

Lysine-Specific Demethylase 1 Is Strongly Expressed in Poorly Differentiated Neuroblastoma: Implications for Therapy

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Abstract

Aberrant epigenetic changes in DNA methylation and histone acetylation are hallmarks of most cancers, whereas histone methylation was previously considered to be irreversible and less versatile. Recently, several histone demethylases were identified catalyzing the removal of methyl groups from histone H3 lysine residues and thereby influencing gene expression. Neuroblastomas continue to remain a clinical challenge despite advances in multimodal therapy. Here, we address the functional significance of the chromatin-modifying enzyme lysine-specific demethylase 1 (LSD1) in neuroblastoma. LSD1 expression correlated with adverse outcome and was inversely correlated with differentiation in neuroblastic tumors. Differentiation of neuroblastoma cells resulted in down-regulation of LSD1. Small interfering RNA-mediated knockdown of LSD1 decreased cellular growth, induced expression of differentiation-associated genes, and increased target gene-specific H3K4 methylation. Moreover, LSD1 inhibition using monoamine oxidase inhibitors resulted in an increase of global H3K4 methylation and growth inhibition of neuroblastoma cells *in vitro*. Finally, targeting LSD1 reduced neuroblastoma xenograft growth *in vivo*. Here, we provide the first evidence that a histone demethylase, LSD1, is involved in maintaining the undifferentiated, malignant phenotype of neuroblastoma cells. We show that inhibition of LSD1 reprograms the transcriptome of neuroblastoma cells and inhibits neuroblastoma xenograft growth. Our results suggest that targeting histone demethylases may provide a novel option for cancer therapy. [Cancer Res 2009;69(5):2065–71]

Introduction

Neuroblastoma, the most common extracranial tumor of childhood, is an embryonal malignancy originating from the neural crest. The clinical course of neuroblastoma is very heterogeneous. While neuroblastoma with favorable biology, as well as the benign variants ganglioneuroblastoma and ganglioglioma, spontaneously regress or differentiate without any therapeutic intervention, neuroblastoma with unfavorable biology

often fatally progresses regardless of multimodal therapy (1, 2). Therefore, the identification of novel drug targets and development of new therapeutic options are urgently needed. Patterns characteristic of aggressive neuroblastoma have been identified via high-throughput analysis, including expression profiling (3, 4) and array CGH (5–7), with *NMYC* amplification, 1p36 and 11q deletion (8), and 17q gain being the most prominent chromosomal alterations. However, pharmacologic intervention to modulate expression patterns has not yet been achieved. Many of the genes discriminating between favorable and unfavorable neuroblastomas on the mRNA level belong to a functional category of transcription factors, among them *MYCN*, the central oncogene in the pathogenesis of *NMYC*-amplified neuroblastoma.

Unfortunately, transcription factors are generally less suited as drug targets. In contrast, enzymes involved in epigenetic gene regulation are suitable drug targets and modulate broad expression patterns. Indeed, histone acetylation and DNA methylation have been shown to specifically regulate central genes in aggressive neuroblastoma (9–11). Treatment with histone deacetylase (HDAC) inhibitors and DNA-demethylating agents has proved effective against neuroblastoma cells *in vitro* (9) and are currently being evaluated for treating neuroblastoma *in vivo*.

In the past, histone methylation was considered to be static and irreversible, thus less important. However, a new class of histone demethylating enzymes was recently identified, with lysine-specific demethylase 1 (LSD1, also identified as AOF2) as its prototype (12, 13). Subsequent to the discovery of LSD1, another family of >30 histone demethylases structurally different from LSD1 was described, all of which sharing a motif designated the jumonji domain. LSD1 specifically interacts with the androgen receptor (AR; ref. 12) or with large chromatin-modifying corepressor complexes, such as the Co-Rest complex (14), thus suggesting that high levels of LSD1 expression suppress neuronal differentiation in neuroblasts. LSD1 allows transcription factors or corepressor complexes to selectively initiate or repress transcription via demethylation of lysine residues 4 or 9 of histone 3, thereby controlling gene expression programs.

Overexpression of LSD1 in prostate carcinoma is sufficient to promote AR-dependent transcription in the absence of androgens (12, 15). Taken into consideration that LSD1 controls broad expression programs and is involved in malignant progression of prostate cancer, we analyzed the role of LSD1 in neuroblastoma in this study. We observed that LSD1 was strongly expressed in undifferentiated neuroblastomas and that *in vitro* differentiation of neuroblastoma cells resulted in the down-regulation of LSD1. LSD1 knockdown using small interfering RNA (siRNA) or inhibition with

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small molecular inhibitors also resulted in growth inhibition of neuroblastoma cells *in vitro*. Finally, we provide evidence that inhibition of LSD1 reduces tumor growth *in vivo*.

