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Lysine-specific demethylase 1 (LSD1) is highly expressed in ER-negative breast cancers and a biomarker predicting aggressive biology

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Breast carcinogenesis is a multistep process involving both genetic and epigenetic changes. Since epigenetic changes like histone modifications are potentially reversible processes, much effort has been directed toward understanding this mechanism with the goal of finding novel therapies as well as more refined diagnostic and prognostic tools in breast cancer. Lysine-specific demethylase 1 (LSD1) plays a key role in the regulation of gene expression by removing the methyl groups from methylated lysine 4 of histone H3 and lysine 9 of histone H3. LSD1 is essential for mammalian development and involved in many biological processes. Considering recent evidence that LSD1 is involved in carcinogenesis, we investigated the role of LSD1 in breast cancer. Therefore, we developed an enzyme-linked immunosorbent assay to determine LSD1 protein levels in tissue specimens of breast cancer and measured very high LSD1 levels in estrogen receptor (ER)-negative tumors. Pharmacological LSD1 inhibition resulted in growth inhibition of breast cancer cells. Knockdown of LSD1 using small interfering RNA approach induced regulation of several proliferation-associated genes like p21, *ERBB2* and *CCNA2*. Additionally, we found that LSD1 is recruited to the promoters of these genes. In summary, our data indicate that LSD1 may provide a predictive marker for aggressive biology and a novel attractive therapeutic target for treatment of ER-negative breast cancers.

Introduction

In the industrialized countries of the Western world, breast cancer is the most common tumor in women and, along with lung cancer, the most important cause of cancer-associated morbidity and mortality. Breast cancer is a heterogeneous disease that encompasses several distinct entities with remarkably different biological characteristics and clinical behavior. Currently, breast cancer patients are managed based on a constellation of clinical and histopathological parameters in conjunction with assessment of hormone receptor [estrogen receptor (ER) and progesterone receptor (PR)] status and HER2 (human epidermal growth factor receptor 2) expression and gene amplification. Breast cancer patients with ER-positive tumors generally have a more favorable prognosis than those with ER-negative tumors. Although effective therapies have been developed for patients with hormone receptor-positive or HER2-positive disease, chemotherapy is the only modality of systemic therapy for patients with breast cancers lacking both hormone receptor and HER2 expression.

Abbreviations: cdk, cyclin-dependent kinase; ChIP, Chromatin immunoprecipitation; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; H3K4, lysine residue 4 of histone H3; H3K9, lysine residue 9 of histone H3; HER2, human epidermal growth factor receptor 2; LSD1, lysine-specific demethylase 1; MAOI, monoaminoxidases inhibitor; MTT, 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PR, progesterone receptor; qRT-PCR, quantitative real-time polymerase chain reaction; siRNA, small interfering RNA.

More recent evidence showed that epigenetic regulation of cell growth and gene expression may provide important predictive information and also novel targets for molecularly directed therapies. Preliminary attempts to profile histone modifications in a range of cell lines suggested that cancer cells frequently reveal loss of monoacetylated and trimethylated forms of histone H4 (1). Further, inhibitors of enzymes controlling epigenetic modifications, specifically DNA methyltransferases and histone deacetylases, showed promising antitumorigenic effects for some malignancies (2).

Histone modifications include acetylation, phosphorylation and methylation, resulting in a combination of histone marks that are collectively referred to as the histone code (3). The combination of chromatin marks at a given promoter specifies whether gene promoters are in an open/active or rather in a closed/repressed conformation (3,4). Histone methylation can be associated with either active or repressive signals and has also recently been discovered to be a dynamic process regulated not only via addition of methyl groups by methylases but also via removal of methylation catalyzed by lysine-specific demethylase 1 (LSD1) and JmjC domain demethylases (5–11). LSD1 specifically interacts with the androgen receptor, the ER or with the large chromatin-modifying corepressor complexes, such as the Co-Rest complex (6,12,13). LSD1 allows transcription factors or corepressor complexes to selectively initiate or repress transcription via demethylation of lysine residue 4 of histone H3 (H3K4) or lysine residue 9 of histone H3 (H3K9), thereby controlling gene expression programs.

LSD1 is essential for mammalian development and probably involved in many biological processes (14). Importantly, we recently showed that LSD1 expression correlates with adverse clinical outcome in neuroblastoma and demonstrated that pharmacological inhibition of LSD1 reduced neuroblastoma growth in xenografted nude mice *in vivo* (15). In addition, we showed previously that high expression levels of LSD1 in prostate cancer predict aggressive tumor biology and early relapse after radical prostatectomy suggesting a tumor-promoting role for LSD1 (6,16). Thus, inhibition of LSD1 might provide a novel epigenetic target for cancer therapy.

Considering recent evidence that LSD1 critically controls hormone-dependent gene expression, cellular growth and malignant progression of prostate cancers, we for the first time investigated here the role of LSD1 in breast cancer. We developed an enzyme-linked immunosorbent assay (ELISA) assay to measure quantitatively LSD1 protein levels in tumor specimens. Thereby, we found that LSD1 expression was increased in breast cancer tissue and particularly high expression levels were observed in ER- and PR-negative and clinically advanced tumors. Further, LSD1 knockdown using small interfering RNA (siRNA) or inhibition with small molecular inhibitors resulted in growth retardation of breast cancer cells *in vitro*. Gene expression in breast cancer cells after LSD1 knockdown using siRNA was analyzed by RNA microarrays and gene expression of several genes related to proliferation was validated by quantitative real-time polymerase chain reaction (qRT-PCR). Chromatin immunoprecipitation (ChIP) analysis confirmed that knockdown of LSD1 decreased the occupancy of LSD1 on the *CDKN1 (*p21) promoter, whereas knockdown of LSD1 decreased the occupancy of LSD1 on *CCNA2* and *ERBB2* promoter regions coinciding with significant increase in the repressive mark of methylated H3K9.

