BIOLOGY OF REPRODUCTION (2013) **88**(4):96, 1–6
Published online before print 6 March 2013.
DOI 10.1095/biolreprod.112.107011

Human Lactate Dehydrogenase A (*LDHA*) Rescues Mouse *Ldhc*-Null Sperm Function[1]

Huanghui Tang,[4] Chongwen Duan,[3,4] Reiner Bleher,[5,6] and Erwin Goldberg[2,4]

[4]*Department of Molecular Biosciences, Northwestern University, Evanston, Illinois*
[5]*Northwestern University Atomic and Nanoscale Characterization Experimental Center (NUANCE Center)/Electron Probe Instrumentation Center (EPIC), Evanston, Illinois*
[6]*Chemistry of Life Processes Institute (CLP)/Quantitative Bioelemental Imaging Center (QBIC), Northwestern University, Evanston, Illinois*

ABSTRACT

By targeted disruption of the lactate dehydrogenase c (*Ldhc*) gene, we demonstrated that spermatozoa require *Ldhc* for capacitation, motility, and fertilizing capacity. *Ldhc* expression is restricted to the developing germ cells that, however, are apparently not compromised by the lack of the LDHC isozyme. Because LDHC is abundant in spermatozoa that utilize aerobic glycolysis for energy requirements, its main function was presumed to be the interconversion of pyruvate to lactate with the concomitant oxidation/reduction of NADH to NAD^+. We found that sperm without LDHC were still able to convert lactate to pyruvate as mediated by LDHA that is tightly bound to the fibrous sheath. It was assumed that the level of glycolysis was insufficient to power motility and the subsequent fertilizing capacity of the mutated sperm. To investigate whether LDHC possesses certain unique characteristics essential for fertility, human *LDHA* was introduced as a transgene to *Ldhc*-null mice. We report here that the exogenous LDHA rescued the phenotype of the *Ldhc*-null males. Sperm from the *LDHA* transgenic males with the *Ldhc* deletion ($LDHA^+/Ldhc^{-/-}$) are motile, capable of protein tyrosine phosphorylation, and able to fertilize, thus restoring these properties to LDHC-null sperm. However, the lactate and ATP levels in the rescued sperm did not differ significantly from sperm lacking LDHC. We suggest that it is the localization of the transgene to the sperm cytosol that is mainly responsible for restoration of sperm function and fertility.

fertility, gene expression, gene regulation, glycolysis, infertility, LDHA*, sperm, spermatogenesis*

INTRODUCTION

Mammalian cells use glycolysis and oxidative phosphorylation to produce ATP for metabolic needs. Glycolysis involves a coordinated series of catalytic events that convert glucose to pyruvate with the coupled production of 2 moles of ATP. Under aerobic conditions, pyruvate is metabolized to CO_2 and H_2O via mitochondrial oxidative phosphorylation. With limiting oxygen, pyruvate conversion to lactate supplies ATP and is catalyzed by lactate dehydrogenase (LDH) with the concomitant oxidation of NADH to NAD^+ [1]. Regeneration of NAD^+ in this step is essential to sustain glycolysis by LDH. The family of lactate dehydrogenases is encoded by three genes *Ldha, Ldhb,* and *Ldhc* for the expression of the LDH subunits A, B, and C. The functional LDH enzyme consists of LDHA and LDHB subunits assembled as homotetramers or heterotetramers that characterize the tissue type in which they are found and presumably is correlated with metabolic needs. Thus, LDHA is most abundant in liver and skeletal muscle and LDHB in heart and red blood cells. Different combinations of tetramers seem to relate to cellular function. LDHC is found in many species of mammalian sperm, including that of human and murine, which rely on aerobic glycolysis to generate the ATP required for motility and capacitation. Targeted disruption of the *Ldhc* gene impairs motility, capacitation is not supported, and the sperm are unable to fertilize eggs. Nevertheless, these sperm are able to convert pyruvate to lactate [2], which is presumably mediated by LDHA that is bound to the fibrous sheath [3]. Here we demonstrate that adding LDHA as a transgene to the null sperm restores motility, capacitation, and fertilizing ability even though the total amount of LDH activity is not significantly increased.

MATERIALS AND METHODS

Generation of Transgenic Mice

All the animal procedures were performed in accordance with National Institute of Health guidelines and approved by the Northwestern University Animal Care and Use Committee. The human *Ldha* transgene was amplified by PCR from cultured HeLa cell mRNA with primer set hldha5′KpnI (5′-GGTACCGCCACCATGGCAACTCTAAAGGATCAGC-3′) and hldha3′NotI (5′-GCGGCCGCTTAAAATTGCAGCTCCTTTTGGATCC-3′). A Kozak sequence was added in front of the start codon ATG. The PCR fragment was cut by restriction enzyme KpnI/NotI and cloned into a vector pCAG-GFP (Addgene Plasmid 11150) in which the GFP sequence was removed by the same restriction enzyme pair. The insert *LDHA* sequence was confirmed by DNA sequencing. The final recombinant DNA was cut by restriction enzyme SpeI/HindIII to release the *LDHA* expression cassette, which includes a CAG promoter, *LDHA* coding sequence, and globin polyA tail (Fig. 1). This cassette fragment was separated from the backbone vector by electrophoresis on an agarose gel, cut, and purified by using a QIAquick Gel Extraction Kit (Qiagen). Transgenic animals were generated at the Northwestern University Transgenic Core Facility by pronuclear injection and screened by PCR primers (5′-AGGCTACACATCCTGGGCTA-3′; 5′-TTTTGGCAGAGGGAAAAAGA-

