

Expression of lactate dehydrogenase C in MDA-MB-231 cells and its role in tumor invasion and migration

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Abstract. The cancer/testis antigen (CTA) lactate dehydrogenase C (LDHC) is a unique LDH isoenzyme associated with glucose and adenosine triphosphate production in mammalian germ cells. However, the role of LDHC in cancer has thus far largely remained elusive. The present study described the expression status of LDHC in human MDA-MB-231 breast cancer cells as well as its role in tumor invasion and migration. Immunohistochemical analysis revealed endogenous LDHC expression in the cytoplasm and nuclei of MDA-MB-231 cells yielded. In addition, *in vitro* cell invasion and migration assays revealed that when LDHC expression was blocked by its specific inhibitor, cell invasion and migration were compromised in MDA-MB-231 cells. Of note, inhibition of LDHC was unable to induce apoptosis in MDA-MB-231 cells. The present study provided evidence that the LDHC enzyme acts as a CTA in breast carcinoma and exerts an essential role in tumor invasion and migration.

Introduction

Lactate dehydrogenase (LDH) is an oxidoreductase widely distributed among vertebrates, plants and bacteria. It catalyzes the interconversion of pyruvate to L-lactate with concomitant reduction of NADH in the glycolytic pathway (1). LDH possesses two sub-units designated as LDH-A and -B (2). Functionally, these two subunits can assemble into catalytically active hetero- or homotetramers and generate five tetrameric isoenzymes (2). The discovery of the sixth isozyme in mature human germ cells indicated the presence of an additional

sub-unit of LDH, described as LDHC or LDHX (3). Of note, the catalytic activity of each LDH isoenzyme is determined by its composition as well as its tissue-specific expression pattern, which varies among mammalian tissues. For instance, LDHA is abundant in anaerobic tissues such as skeletal muscle, where oxygen deficiency following exercise requires glycolysis to meet metabolic requirements (4), whereas LDHB predominately locates in the brain and heart (5). In particular, expression of LDHC is confined to the germinal epithelium of the testes and is mostly contained in mature testis as well as in spermatocytes, spermatids and spermatozoa (6,7). LDHC is associated with glucose and is essential for the maintenance of glycolysis and adenosine triphosphate (ATP) production in sperm flagella (6,8-11).

Cancer/testis antigens (CTAs) are expressed only in the germinal epithelium of the testis as well as in certain types of cancer cell, while being absent in non-cancerous somatic tissues. Among them, the LDHC gene has been shown to be expressed in a large variety of human tumor types, with frequent occurrence in lung cancer, breast cancer and melanoma (12). However, the exact functional role of LDHC in tumors has remained to be elucidated. It is well known that cancer cells produce a substantial amount of energy through aerobic glycolysis even in the presence of adequate levels of oxygen. Tumor metastasis is promoted by lactate-induced secretion of hyaluronan by tumor-associated fibroblasts that create a milieu favorable for migration (13). Of note, lactate itself has been demonstrated to induce the migration of cells and cell clusters (13). As lactate is the substrate of LDHC, it is likely that LDHC activation in cancer may depend on lactate for ATP production. Therefore, the present study hypothesized that LDHC may exert a functional role in breast cancer metastasis. To validate this hypothesis, the present study assessed the expression of LDHC in human MDA-MB-231 breast cancer cells by using immunohistochemistry (IHC) as well as reverse-transcription polymerase chain reaction (RT-PCR) and western blot analyses. In addition, *in vitro* cell invasion and migration assays were performed to examine the functional role of endogenous LDHC in tumor invasion and migration. In a loss-of-function experiment LDHC was blocked with its specific inhibitor *N*-propyl oxamate (14), which reduced the invasion and migration of MDA-MB-231 cells. Furthermore,

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the effects of LDHC inhibition on the apoptotic rate of MDA-MB-231 cells was assessed. The present study enhanced the current understanding of the CTA status of LDHC in breast cancer.

