cumulative population doublings of the indicated genotypes. B, cell cycle distribution for the different MSC genotypes. The tables at the bottom indicate the proportion of dormant ( $G_0/G_1$  phase) and cycling ( $S/G_2/M$ ) MSCs in each genotype and the proportion of diploid versus aneuploid cells. C, distribution of metaphases according to the number of chromosomes in each MSC genotype (representative G-banding karyotypes are depicted in Supplementary Fig. S2). The horizontal line represents the median. D, anchorage-independent growth identifying the ability of different MSC genotypes to form colonies in soft agar. HeLa cells were used as a positive control. Representative images showing typical colonies (top).

neither Wt nor  $Rb^{-/-}$  MSCs developed tumors *in vivo* (Table 1). In contrast, however, both  $p53^{-/-}$  and  $p53^{-/-}Rb^{-/-}$  MSCs generated *in vivo* tumors. Tumor incidence was identical (50%) between  $p53^{-/-}$  and  $p53^{-/-}Rb^{-/-}$  MSCs. Tumors arising from  $p53^{-/-}Rb^{-/-}$  MSCs seemed to be more aggressive, displaying shorter latency period than those from  $p53^{-/-}$  MSCs (52 versus 74 days; Table 1), some level of cellular pleomorphism, and large necrotic areas indicating a higher degree of aggressiveness in these tumors (Fig. 3). These tumors arising from  $p53^{-/-}Rb^{-/-}$  MSCs, however, were not more invasive, with metastases rarely observed. Most importantly, histologic analysis was suggestive of leiomyosarcomas—smooth muscle connective tissue tumors—these leiomyosarcoma-like tumors are composed of sheets of interlacing fascicles of spindle cells with large nuclear size and some degree of cellular pleomorphism with some multinucleated cells. The tumors stained positive for

$\alpha$ -smooth muscle actin and caldesmon but stained negative for myo-D1 and myogenin (typical markers of rhabdomyosarcoma; Fig. 3).

**Cell lines ex vivo-derived from leiomyosarcomas are immortal and show MSC properties in vitro.** To further characterize these experimentally induced leiomyosarcomas, primary tumors were dissected from eight different leiomyosarcoma-carrying mice initially inoculated with either  $p53^{-/-}$  MSCs ( $n = 4$ ) and  $p53^{-/-}Rb^{-/-}$  MSCs ( $n = 4$ ). Tumors were mechanically disaggregated into single cell suspensions which were placed back in MSC culture conditions. After several passages, four immortalized  $p53^{-/-}$  lines (hereinafter termed T-p53 no. 1 to T-p53 no. 4) and four immortalized  $p53^{-/-}Rb^{-/-}$  lines (hereinafter termed T-p53Rb no. 1 to T-p53Rb no. 4) were established. These transformed MSC cell lines display a morphology (Supplementary Fig. S3A), an immunophenotype (Supplementary Fig. S3B), and



**Figure 3.** Histology of tumors developed in NOD/SCID mice infused with  $p53^{-/-}$  and  $p53^{-/-}Rb^{-/-}$  mouse MSCs. Histologic analysis of tumors arising in mice inoculated with either  $p53^{-/-}$  (left) or  $p53^{-/-}Rb^{-/-}$  (right) MSCs. Staining is shown for H&E, caldesmon, smooth muscle actin, myosin, and myogenin. Insets, original magnification,  $\times 20$ ; black arrowheads, necrotic areas; red arrowheads, multinucleated cells.

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a  $p53$  and  $Rb$  gene status (Supplementary Fig. S3C) identical to that of the parental  $p53^{-/-}$  or  $p53^{-/-}Rb^{-/-}$  MSCs. To further investigate their tumorigenic potential, different T-p53Rb-transformed MSC lines were re-inoculated into immunodeficient mice. These transformed MSCs originated leiomyosarcomas in four out of four transplanted mice (100% tumor penetrance) within a 2-week period (Table 2).

***Sca-1 expression does not segregate transformed MSCs into cell fractions with different tumorigenic (sarcoma initiating) potentials.*** As aforementioned, in contrast to Wt MSCs, Sca1 expression is significantly reduced in all mutant MSCs (Fig. 1C) and these levels of Sca1 expression are maintained in the tumor cell lines derived from transformed MSCs (Fig. 4A; Supplementary Fig. S3B). Interestingly, Sca-1 antigen has been suggested as a candidate marker in the

**Table 1.** *In vivo* tumor formation ability of Wt,  $p53^{-/-}$ ,  $Rb^{-/-}$ , or  $p53^{-/-}Rb^{-/-}$  MSCs inoculated in immunodeficient mice

MSC genotype	Tumor incidence	Days to tumor development	Histologic analysis
Wt	0/8*	NT <sup>†</sup>	—
$p53^{-/-}$	4/8*	43–123 (74) <sup>‡</sup>	Leiomyosarcoma
$Rb^{-/-}$	0/8*	NT <sup>†</sup>	—
$p53^{-/-}Rb^{-/-}$	4/8*	43–69 (52) <sup>‡</sup>	Leiomyosarcoma

\*Mice without tumors were sacrificed after 150 days.