Materials and methods

Tissue specimens

Paraffin-embedded tissue specimens and frozen tissue were selected from the archival files of the Institute of Pathology, University of Bonn. Some of the tissue specimens were immediately kept frozen after resection and stored in liquid nitrogen until further use. Tissue processing of all specimens was done in our institution using identical procedures. Clinicopathological variables measured

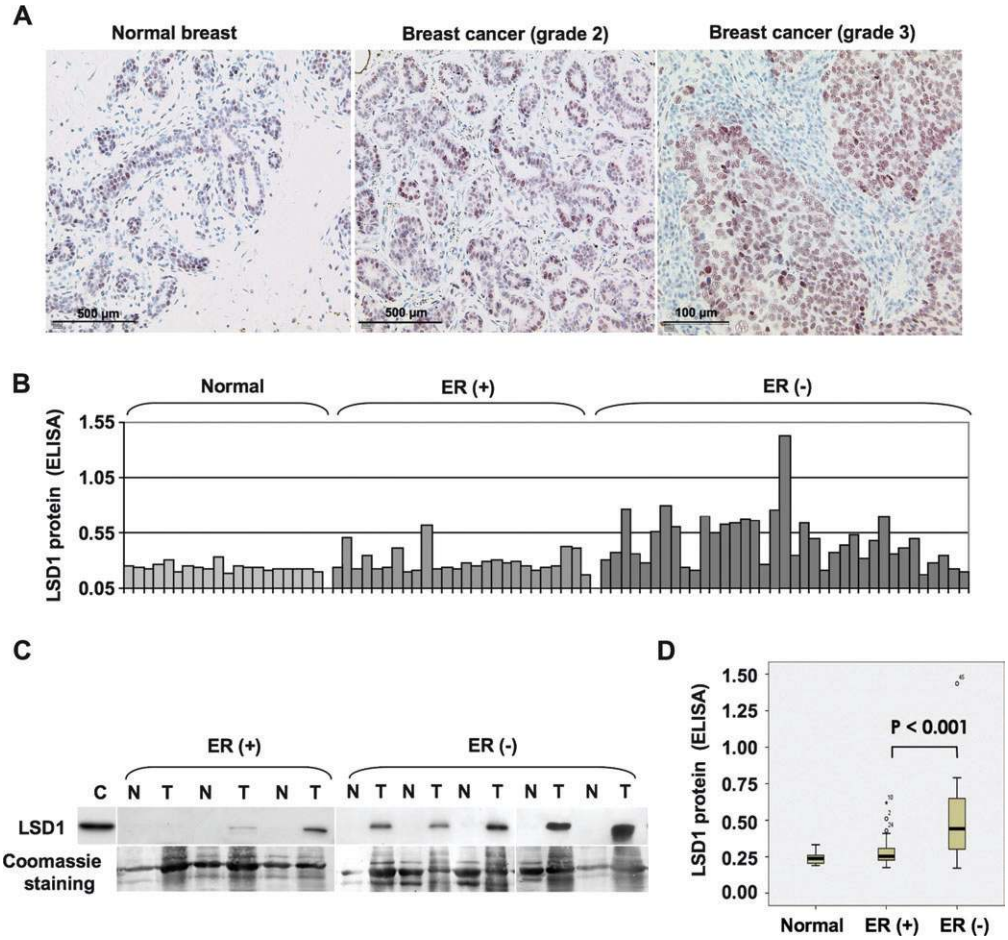


Fig. 1. Overexpression of LSD1 in ER-negative breast tumors. (**A**) Immunohistochemical staining of LSD1 was observed in normal breast tissue and breast cancer (histological grades 2 and 3). (**B**) LSD1 expression level in 20 normal breast tissues samples, 26 ER-positive and 37 ER-negative breast carcinomas was analyzed with ELISA for LSD1. ER (+), ER-positive; ER (−), ER-negative. (**C**) LSD1 expression in normal and tumor tissue extracts was determined by western blot. Coomassie staining was used as the loading control. C, control: *in vitro* translated human LSD1; N, normal breast tissue; T, breast tumor tissue. (**D**) Statistical significance test of ELISA was done by two-sided non-parametrical Mann–Whitney *U*-test to analyze differences in expression levels among normal, ER-positive and ER-negative groups. LSD1 expression was significantly higher in ER-negative breast cancers than in ER-positive cancers or normal tissue ($P < 0.001$).

at diagnosis were obtained from patient records. The study adheres to ethical standards and was approved by the ethics committee (36/08 and 094/09).

Immunohistochemistry

Immunohistochemical staining was done as described previously (15) using an α-LSD1 antibody (catalog No. 100-1762, Novus Biologicals, Littleton, CO) diluted 1:250 or an ERα antibody (clone 6F11; Menarini Diagnostics, Berlin, Germany) diluted 1:75. Nuclear immunostaining results for LSD1 were evaluated using a semi-quantitative Remmele scoring system (17), calculating the staining intensity and the percentage of positive cells. Briefly, the number and intensity of positive cells were counted and scored between 0 and 4 (0 = no positive nuclei, 1 = less than 10% nuclei display intense staining or more nuclei display weak staining, 2 = 11–50% intense staining or more nuclei display moderate staining, 3 = 51–80% nuclei display intensive staining, 4 = 81–100% nuclei display intensive staining). Scoring procedures and controls were described previously (16).

Enzyme-linked immunosorbent assay

For ELISA analysis, 20 normal breast tissues, 26 ER-positive and 37 ER-negative breast tumor tissues were used. Hematoxylin–eosin-stained sections were prepared for assessment of the percentage of tumor cells; only samples with >70% tumor cells were selected. Ninety-six well Maxisorb microplates (Nunc, Wiesbaden, Germany) were incubated with tissue protein lysates (40 µg) in coating buffer (50 mM sodium carbonate buffer, pH 9.2) overnight at 4°C. After removal of the coating solution by inverting the plate, the wells were blocked with 200 µl blocking buffer (Roche, Mannheim, Germany) for 1 h at room

Table I. Correlation between histopathological data and LSD1 expression in tumor specimens from 38 breast cancer patients

	LSD1 low[a], *n* (%) (*n* = 16)	LSD1 high[a], *n* (%) (*n* = 22)	*P*[b]
Size			
pT1	7 (44)	11 (50)	0.752
pT2-4	9 (56)	11 (50)	
Nodal status			
Negative	12 (75)	14 (64)	0.504
Positive	4 (25)	8 (36)	
ER status[c]			
Negative	3 (19)	20 (95)	<0.001
Positive	13 (81)	2 (9)	
PR status[d]			
Low	7 (44)	21 (95)	0.001
High	9 (56)	1 (5)	
Her2/erbB2[e]			
Low	14 (88)	16 (73)	0.426
High	2 (12)	6 (27)	