Materials and methods

Materials. The MDA-MB-231 and MCF-7 human breast cancer cell lines were purchased from the Chinese Academy of Sciences (Shanghai, China). The main reagents used in the present study were as follows: L-15 medium, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) (all from Hyclone, Logan, UT, USA); RT-PCR kit (cat. no. K1622; Fermentas; Thermo Fisher Scientific, Waltham, MA, USA); TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific); PCR kit (cat. no. PC0923; Biomed, Beijing, China); oxamate (Sigma-Aldrich, St. Louis, MA, USA), which was further synthesized to *N*-propyl oxamate by Yuhao Chemical Technology Co., Ltd. (Hangzhou, China); rabbit anti-human LDHC, anti-B-cell lymphoma 2 (Bcl-2) and anti-Bcl-2-associated X protein (Bax; Abcam, Cambridge, MA, USA); rabbit anti-caspase-9 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA); Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis kit (cat. no. KGA107; KeyGEN Biotech, Nanjing, China); EnVision diaminobenzidine (DAB) detection kit (cat. no. KIT-5903; Fuzhou Maixin Biotech, Fujian, China); phosphate-buffered saline (PBS), Tween-20, Bicinchoninic Acid (BCA) Protein Quantification kit (cat. no. P0012S), mouse anti-human β -actin and Colorimetric terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) Apoptosis Assay kit (cat. no. C1098; Beyotime Institute of Biotechnology, Haimen, China).

Cell culture. MDA-MB-231 cells were cultured in L-15 medium and MCF-7 cells were cultured in DMEM medium. Media were supplemented with 10% fetal bovine serum (FBS) and cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. Prior to the assays, a proportion of the cells was treated with *N*-propyl oxamate (0.05 μ M) for 12 h in order to inhibit LDHC.

IHC analysis of LDHC. IHC assays were performed on 4- μ m formalin-fixed, paraffin-embedded tissue/cells using rabbit monoclonal anti-human LDHC primary antibody (cat. no. ab52747) with the EnVision DAB detection kit. Slides were dried overnight at 65°C, de-paraffinized in xylene (Xilong Chemical Co., Ltd., Guangdong, China) and then de-hydrated via a series of graded alcohols (Sinopharm Chemical Regent Co., Ltd., Shanghai, China). Endogenous peroxidase activity was inhibited by incubating the sections in H₂O₂ inhibition buffer (Jianning Pharmacy, Hebei, China) at 37°C for 10 min. Non-specific binding sites were blocked with 10% normal goat serum for 10 min at 37°C. Antigen retrieval was performed with boiling citrate buffer (Fuzhou Maixin Biotech) utilizing in a pressure cooker at 100~120°C for 3 min. Sections were then incubated with LDHC antibody (1:200 dilution) for 90 min at 37°C. The slides were then washed in PBS, followed by incubation with the secondary antibody for 30 min at room temperature. DAB was applied for 2 min and then removed by rinsing with distilled water. Slides were counterstained with

hematoxylin (Fuzhou Maixin Biotech) and observed by light microscopy (Olympus BX40; Olympus, Tokyo, Japan) at x400 magnification.

