Leukemia Research 82 (2019) 29–32

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Letter to the Editor

Lysine-specific demethylase 1 accelerates oncogenesis in p53 heterozygous mice via transcriptional repression of the residual *Trp53* allele

Lysine-specific demethylase 1 (LSD1/KDM1A) bifunctionally regulates the enhancer function of genes by removing the mono- and dimethyl groups from the lysine-4 and lysine-9 residues of histone H3 (H3K4 and H3K9, respectively) [1]. LSD1 has fundamental importance in several biological processes such as ontogenesis, hematopoiesis, oncogenesis and immunity [2]. In a previous study, we demonstrated that LSD1 overexpression underlies the formation of pre-leukemic stem cells, which possess the increased self-renewal potential but retain multi-differentiation ability, using a genetic approach [3]. Transgenic mice harboring LSD1 overexpression in hematopoietic stem cells were ostensibly normal and did not show obvious abnormalities in hemopoiesis but developed T-cell lymphoblastic leukemia/lymphoma (T-LBL) at high frequency after γ-irradiation. The developed T-LBL had point mutations at the heterodimerization and/or PEST domains of the *Notch1* gene, suggesting that aberrant activation of Notch1 acts as a second hit for T-cell leukemogenesis from LSD1-induced pre-leukemic stem cells [3,4]. In contrast, no mutation was detected in the *Trp53* gene of T-LBL developed in γ-irradiated LSD1 transgenic mice. The absence of *Trp53* mutations disagrees with the general consensus that p53 abnormalities play a pivotal role in radiation-triggered oncogenesis [5,6]. We therefore investigated whether p53 dysfunction could be a second hit for the transformation of pre-leukemic stem cells in LSD1 transgenic mice by the aid of developmental genetics.