Materials and Methods

Tissue microarrays. Tissue microarrays (TMA) were prepared from paraffin-embedded tissue specimens of 99 primary neuroblastic tumors selected from the archival files of the Institute of Pathology, University of Bonn Medical School and from the University Children's Hospital Essen. Clinical data were available from the German Neuroblastoma Study for 70 samples. The remaining tumor samples were classified either as ganglioneuroblastomas or ganglioneuromas, which are not included in the German Neuroblastoma Study for follow-up. Three different tissue cores within a single tumor were arrayed from formalin-fixed, paraffin-embedded tissue blocks using a manual device (Beecher Instruments). Two-micrometer paraffin sections were cut from every tissue microarray and used for subsequent immunohistochemical analyses.

Immunohistochemistry. Immunohistochemical staining was done, as previously described (15), using an α -LSD1 antibody (Novus Biologicals) diluted 1:250. Nuclear immunostaining results for LSD1 were evaluated using a semiquantitative scoring system. Briefly, the number and intensity of positive cells were counted and scored between 0 and 3 (0, no positive nuclei; 1, <20% nuclei display intense staining or more nuclei display weak staining; 2, 20–80% intense staining or more nuclei display moderate staining; 3, 80–100% nuclei display intensive staining).

Real-time reverse transcription-PCR. Total RNA was isolated from cells using the RNeasyMini kit (Qiagen), and cDNA synthesis was performed using the SuperScript reverse transcription kit (Invitrogen). LSD1 gene expression was monitored by real-time PCR using "Assays on Demand" (Applied Biosystems). Expression values were normalized to the geometric mean of GAPDH, UBC, and HPRT (16).

Western blot analysis. Protein lysates were extracted from cells and blotted as described in Kahl and colleagues (15). The membranes were incubated for 1 to 2 h using the following antibodies and dilutions: α -LSD1 (Novus Biologicals), 1:1,000; α -diMeK4H3 (Abcam), 1:1,000; β -actin (Sigma-Aldrich), 1:5,000.

Cell culture. Neuroblastoma cells were cultivated in RPMI 1640 supplemented with 10% FCS, L-glutamine, and antibiotics. All-trans retinoic acid (Sigma-Aldrich) was added to the medium at a concentration of 25 μ mol/L to differentiate cells *in vitro*, and the medium was changed daily (17). Number and length of outgrowing neurites were assessed as the markers for differentiation.

siRNA transfection. Cells were seeded with 1×10^5 cells in 24-well plates, then incubated for 2 to 6 d in standard medium in the presence of 10 to 20 nmol/L siRNA directed against LSD1 (DNA target sequence, 5'-AACACAAGGAAAGCTAGAAGA-3') or control siRNA (scrambled) complexed with HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's instructions.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) experiments were performed essentially as described (12). LAN1 and SHEP cells were transfected 6 d before harvesting for ChIP with or without LSD1 siRNA (Qiagen) following the manufacturer's instructions. Immunoprecipitation was performed with specific antibodies to H3K4me2 (Abcam) and LSD1 (Novus Biologicals) on protein A coupled Dynabeads (Invitrogen). Purified DNA were subjected to real-time PCR using a SYBR green probe (Invitrogen) in an ABI Prism 7900 (Applied Biosystems), according to the manufacturer's specified variables. Amplicons were normalized to the 1:100 diluted input DNA. The following TaqMan real-time PCR primers were used for the *TFPI2* proximal promoter region: forward primer, 5'-GCAGTCATTT-CCGTCTAGC-3'; reverse primer, 5'-ACCTGCCTCCCAAACCTTCT-3'. The following primers were used to detect *XRCC5* -300 proximal promoter region: forward primer, 5'-CAATGAGAGAAAAGGGACGTG-3'; reverse primer, 5'-CTCTCCATTCGCCGTAGT-3'.

Microarray analysis. RNA was isolated from 110 primary, untreated tumors, and SH-SY5Y cells were transfected with siRNA directed against

LSD1 or control siRNA from two independent transfection experiments each. Reverse transcription, labeling of total RNA, and subsequent hybridization to Affymetrix U133v2 chips were performed according to the manufacturer's protocols and as previously described (18). Data processing and normalization were performed according to standard procedures using the MAS5 algorithm. The microarray data were released into the GEO-database (accession number GSE13273).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay. Cells were seeded at a density of 2,500 per well and cultured in standard medium, replaced daily. Treatment with clorgyline (Sigma-Aldrich), pargyline (Fluka), or tranlycypromine (Biomol) was accomplished as indicated. An 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed according to the manufacturer's protocol (Roche).

Growth of xenograft tumors in nude mice. SH-SY5Y neuroblastoma cells were cultured to 80% confluency, harvested, and suspended in Matrigel (BD Bioscience). Four-week-old female athymic NCR (*nu/nu*) mice were inoculated s.c. in the flank with 2×10^7 cells in 200 μ L Matrigel. Mice were injected with 2 mg tranlycypromine (in 100 μ L NaCl) or NaCl alone i.p. once per day. Mice were sacrificed at day 21, and tumors were weighed, formalin fixed, and analyzed.

