


## ORIGINAL ARTICLE

# E239K mutation abolishes the suppressive effects of lysine-specific demethylase 1 on migration and invasion of MCF7 cells

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## Abstract

Lysine-specific demethylase 1 (LSD1) is an important histone demethylase that mediates epithelial to mesenchymal transition (EMT). The E239K mutation of LSD1 was identified in a luminal breast cancer patient from the COSMIC Breast Cancer dataset. To investigate the functional effects of the E239K mutation of LSD1, a stable LSD1 knockdown MCF7 cell line was generated. Rescue with WT LSD1, but not E239K mutated LSD1, suppressed the invasion and migration of the LSD1 knockdown cells, indicating that the E239K mutation abolished the suppressive effects of LSD1 on the invasion and migration of MCF7 cells. Further analysis showed that the E239K mutation abolished LSD1-mediated invasion and migration of MCF7 cells through downregulation of estrogen receptor  $\alpha$  (ER $\alpha$ ). Most importantly, the E239K mutation disrupted the interaction between LSD1 and GATA3, which reduced the enrichment of LSD1 at the promoter region of the ER $\alpha$  gene; the reduced enrichment of LSD1 at the promoter region of the ER $\alpha$  gene caused enhanced histone H3K9 methylation, which subsequently suppressed the transcription of the ER $\alpha$  gene. In summary, the E239K mutation abolishes the suppressive function of LSD1 on migration and invasion of breast cancer cells by disrupting the interaction between LSD1 and GATA3.

## KEYWORDS

breast cancer, epithelial to mesenchymal transition, ER $\alpha$ , histone demethylation, LSD1

## 1 | INTRODUCTION

Breast cancer is the most common cancer in women, and metastasis is the major cause of mortality. Epithelial to mesenchymal transition (EMT) plays an essential role in metastasis by promoting the dissemination of solid tumors at primary sites and increasing the migration of tumor cells to distant organs.<sup>1,2</sup> During the progress of EMT, epithelial cells lose cell-cell adhesion and polarity, and acquire properties of migration and invasion. Epithelial to mesenchymal transition

is orchestrated by several critical transcription factors, including Snail, Slug, and several ZEB family members.<sup>1,3,4</sup> These transcription factors collaborate with epigenetic regulators and promote the decreased expression of epithelial markers, such as E-cadherin and  $\alpha$ -catenin, as well as the increased expression of mesenchymal markers, such as Vimentin.<sup>4</sup>

Nearly two-thirds of breast cancer cases, mostly luminal types, express estrogen receptor  $\alpha$  (ER $\alpha$ ),<sup>5,6</sup> a nuclear receptor for estrogen and a critical transcription factor for mammary gland development.<sup>7,8</sup>

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Estrogen receptor  $\alpha$  signaling maintains the proliferation of mammary epithelial cells in collaboration with other transcription factors, such as GATA3 and FOXA1.<sup>9-12</sup> Estrogen receptor  $\alpha$  signaling suppresses EMT by prompting degradation of Smad proteins, and inhibits breast cancer metastasis by downregulating vinculin.<sup>13,14</sup> In addition, ER $\alpha$  signaling suppresses the invasion of breast cancer cells by reducing the expression of Slug.<sup>15</sup> GATA3 is a transcription factor that regulates mammary gland development and luminal cell differentiation, and plays a crucial role in maintaining ER $\alpha$  expression.<sup>10,16,17</sup> Loss of GATA3 promotes progression of breast cancer by initiating tumor dissemination.<sup>18</sup>

Lysine-specific demethylase 1 (LSD1) is an epigenetic regulator that catalyzes the demethylation of di- and monomethyl groups from histones H3K4/9, which is critical for chromosomal remodeling and transcription regulation.<sup>19-21</sup> Demethylation at H3K4 leads to reduced transcription, whereas demethylation at H3K9 results in enhanced transcription.<sup>21</sup> Lysine-specific demethylase 1 is involved in the regulation of embryonic development, cell differentiation, and hematopoiesis.<sup>22-24</sup> Importantly, LSD1 also plays a significant role in regulating EMT.<sup>25-28</sup> Knockdown of LSD1 leads to the loss of epithelial features of luminal breast cancer cells and the promotion of tumor metastasis.<sup>27,29,30</sup>

Lysine-specific demethylase 1 is recruited to DNA by forming protein complexes with transcription factors, such as the CoREST complex.<sup>31</sup> Lysine-specific demethylase 1 contains an unstructured N-terminal region, a SWIRM domain, a Tower domain, and an amino oxidase domain; our previous study found that the SWIRM domain is essential for the interaction between LSD1 and GATA3.<sup>29,32</sup> In this study, we identified an E239K mutation in the SWIRM domain of LSD1 from a COSMIC Breast Cancer dataset, and hypothesized that the E239K mutation might play an important role in the progression of breast cancer by disrupting the interaction between LSD1 and GATA3. Therefore, we examined the functional effects of the E239K mutation of LSD1 and explored in detail the molecular mechanism under which the E239K mutation regulates the metastasis of breast cancer cells.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell culture, generation of stable cell lines, and transfection

MCF7 and HEK293T cells were cultured in DMEM (Gibco) supplemented with 10% FBS and 10 IU/mL penicillin/streptomycin. To generate lentivirus, HEK293T cells were transfected with pLVX-shLSD1-Puro, pLVX-IRES-Neo-LSD1 WT, or E239K (Genaray Biotechnology) together with packaging plasmids. The LSD1 knockdown cells were selected with puromycin, and the rescue cells were selected by both puromycin and G418. Cells were transfected with Lipofectamine 2000 (56532; Invitrogen) or RNAiMax (13778150; Invitrogen) according to the manufacturer's instructions.

### 2.2 | COSMIC and cBioPortal for cancer genomics analysis

COSMIC (<https://cancer.sanger.ac.uk/cosmic/>) and cBioPortal for Cancer Genomics (<http://cbioportal.org>) are Web-based data-mining platforms. We analyzed the information of the E239K mutation of LSD1 from the Sanger, Nature 2012 Breast Cancer cohort.<sup>33</sup>

### 2.3 | Three-dimensional culture

Cells were embedded in Matrigel (356231; Corning) in a 24-well plate, and Advanced DMEM/F12 (12634010; Gibco) supplemented with 1% FBS (Gibco), 5  $\mu$ g/mL insulin (I5500-100mg; Sigma), 10 ng/mL human epidermal growth factor (AF-100-15-100; PeproTech), 0.5  $\mu$ g/mL hydrocortisone (H0888; Sigma), and 20 ng/mL cholera toxin (C8052; Sigma) were added. The culture medium was replaced every 2 days.