Toward this end, we crossed LSD1 transgenic mice C57BL/6J-Tg (Ly6a-KDM1A*v2), lines 6 and 3 carrying 1 copy and ~30 copies of the transgene, respectively [3], with p53 heterozygous mice C57BL-p53$^{+/-}$ (RIKEN CDB0001 K) [7] in the C57BL/6 background. Accumulating evidence indicates that p53 haploinsufficiency reduces life expectancy to < 100 weeks, less than half that of wild-type littermates, because of enhanced tumor development, whereas p53-deficient mice die within 50 weeks by the same cause of death [8,9]. The difference between p53$^{+/-}$ and p53$^{-/-}$ mice is not only the rapid onset of lethal neoplasms in the latter but also the spectrum of developed tumors. The incidences of lymphoma, sarcoma and carcinoma were reported to be approximately 30%, 60% and 10%, respectively, in p53 heterozygous mice, whereas the distribution was changed to 60%, 40% and < 5% in p53 null mice [8,9]. These data suggest that p53 haploinsufficiency reduces the risk of lymphoma at the expense of increased morbidity with sarcoma/carcinoma compared with the complete loss of p53, although the mechanism underlying the altered oncogenic spectrum is largely unknown. Based on previous findings, we speculated that cross-breeding with p53$^{+/-}$ mice induces tumors other than T-LBL in LSD1 transgenic mice due to the instructive effects of p53 haploinsufficiency on pre-leukemic stem cells. As shown in Fig. 1A, cross-breeding with p53$^{+/-}$ mice significantly shortened the median survival of LSD1 transgenic mice: from 105 weeks in line 6 to 77 weeks in line 6 × p53$^{+/-}$ and from 122 weeks in line 3 to 82 weeks in line 3 × p53$^{+/-}$ ($p < 0.05$ in each combination). Among the p53 heterozygous mice (n = 10), two mice developed T-LBL diagnosed by pathological examination and *TCR* gene rearrangement (#1 and #8 in Fig. 1B), whereas the other 8 mice died of pathologically unclassified non-hematological malignancies. The line 6 × p53$^{+/-}$ hybrid mice developed T-LBL (#2), B-LBL (#3, #7 and #10) and myeloid neoplasm with hyperleukocytosis (#9) at frequencies of 10%, 30% and 10%, respectively (Fig. 1B/C/D and Supplementary Figure S1), whereas the other 5 mice died of pathologically unclassified non-hematological malignancies, including possible liposarcoma. The line 3 × p53$^{+/-}$ hybrid mice developed T-LBL, B-LBL (#3, #5, #8 and #10) and myeloid neoplasm with hyperleukocytosis (#2) at frequencies of 0%, 40% and 10%, respectively (Fig. 1B and Supplementary Figure S1), whereas the other 5 mice died of pathologically unclassified non-hematological malignancies including possible germinal tumor in the testis. Overall, the cross-breeding of LSD1-transgenic and p53$^{+/-}$ mice caused malignant lymphoma, mostly of B-cell origin, at a frequency of 40%, and the remaining mice suffered from non-lymphoid malignancies: myeloid neoplasm and possible sarcoma/carcinoma (Fig. 1E). This pattern is completely different from that of γ-irradiated LSD1 transgenic mice, all of which developed T-LBL [3], and rather resembles the spectrum of tumors spontaneously developed in p53 null mice. These results prompted us to speculate that, upon overexpression, LSD1 affected the remaining intact allele of *Trp53*, leading to complete loss of p53 function in heterozygous mice upon cross-breeding. To test this hypothesis, we compared the expression of *Trp53* mRNA in hybrid mice with that of wild-type littermates and LSD1 transgenic mice. As expected, in p53 heterozygous mice, the expression level of *Trp53* mRNA in bone marrow mononuclear cells was approximately half (30–60%) that of wild-type C57BL/6 and LSD1 transgenic mice (Fig. 2A). The abundance of *Trp53* mRNA was further reduced in Line 6 × p53$^{+/-}$ hybrid mice, especially those that developed aggressive T-LBL (#2) and B-LBL (#3) (Fig. 2A). Consistent with the decreased abundance of p53, bone marrow mononuclear cells from these mice failed to up-regulate the expression of downstream effectors of p53, such as p21 and Noxa, in response to a genetic insult by doxorubicin (Fig. 2B). It has been reported that the transcription of *Tp53* is regulated by the binding of CTCF/E2F to promoter regions concordant with the transcription of *Wrap53*, which is essential for the induction of p53 upon DNA damage [10,11]. Forced expression of LSD1 perturbed the induction of both *Tp53* and *Wrap53* in doxorubicin-treated HEK293 cells (Fig. 2C and Supplementary Figure S2A/B). Notably, LSD1 overexpression did not affect the baseline expression of p53 but cancelled the up-regulation of p53 in response to DNA damage (Fig. 2D). This pattern of p53 modulation partly explains the delayed onset of T-LBL in p53-proficient LSD1 transgenic mice [3]. In addition, LSD1 overexpression fails to recapitulate the p53-null condition, underlying the almost identical survival of p53 heterozygous and LSD1 × p53$^{+/-}$ hybrid mice (Fig. 1A). Chromatin immunoprecipitation assays revealed that the E2F1/LSD1 complex was recruited to shared regulatory regions of the

https://doi.org/10.1016/j.leukres.2019.05.008
Received 4 January 2019
Available online 23 May 2019