[1]Supported by National Institutes of Health grant R01 HD005863-36. Partial financial support of this research was provided by the Weinberg College of Arts and Sciences, Northwestern University. The electron microscopic work made use of the EPIC facility (NUANCE Center-Northwestern University), which has received support from the MRSEC program (NSF DMR-0520513) at the Materials Research Center, The Nanoscale Science and Engineering Center (EEC-0118025 / 003), both programs of the National Science Foundation; the State of Illinois; and Northwestern University; and the Northwestern University Quantitative Bioelemental Imaging Center generously supported by the Keck Foundation and the Chicago Biomedical Consortium sponsor #C2006-00997, NU #CNVR48279. The Robert H. Lurie Comprehensive Cancer Center of Northwestern University funds shared facilities and resources used in this research.
[2]Correspondence: Erwin Goldberg, Northwestern University, Department of Molecular Biosciences, 2200 Campus Dr., Evanston, IL 60208. E-mail: erv@northwestern.edu
[3]Current address: Department of Otolaryngology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611

Received: 13 December 2012.
First decision: 6 January 2013.
Accepted: 4 March 2013.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

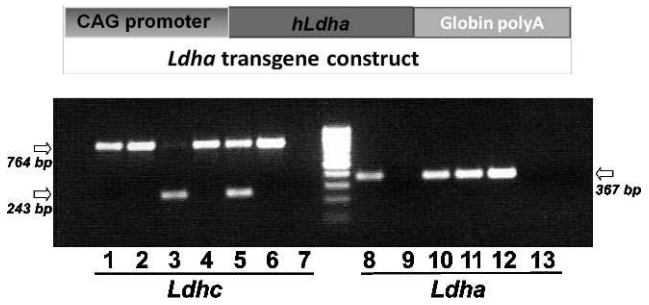

FIG. 1. Transgene construct and PCR genotyping. The human *LDHA* gene was cloned downstream to a synthetic CAG promoter that is composed of a CMV enhancer and a chicken β-actin promoter. Existence of the transgene was detected by PCR. For *Ldhc,* a single high-molecular-weight band represents homozygous ($Ldhc^{-/-}$) (lane 1, 2, 4, and 6), and two bands represent heterozygous ($Ldhc^{+/-}$) (lane 3 and 5). For *LDHA,* a single band confirms transgene expression (lane 8, 10, and 12) while no band is detected if the transgene is not expressed (lane 9). Males with $Ldha^{+}/Ldhc^{-/-}$ genotype were used for the fertility rescue trial. Lane 7 and 13 were PCR-negative controls.

3′). To obtain the *LDHA* transgenic animal with an *Ldhc*-null background ($LDHA^{+}/Ldhc^{-/-}$), the *LDHA* male was first mated with an *Ldhc*-null homozygous female; then offspring with genotype $LDHA^{+}/Ldhc^{+/-}$ were crossed with an *Ldhc*-null female to produce the *LDHA* transgenic animal ($LDHA^{+}/Ldhc^{-/-}$).

Fertility Assay by Natural Mating

Each *LDHA* transgenic male ($LDHA^{+}/Ldhc^{-/-}$) and *Ldhc* knockout (KO) male ($Ldhc^{-/-}$) was mated with two C57/B6 wild-type (WT) females for two or more 4-wk intervals. Females were kept for 4 wk after mating to confirm their pregnancies.

Sperm Preparation

Cauda epididymides were collected in PBS solution; blood and fat pads were carefully removed with forceps and iridectomy scissors. The epididymis was pricked in several locations with a 20-gauge needle to allow the sperm to swim out into the medium. Modified Krebs-Ringer bicarbonate solution (TYH) [2] or M2 medium (Sigma-Aldrich) were used for the sperm capacitation assay. Sperm were incubated at 37°C with 5% CO_2 in a humidified air chamber. For a Western blot assay, the sperm were prepared by washing three times with 0.45% PBS and spun down at 400 × *g*. A Leica Spinning Disk microscope with a DMI6000 SD inverted stand and EMCCD camera was used to record sperm motility.