Semi-quantitative RT-PCR analysis. Total RNA was extracted from treated MDA-MB-231 cells using TRIzol[®] reagent, and the concentration of RNA (80~650 μ g/ml) was determined using the Thermo Multiskan Spectrophotometer Type 1510 (Thermo Fisher Scientific, Inc.). Total RNA was reverse-transcribed into cDNA using the RT-PCR kit (cat. no. K1622; Thermo Fisher Scientific, Inc.). Briefly, 2 μ g total RNA was added to 1 μ l hexamer primer and ddH₂O to a total volume of 12 μ l, and incubated at 65°C for 5 min. Subsequently, 5X Reaction buffer (4 μ l), 1 μ l RNase inhibitor, 10 mM dNTP Mix (2 μ l) and 1 μ l Reverse Transcriptase were added to the reverse transcription reaction mixture to make a total volume of 20 μ l. Amplification of the RNA was achieved utilizing the following reaction conditions: 25°C for 5 min, followed by 45°C for 1 h and 70°C for 5 min. cDNA (0.1 μ g) was then amplified by PCR using the following primers (Sangon Biotech, Co., Ltd., Shanghai, China): LDHC forward, 5'-ACT CTGCCCCGTTTCCGTTACCT-3' and reverse, 5'-CCCCAC TCCATAAGGGGCACACT-3'; GAPDH forward, 5'-CAAGGT CATCCATGACAACCTTTG-3' and reverse, 5'-GTCCACCAC CCTGTTGCTGTAG-3' as described previously (15). PCR was performed using an Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The thermocycling conditions were as follows: Initial denaturation for 5 min at 94°C followed by 30 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec, and a final extension for 7 min at 72°C. The PCR products were separated by 1.5% agarose gel electrophoresis (110 V, 20 min). Images of the PCR products were captured using the Gel Imaging System (Clinx Science Instruments Co., Ltd., Shanghai, China) and densitometry analyses were conducted using ImageJ software, version 1.43b (National Institutes of Health, Bethesda, MA, USA).

Western blot analysis. Cell lysates were prepared using radio-immunoprecipitation assay protein lysis buffer (Beyotime Institute of Biotechnology) and the protein extracts were quantified using a BCA Protein Quantification kit (cat. no. P0012S; Beyotime Institute of Biotechnology). Equal amounts of protein (20 μ g) were then subjected to electrophoresis on a 12% SDS-PAGE gel. The proteins were transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA) and blocked in Tris-buffered saline (TBS) containing 5% non-fat milk powder (Zoman Biotechnology Co., Ltd., Beijing, China). Subsequently, the membranes were incubated with rabbit anti-human LDHC (1:1,000 dilution; cat. no. ab52747) and anti-human Bcl-2 (1:1,000 dilution; cat. no. ab32124) monoclonal antibodies, and rabbit anti-human Bax (1:500 dilution; cat. no. ab53154) and anti-human caspase-9 (1:500 dilution; cat. no. sc-8355) polyclonal antibodies at 4°C overnight. As a loading control, mouse anti-human β -actin monoclonal antibody (1:1,000 dilution; cat. no. AA128) was used. Following washing three times with TBS containing 0.2% Tween-20, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution; cat. no. ab6721; Abcam, Cambridge, MA, USA) and

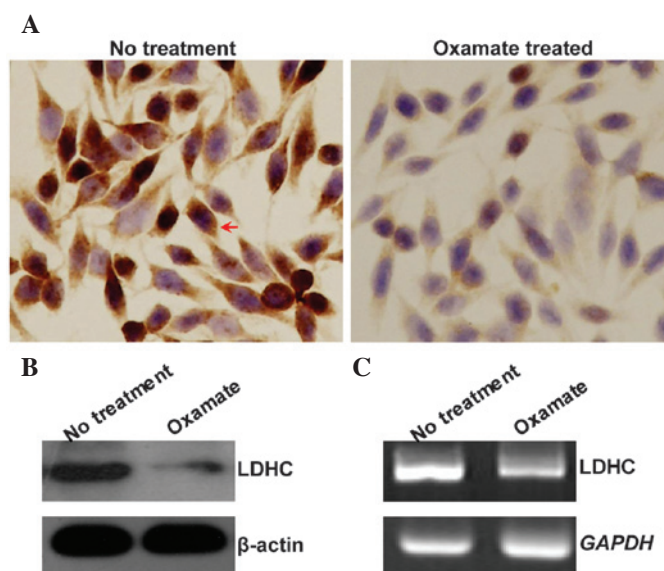


Figure 1. Expression of LDHC in MDA-MB-231 cells. (A) Immunohistochemical detection of LDHC. The red arrow indicates the sub-cellular location of LDHC (magnification, x200). Cells were stained with hematoxylin. (B) Western blot analysis of LDHC with β -actin used as control protein. (C) Reverse-transcription polymerase chain reaction analysis of LDHC. LDHC expression was blocked with the specific inhibitor oxamate ($0.05 \mu\text{M}$) for 12 h. Oxamate, *N*-propyl oxamate; LDHC, lactate dehydrogenase C.