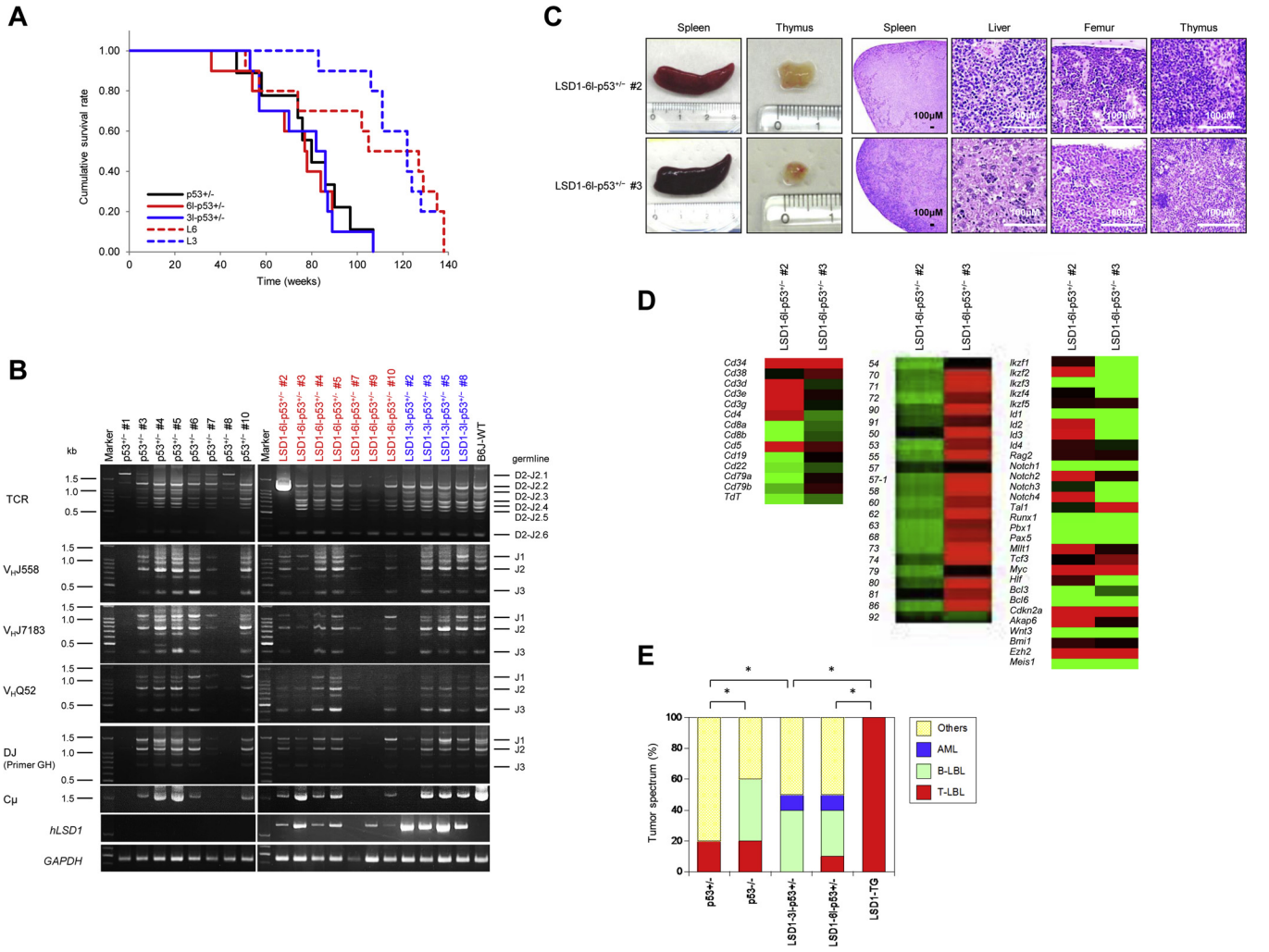

Fig. 1. Cross-breeding of LSD1 transgenic and p53 heterozygous mice causes various types of cancer.
(A) Kaplan-Meier survival curves of line 6 (broken red line) and line 3 (broken blue line) of LSD1 transgenic mice, p53 heterozygous mice (black line), line 6 × $p53^{+/-}$ hybrid mice (red line) and line 3 × $p53^{+/-}$ hybrid mice (blue line) (n = 10 in each group). $P < 0.05$ between line 6 and line 6 × $p53^{+/-}$, and between line 3 and line 3 × $p53^{+/-}$ by the log-rank test. (B) Genomic DNA was extracted from bone marrow mononuclear cells of the indicated mice and subjected to PCR analyses to determine *TCRß* and *IgH* gene rearrangement using the primers depicted in Supplementary Tables S1 and S2. The expression of human LSD1 and murine *Gapdh* transcripts was examined by RT-PCR as described in Supplementary Methods. (C) Representative photographs of dissected organs and histopathological sections, on which the diagnosis of T-LBL (line 6 × $p53^{+/-}$ hybrid mouse #2) and B-LBL (line 6 × $p53^{+/-}$ hybrid mouse #3) was made by outside experts of animal pathology. (D) Total cellular RNA was prepared from bone marrow mononuclear cells from line 6 × $p53^{+/-}$ hybrid mice #2 and #3, and subjected to microarray-based gene expression profiling (see Supplementary Methods for details). Heat maps of lymphoid surface markers (left panel), κ-chain variable 4 regions (middle panel) and some hematopoietic transcription factors (right panel) are exemplified (red: higher expression; green: lower expression). The data have been deposited in the MIAME-compliant GEO database under accession number GSE95119. (E) The spectrum of tumors developed in mice in the present study. AML: acute myeloid leukemia. Others include pathologically unclassified non-hematological malignancies mostly sarcomas. *$P < 0.01$ by the Fisher's exact test.

