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Lactate Dehydrogenase C Produces S-2-Hydroxyglutarate in Mouse Testis

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Abstract

Metabolomics is a valuable tool for studying tissue- and organism-specific metabolism. In normal mouse testis, we found 70 μM S-2-hydroxyglutarate (2HG), more than 10-fold greater than in other tissues. S-2HG is a competitive inhibitor of α-ketoglutarate-dependent demethylation enzymes and can alter histone or DNA methylation. To identify the source of testis S-2HG, we fractionated testis extracts and identified the fractions that actively produced S-2HG. Through a combination of ion exchange and size exclusion chromatography, we enriched a single active protein, the lactate dehydrogenase isozyme LDHC, which is primarily expressed in testis. At neutral pH, recombinant mouse LDHC rapidly converted both pyruvate into lactate and α-ketoglutarate into S-2HG, whereas recombinant human LDHC only produced lactate. Rapid S-2HG production by LDHC depends on amino acids 100–102 being Met-Val-Ser, a sequence that occurs only in the rodent protein. Other mammalian LDH can also produce some S-2HG, but at acidic pH. Thus, polymorphisms in the *Ldhc* gene control testis levels of S-2HG, and thereby epigenetics, across mammals.

Graphical abstract

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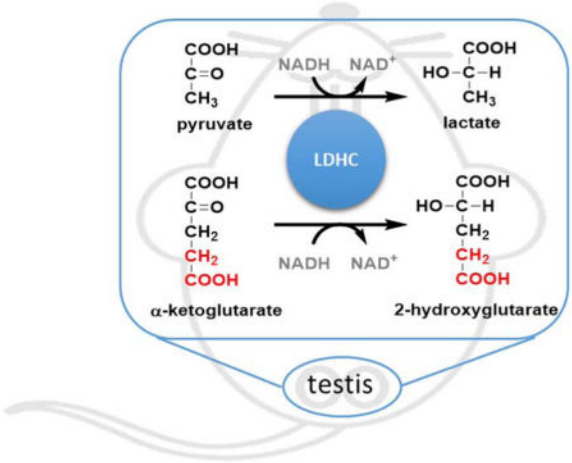
Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschem-bio.6b00290. Figures S1–S4 and Tables S1 and S2 (PDF)



Mammalian tissues have tightly controlled and specialized metabolic functions. Disruption of normal metabolism is a major disease driver. In addition to common metabolic aberrations such as hyperglycemia and hypercholesterolemia, elevations in rare metabolites can also cause disease. One important example is cancer caused by mutant isocitrate dehydrogenase *via* the oncometabolite 2-hydroxyglutarate (2HG).[1] 2HG is a chiral molecule. Both R- and S-2HG are competitive inhibitors of multiple α-ketoglutarate-dependent dioxygenases including histone demethylases and the TET family of 5-methylcytosine (5mC) hydroxylases, with S-2HG being more potent *in vitro* studies.[2,3] Two enantiomers of 2HG, R- and S-, have been associated with a variety of human diseases. Specifically, high levels of R-2HG are produced by mutant isocitrate dehydrogenase 1 and 2 (IDH1/2) in brain cancer and acute myeloid leukemia.[4–7] S-2HG has recently been found to accumulate in renal cell carcinoma.[8] The evolutionarily conserved R-2-hydroxyglutarate (D2HGDH) and S-2-hydroxyglutarate dehydrogenases (L2HGDH) normally prevent accumulation of R-2HG and S-2HG, respectively, by catalyzing their conversion back to α-ketoglutarate.[9] Loss-of-function mutations of D2HGDH or L2HGDH result in 2HG acidurias, which are characterized by systemic elevation of R-2HG or S-2HG, and result in developmental abnormalities including mental retardation and premature death.[10] While the 2HG dehydrogenases typically maintain 2HG at low levels, recent studies showed that hypoxia specifically increases S-2HG in a variety of cell types, including primary cells, immortalized cells, and cancer cells, raising the possibility that S-2HG plays a physiological role in adaptation to hypoxia.[11,12] Nevertheless, the role of 2HG in normal tissue metabolism remains poorly understood.