anti-mouse IgG (1:10,000 dilution; cat. no. ab6789; Abcam) polyclonal antibodies at room temperature for 1-2 h. The immunoreactive bands were detected by the enhanced chemiluminescence luminol reagent (Zoman Biotechnology Co., Ltd.). Images of the membranes were captured using Microtek ScanMaker 4900 (Microtek, Shanghai, China), and the protein bands were quantified by densitometry using ImageJ software, version 1.43b.

Wound healing migration assay. Monolayer cells (5×10^5 cells/well) were seeded in serum-free L-15 medium and incubated in 6 well plates with *N*-propyl oxamate ($0.05 \mu\text{M}$) for 12 h. Following culturing of cells in six-well plates to 90% confluence, a scratch through the cell monolayer was introduced by using a $20 \mu\text{l}$ volume pipette tip (Eppendorf Lab Technologies (Shanghai) Co., Ltd., Shanghai, China). Images were captured immediately following wounding and following 48 h of incubation, and the number of cells that had migrated into the scratched area was determined. This number was normalized to the scratch area. Results were obtained from three independent wound healing experiments.

In vitro cell invasion assay. The invasion assay was performed as described by Ma *et al* (16). Briefly, Millicell Hanging Cell Invasion Chambers with $8\text{-}\mu\text{m}$ pore filters (Millipore Corp., Billerica, MA, USA) were coated with $12 \mu\text{l}$ ice-cold Matrigel (12.0 mg/ml; BD Biosciences, San Jose, CA, USA). MDA-MB-231 cells (1.2×10^5 per well) or MCF-7 cells (1×10^5 per well) were added to the upper chambers of these inserts in $600 \mu\text{l}$ serum-free L-15 or DMEM medium, respectively. The inserts were then placed into 24-well plates containing L-15 or DMEM medium, respectively, containing 10% FBS. Following 48 h of incubation, cells were fixed with methanol (Sinopharm Chemical Reagent Co., Ltd.) and stained