Western Blot Assays

Mouse testes were homogenized in PBS solution mechanically by using a 2-ml Wheaton glass tissue grinder. A 1× proteinase inhibitor cocktail (Sigma-Aldrich) was added to the PBS solution. The protein extracts were centrifuged at 12 000 × *g,* and the supernatant was used for Western blot analysis. Sperm protein extracts were prepared either by using the KSCN buffer (0.6 M KSCN, 0.5 mM Tris-HCI, pH 8.0) [4] or by sonication on ice for 2 × 5 sec and centrifugation at 12 000 × *g*. Protein samples were heated for 5 min at 95°C in a 2× loading buffer with 125 mM Tris-HCl, pH 6.8, 4% SDS, and 100 mM dithiothreitol. Proteins were separated on a 12% polyacrylamide gel and then transferred to a PRO-TRAN Nitrocellulose Transfer Membrane (Whatman Gmbh). The membrane was incubated for 1 h in blocking buffer TBST—6.05 g Tris, 8.76 g NaCl, pH 7.5, with 1 ml Tween 20 in 1 L distilled water (dH2O)—plus 5% fat-free dry milk or 1% bovine serum albumin (BSA; Sigma) for the anti-phosphotyrosine probe. After blocking, the membrane was transferred to a new blocking buffer with addition of primary antibody and incubated overnight at 4°C with shaking. The membrane was washed several times with TBST and incubated for 1 h at room temperature in blocking buffer containing horseradish peroxidase-conjugated secondary antibody. After several washes with TBST, the membrane was incubated in a SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). The membrane was exposed to x-ray film to detect the signal. The dilution factor for each primary antibody was as follows: anti-LDHC, 1:10 000 [5]; anti-human LDHA, 1:2000 (USBiological); and anti-phosphotyrosine, 1:2000 (P4110; Sigma).

LDH Activity Assay Electropherogram

Proteins in extracts of testis or sperm were resolved by PAGE (8% separation gel and 3% stacking gel). The gel was similar to that used for the Western blots except without the addition of SDS to the buffers. The 10× running buffer was composed of 6.0 g Tris, 28.8 g glycine/L dH_2O with the pH adjusted to 8.3 by HCl. PAGE was conducted in 1× running buffer at 4°C overnight at 100 V. The gel was incubated in substrate solution—0.05 M Tris-HCl, pH8.4, 2 mM NAD (N-0632; Sigma), 0.8 mM nitro blue tetrazolium (N6876; Sigma), 62.5 mM D,L-lactate (L7900; Sigma), and a few granules of phenazine methosulfate (P-9625; Sigma)—until the LDH isozyme band pattern was visible.

Sample Preparation for Electron Microscopy

Resin embedding. Sperm cells in PBS (Dulbecco PBS; Cellgro Mediatech, Inc.) were centrifuged at 400 × *g* for 5 min, the supernatant was aspirated, and 1.5 ml of 4% formaldehyde (16% aqueous stock solution [EMS] in PBS, pH 7.4) were added. After fixation for 1 h at 4°C, the pellet was resuspended in 50 μl of 2% low melting point agarose (Fisher Scientific). After a brief rinse in PBS, the hardened agarose with the cells was cut into small pieces, and the sample was rinsed in PBS with 0.05 M glycine (Sigma) for 15 min. The specimens were dehydrated for 20 min each in 25%, 50%, 75%, and 90% ethanol, and two times for 10 min each in 100% ethanol. After infiltration of the samples with a 1:1 mixture of LR-White resin (EMS) and ethanol for 3 h, the samples were infiltrated in pure LR-White resin overnight at 4°C. For polymerization, the samples were transferred into fresh resin in gelatin capsules and cured with ultraviolet light at 4°C for 24 h. The blocks were sectioned using a diamond knife (Diatome) with an ultramicrotome Leica EM UC7 at a nominal thickness of 80 nm, and the sections were collected on 200-mesh nickel grids.

Immunolabeling for electron microscopy. Sections on grids were placed on 100 μl droplets of 0.05 M glycine in PBS for 5 min, followed by transfer to droplets of 5% BSA in PBS for 15 min. Incubation with the primary antibody was performed on 50 μl droplets with goat anti-LDHA (L1011-10; U.S. Biological) diluted 1:50 in PBS with 0.1% BSA for 1 h at room temperature, followed by five washes on PBS droplets for 5 min each. Primary antibodies were labeled with 10 nm gold-conjugated rabbit anti-goat secondary antibodies (EMS) for LDHA in PBS with 0.1% BSA for 1 h at room temperature, and unbound conjugates were removed by five washes on PBS droplets for 5 min. For controls, the primary antibody was replaced with 0.1% BSA in PBS only. The sections were placed on droplets of 2.5% glutaraldehyde (EMS) in PBS for 10 min, rinsed briefly on 3 droplets of MQ water (Millipore), and stained on droplets of 2% aqueous uranyl acetate (EMS) for 5 min. After a final brief rinse with MQ water, the grids were air dried and observed in a scanning transmission electron microscope (HD2300-A; Hitachi) with an acceleration voltage of 80 kV.

Lactate Level Measurements

Sperm were allowed to swim out of the pricked epididymis for 5 min in 2 ml capacitation medium and divided into two aliquots; one aliquot was used for the zero-time assay and the other was incubated in a 5% CO_2 humidified chamber for 4 h. Twenty-microliter samples were taken from both aliquots after 2 h and 4 h incubation, centrifuged at 10 000 × *g,* and the supernatant used to measure the lactate concentration (Biovision) according to manufacturer's instructions.