### 2.4 | Transwell and wound healing assays

The membranes of Transwell chambers (24-well; 8  $\mu$ m) were coated with fibronectin (Sigma-Aldrich). Cells ( $5 \times 10^4$ ) were plated in the top chamber. Cells that invaded to the lower surface of the membrane were fixed and stained. The numbers of cells were counted and analyzed. For wound healing assays, the monolayer of cells was scratched and maintained in serum-free medium. The wounds were photographed at 0, 24, and 48 hours following scraping, and the width of the healing measured with Image-Pro Plus and analyzed with GraphPad Prism software.

### 2.5 | Xenograft tumor model

Cells ( $1 \times 10^7$ ) were injected into the hind limbs of 5-6-week-old female BALB/c nude mice. The xenograft tumors were harvested when the size of tumor reached 0.5 cm. Tumors were fixed with 4% paraformaldehyde, and embedded with paraffin. Tumor sections (5  $\mu$ m thick) were stained with H&E.

### 2.6 | RNA sequencing analysis

Total RNA from the cells was purified with TRIzol reagent (15596-026; Invitrogen) according to the manufacturer's instructions. Sequencing libraries were generated using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs) and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was undertaken on a cBotCluster Generation System using TruSeq PE Cluster Kit v4-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on a Novaseq6000 and paired-end reads were generated. Raw data (raw reads) of fastq

format were first processed through in-house perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N, and low quality reads from raw data. All the analyses were based on clean data with high quality.

## 2.7 | Immunostaining

Cells were seeded on slides at an appropriate density, fixed with paraformaldehyde, treated with 0.3% Triton X-100 for permeabilization and stained with Abs, including LSD1 (ab90996 or ab17721; Abcam), E-Cadherin (14472S; Cell Signaling Technology), Slug (9585S), GATA3 (ab199428), ER $\alpha$  (MA3-310; Thermo Fisher Scientific) and DAPI. The expression of indicated proteins was examined by fluorescence microscopy.

## 2.8 | Quantitative RT-PCR and statistical analysis

Total RNA was extracted and then cDNA was generated with BioTek super RT kit (RR047A; Takara). Quantitative RT-PCR was carried out using 2\*SYBR (RR420; Takara). Student's *t* test (two-sided) was used to calculate the *P* values. The sequences of quantitative RT-PCR primers are listed in Table S1. Data were reported as mean  $\pm$  SEM. Results were considered significant when *P* was less than .05.

## 2.9 | Chromosomal immunoprecipitation

Chromosomal immunoprecipitation and double-ChIP were undertaken as previously described.<sup>29</sup> Briefly, cells were collected and treated with ChIP lysis buffer and then sonicated. The immunoprecipitated Abs (LSD1, ab17721; H3K9me2, ab1220; GATA3, ab199428; ER $\alpha$ , ab32063) and Dynabeads were incubated with the supernatant. Elution buffer was added to wash protein beads, and the supernatant was incubated for reversal of cross-linking. For double-ChIP, the beads complexes were washed by double-ChIP elution buffer, and then incubated with another Ab for second immunoprecipitation. DNA was purified and the enrichment of specific genomic regions was determined by quantitative PCR. Final results were represented as percentage of input chromatin. The sequences of ChIP primers are listed in Table S2.

## 2.10 | Western blot analysis

Cells were collected and lysed as previously described.<sup>29</sup> The proteins were separated and electrotransferred to PVDF membrane. After blocking, primary Abs (E-cadherin, 14472S; Claudin3, ab214487;  $\alpha$ -catenin, ab51032; Vimentin, ab92547; Slug, 9585S; LSD1, ab17721; GATA3, ab199428; ER $\alpha$ , 13258S; GAPDH, Bioworld AP0063; CoREST, Merck 07-455; histone deacetylase 1 [HDAC1], ab7028; and HDAC2, ab12169) were detected using anti-rabbit or anti-mouse Abs, and visualized on a Tanon-5200 Chemiluminescent Imaging System.