Isozymes are the enzymes that catalyze the same chemical reaction but differ in amino acid sequences. Different isozymes may have different kinetic properties and thereby enable differential regulation of the same reaction across tissues. One well-known example is the lactate dehydrogenase (LDH) family, which uses NADH to reduce pyruvate to lactate. There are three isozymes of LDH: LDHA, LDHB, and LDHC. LDHA (M subunit) is most abundant in skeletal muscle and functions optimally in an anaerobic metabolism for pyruvate reduction, while LDHB (H subunit) is highly expressed in cardiac muscle and is geared for aerobic oxidation of lactate. LDHC is a testis-specific isozyme expressed in testis

and spermatozoa, and perhaps to a small extent in oocytes,[13] but not substantially in other tissues or cells.[14] LDHA and LDHB can also assemble into homotetramers or heterotetramers. For example, the major isozyme of skeletal muscle and liver, M_4, has four M subunits, while H_4 is the main isoenzyme in heart and brain, H_2M_2 in lung, and H_1M_3 in kidney and pancreas. Heterotetramers of LDHC have not been found *in vivo*, likely due to its highly specific expression in male germ cells. It was reported that LDHC could not be detected in spermatozoa from some men presenting at a fertility clinic.[15] Also, expression of mLDHC is required for normal mouse male fertility, with the homozygous null mice producing offspring only rarely.[16] Disruption of the *Ldhc* gene results in sperm which cannot reach the capacitation state,[16] which occurs after binding to the oocyte and facilitates eventual sperm entry. The causes of these defects are not fully understood.[14] The *Ldhc*-null sperm, although retaining some catalytic activity for converting pyruvate into lactate (likely due to LDHA expression in the fibrous sheath of the flagellum) are defective in glucose consumption and ATP maintenance.[17] Overexpression of human LDHA in *Ldhc*-null mice did not normalize lactate production or ATP levels but nevertheless largely restored fertility.[17,18] Compared to LDHA and LDHB, LDHC accepts a broader range of substrates —not only pyruvate but some other α-keto acids including α-ketogluta-rate.[14,19] It is possible that a moonlighting activity of mLDHC could play a significant role in regulating spermatogenesis and maintaining sperm function.

Here, during metabolite profiling in normal mouse tissues, we found a markedly high level of S-2HG in mouse testis. To identify the source, we enriched the responsible enzyme by fractionating mouse testicular protein lysate and tracking the 2HG producing activity. Mouse lactate dehydrogenase C (mLDHC) was identified to be the responsible enzyme, and this was confirmed through examination of LDHC knockout mouse testis and the catalytic activity of recombinant mLDHC. However, minimum 2HG producing activity was observed from human lactate dehydrogenase C (hLDHC), and only low levels of S-2HG are found in cat or dog testes. Biochemical studies revealed the enzyme residues Met100-Val101-Ser102 are required for rapid S-2HG production by mLDHC. These residues are found selectively in certain rodent species, raising the possibility that S-2HG is used to shape the testis or sperm epigenome of selected rodents.

RESULTS AND DISCUSSION

S-2-Hydroxyglutarate in Mouse Testis

We used LC-MS to quantitate metabolites in different tissues. More than 100 metabolites were measured, with each tissue showing a distinct metabolite abundances pattern (Figures 1A, S1A). Among the most striking observations was a high level of 2HG selectively in mouse testis (Figures 1A,B, S1A). Reduction of the α-ketone in α-ketoglutarate can produce 2HG. To see if testis contains a soluble enzyme catalyzing such reduction, we generated lysates of testis and other major mouse tissues and incubated them with NADH or NADPH. High NADH-dependent α-ketoglutarate reductase activity was observed selectively in mouse testis lysate (Figure 1C), and no tissue contained high NADPH-dependent activity (Figure S1B). The chirality of testicular 2HG was determined by a two-step derivatization followed by GC-MS analysis[20,21] (Figure 1D). Testicular 2HG coeluted with the spiked-in

S-2HG standard, but not spiked-in R-2HG standard, demonstrating that testis contains S-2HG (Figure 1D).

Enrichment and Identification of the S-2HG Producing Enzyme in Mouse Testis

A major goal of modern metabolic research is to determine the genetic, biochemical, and environmental factors governing phenotypes observed through metabolomics. To this end, it is powerful to integrate metabolomics with lysate biochemistry and proteomics. Specifically, to find the source of an unexpected elevated metabolite (in this case S-2HG in mouse testis), whose production can also be observed in lysates, one can partially purify the lysate and identify the responsible enzyme(s) by proteomics (Figure 2A). We used sequential ion exchange chromatography and size exclusion chromatography, both of which retain an enzyme native state and thus activity, to enrich the 2HG-producing testis enzyme, to the point that it was the predominant band on silver stain SDS-PAGE (Figure 2B). The NADH-dependent α-ketoglutarate reductase activity per milligram of protein increased 1000 times in the final enriched fraction compared to crude mouse testis lysate (Figure 2C). Proteomics identified the enriched enzyme as mLDHC (Table S2). Previous reports showed that human lactate dehydrogen-ase A (hLDHA) produces S-2HG by reducing α-ketoglutarate under oxygen limitation conditions[12] but had not explored the potential for LDHC to make 2HG.