two genes (Supplementary Figure S2C) and significantly reduced the amounts of dimethylated histone H3K4 (Fig. 2E) and CTCF (Supplementary Figure S2D) at the CTCF/E2F-binding sites. These changes may contribute to the transcriptional repression of *Tp53* and *Wrap53* in LSD1-overexpressing cells.

Overall, we conclude that LSD1 overexpression generates pre-leukemic stem cells, which could be transformed to various tumors according to the type of second hit. In a broader sense, the coexistence of LSD1 overexpression and p53 haploinsufficiency, which is often observed in myelodysplastic syndrome and other pre-malignant disorders [12,13], predisposes cells to various types of cancer by mimicking the loss of p53 function. In fact, the inverse correlation was observed between *LSD1* and *TP53* mRNA expressions in primary T-cell leukemia/lymphoma samples (Supplementary Figure S3). Although this finding may advocate a novel cancer-prone condition, validation using large human samples is essential, which is currently underway in our laboratory.

Author contributions

T.W. designed and performed experiments, analyzed data and drafted the manuscript. J.K., D.K. and H.H. provided materials and critically reviewed the manuscript. T.W. and H.H. generated transgenic mice. Y.F. designed and supervised the research, and compiled the manuscript.

Conflict-of-interest statement

The authors declare no conflicts of interest.