## 2.11 | Coimmunoprecipitation and GST pull-down

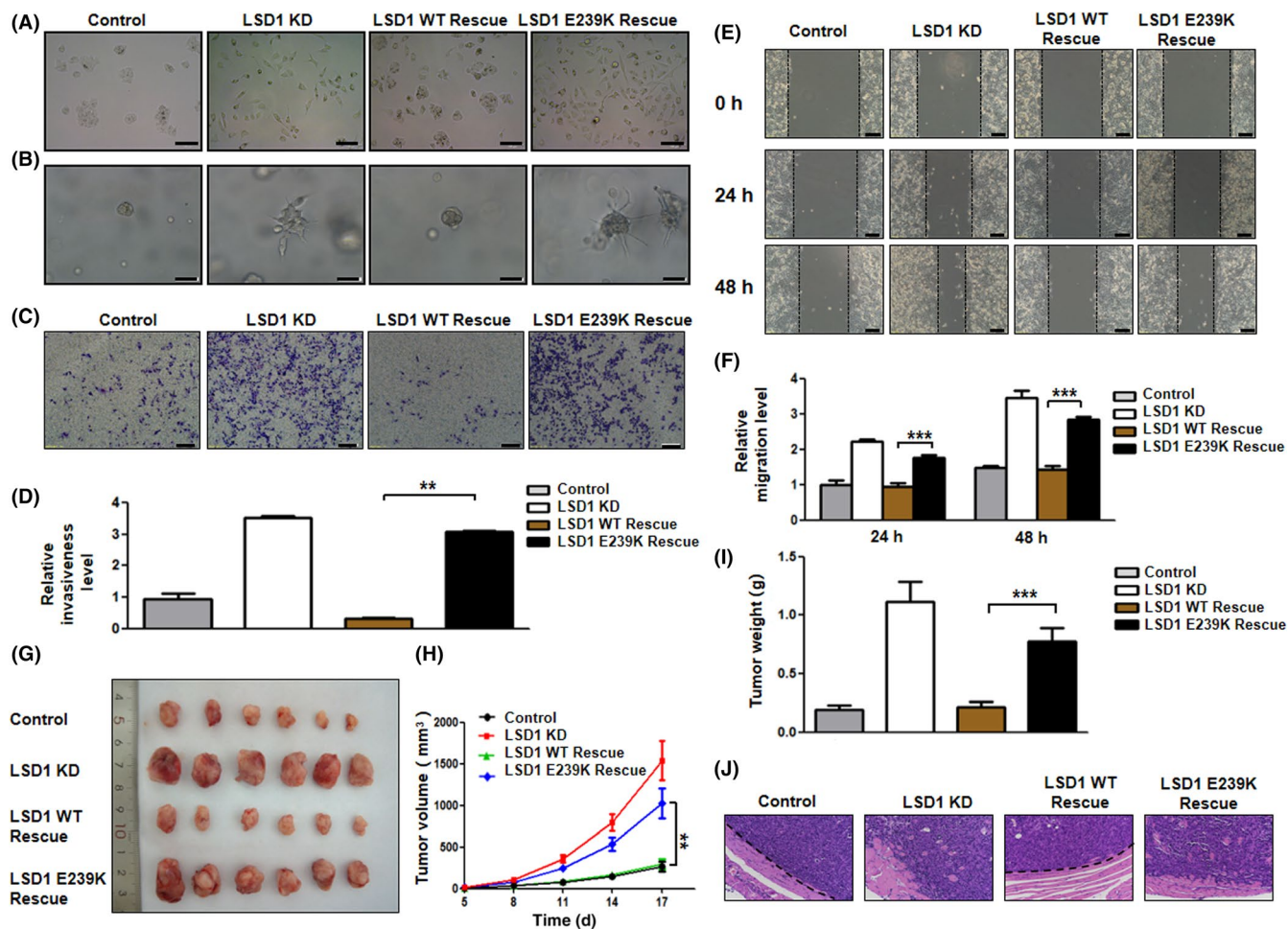
Coimmunoprecipitation and GST pull-down was prepared as previously described.<sup>29</sup> Briefly, 600  $\mu$ g nuclear extract was incubated with LSD1 Abs (ab17721) and Dynabeads. The immunoprecipitated protein complexes were analyzed by western blotting. For GST pull-down assay, the purified GST fusion protein was incubated with MCF7 nuclear extract and analyzed by western blotting.

## 3 | RESULTS

### 3.1 | E239K mutation abolishes suppressive effects of LSD1 on invasion and migration of MCF7 cells

We analyzed the COSMIC Breast Cancer dataset, and identified three missense mutations in the SWIRM domain of LSD1, that is, R187Q, E239K, and R251Q, suggesting that the mutations in the SWIRM domain of LSD1 might be involved in the progression of breast cancer. As the E239K mutation of LSD1 occurred in a patient with ER-positive breast cancer, we chose to investigate the effects of the E239K mutation of LSD1 on ER-positive breast cancer cells<sup>33</sup> (Figure S1A). We established a LSD1 knockdown MCF7 cell line with LSD1 shRNA (LSD1 KD), and restored the expression of WT LSD1 (LSD1 WT Rescue) and E239K mutated LSD1 (LSD1 E239K Rescue) in the LSD1 KD cells with codon-changed LSD1 expression constructs resistant to the LSD1 shRNA (Figure S1B). Compared to the LSD1 WT Rescue cells, the LSD1 E239K Rescue cells showed many distinct mesenchymal features, such as spindle-shaped appearance and forming of filopodia in a 3-D matrix, indicating that the E239K mutation of LSD1 was involved in the modulation of EMT (Figure 1A,B). Furthermore, rescue with the WT LSD1 suppressed the increased invasion of the LSD1 KD cells, whereas rescue with the E239K mutated LSD1 did not suppress the increased invasion (Figure 1C,D). In addition, rescue with the WT LSD1, but not the E239K mutated LSD1, suppressed the migration of the LSD1 KD cells, indicating that the E239K mutation abolished the suppressive effects of LSD1 on the invasion and migration of MCF7 cells (Figure 1E,F).

To examine the influence of the E239K mutation of LSD1 on breast cancer cells in vivo, the Control, LSD1 KD, LSD1 WT Rescue, and LSD1 E239K Rescue cells were subcutaneously injected into immunodeficient mice to evaluate their ability to form xenograft tumors. Rescue with the WT LSD1, but not the E239K mutated LSD1, repressed the growth of tumors (Figure 1G,H). To clarify the role of cell growth in tumor development, we examined the proliferation of cells in each group in vitro, and found that LSD1 E239K Rescue cells proliferated faster than LSD1 WT Rescue cells (Figure S2). Consistently, the xenograft tumors from the mice injected with the LSD1 E239K Rescue cells were significantly heavier than those from the mice injected with the LSD1 WT Rescue cells (Figure 1I). Most importantly, although the xenograft tumors from the mice injected with the LSD1 WT Rescue cells showed intact basement membrane, the xenograft tumors from the mice injected with the LSD1 E239K Rescue cells showed infiltration into neighboring tissues, showing



**FIGURE 1** E239K mutation abolished the suppressive effects of lysine-specific demethylase 1 (LSD1) on the invasion and migration of MCF7 cells. A, Morphology of Control, LSD1 knockdown (KD), LSD1 WT Rescue, and LSD1 E239K Rescue cells. Scale bars, 100  $\mu$ m. B, Morphology of Control, LSD1 KD, LSD1 WT Rescue, and LSD1 E239K Rescue cells in 3-D gel. Scale bars, 50  $\mu$ m. C, Representative images of the staining of Control, LSD1 KD, LSD1 WT Rescue, and LSD1 E239K Rescue cells that invaded through the matrix layer. Scale bars, 100  $\mu$ m. D, Quantification of the relative invasion of the cells from (C) ( $n = 3$ ). E, Representative images of Control, LSD1 KD, LSD1 WT Rescue, and LSD1 E239K Rescue cells in wound-healing assays. Scale bars, 100  $\mu$ m. F, Quantification of the relative migration of Control, LSD1 KD, LSD1 WT Rescue, and LSD1 E239K Rescue cells from (E) ( $n = 9$ ). G, Control, LSD1 KD, LSD1 WT Rescue, and LSD1 E239K Rescue cells were subcutaneously injected into female BALB/C-nu/nu mice ( $n = 6$ ). Tumors formed in these mice are shown. H, Sizes of xenograft tumors were measured every 3 d, and growth of tumors is shown. I, Mean of tumor weight is shown ( $n = 6$ ). J, Xenograft tumors were stained with H&E, and representative images are shown.  $**P < .01$ ,  $***P < .001$

that the LSD1 KD cells rescued with the E239K mutated LSD1 have enhanced invasion capability (Figure 1J).