Promiscuity of Mouse LDHC

The favored LDH substrate, pyruvate, is structurally similar to α-ketoglutarate (Figure 3A). We examined the NADH-dependent reducing activity of recombinant LDHC (N-terminus Streptavidin-tagged) with pyruvate or α-ketoglutarate as the substrate. At pH 7.4 and 37 °C, NADH oxidation, monitored by 340 nm absorption, was observed in the presence of both pyruvate and α-ketoglutarate (Figure 3B). LC-MS analysis confirmed that the reduction produces lactate (not shown) and 2HG (Figure 3C), respectively. The pyruvate reduction activity catalyzed by mLDHC exhibited a striking substrate inhibition as previously reported,[22] while the α-ketoglutarate reduction saturation curve increased monotonically (Figure 3D). The primary role for LDHC in making testis 2HG *in vivo* was confirmed by markedly decreased 2HG concentration (Figure 3E) and minimum enzymatic activity (Figure 3F) in testis from *Ldhc*-null mice.

Substrate Specificity of Mouse and Human LDHC

To investigate the extent to which S-2HG production was a generic feature of LDH, we examined the α-ketoglutarate reductase activity of different recombinant LDH isozymes. Surprisingly, minimal activity was observed with human lactate dehydrogen-ase C (hLDHC; Figure 4A, Table 1, Figure S2). In addition, activity was minimal with human lactate dehydrogenase A (hLDHA; Table 1), which was previously reported to produce S-2HG under hypoxia.[12] To identify candidate LDH residues that might control substrate specificity, we performed sequence alignment for vertebrate LDHC enzymes (Figure S3). A distinctive three residue sequence Met100-Val101-Ser102 (MVS) was observed in mLDHC, whereas Gln100-Gln101-Glu102 (QQE) was observed in mouse LDHA and human LDHA and LDHC (Figure S3A). The QQE sequence was also observed in LDHCs of most vertebrates except some rodents (Figure S3B). Consistent with LDHC being the primary source of 2HG

in mouse testis, and with nonrodent LDHC lacking this activity, minimal 2HG and α-ketoglutarate reductase activity was observed in cat or dog testes (Figure 4B,C, S3B).

Structure–Function Relationships

Mouse LDHC, as well as several other mammalian LDH isozymes, have been crystallized. These structures show that amino acid residues 100–102 are located on the upper jaw of the substrate binding pocket, within a mobile loop that folds over the active site upon substrate binding.[23,24] Functional studies have shown that these residues can control substrate specificity.[25] Consistent with this, mutation of the QQE loop in hLDHC to MVS resulted in significant α-ketoglutarate reductase activity (Figure 4A, Table 1).

The relatively smaller side chains of MVS compared to QQE may lead to a larger vacuole in the catalytic closed conformation which can accommodate α-ketoglutarate better. The negative charge on Glu102 is positioned to repel α-ketoglutarate electrostatically. Mutation of Glu102 or Gln101 to a neutral or cationic residue (Figure 4D) increased k_{cat}/K_m with α-ketoglutarate as a substrate by 3- to 7-fold, and the effect of mutating both residues was additive (Figure 4E). A substantially larger effect was observed, however, when Gln100 was changed to Met. A previous study of substrate specificity of LDH from *Bacillus stearothermophilus* showed that mutation of the corresponding Gln to Arg dramatically enhanced oxaloacetate reduction.[25,26] One possible explanation for the key role of Gln100 in enforcing pyruvate substrate specificity involves a hydrogen bond between Gln100 and NADH, which promotes the formation of a tight closed catalytic vacuole.[27] Mutation of Gln100 disrupts this hydrogen bond and enables reduction of a broader diversity of α-ketoacids.

Mammalian lactate dehydrogenase activity generally reaches its maximum value at around pH 7.4.[28–31] This is consistent with evolutionary selection for maximal activity at physiological pH. In contrast, the α-ketoglutarate reductase activity of hLDHA and hLDHC increases with decreasing pH, as may occur in hypoxic tissue. In contrast, α-ketoglutarate reduction by mLDHC decreases with decreasing pH (Figure 4F). Mutagenesis studies showed that Gln100 is the key residue controlling the pH-dependence of α-ketoglutarate reduction (Figure 4G). Thus, due substantially to its MVS sequence at residues 100–102, mouse LDHC (unlike most other mammalian LDH) rapidly produces not only lactate but also 2HG at physiological pH.

Biological Significance

2HG is normally considered to be a metabolic error product which can potentially alter DNA and histone methylation and induce malignancy.[1,8,32,33] Recently, human LDHA and LDHC have been shown to produce 2HG in hypoxia, raising the potential that 2HG may play a physiological role.[12] Here, we find substantially more rapid 2HG production than is observed with human LDH with mouse LDHC at physiological pH, while human LDHA produces some 2HG under acidic conditions, as may occur in hypoxia. LDHC is expressed almost exclusively in testes and sperm. The rapid 2HG production at neutral pH by mouse LDHC results from the sequence of a short loop near the active site, Met100-Val101-S102. This sequence occurs only in rodent LDHC protein.