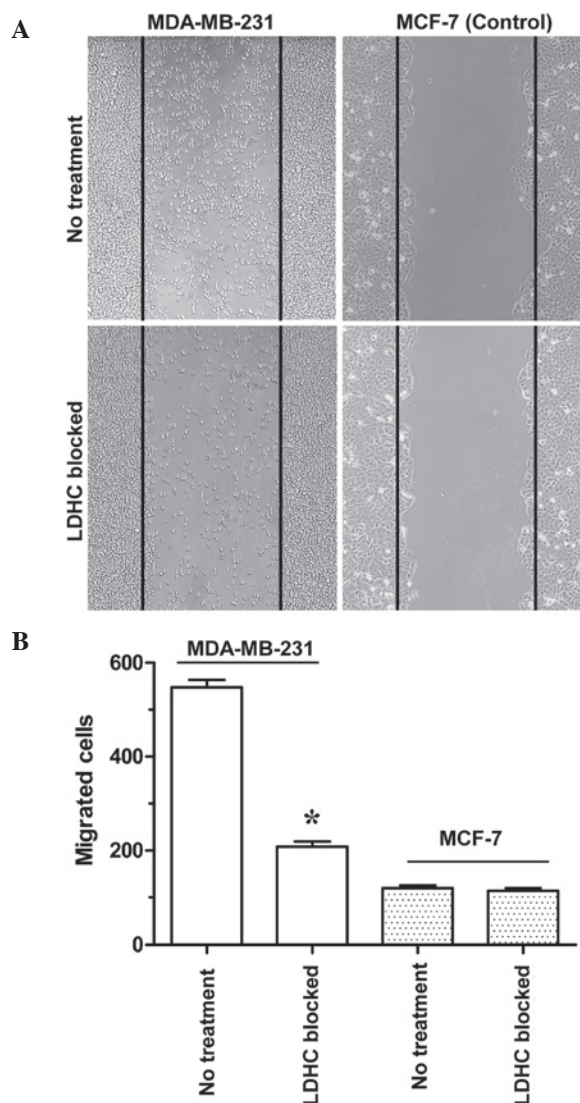


Figure 2. Wound healing migration assay. (A) Relative motility was indicated by the ability of the cells to migrate into a scratch wound of the confluent cell monolayer (magnification, x200). (B) Cell migration was assessed at 48 h by counting the number of cells that had migrated into the wounded area. MCF-7 cells (LDHC-negative) were applied as a control. Values are expressed as the mean \pm standard deviation ($n=3$). * $P<0.05$ vs. paired untreated group. LDHC, lactate dehydrogenase C.

with Giemsa (Sigma-Aldrich). Cells on the lower side of the filter were counted under a light microscope (Olympus) at x200 magnification and images were captured.

TUNEL analysis. The TUNEL assay detects nucleosome-sized DNA fragments by labeling their 3'-OH ends with digoxigenin nucleotides using terminal deoxynucleotidyl transferase (TdT) (17). In the present study, a Colorimetric TUNEL Apoptosis Assay kit was used following the manufacturer's instructions. Briefly, following incubation with $0.05 \mu\text{M}$ *N*-propyl oxamate for 48 h, the cells were fixed with 4% paraformaldehyde and incubated with TdT buffer. The numbers of TUNEL-positive cells were counted under a microscope (Olympus) at x400 magnification.

Flow cytometric analysis. The apoptotic rate (%) was assessed by flow cytometry according to a method described in a

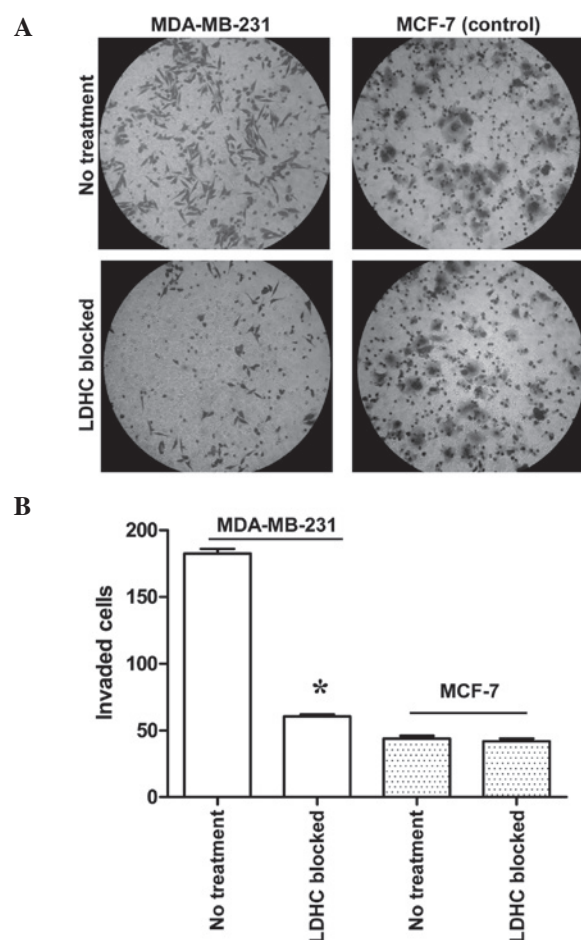


Figure 3. LDHC enhances cell invasion as indicated by a Transwell assay. (A) Cells on the lower side of the filter observed at 48 h (magnification, x200). Cells were stained with Giemsa. (B) The number of invaded cells was determined by counting cells on the lower side of the filter. MCF-7 cells (LDHC-negative) were applied as a control. Values are expressed as the mean \pm standard deviation (n=3). *P<0.05 vs. paired untreated group. LDHC, lactate dehydrogenase C.

previous study by our group (18). In brief, cells were collected and stained with Annexin V-FITC and PI following the manufacturer's instructions, and analyzed using a BD FACSCalibur flow cytometer (BD Biosciences).