ATP Level Measurements

Sperm collected at time 0 and after 4 h incubation were used to measure ATP level as described in Miki et al. [3]. Briefly, after centrifugation at 500 × *g* for 3 min, sperm were resuspended in 450 μl boiling extraction buffer (100 mM Tris-HCl, 4 mM ethylenediaminetetraacetic acid, pH7.8) and incubated for 2 min at 100°C. Samples were then centrifuged at 10 000 × *g* for 3 min, and 50 μl duplicate aliquots of the supernatant were used for ATP measurement with the ATP Bioluminescence Assay Kit CLS II (Roche Applied Science) according to manufacturer's instructions.

Data Analyses

Data analyses were performed by using GraphPad Prism 5 (GraphPad Software Inc.). One-way analysis of variance and Bonferroni multiple

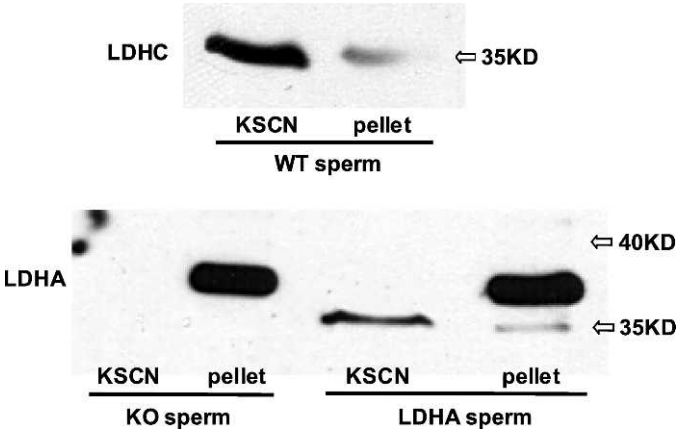

FIG. 2. Transgenic and endogenous LDHA in sperm. Sperm were collected from the cauda epididymis of wild-type mice (WT), the *Ldhc* knockout animal (KO), and *LDHA* transgenic animal with the *Ldhc*-null background (LDHA). Sperm were extracted in 0.6 M KSCN buffer and centrifuged. The soluble fraction and sperm pellet were dissolved in SDS loading buffer and run on 12% SDS-PAGE followed by Western blot analysis with anti-human LDHA antibody (1×10^6 sperm/lane). LDHC and transgene LDHA have a molecular weight of 35 kDa and are soluble in the KSCN fraction; in contrast, endogenous LDHA has a higher molecular weight (38 kDa) and is present only in the pellet fraction.

comparsion test or pairwise *t*-test were used to evaluate the data. Differences were considered significant at $P < 0.05$.

RESULTS

To ensure robust expression of the transgene, we used the synthetic CAG construct that consists of a CMV enhancer and a chicken β-actin promoter (Fig. 1). We obtained a single transgenic line. Genotyping was confirmed by PCR (Fig. 1). For the *Ldhc* locus, a single band in the gel image indicated homozygosity (–/–) and double bands represented heterozygosity (+/–); the *LDHA* transgene positive signal appeared as a single PCR band. LDHA in sperm from the transgenic animal was confirmed by comparison with that in sperm from the *Ldhc* KO male by Western blot analysis (Fig. 2). We visualized the human LDHA and a higher molecular-weight signal for mouse sperm LDHA by Western blot analysis of sperm extracts (Fig. 2). Human LDHA with a molecular weight of 35 kDa was mainly present in the KSCN-solubilized fraction with a trace amount remaining in the sperm pellet. The high molecular-weight band presumably is the LDHA tightly bound to the fibrous sheath [4] and therefore only appears in the pellet fraction. The molecular weight is close to 38 kDa.

The transgene subunit formed enzymatically active hetero-tetramers with murine LDHA and B subunits in several tissues including testes of the mutant mice (Fig. 3). The transgenic hLDHA activity was detected in sperm extracts and migrated more cathodally during PAGE, which is comparable to that in the testis electropherogram, and absence of LDHC in these animals was confirmed by Western blot analysis (Fig. 4). Trace amount of murine LDHA was identified in the PBS fraction; it possibly came from the small fragments of sperm tails resulting from the sonication treatment that usually cannot be precipitated efficiently by centrifugation at $13\,000 \times g$. The results of immunogold-labeling electron microscopy confirmed the presence of the transgene in the sperm (Fig. 5). Sperm from transgenic mice contained LDHA immunogold particles that were densely distributed in the principal piece of the flagellum (right panel). In contrast, only a few immunogold particles barely above background were observed in KO spermatozoa