An important question is whether the concentration of S-2HG in mouse testis is biologically significant. IDH1-mutated gliomas accumulate R-2HG to an extremely high level, between 5 and 35 mM.[1] Hypoxia induces S-2HG levels of 50–300 μM in a variety of cultured cell types, due to a combination of increased production (perhaps as a result of elevated NADH or acidic pH) and decreased consumption due to lower levels of L2HGDH.[11,12] We observed about 70 μM 2HG in normal mouse testis, which is more than 10-fold higher than any other normal tissues and within the range observed in hypoxic cultured cells. The fold change between testis and other tissues is similar to that in renal cell carcinoma compared to normal kidney.[8] RCC tumors, with S-2HG concentrations comparable to those observed here in mouse testis, demonstrated a reduced level of 5-hydroxymethylcytosine (5hmC), consistent with 2HG-mediated inhibition of TET enzymes, which convert 5-methylcytosine (5mC) to 5hmC.[8] Moreover, S-2HG promotes histone methylation in hypoxic cells in culture.[12] Given that LDHC is localized spatially and temporally in the germinal epithelium of mouse testis,[34] the 2HG level in these LDHC expressing cells is likely substantially higher and biologically significant, increasing histone and/or DNA methylation in mouse testis.

We assessed 5hmC, 5mC, and histone methylation in a pair of wild-type and *Ldhc*-null testes (Figure S4). While histone methylation did not change, there was a trend toward increased 5hmC and decreased 5mC in the *Ldhc*-null testes, consistent with S-2HG produced by LDHC inhibiting TET enzymes and thereby promoting DNA methylation (Figure S4).

It is intriguing to speculate that the epigenetic effects of S-2HG may impact mouse sperm function. Homozygous deletion of *Ldhc* severely impairs male mouse fertility, without altering testis morphology or sperm production.[16] The fertility of female *Ldhc*-null mice appears to be normal.[16] *Ldhc*-null sperm exhibit canonical metabolic effects associated with less lactate dehydrogenase activity, such as decreased glucose consumption, lactate secretion, and ATP levels. Intriguingly, however, their NADH/NAD^+ ratio and pyruvate levels were unchanged, and pyruvate was rapidly metabolized to lactate as in wild-type sperm, putatively by endogenous LDHA tightly bound to the fibrous sheath.[17] Furthermore, a loss of *Ldhc* resulted in more severe ATP and sperm function defects than LDH inhibitor treatment (oxamate) despite oxamate having greater redox effects.[17] The fertility defect caused by *Ldhc* loss was substantially rescued by human LDHA, surprisingly without restoration of normal lactate production or ATP levels.[18] Collectively, these observations suggest that LDHC contributes to basal sperm metabolism, perhaps also plays structural or signaling roles unrelated to 2HG that are important for sperm function, and may also impact testes or sperm epigenetics *via* S-2HG. Given that nonrodents do not require S-2HG producing lactate dehydrogenase in testis for male fertility, and that LDHA substantially rescues fertility in *Ldhc* null mice, it is likely that the role of the S-2HG-producing LDHC in certain rodents involves epigenetic fine-tuning that is selectively advantageous in their lifecycle, perhaps by enhancing male reproductive success or pup viability in the competitive nonlab environment. The exact physiological role of such fine-tuning, either in the function of sperm itself or in development of the resulting embryo, is an important topic for future investigation.

METHODS

Tissue Collection and Metabolite Extraction

C57BL/6 mice including C57BL/6 *Ldhc*$^{-/-}$ from which testes were harvested[16] were euthanized by cervical dislocation and tissues isolated and immediately frozen in liquid nitrogen. Tissue samples were weighed and then pulverized by agitation with stainless steel balls at liquid nitrogen temperature (CryoMill, Retsch, 25 Hz for 1 min). The pulverized tissue was vortexed with 1 mL of −80 °C extraction buffer (80:20 methanol/water, 0.1% formic acid (v/v)) and placed on dry ice to extract for 5 min. Each sample was centrifuged to isolate the soluble extract, and insoluble material was re-extracted, this time with 1 mL of 40:40:20 methanol/acetonitrile/water (v/v) for 5 min at −20 °C. The supernatants were combined, dried under a nitrogen gas flow, and reconstituted in LC-MS grade water (1 mL of water per 25 mg tissue wet weight). Absolute concentrations were quantified by spiking in an unlabeled standard in a known concentration and monitoring the change in signal intensity.