Statistical analysis. Values are expressed as the mean \pm standard deviation. Student's *t*-test was used for comparison of differences between groups using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

LDHC is expressed in MDA-MB-231 cells. LDHC has been shown to be expressed in certain types human cancer and is likely to be linked with cancer cell growth and metastasis (12). To investigate whether LDHC was expressed in breast cancer cell lines, MDA-MB-231 cells were examined by IHC using an antibody specific for LDHC. As displayed in Fig. 1A, positive LDHC staining was detected in the cytoplasm and nuclei of MDA-MB-231 cells. Furthermore, when MDA-MB-231 cells were treated with *N*-propyl oxamate, an

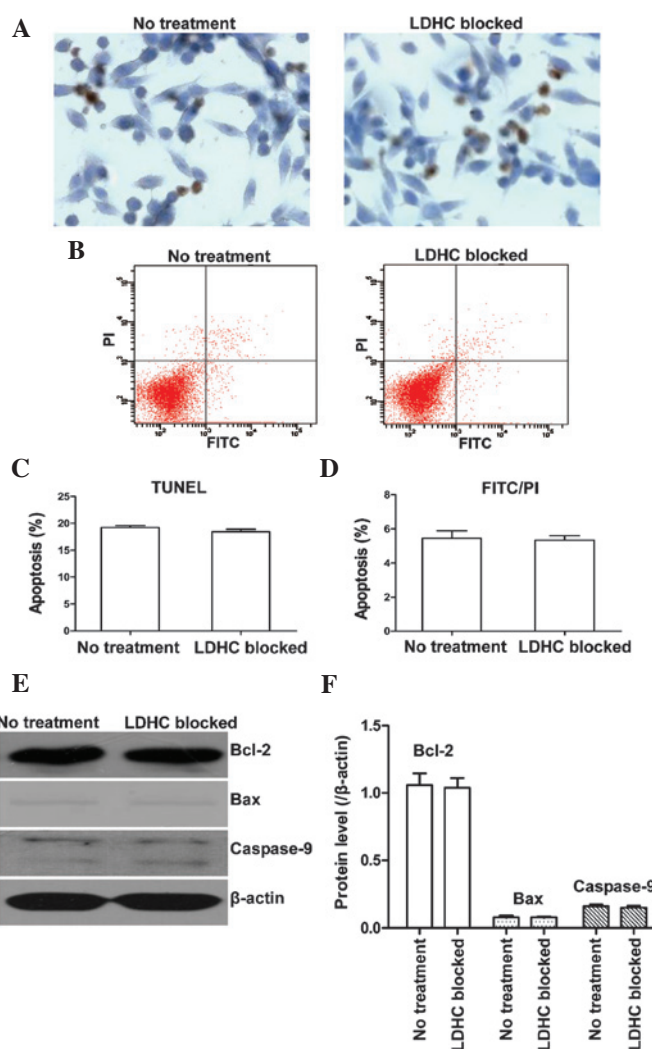


Figure 4. Effects of LDHC inhibition on apoptosis of MDA-MB-231 cells. (A) TUNEL staining (magnification, x400). (B) Flow cytometric dot plots showing Annexin V-FITC and PI staining. (C and D) The apoptotic rates were determined by quantification of A and B, respectively. (E) Expression of apoptosis-associated proteins was assessed by western blot analysis at 48 h. (F) Protein levels were quantified by densitometric analysis of band intensities with normalization to β -actin. Values are expressed as the mean \pm standard deviation (n=2). LDHC, lactate dehydrogenase C; FITC, fluorescein isothiocyanate; PI, propidium iodide; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

LDHC-specific inhibitor, the expression of LDHC was down-regulated (Fig. 1A). LDHC mRNA and protein expression in MDA-MB-231 cells and its downregulation by *N*-propyl oxamate were further confirmed by RT-PCR and western blot analyses (Fig. 1B and C).