<sup>†</sup>NT, no tumor was detected.

<sup>‡</sup>Range of days for tumor development (median).

search for tissue-resident and cancer-initiating cells (41). We then wanted to test whether Sca-1 expression allows the segregation of transformed MSCs into fractions with different tumorigenic (sarcoma initiating) potentials. Sca1+ and Sca1 low/- cell fractions were FACS-purified from T-p53Rb MSCs as described in Fig. 4A. Purity was consistently >80% for Sca1+ fraction and >94% for Sca1 low/- cell subset. Interestingly, both cell fractions, Sca1+ and Sca1 low/-, originated leiomyosarcomas in all transplanted mice (Table 2) with no differences in tumor penetrance (100%) tumor weight (0.82 versus 0.67 g) or tumor latency (all tumors appeared within 2 weeks period after inoculation; Table 2). Finally, all leiomyosarcoma-like tumors arising from Sca1+ and Sca1 low/- cell subsets were histologically very similar. Similar to the tumors initially obtained from  $p53^{-/-}Rb^{-/-}$  MSCs, these tumors stained positive for  $\alpha$ -smooth muscle actin but stained negative for myo-D1 and myogenin (Fig. 4B).

## Discussion

Recent evidence suggests that MSCs might constitute a target cell for transforming mutations responsible for the formation of sarcomas (14–18). Thus, MSCs could become an

**Table 2.** *In vivo* tumor formation ability of T-p53Rb cells sorted according to Sca1 expression

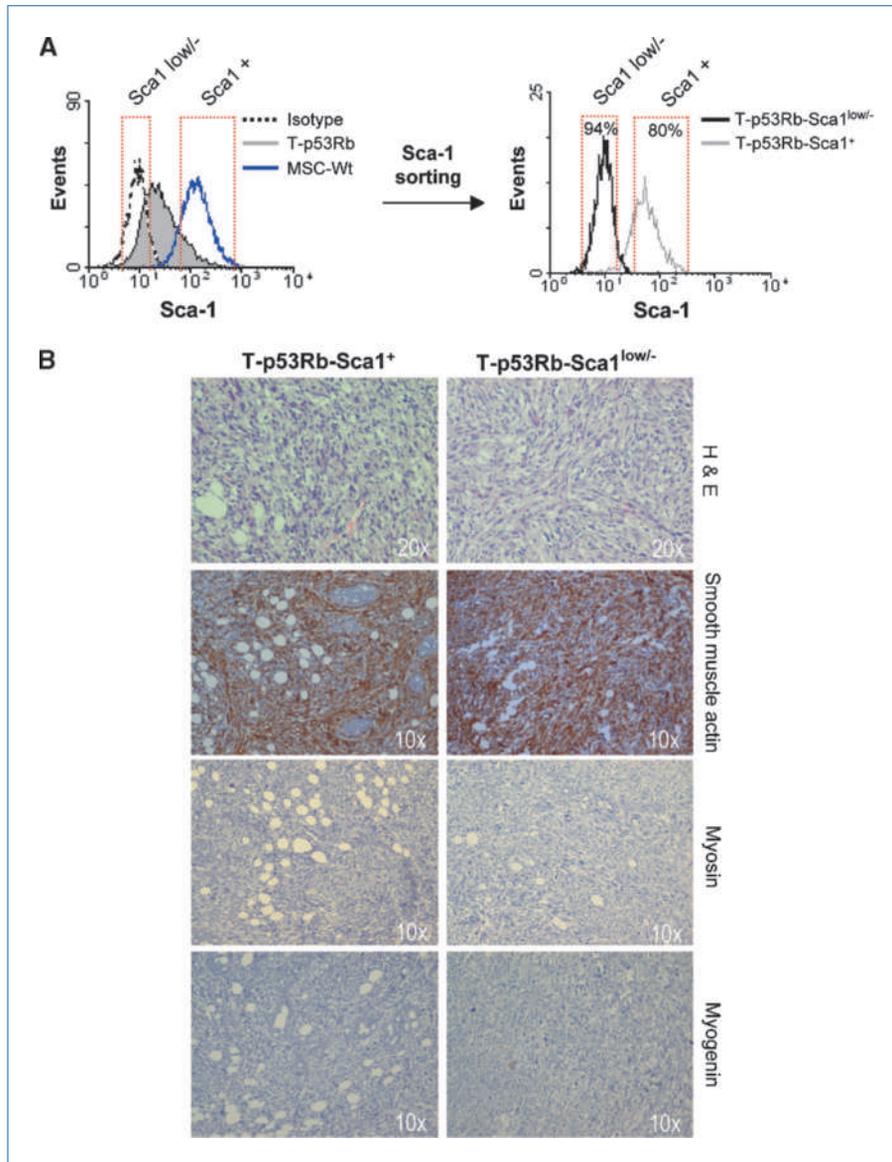
Cell type*	Tumors/mice	Tumor weight (g $\pm$ SD) <sup>†</sup>	Histologic analysis
Unsorted	4/4	0.719 $\pm$ 0.16	Leiomyosarcoma
Sca1-	7/7	0.675 $\pm$ 0.26	Leiomyosarcoma
Sca1+	7/7	0.820 $\pm$ 0.56	Leiomyosarcoma