Results

LSD1 is strongly expressed in poorly differentiated neuroblastomas. We analyzed LSD1 expression in primary neuroblastic tumors, including malignant neuroblastomas, benign ganglioneuroblastomas, and ganglioneuromas. A tissue microarray was prepared for this purpose, incorporating 99 primary, untreated tumors, of which 77 were neuroblastomas and 22 were ganglioneuroblastomas and ganglioneuromas. LSD1 expression was significantly higher in poorly differentiated than in differentiated neuroblastomas (Mann-Whitney test, $P = 2.6 \times 10^{-5}$; Fig. 1B). LSD1 expression was also higher in differentiated neuroblastomas than in ganglioneuromas and ganglioneuroblastomas (Mann-Whitney test, $P = 8.2 \times 10^{-5}$; Fig. 1B). LSD1 was not expressed in nonmalignant cells, such as stromal tissue or infiltrating leukocytes (Fig. 1A). Similar results were obtained in an independent cohort of 102 neuroblastic tumors previously analyzed on Affymetrix microarrays when these data were reanalyzed for LSD1 mRNA levels (Fig. 1C). Kaplan-Meier analysis revealed that a low LSD1 mRNA expression level was predictive of event-free survival (EFS) in the latter cohort (log-rank test, $P = 0.021$; Fig. 1D). LSD1 protein levels were semiquantitatively measured on the TMA using immunohistochemistry. In contrast to mRNA expression, LSD1 protein expression did not prove to be a statistically significant predictive variable of survival, relapse or progression, and did not correlate with amplification of the *MYCN* oncogene.

LSD1 expression in neuroblastoma cell lines. LSD1 protein expression was assessed in neuroblastoma cell lines using Western blotting. All cell lines strongly expressed LSD1 (Fig. 2A). As all existing neuroblastoma cell lines were established from undifferentiated, aggressive tumors, this result was consistent with our data from primary tumors, wherein aggressive tumors showed high LSD1 expression. We then asked if induction of differentiation might result in the down-regulation of LSD1. We, therefore, treated SY5Y and BE2C neuroblastoma cells with all-trans retinoic acid, a drug known to induce differentiation of neuroblastoma cells. As expected, morphologic differentiation resulted in neurite development and reduced proliferation (Fig. 2B). Upon differentiation, a significant down-regulation of LSD1 was also detected (Fig. 2C).

Inhibition of LSD1 impairs neuroblastoma growth *in vitro*. To further analyze the functional relevance of LSD1 in neuroblastic