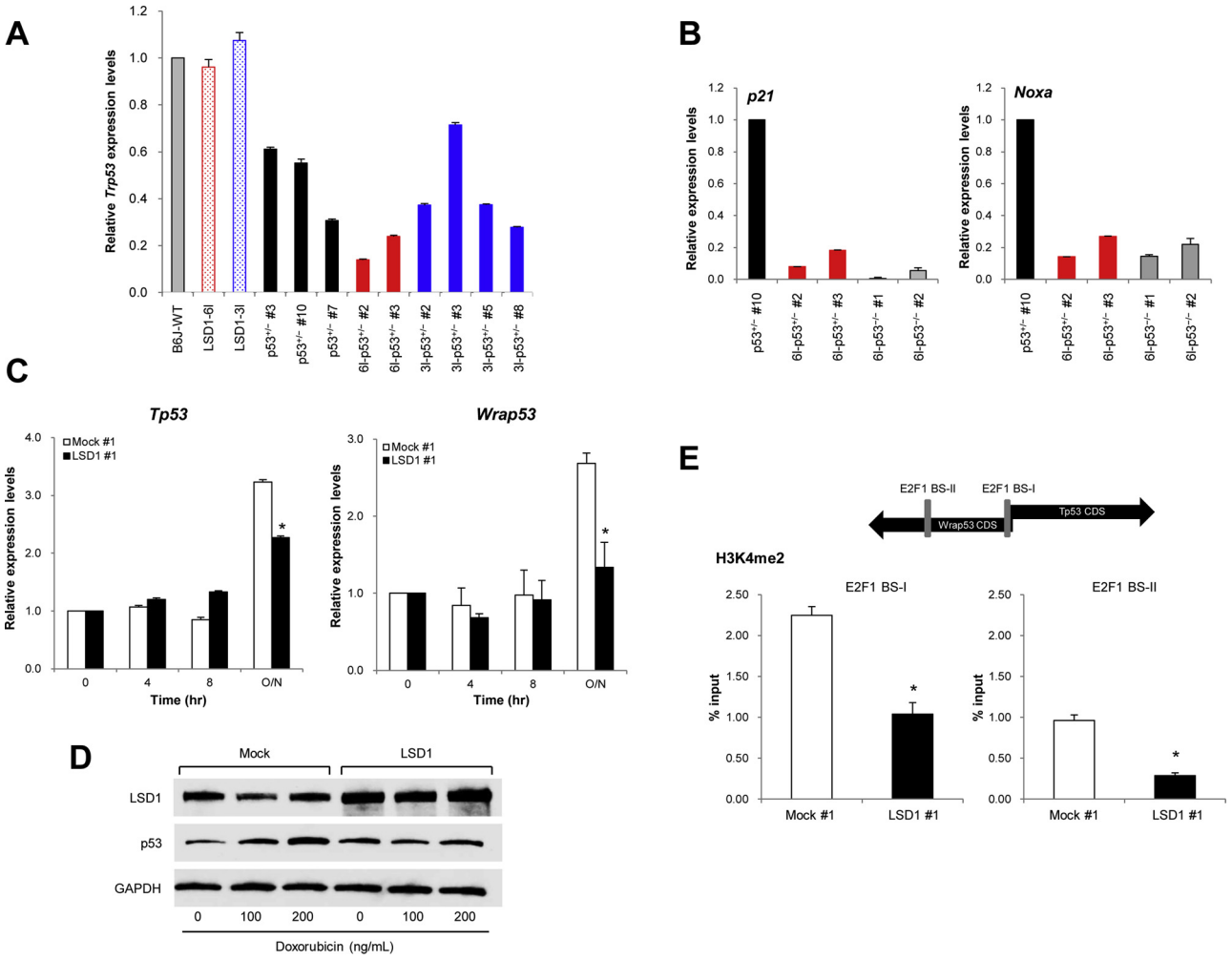

Fig. 2. LSD1 represses transcription of the *Tp53* and *Wrap53* genes by binding to the CTCF/E2F consensus sites of the *Tp53* promoter.
(A) Total cellular RNA was prepared from bone marrow mononuclear cells of the indicated mice and subjected to real-time quantitative RT-PCR for *Trp53* mRNA expression. The data were quantified by the $2^{-\Delta\Delta Ct}$ method using *Gapdh* as a reference and are shown as relative values of the expression level in wild-type C57BL/6 mice (B6J -WT) [14]. (B) Bone marrow mononuclear cells were isolated from the indicated mice and cultured with 1 μM doxorubicin for 16 h. Total cellular RNA was subjected to real-time quantitative RT-PCR to measure the expression of *p21* and *Noxa* transcripts. The data were quantified by the $2^{-\Delta\Delta Ct}$ method using *Gapdh* as a reference and are shown as relative values of the expression levels in p53 heterozygous mouse #10. (C) Stable transformants were established by transducing HEK293 cells with either an empty vector (Mock #1) or an LSD1 expression vector (LSD1 #1). The expression of *Tp53* and *Wrap53* transcripts was determined by real-time quantitative RT-PCR at the indicated time points after culture of these cells with 1 μM doxorubicin (O/N; approximately 16 h). Data were quantified by the $2^{-\Delta\Delta Ct}$ method using *GAPDH* as a reference and are shown as relative values of the expression levels in uncultured cells (T-0). The means ± S.D. (bars) of three independent experiments. $^*P < 0.05$ between Mock #1 and LSD1 #1 by Student's *t* test. (D) HEK293 cells were transfected with either an empty vector (Mock) or an LSD1 expression vector (LSD1) and cultured with the indicated concentrations of doxorubicin for 24 h. Whole cell lysates were subjected to immunoblotting for the expression of LSD1, p53 and GAPDH (loading control). (E) HEK293 transformants (Mock #1 and LSD1 #1) were cultured with 1 μM doxorubicin for 16 h. We fixed the cells in 1% formaldehyde at room temperature for 10 min and isolated chromatin fractions using enzymatic shearing [15]. After centrifugation, the supernatants were incubated with either specific antibody against histone H3 dimethylated at lysine-4 or isotype-matched control (IgG) in the presence of protein G magnetic beads at 4 °C overnight. We purified DNA fragments from the mixture and subjected them to real-time quantitative PCR using primers spanning the CTCF/E2F-binding sites (E2F1 BS-I and E2F1 BS-II) in the promoter regions of the *Tp53* and *Wrap53* genes (see Supplementary Table S3 for primer sequences). Immunoprecipitated DNA is expressed as a percentage of input DNA. The means ± S.D. (bars) of three independent experiments. $^*P < 0.05$ by Student's *t* test.

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