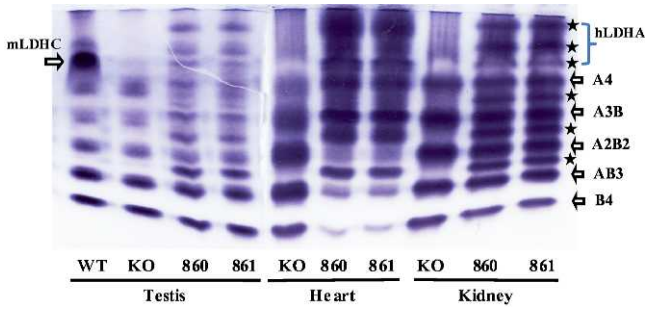

FIG. 3. LDH Isozyme activities in wild-type (WT), *Ldhc* knockout (KO), and *Ldha* transgenic mice 860 and 861 ($LDHA^+/Ldhc^{-/-}$) males. These animals were from a single founder. Protein extracts from testis, heart, and kidney tissues were run on a native 8% acrylamide gel and incubated in reaction mixture for LDH activity. Five mouse isozymes (A4, A3B1, A2B2, A1B3, B4) were as labeled. The human LDHA subunit was identified in the three bands above the A4 isozyme and three other heterotetramer hybrid bands with mouse LDHA and LDHB subunits (labeled with star). LDHC, as expected, was seen only in the WT but not in the KO and LDHA transgenic animals (860 and 861).

(left panel). Most importantly, these electron micrograph sections show the abundance of immunogold particles in sperm from the transgenic animals compared to those from the *Ldhc* KO.

Cauda epididymal sperm were collected from WT, *Ldhc* KO, and *LDHA* transgenic (LDHA) animals using M2 medium. Sperm motility was compared and recorded in movie

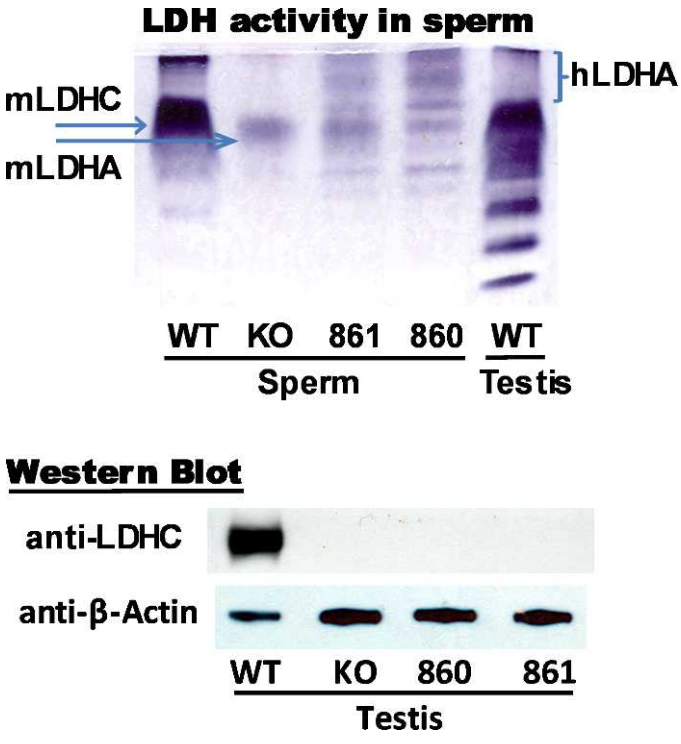

FIG. 4. LDH expression in spermatozoa. Cauda epididymal sperm were collected from WT, KO, and LDHA transgenic animals and sonicated in PBS buffer. The PBS soluble fraction was run on a native gel and stained for LDH activity (top panel); for each lane, 20 μg protein was loaded; and endogenous LDHA was identified in KO sperm as a single band. Transgene human LDHA was detected in samples 860 and 861. LDHC enzymatic activity is much greater compared to transgene human LDHA in sperm. LDHC expression was examined in WT, KO, 860 and 861 testes to confirm the *Ldhc*-null status by Western blot analysis (bottom panel).

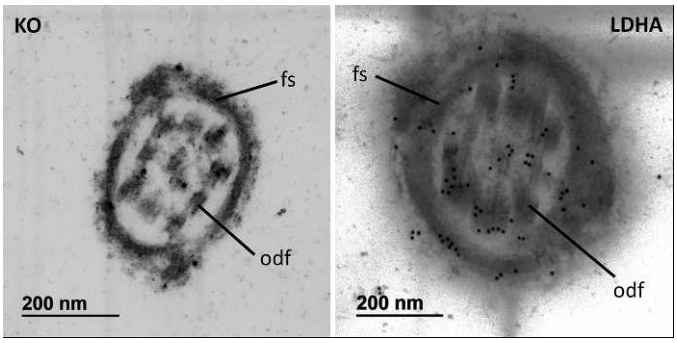

FIG. 5. Electron microscopic images of LDHA distribution in cross sections of the principal piece of the sperm flagellum. *Ldhc* knockout sperm (KO) (left panel) and LDHA transgenic sperm (LDHA) (right panel). Sections of resin embedded spermatozoa were stained with anti-LDHA primary and gold-conjugated secondary antibodies: Odf, outer dense fibers; fs, fibrous sheath. There are few barely above background immunogold particles in KO sperm compared to the abundant number in the TG sperm section. It is possible that epitopes recognized by this antibody are not accessible, i.e., buried in the Fsc.

format (Fig. 6; Supplemental Movies are available online at www.biolreprod.org). Initially, KO sperm motility was sluggish compared to WT. Motility of sperm containing the transgene was comparable to that of WT sperm (Supplemental Movies). After 4 h in capacitation medium, TG (transgenic) sperm were still motile while KO sperm were nonmotile.