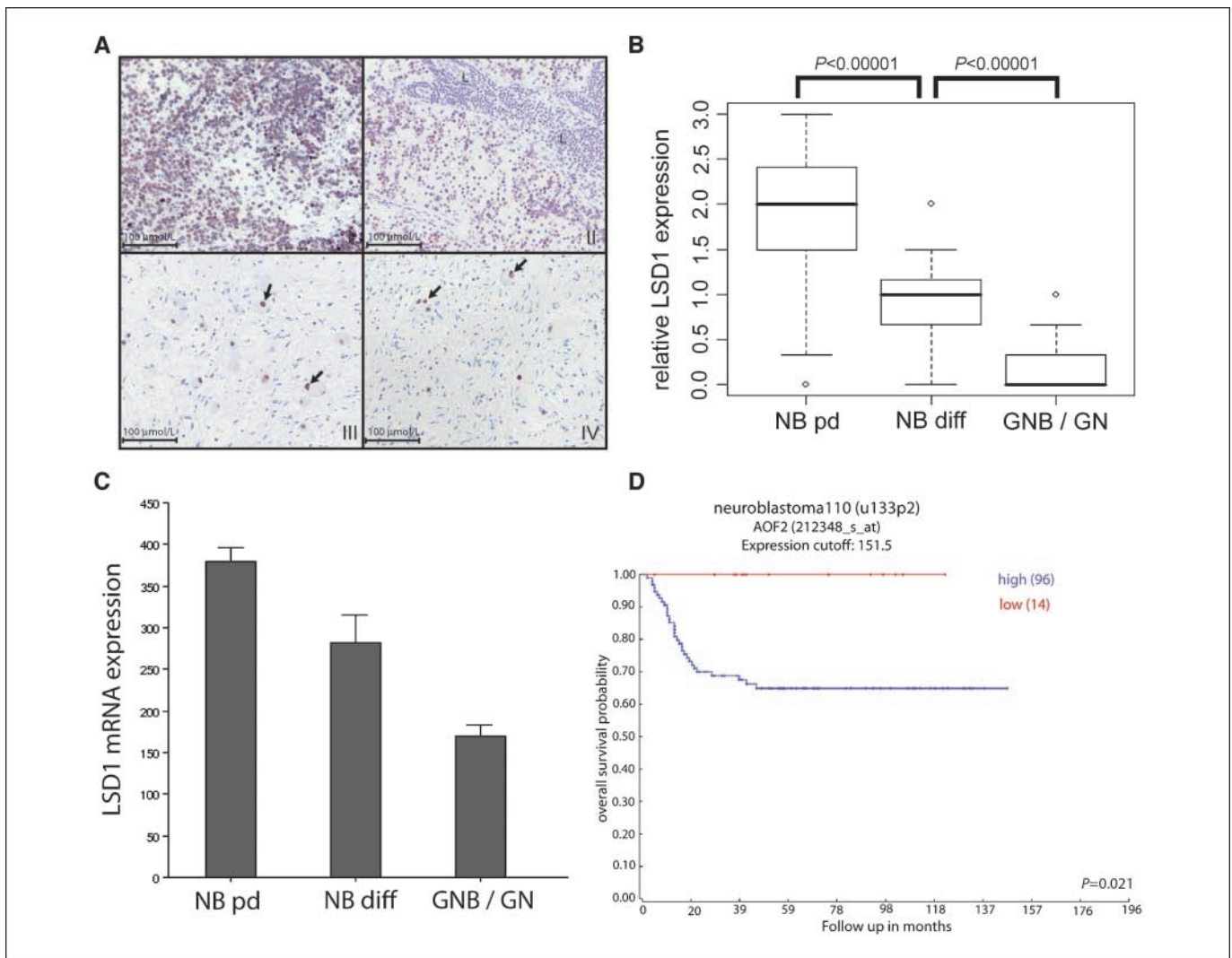


Figure 1. A, immunohistochemical staining of LSD1 in neuroblastic tumors. In poorly differentiated neuroblastomas (I and II), nuclear LSD1 staining is observed in almost all tumor cells. In contrast, in benign ganglioneuromas (III)/ganglioneuroma (IV), LSD1 staining is mild or absent. The few signals marked by arrows in III and IV represent nucleoli of differentiated ganglia. Infiltrating leukocytes (L) in II, and Schwannian stroma do not display any immunoreactivity for LSD1 (III/IV). B, a tissue microarray with 99 primary neuroblastic tumors was used to analyze LSD1 expression in neuroblastoma and its benign derivatives. Expression was significantly higher in poorly differentiated neuroblastomas (NB pd) than in differentiated neuroblastomas (NB diff) or ganglioneuromas/ganglioneuromas (GNB/GN). C, LSD1 mRNA expression in an independent cohort of 110 neuroblastic tumors analyzed with Affymetrix microarrays. D, Kaplan-Meier analysis of 110 neuroblastic tumors shows that low LSD1 mRNA expression levels are predictive of EFS.

tumors, SH-SY5Y cells were transiently transfected with siRNA directed against LSD1 or with a scrambled control siRNA. A significant LSD1 knockdown was detected on both mRNA and protein levels after transfection with either 10 or 20 pmol siRNA (Fig. 3A and Supplementary Fig. S1).

To determine whether LSD1 knockdown influences gene specific methylation status, SHEP and LAN1 cells treated with siRNA directed against LSD1 or with a scrambled control siRNA were subjected to ChIP. After LSD1 knockdown, genomic DNA corresponding to the *TFPI2* and *XRCC5* proximal locus (*TFPI2* and *XRCC5* are regulated by siRNA approach; see below) was enriched with anti-diMeK4H3 antibody (Fig. 3B).

Upon siRNA-induced knockdown of LSD1, a significant decrease in cell viability was detected in MTT assays (Fig. 3C, left). Decreased viability was accompanied by the appearance of morphologic features indicating differentiation, such as outgrowth of neurite-like structures (Fig. 3C, right). Microarray analysis (Fig. 3D) revealed

changes in expression that were consistent with these observations 72 hours after LSD1 knockdown. At this time, 28 genes were significantly induced at least 1.5-fold and 29 genes were significantly repressed at least 1.5-fold. Among the 28 induced genes, four are markers of cytoskeletal remodeling (*TNS1*, *TPM1*, *DNM2*, *DNAL4*), indicating differentiation, and three (*TPM1*, *DNM2*, and *SHANK2*) are functionally linked to neurite dynamics and synaptic trafficking (19–21). TaqMan quantitative reverse transcription-PCR confirmed the expression changes detected via microarray analysis for LSD1, *DNAL4*, *DNM2*, *TNS1*, and *TPM1* (Supplementary Fig. S2). *TNS1* is induced upon siRNA mediated LSD1 knockdown *in vitro*, we analyzed *TNS1* expression *in vivo*. *TNS1* was significantly down-regulated in primary tumors with low LSD1 expression, as well as in ganglioneuroma and ganglioneuromatoma (Supplementary Fig S3).