\*Cells ( $2 \times 10^5$ ) were inoculated into NOD-SCID mice.

<sup>†</sup>All mice were sacrificed 15 days after inoculation.

important instrumental tool in studies aimed at dissecting the pathogenesis and cellular origin of sarcomas. Nevertheless, little is known about the mechanistic basis of MSC transformation, which has often been linked to the accumulation of chromosome instability (23–27). This observation suggests that the loss of accurate regulation of the cell cycle may be key in the transformation process. In fact, alterations in p16, p53, and p21 have been detected in transformed MSCs (23, 24, 42). We have previously reported that the loss of heterozygosity of  $p53$  induced tumoral transformation in  $p21^{-/-}$  MSCs, and that this process was accompanied by karyotypic instability and the loss of p16 (26). In line with this report, recent studies confirmed that the loss of p53 (43) and p16 (25) are relevant events in the transformation process of murine bone marrow-derived MSCs.

Alterations in the p53 and/or Rb pathways are present in virtually all human sarcomas (29). With the goal of generating a mouse model for sarcomas, we have assessed for the first time the effect of p53 and/or Rb depletion in fat-derived MSCs.  $Rb^{-/-}$ ,  $p53^{-/-}$ , and  $p53^{-/-}Rb^{-/-}$  MSCs, in contrast with Wt cultures, showed severe alterations in culture homeostasis, including highly increased levels of aneuploidization. Cancer is believed to be the result of a multistep process with specific genetic insults affecting proliferation/survival advantage and other genetic hits impairing differentiation. In fact,  $p53^{-/-}$  and  $p53^{-/-}Rb^{-/-}$  MSCs displayed a robust impairment in adipogenic differentiation whereas osteogenic differentiation was enhanced as previously described in  $p53^{-/-}$  MSCs (44). In line with this impairment in their differentiation potential,  $p53^{-/-}$  and  $p53^{-/-}Rb^{-/-}$  MSCs grew faster and, as previously reported, higher proliferation rates seem to correlate with a poor adipogenic differentiation and an increased differentiation to osteogenic lineage (45). More importantly,  $p53^{-/-}$  and  $p53^{-/-}Rb^{-/-}$  MSCs, but not  $Rb^{-/-}$ , were capable of both *in vitro* transformation and *in vivo* tumor development. In all cases, either  $p53^{-/-}$  or  $p53^{-/-}Rb^{-/-}$  MSC-derived tumors showed characteristics typical of leiomyosarcomas, including morphologic features and positive staining for smooth muscle actin and caldesmon. Nevertheless,  $p53^{-/-}Rb^{-/-}$  MSC-derived tumors are associated with shorter latency periods and displayed large areas of necrosis indicating a higher degree of aggressiveness. Interestingly, these  $p53^{-/-}Rb^{-/-}$  tumors, but not the  $p53^{-/-}$  tumors, showed negative staining for desmin indicating a lower level of differentiation in these tumors (data not shown). Thus, this data underscores not only the key role of p53 as a leading oncogenic hit in leiomyosarcoma-like development, but also the fact that the concurrent loss of Rb could potentiate and modulate the transformation of MSCs. Previous reports suggest that the loss of Rb and p53 seems to contribute to the initiation and/or progression of leiomyosarcoma in humans (46, 47). In a previous report, in which the same mice strain was used, somatic inactivation in the epidermis of  $p53$  but not in  $Rb$  produced spontaneous tumor development. The simultaneous inactivation of  $Rb$  and  $p53$  did not aggravate the phenotype observed in  $p53$  single mutants, but accelerated the development of squamous cell carcinoma, indicating that  $p53$  is the predominant tumor suppressor acting in mouse epidermis (34).



**Figure 4.** Sca1<sup>+</sup> and Sca1<sup>low/-</sup> cell fractions FACS isolated from T-p53Rb mesenchymal cell lines display identical sarcomagenesis potential. A, FACS sorting strategy for Sca1<sup>+</sup> and Sca1<sup>low/-</sup>. B, histologic analysis of tumors arising in mice inoculated with either T-p53Rb Sca1<sup>+</sup> or T-p53Rb Sca1<sup>low/-</sup> tumoral MSCs. Staining is shown for H&E, smooth muscle actin, myosin, and myogenin. Original magnification is indicated.