After confirmation of human *LDHA* expression, matings were set up between transgenic males ($LDHA^{+}/Ldhc^{-/-}$) and C57/B6 WT females, while the $Ldhc^{-/-}$ KO males were used in control matings that confirmed infertility. Seven transgenic males produced 10 pregnancies in 13 matings while six matings by KO males were nonproductive (Table 1). In our previous trials 100% of WT and $Ldhc^{+/-}$ males were fertile [2]. The litter size was approximately the same for all the matings.

LDH activity was measured in extracts from transgenic animals (LDHA), KO, and WT sperm (Fig. 7) that were incubated in capacitation medium. Lactate production by sperm was significantly higher in WT compared to LDHA and KO at 2 and 4 h ($P < 0.01$); The sperm lactate production by LDHA

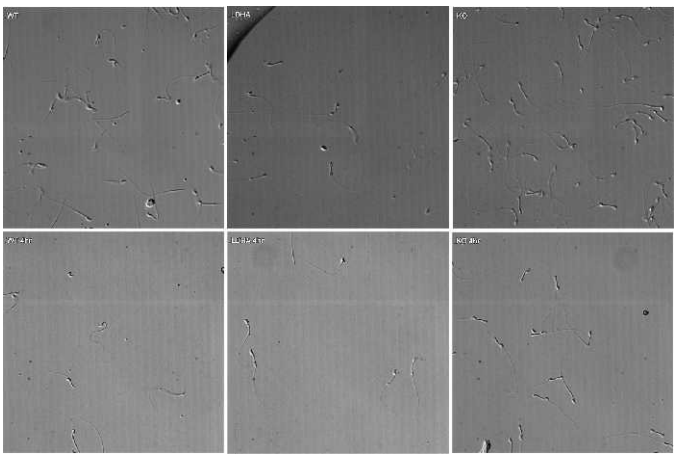

FIG. 6. Frame capture movie clips of sperm motility for WT, KO, and LDHA animals at times 0 and after 4h incubation in capacitation medium. Sperm motility was compared and recorded in movie format. Initially KO sperm motility was sluggish compared to the WT. Motility appeared comparable between WT and LDHA TG sperm. At 4 h, KO sperm were nonmotile while TG sperm were still motile.

TABLE 1. Fertility of WT (C+/+), heterozygous *Ldhc*+/– (C+/–), KO *Ldhc*–/– (C–/–), and transgenic *Ldha*+/Ldhc–/– (A+C–) mice.

Genotype	C+/+ [a]	C+/– [a]	C–/– [a]	C–/–	A+C–
Male fertility[b]	6/6	12/12	2/14	0/6	10/13
Litter size	6.0	6.7	>1	N/A	5.2

[a] Data cited from previous experiments [2].
[b] The number of pregnancies/number of mating groups. Fertility of *Ldhc* heterozygous males was comparable to WT. For *Ldhc* homozygous males, only 2 out of 14 mating pairs sired single litters of one and three pups, which was considered as being subfertile or infertile; in the current mating trial, six homozygous null males sired no pups. Introducing human *LDHA* to the *Ldhc*-null mice increased the pregnancy rate to 10 in 13 matings with an average of 5.2 pups per litter.

transgenic mice was significantly higher than KO at 2 h ($P < 0.05$) but the difference was not significant at 0 and 4 h ($P > 0.05$).

Sperm ATP levels were measured in LDHA, KO, and WT animals after 0, 90, and 240 min incubation in capacitation medium (Fig. 8). ATP production was significantly higher in WT compared to LDHA and KO animals at all three points ($P < 0.01$). There was no significant difference in ATP levels between LDHA and KO sperm at 0, 90, and 240 min ($P > 0.05$).

A distinguishing characteristic for sperm capacitation is protein tyrosine phosphorylation [6–8]. The tyrosine phosphorylation profiles were examined in WT, KO, and LDHA sperm after 90 min incubation in capacitation medium (Fig. 9). Compared to the lack of phosphorylation in KO, LDHA sperm exhibited recovery of tyrosine phosphorylation, which apparently is sufficient for capacitation.