Inhibition of LDHC reduces the migration and invasion of MDA-MB-231 cells. In order to eliminate the confounders that *N*-propyl oxamate may either be naturally implicated tumor cell metabolism or has an impact on cell migration and invasion, the LDHC-negative cell line MCF-7 was used as a negative control. The wound healing and Transwell assays showed that inhibition of endogenous LDHC decreased the migration and invasion of MDA-MB-231 cells by 2- to 3-fold (P<0.05) (Figs. 2 and 3), while *N*-propyl oxamate had no impact on MCF-7 cell migration and invasion (P>0.05).

These results strongly suggested that LDHC is linked with MDA-MB-231 cell migration and invasion.

Inhibition of LDHC does not affect the apoptotic rate of MDA-MB-231 cells. To investigate whether LDHC inhibition induces apoptosis in MDA-MB-231 cells, TUNEL and flow cytometric assays were performed. The results indicated that a inhibition of endogenous LDHC in MDA-MB-231 cells did not trigger apoptosis, as the apoptotic rate of the control cells was similar to that of *N*-propyl oxamate-treated cells ($P>0.05$) (Fig. 4A-D). In parallel, western blot analysis revealed that the levels of apoptosis-associated proteins in MDA-MB-231 cells, including Bcl-2, Bax and caspase-9, were not significantly altered by *N*-propyl oxamate ($P>0.05$) (Fig. 4E and F). These results indicated that at the used concentration, *N*-propyl oxamate, and therefore the inhibition of LDHC, did not induce apoptosis in breast cancer cells.

Discussion

The present work study demonstrated for the first time that LDHC was expressed in MDA-MB-231 cells in the cytoplasm and in the nucleus. In addition, it was indicated that endogenous LDHC may be involved in the metastasis of breast cancer. However, inhibition of LDHC did not induce apoptosis in MDA-MB-231 cells.

CTAs, which are expressed in healthy individuals in the germinal epithelium of the testis only, have also been observed to be expressed in certain types of cancer. At present, CTAs are considered to be promising candidate diagnostic markers and immunotherapeutic targets for cancer. As one of these CTAs, the testis-specific LDHC gene has been shown to be expressed in a broad spectrum of human carcinomas, with a high frequency in lung cancer, breast cancer and melanoma (12). Consistent with these data, the present study revealed that endogenous LDHC was expressed in the MDA-MB-231 breast cancer cell line. IHC analysis demonstrated that the LDHC enzyme was located in the cytoplasm and the nuclei of MDA-MB-231 cells. While the testis-specific status of LDHC has been documented by a large number of studies (6-11), LDHC also acts as a CTA in certain types of cancer cell (12). The mechanism of LDHC activation in cancers is likely to be highly complex. De Smet *et al* (19) previously highlighted that CTA genes, including LDHC, may escape transcriptional repression in adult somatic tissues in the course of malignant transformation by promotor de-methylation. However, Koslowski *et al* (12) found that treatment with a genome de-methylation agent did not induce LDHC expression in tumor cells.