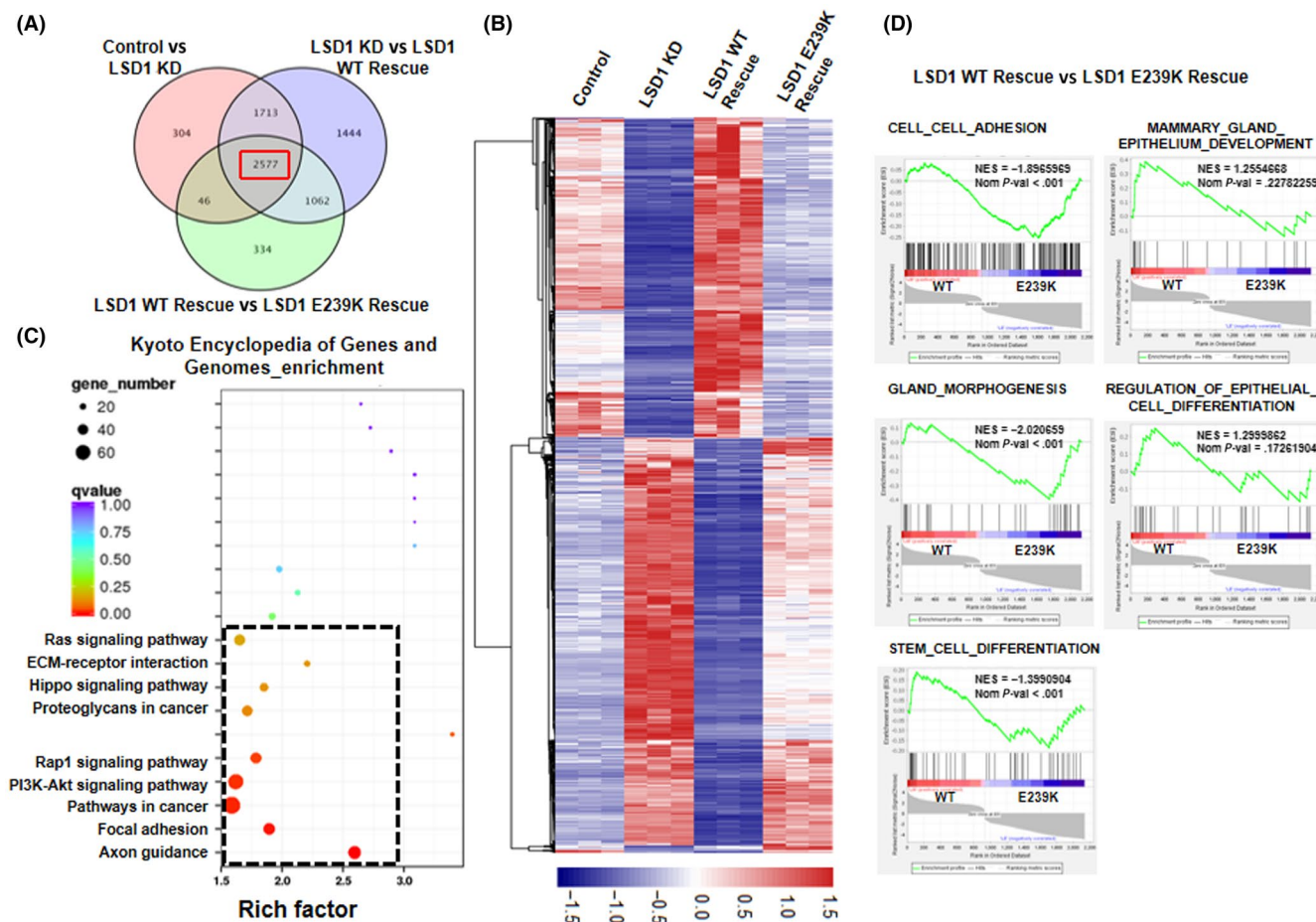
### 3.2 | E239K mutation affects expression of genes associated with mammary gland development

To investigate the molecular mechanism underlying the E239K mutation-mediated invasion and migration, we compared gene expression in the Control, LSD1 KD, LSD1 WT Rescue, and LSD1 E239K Rescue cells, and found that the expression levels of 2577 genes were significantly affected following LSD1 knockdown and the E239K mutation of LSD1 (Figure 2A,B). The Kyoto Encyclopedia of Genes and Genomes analysis demonstrated that these genes were involved in pathways associated with cancer, such as focal adhesion

and PI3K-AKT signaling pathways (Figure 2C). Similarly, the Gene Set Enrichment Analysis showed that several gene sets, including cell-cell adhesion, gland morphogenesis, stem cell differentiation, mammary gland epithelium development, and epithelial cell differentiation, were affected by the E239K mutation of LSD1 (Figure 2D).

### 3.3 | E239K mutation abolishes LSD1-mediated cell adhesion and EMT

As the E239K mutation abolished the suppressive effects of LSD1 on the invasion and migration of MCF7 cells, we examined the expression of genes associated with cell adhesion (Figure 3A). Rescue with WT LSD1 restored the expression of genes involved in EMT in LSD1 KD cells, such as *CLDN3*, *CDH1*, *SNAI2*, *VIM*, and *CTNNA1*, whereas