[a]LSD1 low, 0 ≤ score ≤ 9; LSD1 high, 9 < score ≤ 12.
[b]Fisher's exact test (two-sided).
[c]ER negative, score = 0; ER positive, score =12.
[d]PR low, 0 ≤ score ≤ 6; high, 6 < score ≤ 12.
[e]Her2/erbB2, low, score 0 or 1; high, score 2 or 3.

temperature. After rinsing with washing buffer (0.05% Tween in phosphate-buffered saline), the wells were incubated with α-LSD1 solution (1: 400, Novus Biologicals, catalog No. NB 100-1762) in 100 µl blocking buffer for 1 h at 25°C followed by three washing steps with 200 µl 0.05% of Tween in phosphate-buffered saline. After addition of 100 µl horseradish peroxidase-labelled α-mouse (1:1000; DAKO, Glostrup, Denmark, catalog No. P-0448), the wells were incubated for 0.5 h and washed three times. Finally, 100 µl of the 3, 3′, 5, 5′- tetramethylbenzidine substrate solution (1 Step Ultra 3, 3′, 5, 5′- tetramethylbenzidine; Thermo Scientific, Rockford, IL) were added to each well. The conversion of substrate was stopped by addition of 100 µl of 2 N sulfuric acid solution. The optical density was determined in an ELISA reader (ELx 800 Universal; BIO-TEK Instruments, Winooski, VT) at 450 nm.

Cell culture and proliferation assays

MCF7, MDA-MB 453 and MDA-MB 231 breast cancer cells were cultivated in Dulbecco's modified Eagle's medium and T47D cells were cultivated in RPMI. All media were supplemented with 10% fetal calf serum, L-glutamine and antibiotics.

To measure cell growth, cells were seeded at a density of 2500 cells per well in 96 well microplates and cultured in standard medium. Treatment with clorgyline (Sigma-Aldrich, Hamburg, Germany) or tranylcypromine (Biomol, Hamburg, Germany) was done as indicated. A 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed according to the manufacturer's protocol (Roche). To perform proliferation assays in the presence of 17β-estradiol (E_2; Sigma-Aldrich, München, Germany) and tranylcypromine, T47D cells were hormone deprived for 4 days in phenol-free medium with 10% charcoal-treated fetal bovine serum (Invitrogen, Karlsruhe, Germany) and then treated with 100 nM either in the presence or absence of tranylcypromine.

SiRNA transfection

Cells were seeded with 1×10^5 cells in 24 well plates and then incubated for 3–12 days in standard medium in the presence of 10–20 nM siRNA directed against LSD1 (targeted on exon 8; Ambion, Austin, TX) or control siRNA (scrambled) complexed with HiPerFect Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