LC-MS Analysis

Metabolite analysis was performed by reversed-phase ion-pairing chromatography coupled by negative mode electrospray ionization to a stand-alone orbitrap mass spectrometer (Thermo Scientific) scanning from *m*/*z* 85 to 1000 at 1 Hz at 100 000 resolution. LC separation was on a Synergy Hydro-RP column (100 mm × 2 mm, 2.5 μm particle size, Phenomenex, Torrance, CA) using a gradient of solvent A (97:3 H_2O/MeOH (v/v) with 10 mM tributylamine and 15 mM acetic acid) and solvent B (100% MeOH).[35] Data were analyzed using MAVEN software.[36]

Separation of S- and R-2-Hydroxyglutarate

Dried tissue extracts were resuspended in (R)-2-butanol with 1 M HCl and heated for 2 h at 100 °C. Samples were then dried down under nitrogen gas flow and resuspended in 1:1 pyridine/acetic anhydride (v/v) and heated for 0.5 h at 100 °C.[20,21] The resulting product was dried down, dissolved in dichloromethane, and analyzed by an Agilent 7890A GC system with an Aux EPC column (30 m × 0.25 mm, 0.25 um film thickness) coupled with a TOF mass spectrometer in negative mode. Standards of S- and R-2-HG and all reagents were purchased from Sigma.

Expression of Strep-Tagged Recombinant LDHs

hLDHC, mLDHC, and hLDHA genes were amplified from clones obtained from GeneCopoeia. hLDHC mutant genes were constructed by the Gibson Assembly method, using primers listed in Table S1. These genes were amplified by PCR with the primers in Table S1A and cloned into pET 51b(+) using the *Kpn*I/SacI cloning site. The resulting constructs were transformed in *E. coli* BL21(DE3) cells, and protein expression was carried out at 30 °C in the presence of 1 mM IPTG and 100 μg/mL ampicillin for 5 h. The recombinant soluble protein supernatant was purified using Strep-Tactin Superflow Plus (QIAGEN) affinity chromatography. The purity of each recombinant enzyme was determined by SDS-PAGE.

Enzyme Activity Measurements

Enzyme activity in tissue extracts was determined by mixing the pulverized tissue (generated as above) with 200 μL of 100 mM Tris-HCl, at a pH of 7.4, with protease inhibitor (Thermo Scientific Halt Protease Inhibitor Cocktail). The resulting mixture was centrifuged to isolate the soluble protein. The insoluble material was re-extracted using the same procedure, and the supernatants were combined. The enzyme activity was measured using 1 mg mL^{-1} tissue lysate protein, with 0.5 mM NADH and 5 mM α-ketoglutarate in 50 mM Tris-HCl, at a pH of 7.4 and a temperature of 37 °C. Reactions were initiated by adding α-ketoglutarate and quenched with four volumes of methanol at different time points. The quenched solutions were centrifuged to remove protein and diluted for LC-MS measurement.

The kinetic parameters of different LDH variants were measured using purified recombinant LDH enzymes in 100 mM Tris-HCl, at a pH of 7.4, with 0.5 mM NADH and varying concentrations of pyruvate or α-ketoglutarate at 37 °C. The pH dependent enzymatic activity was carried out in 50 mM sodium phosphate buffer at a pH of 5.8–8.0 and in the presence of 20 mM α-ketoglutarate and 0.5 mM NADH. The reaction was monitored as NADH consumption, measured by absorbance at 340 nm using a plate reader (BioTek, Synergy HT). All reagents were purchased from Sigma.

Enrichment of 2HG Producing Enzyme from Mouse Testis Extract

Protein with α-ketoglutarate reducing activity was purified using sequential ion-exchange chromatography and size exclusion chromatography on an ÄKTA FPLC apparatus (Amersham Pharmacia), by tracking the enzymatic activity in different fractions. Mouse testis protein lysate was prepared as described above in buffer A (50 mM Tris-HCl, pH 8.0) and applied to a 5 mL HiTrap Q column (GE Healthcare) pre-equilibrated with buffer A and washed with 20 mL of buffer A. Protein was eluted with a gradient of 1–100% buffer B (50 mM Tris-HCl, pH 8.0, 1 M NaCl) over 25 mL (five column volumes) at flow rate of 3 mL/min, and fractions were collected in a 96-well plate at 250 μL/fraction. Enzymatic activity in each fraction was measured as described previously by monitoring absorbance at 340 nm. Fractions with α-ketoglutarate reducing activity (nine fractions) were pooled, concentrated by centrifugal filter unit (3 kDa Nominal Molecular Weight Limit, Amicon) and loaded on a Superdex 200 10/300 GL (GE Healthcare), pre-equilibrated with buffer C (50 mM Tris-HCl, pH 7.4, 150 mM NaCl). The protein was then eluted with 30 mL of buffer C at a flow rate of 0.5 mL/min and collected in 96-well plates at 250 μL/fraction. Fractions with α-ketoglutarate reducing activity (5 fractions) were pooled and concentrated as described.