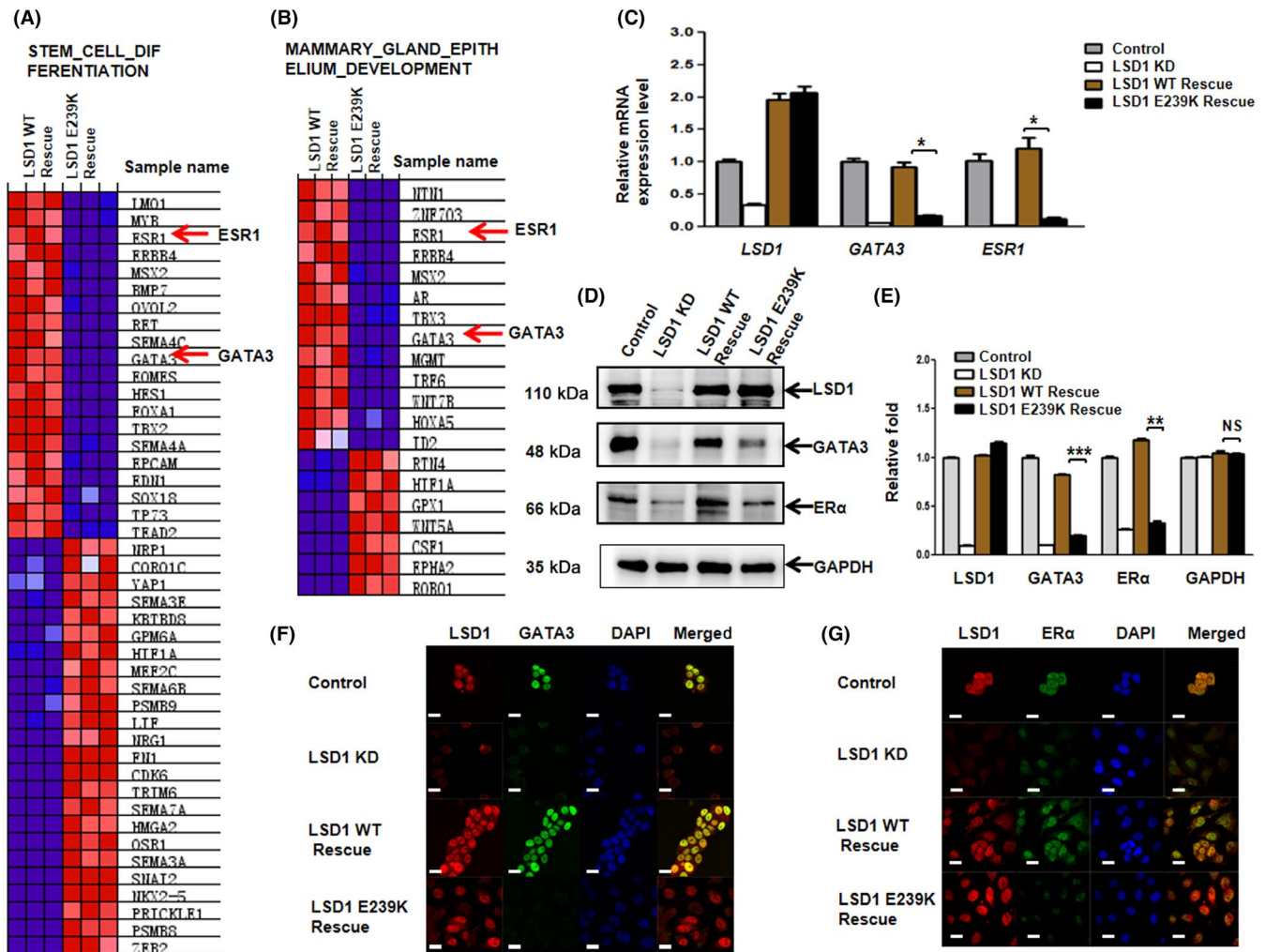
**FIGURE 2** E239K mutation of lysine-specific demethylase 1 (LSD1) affects the expression of genes involved in mammary gland development and epithelial cell differentiation. A, Venn diagrams of RNA sequencing analysis of the genes affected by LSD1 knockdown (KD) and E239K mutation. B, Hierarchical clustering of the 2577 genes in the Control, LSD1 KD, LSD1 WT Rescue, and LSD1 E239K Rescue cells. C, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the 2577 genes affected by both LSD1 knockdown and E239K mutation. D, Gene Set Enrichment Analysis of representative gene sets affected by E239K mutation. NES, normalized enrichment score

rescue with E239K mutated LSD1 did not restore the expression of these genes (Figure 3B). Expression of epithelial markers, including E-cadherin (encoded by *CDH1*), Claudin 3 (encoded by *CLDN3*), and  $\alpha$ -catenin (encoded by *CTNNA1*), were much lower in the LSD1 KD cells rescued with E239K mutated LSD1 than in the LSD1 KD cells rescued with WT LSD1 (Figure 3C). Conversely, the expression of genes that activated EMT, including Slug (encoded by *SNAI2*) and Vimentin (encoded by *VIM*), were much higher in the LSD1 KD cells rescued with E239K mutated LSD1 than in the LSD1 KD cells rescued with WT LSD1 (Figure 3C). Consistently, the immunofluorescent staining showed a decreased expression of E-cadherin and an increased expression of Slug in the LSD1 E239K Rescue cells compared with the LSD1 WT Rescue cells (Figure 3D,E). As Slug was a master regulator of EMT,<sup>15</sup> these results indicated that the E239K mutation might abolish LSD1-suppressed EMT by increasing the expression of Slug. Together, results indicated that the E239K mutation is crucial for LSD1-mediated cell adhesion and EMT.

### 3.4 | E239K mutation abolishes LSD1-mediated regulation of ER $\alpha$ expression

The expression of genes essential for stem cell differentiation and mammary gland epithelium development were affected by the E239K mutation of LSD1 (Figure 4A,B). The expression of ER $\alpha$  (encoded by *ESR1*) and GATA3 was significantly decreased following LSD1 knockdown, and rescue with WT LSD1, but not E239K mutated LSD1, restored the expression of these genes (Figure 4C-E). Consistently, immunofluorescence staining verified the lower level of GATA3 and ER $\alpha$  in cells rescued with E239K mutated LSD1 than in the cells rescued with WT LSD1 (Figure 4F,G). As it has been reported that ER $\alpha$  signaling suppressed EMT in breast cancer by inhibiting the expression of Slug,<sup>9,15</sup> these results indicated that the E239K mutation might abolish LSD1-suppressed EMT by reducing the expression of ER $\alpha$ .