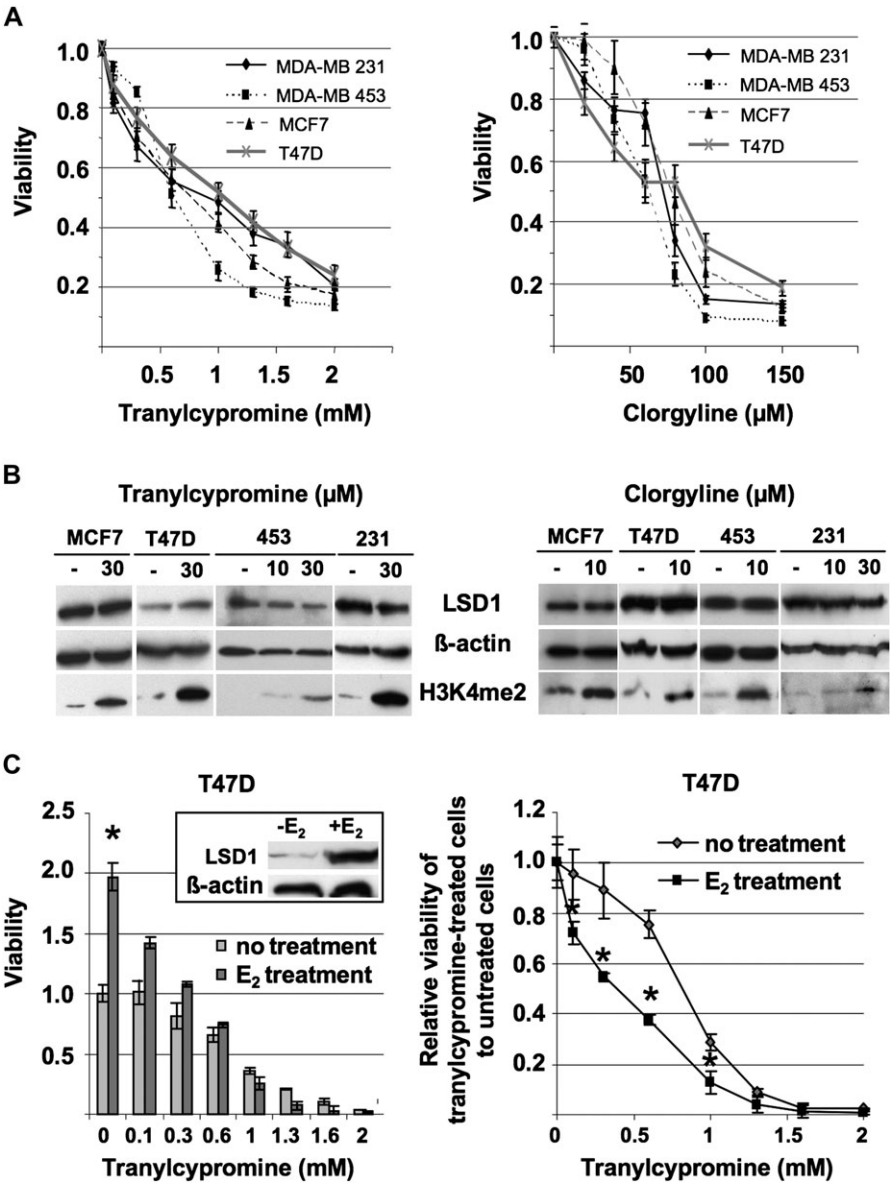


Fig. 2. Reduction in cell growth and increase of global H3K4 methylation upon MAOIs treatment. (**A**) Four different breast cancer cells were treated with tranylcypromine and clorgyline for 72 h for MTT assay. MAOIs treatment resulted in extensive reduction of cell numbers. (**B**) Western blot analysis confirmed an accumulation of H3K4 dimethylation upon treatment with 10 or 30 µM tranylcypromine and 10 or 30 µM clorgyline for 24 h in all breast cancer lines. In contrast, LSD1 protein levels were not affected. β-actin served as the loading control. (**C**) Effect of estrogen treatment on the sensitivity to MAOIs was tested in T47D (ER+) cells. Cells were treated with different concentrations of tranylcypromine in the absence or presence of 17β-estradiol (E_2). Western blot analysis showed that E_2 induced LSD1 protein. The unpaired *t*-tests were performed to show that the differences between two groups are significant ($P < 0.05$).

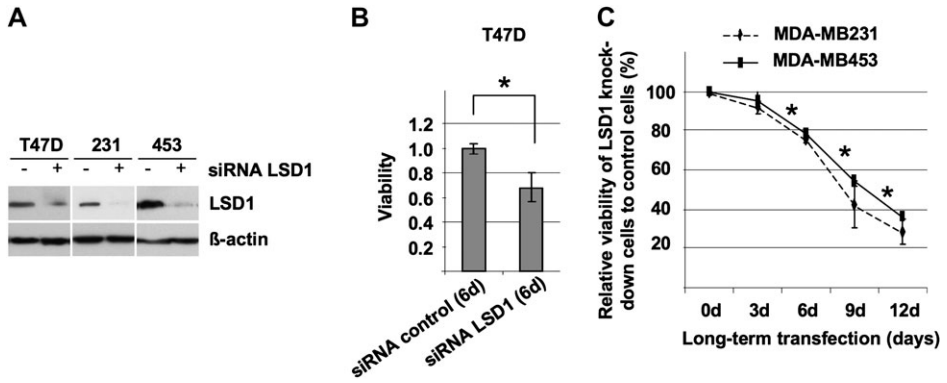


Fig. 3. Decreased cellular growth upon siRNA-mediated knockdown of LSD1. (**A**) Knockdown of LSD1 protein levels was determined 6 days after transfection by western blot. β-Actin served as the loading control. A significant reduction in cell number was observed in MTT assay upon knockdown of LSD1 in T47D (**B**) for 6 days. Decreased cellular growth upon siRNA-mediated knockdown was observed in MDA-MB231 and MDA-MB 453 (**C**). MDA-MB231 and MDA-MB cells were treated with siRNA against LSD1 for 12 days.

RNA extraction and affymetrix microarray procedures

To identify changes on gene expression caused by treatment with siRNA directed against LSD1 or control siRNA in MDA-MB-231 cells, total RNA was purified from the cells after treatment for 6 days (two rounds of transfection) using the RNeasy Mini kit from Qiagen, as specified by the manufacturer. RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany).

For microarray analyses, we used the Affymetrix GeneChip platform employing a standard protocol for sample preparation and microarray hybridization. Total RNA (2.5 μg) was converted into biotinylated complementary RNA according to the Affymetrix standard protocol version 2, purified, fragmented and hybridized to HG-U133Plus_2.0 microarrays (Affymetrix, Santa Clara, CA). The arrays were washed and stained according to the manufacturer's recommendations and finally scanned in a GeneChip scanner 3000 (Affymetrix). Three independent arrays, derived from independent cell samples, were analyzed for each experimental group. Raw data representing the signal values of gene expression were preprocessed with the GeneChip Operating Software 1.4. Expression console 1.1 was used to obtain quality control data after MAS 5.0 statistical algorithm. Only candidate genes differentially expressed >2-fold with a $P < 0.05$ were selected.

RNA isolation and qRT-PCR

Total RNA was isolated from cells using the RNeasyMini kit (Qiagen), and complementary DNA synthesis was performed using the SuperScript Reverse Transcription kit (Invitrogen). Gene expression was monitored by qRT-PCR (Applied Biosystems, Foster City, CA). Expression values were normalized to the mean of 18S ribosomal RNA. A list of primers used for qRT-PCR validation is available in supplementary data (available at *Carcinogenesis* Online).

Western blot analysis

Protein lysates were extracted from cells and blotted as described in Schulte *et al*. (15). The membranes were incubated for 1–2 h using the following antibodies and dilutions: α-LSD1 (Novus Biologicals) 1:1000; α-K4H3me2 (Abcam, Cambridge, UK) 1:1000; β-actin (Sigma-Aldrich, Hamburg, Germany) 1:5000. Coomassie staining was used as a loading control since the frequently used reference protein ß-actin was clearly upregulated in the cancer specimens (18).

Chromatin immunoprecipitation

ChIP experiments were performed essentially as described (15). MCF7 cells were transfected 6 days before harvesting for ChIP with or without LSD1 siRNA (Ambion) following the manufacturer's instructions. Immunoprecipitation was performed with specific antibodies to H3K4me2 (Abcam), H3K9me2 (Abcam) and LSD1 (Novus Biologicals) on protein A coupled Dynabeads (Invitrogen). Purified DNA specimens were subjected to qRT-PCR using a SYBR green probe (Invitrogen) in an ABI Prism 7900 (Applied Biosystems) according to the manufacturer's specified parameters. Amplicons were normalized to the DNA immunoprecipitated with antibody to histone H3 (Abcam). Recovered DNA was analyzed by TaqMan qRT-PCR using the following loci-specific primers: *CCNA2* (−137 to −30) proximal promoter region: forward primer, 5′-CCTGCTCAGTTTCCTTTGGT-3′; reverse primer, 5′-ATCCCGCGACTATTGAAATG-3′; *ERBB2* (−309 to −220) proximal promoter region: forward primer, 5′-GGCTTGGGATGGAGTAGGAT-3′; reverse primer, 5′-TCCCTAGGCTGCCACTCTTA-3′. *CDKN1A* (p21) (−48 to 32) proximal promoter region: forward primer, 5′-GGGGCGGTTGTATATCAGG-3′, reverse primer, 5′-GGCTCCACAAGGAACTGACT-3′, (−418 to −348) proximal promoter region: forward primer, 5′-CTCTCCAATTCCCTCCTTCC-3′, reverse primer, 5′-AGAAGCACC-TGGAGCACCTA-3′.