We also analyzed the effect of small molecule inhibitors of LSD1. The sequence of the catalytic domain of LSD1 has homology to

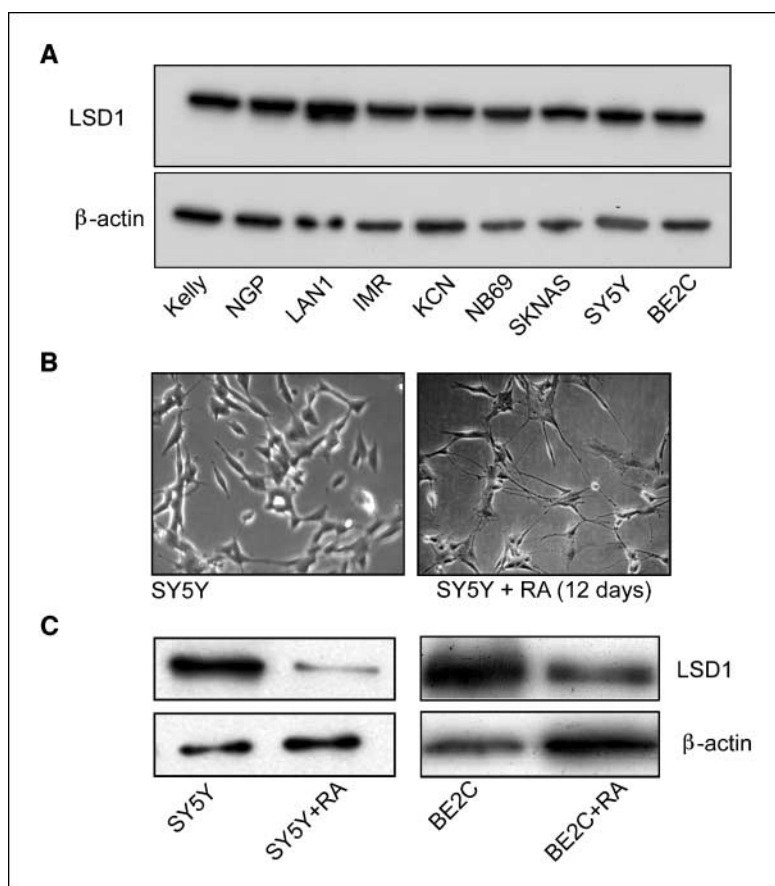


Figure 2. A, LSD1 protein expression in different neuroblastoma cell lines. β -Actin was used as the loading control. B, treatment of SH-SY5Y cells with all-trans retinoic acid (RA) resulted in a significant increase in the number and length of neurites, which served as an indicator of differentiated phenotype. C, after 12 d, retinoic acid treatment induced differentiation of SH-SY5Y and BE(2)-C neuroblastoma cells. LSD1 protein levels were significantly reduced in both cell lines. β -Actin served as the loading control.

monoaminoxidase (MAO), and uses the same demethylating mechanism. Importantly, MAO inhibitors (MAOI) were shown to inhibit LSD1 (22). Treatment of neuroblastoma cell lines with the reversible MAOIs,pargyline and clorgyline, or with the irreversible MAOI, tranylcypromine, impaired growth of neuroblastoma cells in a dose-dependent manner (Fig. 4A and Supplementary Fig. S4). Reduced viability was accompanied by increased global dimethylation of lysine 4 in histone 3 (diMeH3K4; Fig. 4B).

Small molecule inhibitors of LSD1 inhibit xenograft tumor growth. A xenograft mouse model was used to assess the potential therapeutic value of small molecule inhibitors targeting LSD1 against neuroblastic tumors *in vivo*. Nude mice (nu/nu) were s.c. injected with 2.0×10^7 SH-SY5Y neuroblastoma cells in the flank. Tranylcypromine treatment by i.p. injection of 2 mg tranylcypromine once daily was started at the time of xenograft injection, and sodium chloride was injected into control animals. During treatment, one mouse died of peritonitis and six mice died from tranylcypromine-induced seizures. Surviving mice were sacrificed 21 days after injection of tumor cells. Xenograft tumors from mice treated with tranylcypromine were significantly smaller than control-treated animals (*t* test, $P = 0.044$; Fig. 4C). Histologic examination revealed that tranylcypromine treatment resulted in xenograft tumors containing a higher content of fibrosis and extensive necrotic areas (Fig. 4D).