Compared with normal cells, the metabolism is known to be altered in cancer cells, shifting away from the tricarboxylic acid cycle toward glycolysis, which increases glucose consumption and lactate production. Of note, metastasis of tumors is promoted by lactate-induced secretion of hyaluronan by tumor-associated fibroblasts to create a milieu favorable for migration. Thus, lactate itself has been found to induce the migration of cells and cell clusters (13). The LDHC enzyme is associated with glucose and has an essential role in the maintenance of glycolysis and ATP production in sperm cells (6,8-11). As lactate is the substrate

of LDHC, the present study hypothesized that LDHC is associated with metastasis of breast cancer. To gain insight into the possible function of LDHC in breast cancer metastasis, endogenous LDHC was inhibited in MDA-MB-231 cells using its specific inhibitor *N*-propyl oxamate as described previously (14). As expected, migration and invasion assays revealed that the inhibition of endogenous LDHC expression compromised the metastatic potential of MDA-MB-231 cells. To the best of our knowledge, the present study demonstrated for the first time that the presence of endogenous LDHC enhanced MDA-MB-231 cell migration and invasion. To exclude the possibility that inhibitor *N*-propyl oxamate itself may naturally yield anti-cancer effects or influence cell metastasis, the LDHC-negative cell line MCF-7 was used as a control. The results showed that *N*-propyl oxamate (0.05 μ M) did not affect the migration and invasion of MCF-7 cells, while it reduced that of MDA-MB-231 cells. In support of this finding, Miskimins *et al* (20) showed that *N*-propyl oxamate alone at concentrations <80 mM only had a weak cytotoxic effect on cancer cell lines, including MCF-7. Therefore, it may be hypothesized that LDHC exerts an essential role in the invasion and migration of MDA-MB-231 cells and accordingly in the metastasis of breast cancer.

Studies have indicated that another LDH isoenzyme, LDHA, is overexpressed in a wide range of tumor types, including breast cancer (4,5,21), and that the inhibition of LDHA resulted in tumor-cell apoptosis (22). The LDHC-specific inhibitor *N*-propyl oxamate, which was used in the present study, is 146 and 74 times more selective for LDHC than for LDH-A4 and LDH-B4, respectively (14), differs from sodium oxamate, a broad-spectrum LDH inhibitor used for LDHA inhibition. Furthermore, the concentration of *N*-propyl oxamate (0.05 μ M) used in the present study was markedly lower than the concentration of sodium oxamate ($IC_{50}>90$ mM) required for LDHA inhibition. Correspondingly, the apoptosis assays performed in the present study further confirmed that inhibition of LDHC by *N*-propyl oxamate in MDA-MB-231 cells had no obvious apoptotic effects.

To a certain extent, the experimental design of the present study, in which LDHC-negative MCF7 cells were used as a negative control, may have eliminated the possible confounding effects of *N*-propyl oxamate on the metastasis of MDA-MB-231 cells. Despite the promising results, the present study had certain limitations: First, LDHC expression was downregulated using a pharmacological inhibitor instead of RNA interference; secondly, the MDA-MB-231 cell line used in the present study was not primarily cultured, and thirdly, the repetition size in our blot analysis ($n=2$) was relatively small. Finally, ATP and lactate levels were not assessed in LDHC-blocked MDA-MB-231 cells.

Previous studies have shown that LDHA inhibition resulted in cell apoptosis in both breast cancer and non-small cell lung carcinoma (21,22); however, in the present study, LDHC inhibition failed to promote apoptosis in MDA-MB-231 cells, as indicated by TUNEL and flow cytometric analyses. Correspondingly, expression of Bcl-2, Bax and caspase-9 in MDA-MB-231 cells was not significantly altered by *N*-propyl oxamate treatment. It remains to be fully elucidated whether LDHC inhibition is able to trigger apoptosis in tumor cells. A previous study reported that LDHA

inhibition with sodium oxamate resulted in ATP reduction and a reactive oxygen species burst in cancer cells, which lead to apoptosis and G2/M-phase arrest in H1395 non-small cell lung cancer cells (22). However, as the unique biological effects of LDHC differ from those of LDHA, they require further elucidation.

In conclusion, the present study was the first to demonstrate that endogenous LDHC was expressed in MDA-MB-231 breast cancer cells and implicated in cancer metastasis. However, inhibition of LDHC did not affect the apoptotic rate of breast cancer cells. Future studies by our group will assess the biological significance of LDHC in specific metabolic processes in tumors.

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