Statistical analysis

Statistical significance of the ELISA results was tested by two-sided non-parametrical Mann–Whitney *U*-test to analyze differences in protein levels among distinct groups using SPSS 17.0 program (SPSS, Zürich, Switzerland). Association between categorical variables was assessed by two-sided Fisher's exact test using GraphPad Prism 5 (La Jolla, CA).

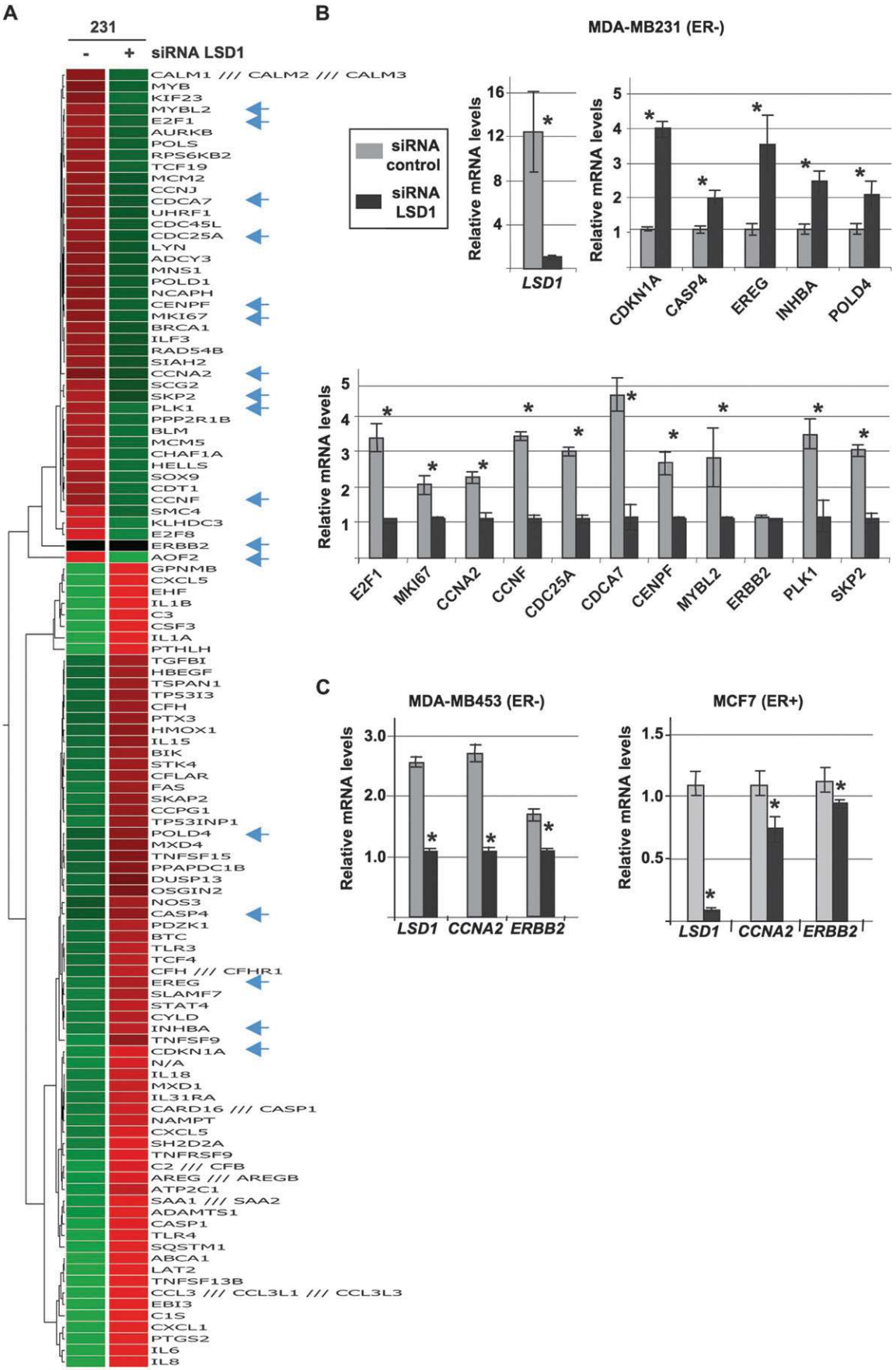
Results

LSD1 is strongly expressed in ER-negative breast cancer

For this study, we retrospectively analyzed LSD1 expression both in fresh-frozen and in formalin-fixed paraffin-embedded tissue specimens of ductal and lobular breast cancer. Initial immunohistochemical staining revealed moderate nuclear expression in luminal cells of normal breast glands and ER-positive cancers (histological grade 2). Significantly more intense staining was observed in ER-negative breast cancers (histological grade 3), in which every tumor cell showed a strong and specific nuclear staining pattern (Figure 1A, supplementary Figure 1A is available at *Carcinogenesis* Online).

Therefore, we aimed to measure LSD1 expression levels by a quantitative LSD1 ELISA. The assay was validated by recombinant LSD1 protein and performed in a quantitative manner over a broad spectrum of LSD1 protein concentrations between 1 and 250 μg/l and also after serial dilution of protein lysates from breast cancer tissue specimens [supplementary data and supplementary Figure 1B (available at *Carcinogenesis* Online)]. In protein lysates of snap-frozen primary breast tissues, including 20 normal breast tissues, 26 ER-positive and 37 ER-negative breast tumors, LSD1 protein was significantly stronger expressed in ER-negative breast cancers than in ER-positive cancers or normal tissue (Mann–Whitney *U*-test, $P < 0.001$, Figure 1B and D). There was a trend of slightly higher expression comparing ER-positive breast cancer and normal breast tissue, but this did not reach statistical significance. Similar results were seen in a small set of breast cancer specimens analyzed by western blot analysis. LSD1 was strongly expressed in ER-negative breast tumors compared with normal breast tissues and ER-positive tumors (Figure 1C).