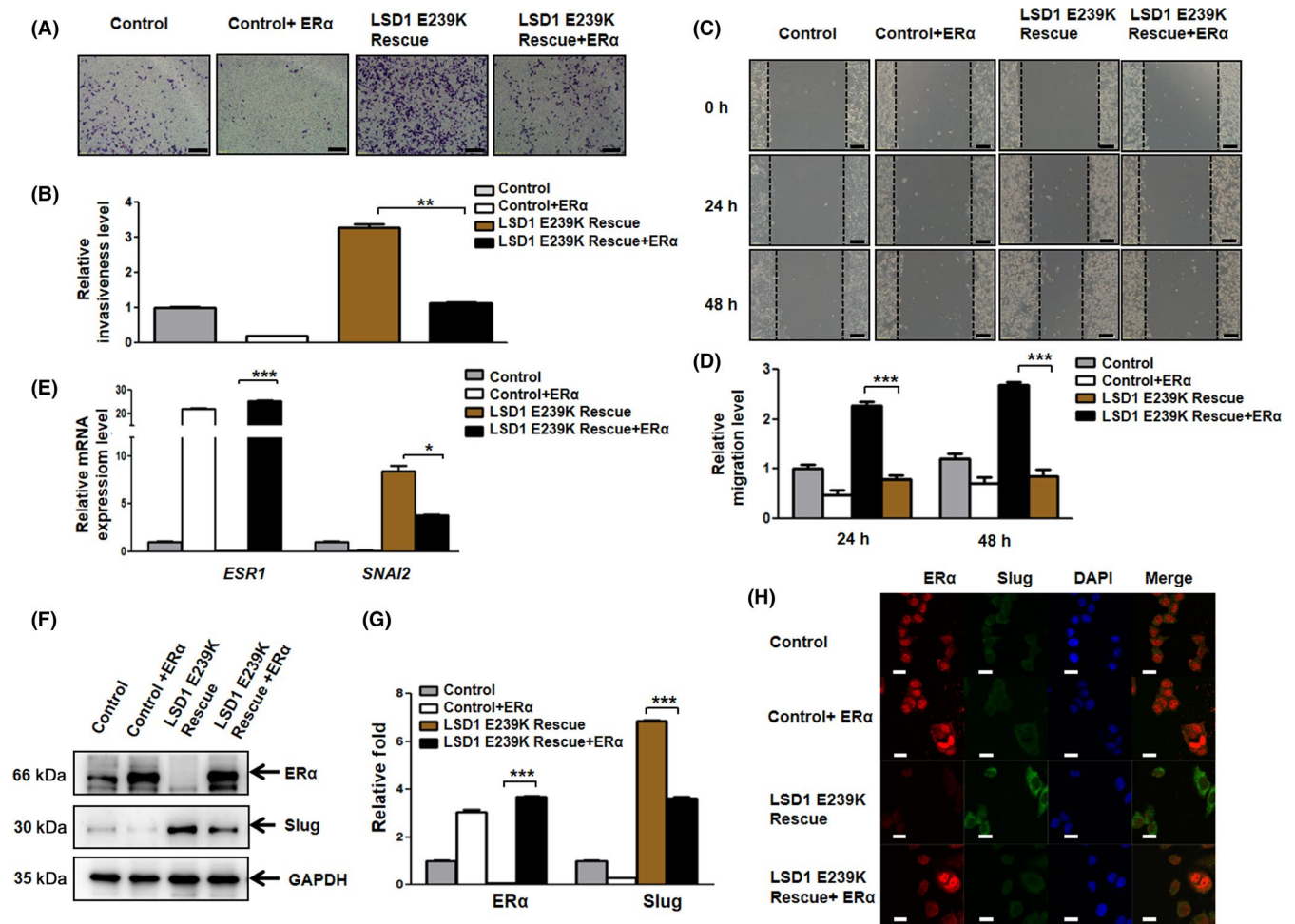




**FIGURE 4** E239K mutation abolishes lysine-specific demethylase 1 (LSD1)-mediated regulation of estrogen receptor  $\alpha$  (ER $\alpha$ ). A, B, Heatmap showing genes with changed expression in LSD1 WT Rescue and LSD1 E239K Rescue cells from the STEM\_CELL\_DIFFERENTIATION gene set (A) and MAMMARY\_GLAND\_EPITHELIUM\_DEVELOPMENT gene set (B). Important breast cancer-related genes are indicated with red arrows. C, D, Quantitative RT-PCR (C) and western blot analysis (D) of the expression of *LSD1*, *GATA3*, and *ESR1* in Control, LSD1 knockdown (KD), LSD1 WT Rescue, and LSD1 E239K Rescue cells. E, Quantification of the relative expression of *LSD1*, *GATA3*, and ER $\alpha$  expression from (D) ( $n = 3$ ). F, Representative images of immunostaining of LSD1 (red) and *GATA3* (green) in the indicated cells. Scale bars, 20  $\mu$ m. G, Representative images of immunostaining of LSD1 (red) and ER $\alpha$  (green) in the indicated cells. Scale bars, 20  $\mu$ m. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ . NS, not significant

mutated LSD1, restored the enrichment of LSD1 at the promoter region of *ESR1*, indicating that the E239K mutation abolished the binding of LSD1 to the promoter of *ESR1* (Figure 6A). Furthermore, LSD1 E239K Rescue cells showed a higher H3K9me2 level at the promoter region of *ESR1* than LSD1 WT Rescue cells, which indicated an increased transcriptional repression state of *ESR1* in cells with E239K mutated LSD1 (Figure 6B). Lysine-specific demethylase 1 required interactions with other chromatin regulators and transcription factors to bind to DNA. Our previous study showed that *GATA3* recruited LSD1 to the promoter region of *TRIM37* in luminal breast cancer cells; in this study, we examined whether the binding of LSD1 to the promoter region of *ESR1* also required *GATA3*.<sup>29</sup> The double-ChIP assay showed that LSD1 and *GATA3* bound to the same region of the *ESR1* promoter (Figure 6C).

Moreover, *GATA3* knockdown caused a reduced binding of LSD1 at the promoter region of *ESR1* (Figure 6D). In addition, the H3K9me2 level at the promoter region of *ESR1* was significantly increased following *GATA3* knockdown (Figure S4). Collectively, those results suggested that *GATA3* recruited LSD1 to the promoter region of *ESR1* and activated the transcription of ER $\alpha$  through demethylation of H3K9me2. As the E239K mutation was located in SWIRM domain of LSD1, which was required for the interaction between LSD1 and *GATA3*,<sup>29</sup> it might disrupt the interaction between LSD1 and *GATA3*. Indeed, the IP results showed that the E239K mutation abolished the interaction between LSD1 and *GATA3* (Figure 6E). Furthermore, the GST pull-down assay also confirmed the reduced interaction between *GATA3* and E239K mutated LSD1 (Figure 6F).