Discussion

LSD1 expression correlates with differentiation and cell growth in neuroblastoma. Our data clearly indicate that LSD1

expression inversely correlates with differentiation and adverse outcome of primary neuroblastic tumors. Similar results were recently reported by Kahl and colleagues for prostate cancer (15). Moreover, Wang and colleagues (23) reported that, under physiologic conditions, high LSD1 expression is characteristic of undifferentiated progenitor cells. Consistently, *in vitro* differentiation of neuroblastoma cells resulted in the down-regulation of LSD1, and inhibition or knockdown of LSD1 resulted in differentiation and reduced cell viability. Although the precise molecular mechanisms are not yet fully understood, these data suggest that aggressive, undifferentiated cancer cells retain high LSD1 expression characteristic of undifferentiated progenitor cells.

LSD1 specificity and mechanism of action are complex-dependent. LSD1 modulates tumor cell biology by demethylating monomethyl and dimethyl lysines 4 or 9 in histone, H3 (24). Demethylation specificity is governed by the interaction partners of LSD1. LSD1 was previously found to be part of the chromatin-modifying Co-Rest complex. The REST/Co-Rest complex, which includes LSD1, as well as HDAC1/2, is recruited to the promoters of neuronal-specific genes and specifically represses the respective genes by epigenetic silencing (25, 26). Recently, REST was shown to be down-regulated upon neuroblastoma cell differentiation (27). Therefore, highly active LSD1 in the REST/Co-Rest complex might repress a neuronal differentiation program in neuroblastoma, and this differentiation program might be, at least in part, reexpressed upon decreasing LSD1 expression and REST/Co-Rest activity. Depending on cellular context, LSD1 also directly interacts with transcription factors to specifically demethylate lysine residues in the promoter regions of target genes. This has been shown for the

interaction of LSD1 with AR (28). Thus, suppression of the neuronal differentiation program of undifferentiated neuroblastoma cells by high LSD1 expression might be accomplished both by recruiting transcription factors and corepressors.

Epigenetic therapy may serve as an alternative to targeting transcription factors. As discussed above, LSD1 is involved in the

regulation of broad gene expression programs that maintain the aggressive, undifferentiated phenotype in neuroblastoma. Whereas this functionality is shared by LSD1 and its cooperating transcription factors, LSD1 and other histone-modifying enzymes additionally have intrinsic enzymatic activities. Thus, they can be considered as pharmacologic targets for small molecule inhibitors.