Significant inverse correlation between LSD1 expression and ER status was also seen in detailed immunohistochemical analysis. To statistically calculate the association between histopathological parameters and LSD1 expression levels (Table I), tumor specimens were classified into a group with low LSD1 expression ($n = 16$) and a second group with high LSD1 expression ($n = 22$). Results in Table I



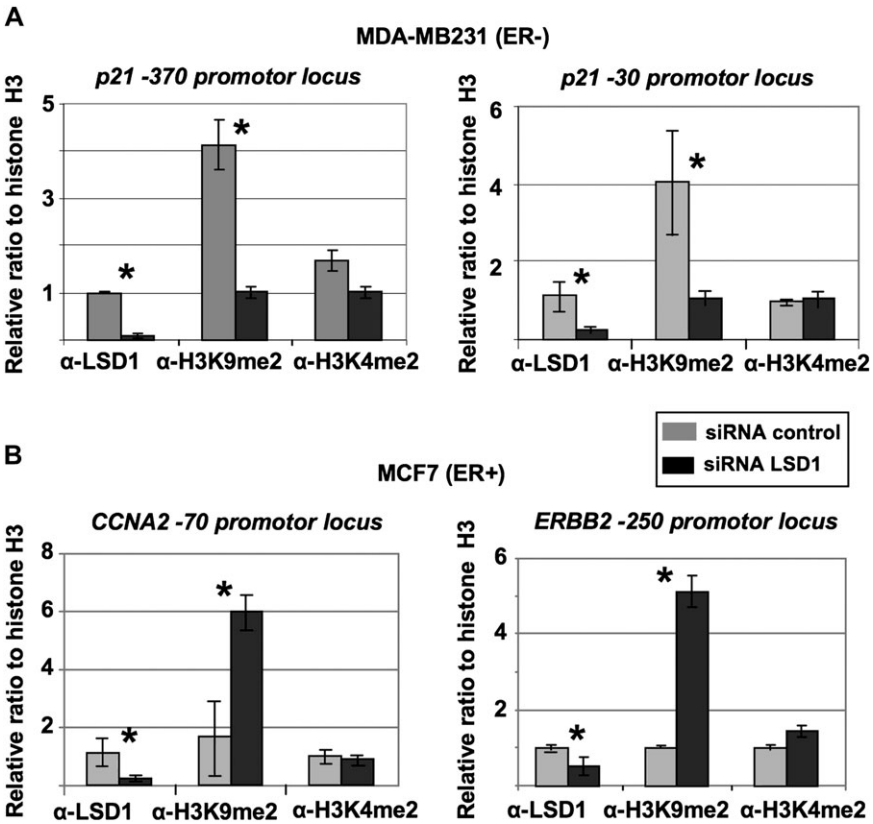


Fig. 5. Panels show ChIP/qPCR occupancy analysis of LSD1, H3K9me2 and H3K4me2 on *p21*, *CCNA* and *ERBB2* genomic loci after treatment of cells with siRNA directed against LSD1 or with scrambled control siRNA. (**A**) LSD1 binds specific regions of the *p21* promoter in MDA-MB 231 cells. The sites are located 370 bp (−370) and 30 bp (−30) upstream of the transcriptional start site. (**B**) Enrichment of H3K9 dimethylation in the proximal promoter region of *CCNA2* or *ERBB2* was observed upon knockdown of LSD1. In ChIP experiments, the sonicated chromatin of MDA-MB 231 and MCF7 cells was immunoprecipitated with α-LSD1, α-H3K9me2 and α-H3K4me2. The precipitated DNA was amplified by polymerase chain reaction using primers flanking the *p21* (−370), *p21* (−30), *CCNA2* (−70) or *ERBB2* (−250) genomic loci upstream of the transcriptional start site. Normalized values were calculated as ratio to histone H3. Significant differences are indicated with $^{*}P < 0.05$.

clearly indicated that strong nuclear LSD1 staining (score >9) was associated with negative ER status (score = 0) (Fisher's exact test, $P < 0.001$, Table I). Consistently, high LSD1 expression also correlated with low PR expression (score ≤6) ($P = 0.001$). Neither tumor size and nodal status nor Her2/erbB2 status showed any correlation with LSD1 expression.

Considering that hormone receptor expression in breast cancer is associated with a significantly better prognosis (19), high LSD1 expression appears to provide a biomarker for aggressive tumor biology associated with hormone receptor-negative breast cancer.

LSD1 inhibition using monoaminoxidase inhibitors confers growth inhibition and increase of global H3K4 methylation in breast cancer cell lines

The catalytic domains of LSD1 and monoaminoxidases share structural homology and make use of the same catalytic mechanism (20). Therefore, we used the monoaminoxidases inhibitors (MAOIs) tranylcypromine and clorgyline to inhibit LSD1 in breast cancer cell lines *in vitro*. Four different breast cancer cell lines, all of which strongly expressed LSD1 (Figure 2B), were tested. Treatment with tranylcypromine and clorgyline for 72 h impaired cell growth in a dose-dependent manner (Figure 2A) in all four cell lines. To address whether reduced cell viability after treatment with MAOIs correlates with LSD1 inhibition, we analyzed the methylation status of H3K4 in cells before and after treatment. Upon treatment of MAOIs, global dimethylation of H3K4 increased, whereas LSD1 enzyme levels were not altered (Figure 2B).