Identification of the Enriched 2HG Producing Enzyme

A total of 10 μg partially purified protein in 100 μL buffer C was reduced and alkylated as follows: TCEP was added to a 5 mM final concentration and left to incubate at 60 °C for 15 min. Samples were cooled to RT, and 10 mM chloroacetamide was added and left to incubate in the dark at RT for further 30 min. A total of 500 ng of trypsin (MS grade, Promega) was added to each reaction and incubated end-over-end at 37 °C for 16 h. Digested samples were desalted using C18 stage-tips.[37] Samples were dried completely in a speedvac and resuspended with 20 μL of 0.1% formic acid at a pH of 3 and injected onto

LC-MS/MS. The LC-MS/MS system involved an Eskigent nano-HPLC coupled to a Thermo Orbitrap Elite MS/MS using a PicoTip electrospray source (New Objective). The mass spectrometer was operated in data dependent mode with dynamic exclusion with the 120 000 resolution MS1 scan (400–1800 m/z) in the Orbitrap followed by up to 20 MS/MS scans in the ion trap. MS/MS spectra were analyzed using SequestHT (Thermo Fisher Scientific, version 1.4.1.14) within the Proteome Discoverer 1.4 suite (Thermo Scientific). Sequest was set up to search Uniprot Mouse database assuming the digestion enzyme trypsin, carbamidomethylation of cysteine, fragment ion mass tolerance of 0.60 Da, and a parent ion tolerance of 10 ppm. Deamidation of asparagine and glutamine, oxidation of methionine, and acetyl of the n-terminus were specified in Sequest as variable modifications. Scaffold (version Scaffold_4.4.5, Proteome Software Inc.) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.[38] Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped.

Determination of DNA and Histone Methylation

A total of 10 μg of genomic DNA of wildtype and *Ldhc*-null mouse testis were digested by 20 U DNA degradase (Zymo Research) in 50 μL of reaction volume at 37 °C for 1 h. The enzyme was removed by passing it through a centrifugal filter unit (10 kDa Nominal Molecular Weight Limit, Amicon), and the resulting free nucleotide monophosphates were measured by LC-MS as described above. The following antibodies were used for immunoblot: H3 (Abcam, 1:1000 dilution), H3K9Me3 (Active Motif, 1:1000 dilution), H3K27Me3 (Millipore, 1:2000 dilution), H3K27Me3 (Active Motif, 1:1000 dilution), and LDHC (Abcam, 1:1000 dilution).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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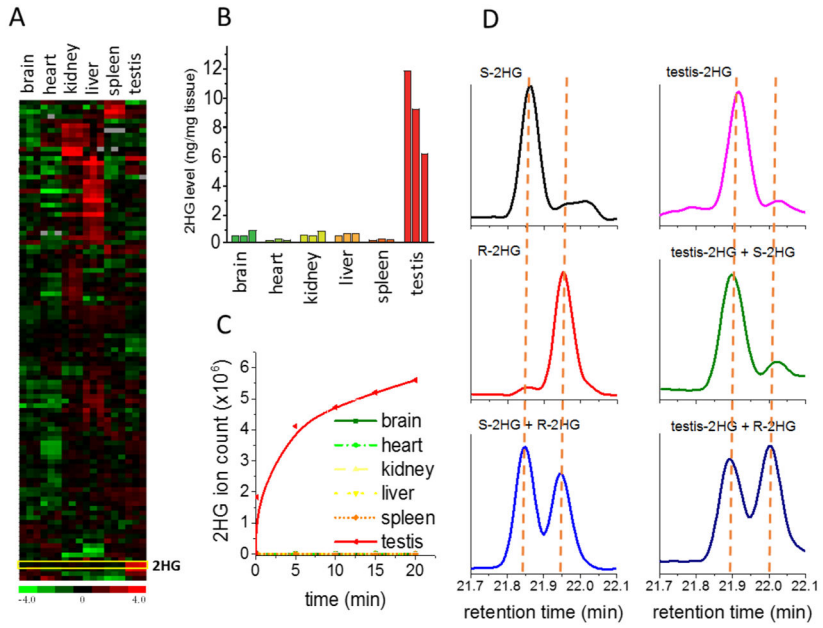


Figure 1.