**FIGURE 5** E239K mutation abolishes lysine-specific demethylase 1 (LSD1)-mediated migration and invasion of luminal breast cancer cells through estrogen receptor  $\alpha$  (ER $\alpha$ ). A, Representative images of the staining of Control, Control + ER $\alpha$ , LSD1 E239K Rescue, and LSD1 E239K Rescue + ER $\alpha$  cells that invaded through the matrix layer. Scale bars, 100  $\mu$ m. B, Quantification of the relative invasion of cells from (A) ( $n = 3$ ). C, Representative images of Control, Control + ER $\alpha$ , LSD1 E239K Rescue, and LSD1 E239K Rescue + ER $\alpha$  cells in wound-healing assays. Scale bars, 100  $\mu$ m. D, Quantification of the relative migration of cells from (C) ( $n = 9$ ). E, F, Quantitative RT-PCR (E) and western blot analysis (F) of the expression of *ESR1* and *SNAI2* in Control, Control + ER $\alpha$ , LSD1 E239K Rescue, and LSD1 E239K Rescue + ER $\alpha$  cells. G, Quantification of the relative expression of ER $\alpha$  and Slug from (F) ( $n = 3$ ). H, Representative images of immunostaining of ER $\alpha$  (red) and Slug (green) in the indicated cells. Scale bars, 20  $\mu$ m. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$

## 4 | DISCUSSION

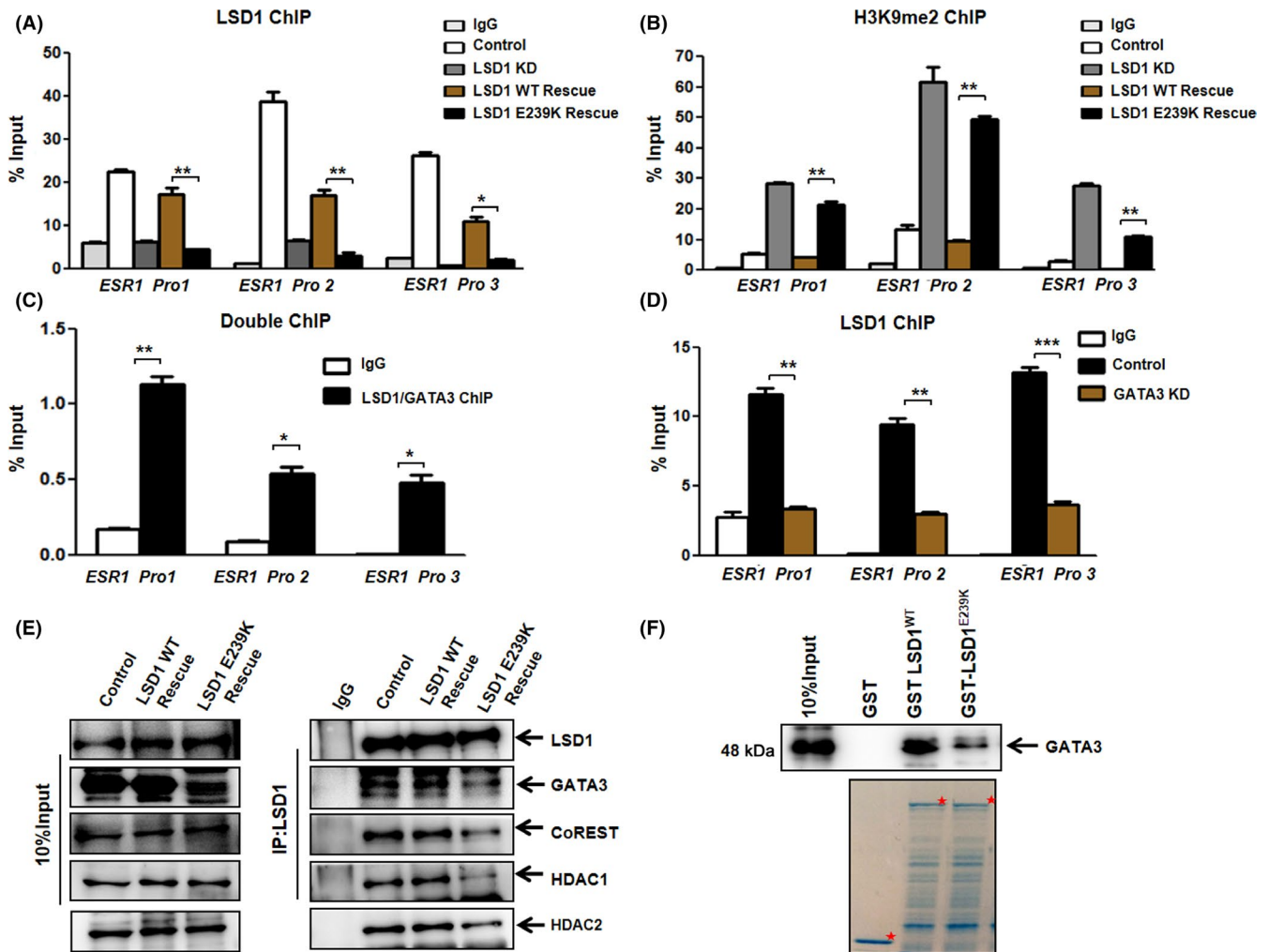
In this study, we showed that the E239K mutation of LSD1 abolished the interaction between LSD1 and GATA3, which led to a decreased expression of ER $\alpha$  through reduced enrichment of LSD1 at the promoter region of *ESR1*. The reduced ER $\alpha$  expression led to decreased epithelial characteristics of MCF7 cells (Figure 7).