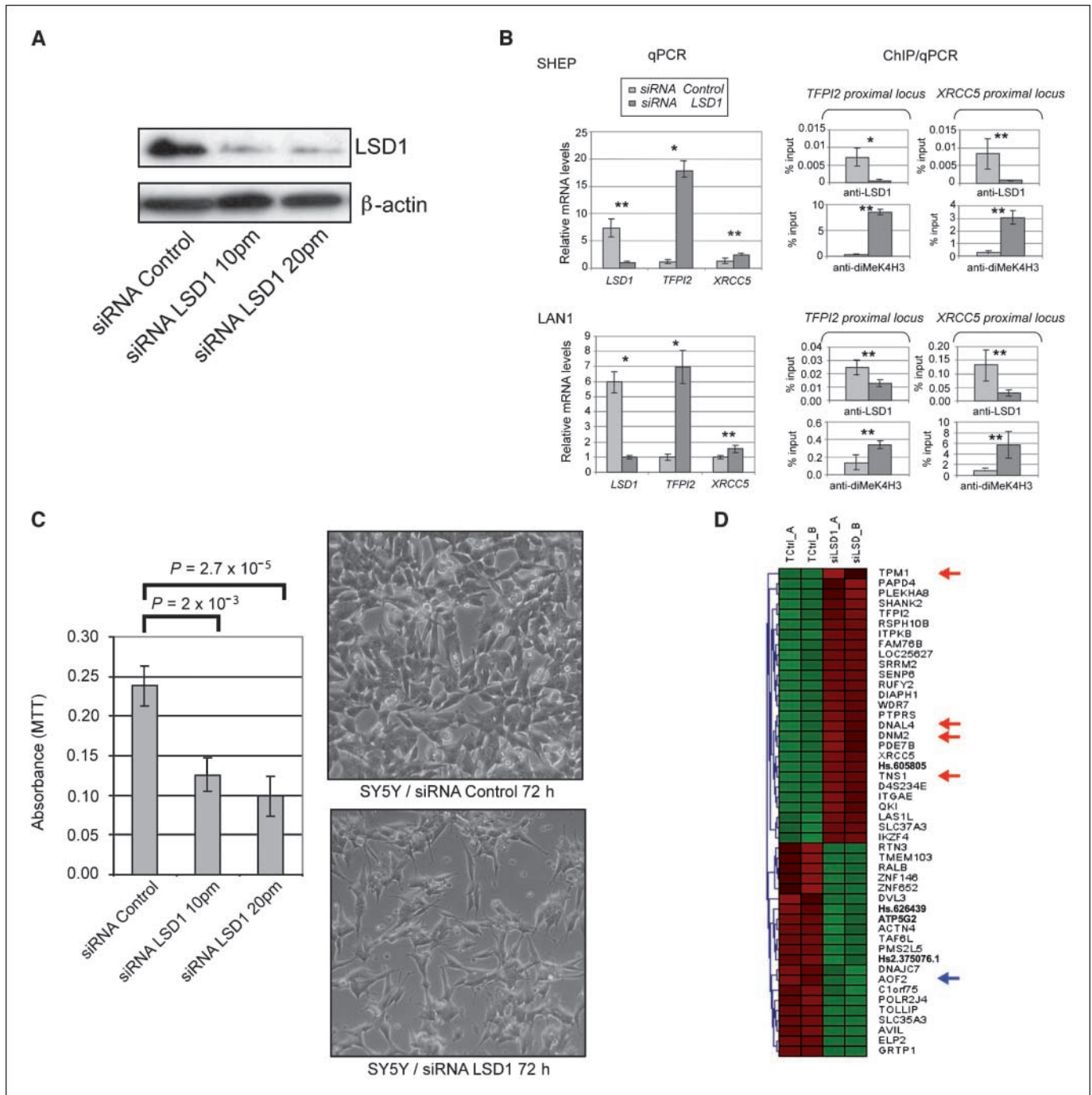


Figure 3. A, transfection of SH-SY5Y neuroblastoma cells with LSD1-directed siRNA resulted in reproducible knockdown of LSD1 protein levels. β -Actin served as the loading control. B, SHEP and LAN1 cells were transfected with LSD1-directed siRNA. ChIP (left) was performed with the indicated antibodies. The precipitated DNA was amplified by PCR using primers flanking the *TFPI2* proximal locus or *XRCC5* proximal locus. siRNA-mediated knockdown of LSD1 was verified by quantitative PCR analysis (right). C, MTT assay of SH-SY5Y cells transfected either with siRNA against LSD1 or scrambled control siRNA. Phenotypic changes were observed 72 h posttreatment (right). D, microarray analysis of SH-SY5Y cells treated with siRNA directed against LSD1 or with scrambled control siRNA revealed an induction of genes involved in differentiation and neurite dynamics (red arrow). siRNA-mediated knockdown of LSD1/AOF2 resulted in reduction of LSD1 mRNA as expected (blue arrow).