Mouse testis contain abundant S-2HG. (A) Heat map of metabolite levels in different tissue reveals high 2HG level in testis (yellow box). Metabolite levels were normalized to tissue mass then normalized to the median of each metabolite across tissues. Results are log2 transformed and clustered. Data are collected from three mice. (B) 2HG level in different tissues. Data are collected from three mice. (C) 2HG producing activity in tissue lysates with 0.5 mM NADH and 5 mM α-ketoglutarate. (D) Mouse testis produces the S- enantiomer of 2HG. GC-MS traces for the S-2HG standard, R-2HG standard, a mixture of the S- and R-2HG standards, testis 2HG, and testis 2HG spiked with S- or R-2HG standard.

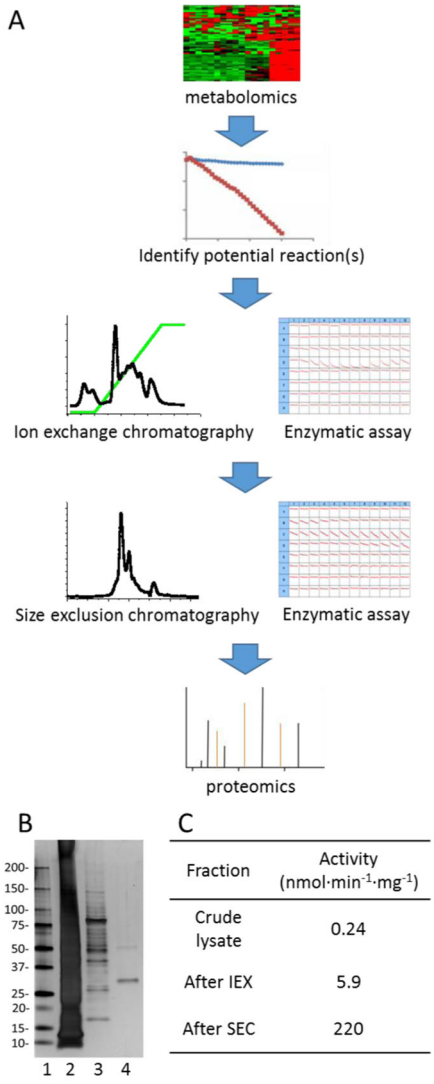


Figure 2.

Enrichment and identification of 2HG producing enzyme in mouse testis. (A) Scheme of experimental procedure. (B) Silver stain of testis lysate at different enrichment stages. Lane 1, protein ladder; lane 2, crude mouse testis lysate; lane 3, proteins after ion exchange chromatography (IEX); lane 4, proteins after size exclusion chromatography (SEC). (C) 2HG producing activity at different enrichment stages.

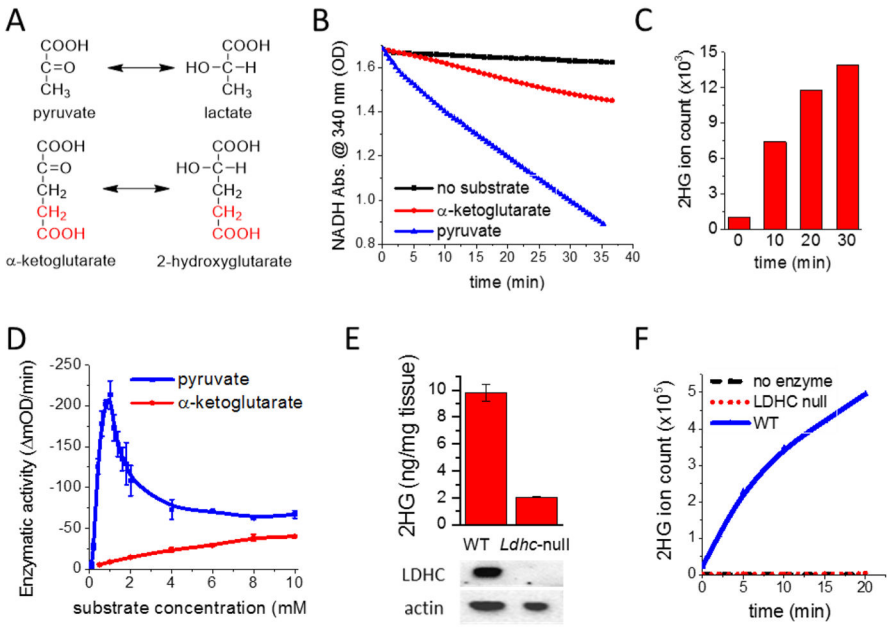
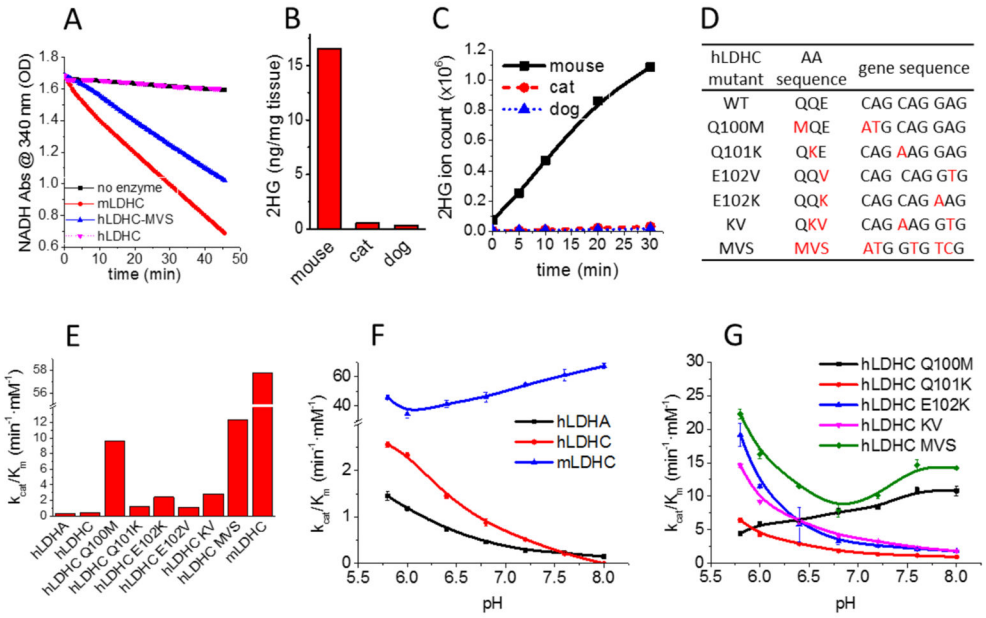