Next, we investigated the effect of estrogen treatment on the sensitivity to MAOIs. For this purpose, we treated ER-positive cells T47D with different concentrations of tranylcypromine in the absence or presence of 10 nM 17β-estradiol (E_2). Western blot analysis revealed that E_2 results in induction of LSD1 and that increased LSD1 levels coincided with increased sensitivity to tranylcypromine (Figure 2C).

siRNA-mediated knockdown of LSD1 reduces cellular growth

To analyze the consequences of reduced LSD1 expression, T47D, MDA-MB 453 and MDA-MB 231 cells were transiently transfected with 15 nM siRNA directed against LSD1 or with 15 nM scrambled control siRNA. Significant LSD1 knockdown was detected measuring

Fig. 4. LSD1 regulates a set of genes involved in proliferation. (**A**) Functional annotation based on gene ontology revealed that downregulation of LSD1 in MDA-MB 231 cells leads to differential expression of 113 genes encoding for a variety of proteins involved in differentiation, proliferation and cell cycle, respectively. (**B**) In MDA-MB 231 cells, transcript levels for *CDKN1A* (*p21*), *CASP4*, *EREG*, *INHBA* and *POLD4* were significantly upregulated after treatment with LSD1-specific siRNA. In contrast, most of the proliferation-associated genes were downregulated like *E2F1*, *MKI67*, *CCNA2*, *CCNF*, *CDC25A*, *CDCA7*, *CENPF*, *MYBL2*, *ERBB2* and *SKP2*. (**C**) *CCNA2* and *ERBB2* mRNA expressions in MDA-MB 453 (ER−) as well in MCF7 (ER+) cells were downregulated 6 days after knockdown of LSD1. qRT-PCR analysis was done in three different breast cancer cells treated with siRNA directed against LSD1 or with scrambled control siRNA. In all experiments, 18S ribosomal RNA was used as the endogenous reference gene. Significant differences are indicated with $^{*}P < 0.05$.

protein levels 6 days after transfection by western blot (Figure 3A). MTT assays indicated that the silencing of LSD1 caused a significant decrease in cell growth and viability in ER-positive and ER-negative cell lines (Figure 3B and C). Morphologically, no sign of apoptosis was detected. LSD1 inhibition appeared to affect the number of dividing cells consistent with a previous report that inhibition of LSD1 leads to G_2/M cell cycle arrest (21).

Knockdown of LSD1 induces downregulation of proliferation-associated genes and alters gene-specific H3K9 methylation

Considering that LSD1 regulates gene expression through modification of histone methylation in gene promoter regions and previous evidence that silencing of LSD1 decreased cellular proliferation, we further analyzed expression of LSD1 target genes. To identify targets of LSD1 in ER-negative breast cancer cells, we performed microarray analysis from MDA-MB 231 (ER−) cells treated with either LSD1-specific siRNA or a scrambled control. We identified by this approach 113 genes, which might represent putative proliferation-related targets of LSD1 (Figure 4A) and we validated several genes by qRT-PCR. Steady-state transcript levels for *CDKN1A (p21)*, *CASP4 (*caspase 4), *EREG* (epiregulin), inhibinβ4 (*INHBA*) and polymeraseδ4 (*POLD4*) were significantly upregulated in MDA-MB 231 cells (Figure 4, upper panel) after treatment with LSD1-specific RNAi. In contrast, several of the proliferation-associated genes were downregulated like *E2F1* (E2F transcription factor 1), *MKI67* (Ki-67), *CCNA2* (cyclin A2), *CCNF* (cyclin F), *CDC25A* (cell division cycle 25 homolog A), *CDCA7* (cell division cycle associated 7 protein), *CENPF* (centromere protein F, mitosin), *MYBL2* [v-myb myeloblastosis viral oncogene homolog (avian)-like 2], *ERBB2* (Her2/erbB2) and *SKP2* (*S-phase kinase-associated protein 2*, *p45*). All of these genes encode proteins that play important roles in proliferation, cell cycle control and/or tumorigenesis (22–31). In addition, as illustrated in Figure 4C, *CCNA2* and *ERBB2* were downregulated after LSD1 knockdown also in ER-negative MDA-MB 453 cells as well as in ER-positive MCF7 cells.

To assess whether the promoters of *CDKN1A* (p21), *CCNA2* and *ERBB2* are direct or rather indirect targets of histone modification by LSD1, MCF7 and MDA-MB 231 cells treated with siRNA directed against LSD1 or with a scrambled control siRNA were subjected to ChIP using α-LSD1, α-K9H3me2 and α-K4H3me2 antibodies. Indeed, we found that knockdown of LSD1 abolished the binding of LSD1 on two regions of the p21 promoter (Figure 5A). These regions include the sites −370 bp and −30 bp upstream the transcriptional start site. We found significantly reduced levels of H3K9me2 at the p21 locus and a nearly unchanged H3K4me level when LSD1 is downregulated (Figure 5A).

In addition, ChIP analysis confirmed that LSD1 is present at the proximal promoter of the *CCNA2* and *ERBB2* gene. Knockdown of LSD1 decreased the occupancy of LSD1 on *CCNA2* (−70) and *ERBB2* (−250) promoter regions (Figure 5B, left panel). This was accompanied by significant increase in dimethylation on H3K9, which has been shown previously to result in transcriptional repression (Figure 5B, middle panel). In contrast, after LSD1 knock down, genomic DNA corresponding *CCNA2* and *ERBB2* proximal locus were not enriched with α-H3K4me2 antibody (Figure 5B, right panel).

Discussion

LSD1 is highly expressed in hormone receptor-negative breast cancers

Several prognostic and predictive biomarkers are currently used to stratify patients with breast cancers for appropriate chemotherapies. Established biomarkers such as ER and PR already play a significant role in the selection of patients for endocrine therapy. In this study, we demonstrate that high expression levels of LSD1 may serve as a novel molecular marker for breast cancers. LSD1 is significantly strongly expressed in ER-negative breast cancers, which are well known to carry a poorer prognosis than ER-positive tumors (19). ER-negative tumors are characterized by their rapid growth, loss of differentiation and acquisition of invasive and metastastic capability. The more aggressive biology of ER-negative breast cancers is accompanied by specific changes in gene expression patterns. Microarray expression studies clearly provide evidence that ER-positive and ER-negative breast tumors reveal different gene expression profiles (32). In addition, recent studies have implicated LSD1 to certain high-risk tumors (14–16,33).

The detailed mechanism by which LSD1 overexpression *in vivo* contributes to neoplastic conversion of tumor cells remains to be elucidated. A recent study indicated that LSD1 might promote G_2-M phase transition and cell proliferation, which is one way in which its overexpression might promote tumorigenesis (21). Interestingly, a reduction of the level of H3K4 monomethylation concomitant with an alteration of the subcellular localization of LSD1 is one of the early events in the cellular response to chemical carcinogens, suggesting a critical role for increased LSD1 activity in oncogenic transformation (34).