DISCUSSION

The phenotype for *Ldhc* KO mice is male infertile although spermatogenesis is normal. Our strategy to rescue this

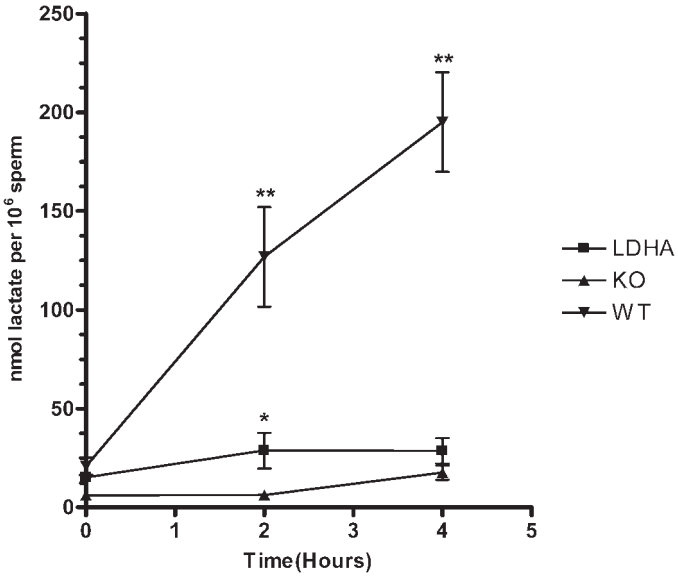

FIG. 7. Lactate level in capacitation medium containing wild-type (WT), *Ldhc*-null (KO), and *LDHA* transgenic sperm (LDHA) that were incubated for 0, 2, and 4 h. The lactate level was significantly higher in WT at 2 and 4 h compared to KO and LDHA transgenics ($P < 0.01$). The lactate level at 2 h in LDHA transgenic sperm is significantly higher than KO ($P < 0.05$), but the difference at time 0 and 4 h was not significant ($P > 0.05$). The bar represents means ± SEM, n = 3; *$P < 0.05$, **$P < 0.01$.

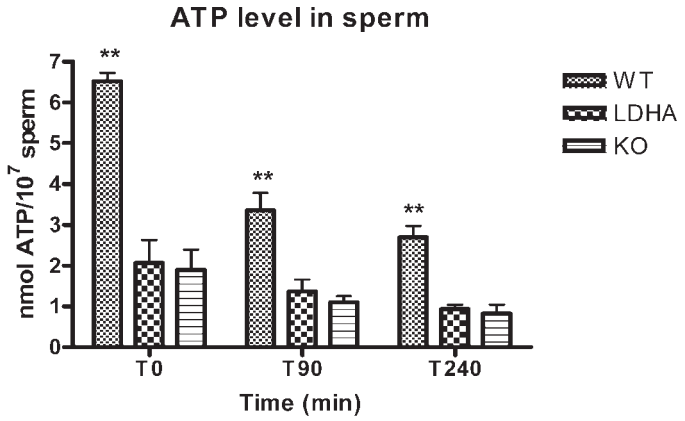

FIG. 8. Sperm ATP level in *Ldha*$^{+}$/*Ldhc*$^{-/-}$ (LDHA), wild-type (WT), and knockout *Ldhc*$^{-/-}$ (KO) mice. The WT sperm ATP level is significantly higher than in LDHA and KO ($P < 0.01$) at 0, 90, and 240 min. Also, there is no significant difference between LDHA and KO sperm at all three points. The bar represents the mean ± SEM, n = 3; $^{**}P < 0.01$.

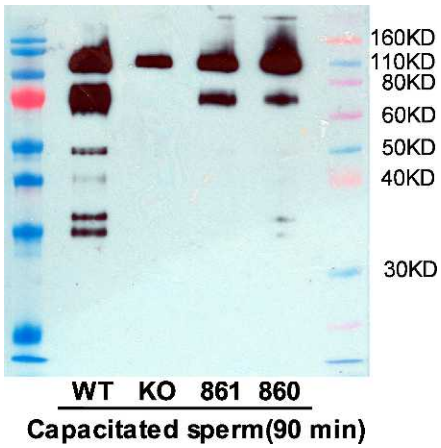

FIG. 9. Protein tyrosine phosphorylation in wild-type (WT), Ldhc$^{-/-}$ (KO), and Ldha^{+}/Ldhc$^{-/-}$ (860 861) sperm. Sperm were incubated for 90 min in capacitation medium. Protein was extracted in an SDS loading buffer. A phosphotyrosine antibody was used for the Western blot analysis; 2×10^6 sperm were loaded for each lane.

phenotype was to target the sperm cell by increasing the LDH level with a transgene encoding LDHA. In a previous report, we demonstrated that a reporter gene, *β-gal*, driven by the *Ldhc* promoter did not express beyond the pachytene stage [9, 10]. Therefore a composite cytomegalovirus-immediate early enhancer/chicken β-actin promoter (CAG), designed to provide ubiquitous expression [11–13], was used to drive the transgene human *LDHA* for the rescue trial. Because endogenous *Ldhc* expression is robust in germ cells, our purpose in using the CAG promoter was to try to restore a comparably high level of LDH activity in sperm. In fact, the relative enzymatic activity of the added LDHA was much lower in sperm compared to the native LDHC in WT sperm (Fig. 4) and barely increased this activity in KO sperm. This is consistent with the intensity of the bands on the Western blot (Fig. 2), suggesting only a small fraction of protein compared to the endogenous situation was present in these sperm. Nevertheless, this strategy was successful in rescuing sperm motility and fertilizing capacity, suggesting that perhaps it was not total LDH catalytic activity that was the driving force behind sperm function.