Recent genetically engineered mouse models have linked the loss of p53 and Rb in osteoblast precursors, bone marrow-derived MSCs, or MSCs of the limb bud with the development of osteosarcomas (31–33). Our results, based on fat-derived MSCs, along with these previous reports, suggest a strong relationship between the tissue of origin of the target MSC undergoing transformation and the type of sarcoma obtained. Thus, leiomyosarcoma would be linked with the loss of p53 (and Rb) in fat tissue-derived MSCs, whereas the loss of these cell cycle regulators in bone marrow MSCs or their derived osteogenic lineage would result in osteosarcoma development. In these studies, p53 is again the main hit needed to achieve cell transformation and, as in our model, Rb deletion does not initiate sarcoma formation although it can potentiate tumor development (31, 33) making these tumors less differentiated (32).

Then, we established *ex vivo* immortal *p53*<sup>-/-</sup> and *p53*<sup>-/-</sup>*Rb*<sup>-/-</sup> MSC lines (T-p53 and T-p53Rb). These tumor cell lines maintained MSC properties and were capable of reinitiating aggressive secondary tumors. In contrast with Wt MSCs, Sca1 expression was significantly reduced in all mutant MSCs and these levels of Sca1 expression were maintained in the tumor cell lines derived from transformed MSCs. We have previously shown that there is a relation between the level of p53 and Sca-1 expression in fat-derived MSCs (26). Similar downregulation of Sca1 has been previously described in bone marrow MSCs and in osteoblast precursors deficient for Rb and p53 which give rise to osteosarcomas (31). By FACS sorting experiments, these authors isolated both populations and assayed their different *in vivo* tumorigenic potentials showing that the Sca-1<sup>+</sup> cells seemed to be enriched for tumor-initiating cells. We also undertook similar sorting and transplantation

experiments to address whether Sca1+ cells could be enriched in sarcoma-initiating cells in our model of *p53<sup>-/-</sup>Rb<sup>-/-</sup>* leiomyosarcoma cell lines. Interestingly, both Sca1+ and Sca1 low/- cells originated leiomyosarcomas in all transplanted mice with no differences in tumor penetrance, tumor weight, or tumor latency. All leiomyosarcoma-like tumors arising from Sca1+ and Sca1 low/- cell subsets were histologically very similar, indicating that Sca-1 expression does not segregate transformed MSCs into cell fractions with different tumorigenic (sarcoma initiating) potentials. Therefore, in contrast with the studies undertaken by Berman and colleagues (31) we show that loss of p53 in fat tissue-derived MSCs is sufficient to yield transformed MSCs that can initiate leiomyosarcoma formation *in vivo*, regardless of the status of Rb and the expression of Sca1 antigen, linking this type of smooth muscle sarcoma with p53 deficiency in fat tissue-derived MSCs.

It is worth noting that the Rb protein is heavily downregulated in *p53<sup>-/-</sup>* MSCs as compared with Wt MSCs. It is well known how the p53/p21 pathway could regulate the levels of phosphorylation of Rb, thereby preventing cells from exiting the G<sub>1</sub> phase of the cell cycle (48) but the mechanism by which p53 can influence the total protein levels of Rb has not been so well reported. These results suggest that certain p53 background levels are needed to maintain normal levels of Rb, explaining in part why p53 deficiency alone could promote MSC tumoral transformation and why concurrent Rb deletion has only a marginal contribution. Conversely, p53 protein was upregulated in *Rb<sup>-/-</sup>* MSCs as compared with Wt MSCs. The connection between the Rb and the p53 pathways through the E2F1-mediated expression of p19 has been extensively reported (49). The strong activation of p53 tumor suppressor pathways may help to explain the lack of transformation of these *Rb<sup>-/-</sup>* MSCs. In this sense, we have previously reported the overactivation of p53 and its dependent

apoptotic pathway in *Rb<sup>-/-</sup>* mouse models of skin carcinogenesis (50).

We present data supporting the proof-of-principle that MSCs might be the target cell for transformation or the cell of origin in sarcomas. We show for the first time that p53, but not Rb deficiency in fat tissue-derived MSCs, drives *in vitro* transformation and could initiate leiomyosarcoma-like tumor formation *in vivo*. We also show that, at least in fat tissue-derived MSCs, the expression of Sca1 does not seem to enrich for sarcoma-initiating cells. The model we report provides new opportunities for the exploration of the molecular pathogenesis of leiomyosarcomas and could constitute a model for the screening of new therapeutic approaches for the treatment of this cancer.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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