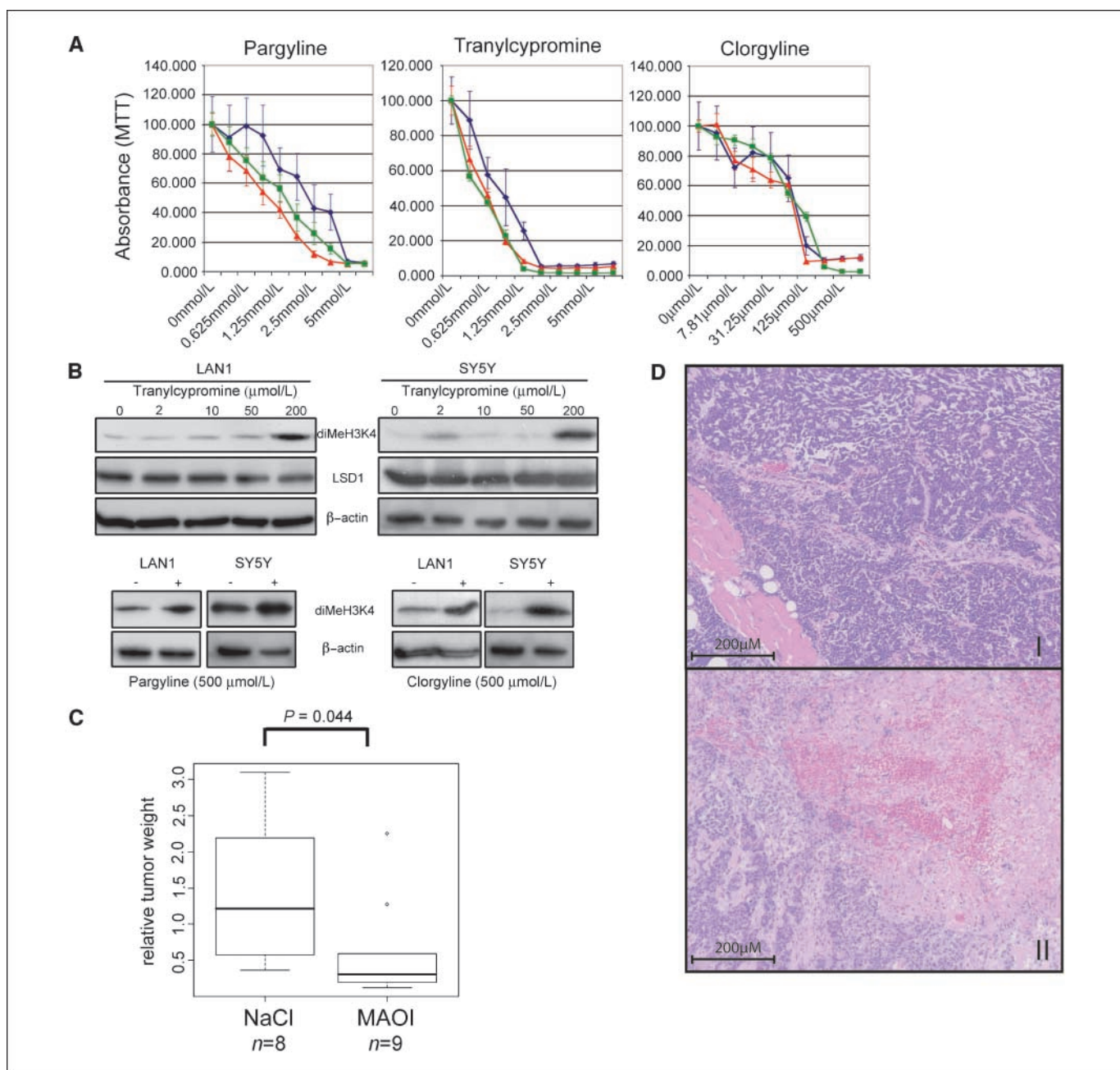


Figure 4. A, treatment of SHEP (red), SH-SY5Y (green), or LAN-1 (blue) neuroblastoma cells with pargyline, tranylcypromine, or clorgyline resulted in extensive reduction of cell numbers and MTT uptake. B, Western blot analysis confirmed an accumulation of H3K4 dimethylation upon treatment with MAOIs. In contrast, LSD1 protein levels were not affected. β -Actin served as the loading control. C, relative tumor weight of SH-SY5Y xenografts in nude mice treated with 2 mg tranylcypromine (MAOI) or control (NaCl). Mice were sacrificed, and the tumors were weighed 21 d after s.c. tumor cell inoculation. Tumors in mice receiving tranylcypromine were significantly smaller than mice receiving saline only. D, histologic appearance of tumors treated without MAOI (saline, I) or with 2 mg tranylcypromine (II) for 21 d. Sections were stained with H&E. Note the massive necrosis and hemorrhage of tumors in the MAOI-treated xenografts (II).

LSD1 has recently been identified as a target for MAOIs, including tranylcypromine, which inhibit the oxidative deamination of neurotransmitters (22, 24, 29, 30). MAOIs are already in clinical use to treat depression.

Do MAOIs qualify as LSD1 inhibitors in a clinical setting? As MAOIs are the first available small molecular inhibitors of LSD1 (12, 29–31), we analyzed their effectiveness against neuroblastoma cells. Whereas they seemed to be very effective *in vitro*, only relatively high doses reduced xenograft tumor growth *in vivo* in a

prevention model. The doses required were higher than those effectively inhibiting neurotransmitter deamination, resulting in excessive side effects, such as seizures in the treated mice. For this reason, we do not expect the currently available MAOIs to be clinically applicable as LSD1 inhibitors. Instead, specific LSD1 inhibitors must be developed, which do not inhibit the types A and B MAOs. The development of small molecules modulating substrate specificity seems plausible when the surprising capability of LSD1 to change substrate specificity between H3K4 and H3K9 is

taken into consideration, which might well be the effect of allosteric conformational switches (24).

Multimodal epigenetic therapy might be applicable as targeted therapy. At present, rational tumor therapy aims to target the hallmarks of cancer cells by inhibiting angiogenesis, blocking antiapoptotic proteins, and inhibiting tumor-associated receptor tyrosine kinases that provide survival signals. Most often, tumor cells circumvent such therapeutic interventions, either by mutation of the target structure or by activation of alternative pathways. We have observed such secondary mutations in response to imatinib therapy of gastrointestinal tumors (32). The problem of resistance to targeted therapies certainly needs to be addressed in multimodal strategies. Therefore, it is of great importance that reprogramming of tumor cells seems possible via interference with enzymes manipulating epigenetic patterns. A combination of histone demethylases and HDAC inhibitors might prove useful to prevent the development of resistance to treatment and achieve a maximal effect. Indeed, inhibition of LSD1 and HDAC turned out to be synergistic for epigenetic regulation (22). Furthermore, LSD1 and the jumonji domain family of proteins were reported to cooperate to stimulate AR-dependent gene expression (28). Taken together, this suggests that the combination of inhibitors of different histone demethylases might act synergis-

tically to reprogram gene expression signatures underlying the malignant phenotype of tumor cells. In summary, we provide the first evidence that LSD1 may serve as a druggable target in neuroblastoma and that LSD1 inhibitors alone or in combination with other chromatin-modifying agents may provide potential therapeutic options to reprogram the malignant phenotype of neuroblastoma and possibly other aggressive cancers with features of undifferentiated progenitor cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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