Slug is a master regulator of cell differentiation during mammary gland development and the EMT process during progression of breast cancer.<sup>34,35</sup> During mammary gland development, Slug regulates mammary epithelial cell differentiation and lineage commitment programs, and the inhibition of Slug promotes luminal cell differentiation. In breast cancer, Slug promotes EMT by repressing the expression of epithelial markers and activating the expression of luminal markers.<sup>36</sup> In the present study, we found that E239K mutation abolishes LSD1-mediated repression of Slug,

and overexpression of ER $\alpha$  in the LSD1 E239K Rescue cells suppresses the increased expression of Slug, suggesting that LSD1 E239K mutation abolished LSD1-mediated repression of Slug through ER $\alpha$ . This observation is consistent with a previous study, which reported that ER $\alpha$  could bind to the promoter region of Slug and suppress the expression of Slug through HDAC1 or glycogen synthase kinase-3 $\beta$ .<sup>15</sup>

The transcription factor GATA3 regulates the expression of ER $\alpha$ .<sup>16,17</sup> GATA3 binds to two cis-regulatory elements located within the ER $\alpha$  gene, and is required for the recruitment of RNA polymerase II to the ER $\alpha$  gene.<sup>10</sup> Our study shows that GATA3 recruits LSD1 to the promoter region of ER $\alpha$  gene and regulates the expression of ER $\alpha$  by demethylation of H3K9me2, which reveals a novel epigenetic mechanism under which GATA3 regulates ER $\alpha$  expression. FOXA1 is another important transcription factor that regulates the expression of ER $\alpha$  during mammary gland





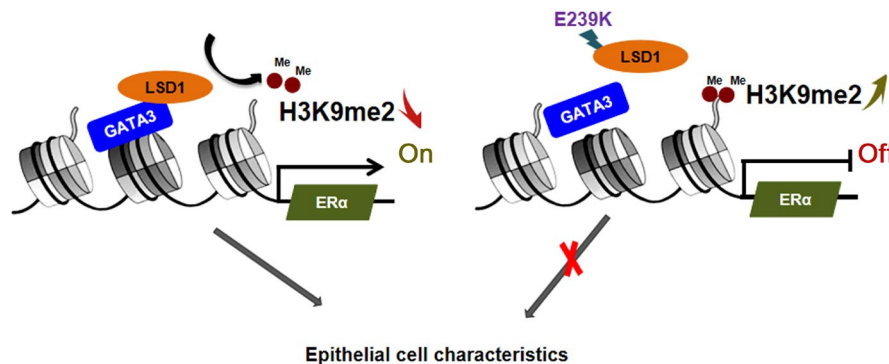
**FIGURE 6** E239K mutation abolishes the binding of lysine-specific demethylase 1 (LSD1) on ESR1 promoter. A, B, ChIP analysis of LSD1 enrichment (A) and H3K9me2 level (B) at the promoter region of the *ESR1* gene in Control, LSD1 knockdown (KD), LSD1 WT Rescue, and LSD1 E239K Rescue cells ( $n = 3$ ). C, Double ChIP analysis showing that LSD1 and GATA3 bind at the promoter region of the *ESR1* gene ( $n = 3$ ). D, ChIP analysis showing decreased enrichments of LSD1 at the promoter region of the *ESR1* gene following GATA3 knockdown ( $n = 3$ ). E, Nuclear extracts from Control, LSD1 WT Rescue, and LSD1 E239K Rescue cells were immunoprecipitated by an anti-LSD1 Ab and analyzed by western blotting with the indicated Abs. F, GST pull-down assays showing the reduced interaction between GATA3 and E239K mutated LSD1. A Coomassie blue staining image of PAGE gel is shown to confirm the similar expression level of LSD1. Asterisks indicate the positions of GST-tagged LSD1. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$

development, which binds to the promoter region of the ER $\alpha$  gene and recruits RNA polymerase II for transcription.<sup>11</sup> Interestingly, LSD1 E239K mutation also causes a decreased expression of FOXA1 (Figure 4A). Further study is needed to investigate whether FOXA1 is also involved in the E239K mutation-mediated downregulation of ER $\alpha$ .

As LSD1 does not have a DNA-binding domain, it requires the interaction with other transcription factors to regulate gene expression.<sup>37</sup> Our previous study showed that LSD1 interacts with GATA3 through the SWIRM domain of LSD1 and this interaction is crucial to the progression of luminal breast cancer cells.<sup>29</sup> The present study shows that the cancer-associated E239K mutation in the SWIRM domain of LSD1 abolishes the interaction between LSD1 and GATA3, which confirms the crucial role of the SWIRM domain on forming the functional LSD1/GATA3 protein complex. In addition to the E239K

mutation, we identified R187Q and R251Q mutation in LSD1 from the COSMIC Breast Cancer dataset. These mutations were also in the SWIRM domain of LSD1; therefore, mutation in the SWIRM domain might be a common type of mutation that disrupts the function of LSD1 in breast cancer.

To investigate the functional effects of E239K mutated LSD1 on EMT in the presence of the WT LSD1, we tried overexpression of E239K mutated LSD1 in MCF7 cells (Figure S5A). Overexpression of WT LSD1 suppressed the invasion and migration of MCF7 cells, whereas overexpression of E239K mutated LSD1 did not significantly affect the invasion and migration of MCF7 cells (Figure S5B-E). Consistently, overexpression of E239K mutated LSD1 did not affect the expression of GATA3, ER $\alpha$ , or EMT-related genes (Figure S6). Collectively, these results indicated that E239K mutated LSD1 do not have dominant effects over WT



**FIGURE 7** Schematic representation of the functional effects of E239K mutation on the epithelial to mesenchymal transition (EMT) of luminal breast cancer cells. E239K mutation abolishes the interaction between lysine-specific demethylase 1 (LSD1) and GATA3, which leads to decreased enrichment of LSD1 at the promoter region of the estrogen receptor  $\alpha$  ( $ER\alpha$ ) gene that reduces the expression of  $ER\alpha$  by enhancing the H3K9me2 level. Reduced  $ER\alpha$  expression promotes the EMT of luminal breast cancer cells by blocking the epithelial characteristics of cells

LSD1. However, we still think that the heterozygous E239K mutation of LSD1 might have an impact on EMT in breast cancer. First, the heterozygous E239K mutation of LSD1 might reduce the expression of WT LSD1, which could potentially alter the expression of many genes. Second, patients with the heterozygous mutation of LSD1 could have mutations on other epigenetic regulators, such as GATA3, which might disrupt the epigenetic regulation in the mammary gland epithelial cells further, and collaboratively promote tumorigenesis.

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#### DISCLOSURE

Authors declare no conflicts of interest for this article.

#### ETHICS STATEMENT

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Jilin University and approved by the Animal Ethics Committee of Department of Science and Technology of Jilin Province.

#### DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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#### SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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