Given that LSD1 is involved in the regulation of broad gene expression programs by changing epigenetic histone marks in gene promoters, aberrant overexpression of LSD1, possibly in concert with other genetic/epigenetic factors, can contribute to reprogram the gene expression profile and promote neoplastic conversion of breast tumor. In recent years, it has become increasingly evident that epigenetic changes play a key role in carcinogenesis. Hypermethylation of the CpG islands in the promoter regions of tumor-suppressor genes are the best-studied epigenetic alterations and an early event in malignant transformation of many tumors (35). Further, aberrant changes in the expression of histone modifying enzymes, such as enhancer of zeste drosophila homologue 2 (EZH2), a component of polycomb protein, were observed to be strongly associated with the metastasis of prostate cancer (36).

Alteration in LSD1 expression appears not to be linked specifically to breast cancer. Recently, our group found that LSD1 expression is upregulated in high-risk prostate cancers with aggressive biology (16). In neuroblastomas, LSD1 expression was strongly associated with adverse outcome and inversely correlated with differentiation (15). Taken together, this study provides additional evidence that LSD1 can serve as a promising molecular marker for aggressive tumor biology.

LSD1 contributes to cell proliferation through regulation of cell cycle-regulatory genes

LSD1, a histone H3K4me1/2 and H3K9me1/2 demethylase, acts as a functional component of either coactivator or corepressor complexes and regulates activation and repression programs. Transcriptional regulation by LSD1 is known to be cell type specific and modulated by its associated partners (13).

In this study, we identified candidate targets of LSD1 by comparing the gene expression profiles of MDA-MB 231 cells treated with LSD1-specific siRNA. Among those were several genes involved in regulation of proliferation and cell cycle regulation. We further demonstrated that three of the candidate genes were indeed direct targets of LSD1 and that downregulation of LSD1 resulted in increased (p21) or decreased gene expression (*CCNA2* and *ERBB2*).

$p21^{WAF1/CIP1}$ is a cyclin-dependent kinase (cdk) inhibitor and is a key mediator of p53-dependent cell cycle arrest after DNA damage (37). p21 belongs to the Cip/Kip family of cdk inhibitors and it inhibits proliferation mainly by interfering with cyclin E/cdk2 activity (31,38). Our data clearly show that *in vitro* silencing of LSD1 by an RNAi approach leads to induction of p21 and subsequent inhibition of cellular proliferation.

In addition, silencing of LSD1 was shown to inhibit tumor cell growth by downregulating genes involved in proliferation. ChIP revealed that LSD1 was recruited to the *CCNA2* and *ERBB2* promoter, accompanied by a significant and highly reproducible increase in histone H3K9me2 upon knockdown of LSD1. Therefore, *CCNA2* and *ERBB2* seem to be direct positively regulated targets of LSD1 in breast cancer cells. *CCNA2* encodes Cyclin A2 that functions as an activator of CDK2 kinase and thus promotes both cell cycle G_1/S and G_2/M transitions. Abnormal overexpression of cyclin A2 corresponds

to an increase in the proliferative status of tumor cells and may play an important role in tumor development and progression (39,40). Hence, *CCNA2* upregulation by LSD1 may be one important mechanism by which LSD1 drives tumorigenesis and aggressive biology of breast cancers. In addition, overexpression of cyclin A is associated with poor prognosis in breast cancer patients (30). However, as we did not find a significant correlation of LSD1 and HER2 overexpression in breast tumor specimens *in vivo*, it appears that additional mechanisms, such as gene amplification (41), are active in HER2-positive tumors.

Importantly, silencing of LSD1 caused a partial repression of gene transcription as shown by qRT-PCR analysis (66–83% decrease in *ERBB2* messenger RNA level; 40–65% decrease in *CCNA2* messenger RNA level). LSD1 functions in association with other transcriptional cofactors/epigenetic enzymes and the activation status of the transcriptional complex is regulated by specific signaling pathways (10). In case of *CCNA2* or *ERBB2*, E2F and AP2 transcription factors are known to be positive regulators of gene transcription, respectively (42,43). It is therefore possible that in the presence of corresponding stimuli and/or in combination with knockdown of other epigenetic enzymes, silencing of LSD1 would induce even stronger downregulation of its target genes. Therefore, the mechanism of LSD1 activity on H3K9 in partnership with other transcriptional cofactors needs to be addressed by further experiments.

Targeting LSD1 in breast cancer: a novel therapeutic option

Aberrant expression of LSD1 in ER-negative breast tumor and its function in driving *CCNA2* overexpression suggest that LSD1 may not only serve as a biomarker for malignant breast tumors but also as a therapeutic target in cancer treatment. Although ER-positive breast tumors respond well to anti-hormonal therapy, the treatment of ER-negative breast tumors usually includes chemotherapy by non-selective cytotoxic drugs. Targeting LSD1 in ER-negative breast cancer might provide an alternative and more specific treatment.

Both MAOI and polyamine analogues have been shown to inhibit LSD1 enzymatic activity (6,20,44). Polyamine analogues cause reexpression of aberrantly silenced genes that are important in the development of colon cancer (44). The level of reexpression of these otherwise silenced genes was almost 30% of that observed after treatment with DNA-methyltransferase inhibitors, which are of great therapeutic interest but have many side effects. If LSD1 inhibition leads to significant derepression of some of the same genes that are reactivated by DNA-methyltransferase inhibitors, LSD1 might be an important alternative target for therapy. Consistently, we recently provided direct evidence that LSD1 is indeed a target in cancer therapy. In a xenograft mouse model, MAOIs significantly decreased neuroblastoma tumor growth (15).

It was recently shown that patterns of histone methylation are important for establishing patterns of DNA methylation, indicating that these types of epigenetic regulation are highly interdependent. Notably, it was shown that LSD1 is required for maintenance of global methylation by demethylating and stabilizing Dnmt1 (45). In addition, expression of several LSD1 regulated genes like p21, TGFβ1 and p53 is also found to be altered by treatment with histone deacetylase inhibitors (46). Therefore, we hypothesize that LSD1 inhibitors alone or in combination with DNA demethylating drugs or/and chromatin-modifying agents might prove effective for treatment of hormone receptor-negative aggressive breast cancer.

Supplementary material

Supplementary data and Figure 1A and 1B can be found at http://carcin.oxfordjournals.org/

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