Figure 3.

Mouse lactate dehydrogenase C produces 2HG. (A) Structures of the primary mLDHC substrate/product and α-ketoglutarate/2HG as alternative endogenous substrate/product. (B) mLDHC catalyzed NADH-driven reduction of pyruvate and α-ketoglutarate. A total of 3 mM of α-ketoglutarate (red), pyruvate (blue), or no ketoacid control (black) was incubated with 0.5 mM NADH. The reaction was monitored by a decrease in NADH absorbance at 340 nm. (C) LC-MS analysis of the mLDHC-catalyzed α-ketoglutarate reduction product 2HG over time. (D) Enzyme activity as a function of pyruvate and α-ketoglutarate concentration. Assay conditions were as described in B except the concentration of the keto-acid was varied as shown. (E) 2HG level in WT and *Ldhc*-null mouse testis. Mean ± SD, N = 3 technical replicates. LDHC expression by Western is shown on the bottom. (F) 2HG producing activity in lysate from WT testis (blue), *Ldhc*-null testis (red), and no lysate control (black).



hLDHC mutant	AA sequence	gene sequence
WT	QQE	CAG CAG GAG
Q100M	MQE	ATG CAG GAG
Q101K	QKE	CAG AAG GAG
E102V	QQV	CAG CAG GTG
E102K	QQK	CAG CAG AAG
KV	QKV	CAG AAG GTG
MVS	MVS	ATG GTG TCG

Figure 4.

LDH substrate selectivity is controlled by a short loop near the active site. (A) α-Ketoglutarate reduction activity catalyzed by recombinant strep-tagged mLDHC (red), hLDHC (pink), hLDHC MVS mutant (blue), and no enzyme control (black). The reaction was monitored by a decrease in NADH absorbance at 340 nm. (B) Testis 2HG level in different species. (B) Enzymatic assay of 2HG producing activity in testis lysate of mouse (black), cat (red), and dog (blue) with 0.5 mM NADH and 3 mM α-ketoglutarate. (D) hLDHC mutants and their corresponding amino acid sequences and gene sequences. (E) Apparent second order rate constant (k_{cat}/K_m) of different LDH variants with α-ketoglutarate as the substrate. (F) pH dependence of k_{cat}/K_m of different LDHC variants with α-ketoglutarate as the substrate. k_{cat}/K_m of hLDHA and hLDHC, but not mLDHC, markedly increases at lower pH, (G) and the pH dependence requires Gln100.

Table 1

Michaelis–Menten Parameters for Pyruvate and α-Ketoglutarate Reduction by Different LDH Variants

	pyruvate		α-ketoglutarate	
	k_{cat} (min^{-1})	K_m (mM)	k_{cat} (min^{-1})	K_m (mM)
mLDHC	1730 ± 151	0.76 ± 0.21	433 ± 32	7.5 ± 0.7
hLDHC	2210 ± 143	0.14 ± 0.02	7.6 ± 1.0	21.4 ± 3.0
hLDHC-MVS	377 ± 50	0.12 ± 0.02	161 ± 7	13.6 ± 0.7
hLDHA	9455 ± 304	0.38 ± 0.02	3.0 ± 0.3	11.8 ± 1.2