The LDH complement of sperm consists of LDHC in the cytoplasm of the sperm tail, an isozyme of LDHA tightly bound to the fibrous sheath [4], and an LDHA-like isoform (LDHAL6B) isolated from lipid rafts associated with the sperm membrane [14]. The abundance of LDHC in terms of activity suggested that this isozyme was important for sperm function. Our hypothesis was supported by targeted disruption of the *Ldhc* gene [2]. Both the Western blots and electron micrograph images (Figs. 2 and 5) confirm that the transgene increased the amount of LDH protein in the KO sperm, particularly that the LDHA from the transgene was soluble in the KSCN fraction of the sperm extract whereas endogenous LDHA was not solubilized by this reagent. Endogenous LDHA in sperm is bound tightly to the fibrous sheath [4] and was detected as a 38-kDa protein by Western blot analysis (Fig. 2). An LDHA isoform was previously identified from testis mRNA [15, 16] as containing a leader peptide sequence and estimated molecular weight of 38 kDa. The genebank database lists this protein as LDHA isoform 2 (accession number NP_001129541).

Why *Ldhc* gene expression has been conserved in mammalian germ cells where *Ldha* and *Ldhb* genes are also transcribed now becomes an enigma. From an evolutionary perspective, a gene duplication event (here *Ldha* giving rise to *Ldhc*) [17] would not be expected to persist unless it confers a selective advantage to the organism/tissue/cell. Not only has *Ldhc* survived as a testis-specific gene from marsupials to mammals, the protein is highly abundant in sperm and provides approximately 82% of the catalytic activity of this enzyme in these cells. This would seem to be advantageous because the primary metabolic path to ATP production is aerobic glycolysis. In fact, metabolism in sperm is not perturbed when they are treated with inhibitors of oxidative phosphorylation or when their mitochondria lack cytochrome c_t [2, 18]. However, as we demonstrated here, exogenous LDHA is as good as LDHC in maintaining fertilizing ability, and this occurs without an appreciable increase in null sperm glycolysis, which perhaps is related to our observation that *Ldhc*-null sperm were still able to convert pyruvate to lactate efficiently, being catalyzed by LDH that is tightly bound to the fibrous sheath of the sperm principal piece [2–4]. When the phenotype for the *Ldhc* KO mouse was identified to be male infertile, our first assumption was that it was due to a glycolytic deficit in energy production (ATP) required for motility and capacitation.

Capacitation is accompanied by a cAMP-protein kinase-dependent rise in tyrosine phosphorylation [19–21]. Protein phosphorylation mediated by kinases is involved in regulation of many cellular events such as transduction of extracellular signals and intracellular transport. During sperm capacitation, the cellular ATP level is considered to be critical for flagellar movement and signal transduction via protein phosphorylation [22, 23]. In *Ldhc* KO sperm, both motility and tyrosine phosphorylation are impaired and cannot be induced by in vitro capacitation. Additionally, *Ldhc* KO sperm are unable to take up glucose, which reportedly is necessary for protein tyrosine phosphorylation [24]. Protein is tyrosine phosphorylated in these sperm containing the LDHA transgene even though the cellular ATP level is not significantly different from the *Ldhc* KO sperm. This is further support for the argument that a soluble LDH must reside in the sperm cytoplasm to maintain this function.

We cannot rule out the possibility that the small increase in LDH activity is sufficient for rescue because there are differences in kinetic properties between LDHA and LDHC [25–29]. For example, the Michaelis-Menten Km for LDHA differs by an order of magnitude showing higher affinity for

pyruvate compared to LDHC. In addition, LDHC shows significant inhibition by high substrate concentration whereas LDHA is not inhibited by pyruvate. There was an initial increase in lactate production by the LDHA transgenic sperm. If a metabolic deficit is not partially or completely responsible for the KO phenotype, the key is likely to be cytoplasmic localization of the LDH. The endogenous LDHA in sperm has a higher molecular weight (Fig. 2), consistent with a leader sequence binding the protein tightly to the fibrous sheath, which is similar with what has been observed with GAPDHs [3]. The addition of LDHA to the cytoplasm restored motility, capacitation, and fertilizing capacity. Obviously, the LDH isozyme remaining in sperm lacking LDHC cannot preserve these functions. The transgenic LDHA, like LDHC, localizes to the cytoplasm, providing sufficient metabolic energy credits or perhaps just enabling glucose uptake to rescue sperm function. Alternatively, glycolysis mediated by LDH may be only partially responsible for sperm function. There may be a role for cytoplasmic LDH as suggested by the detection of LDHC protein-binding partners in WT sperm [30] that is required for the fertilizing capacity of spermatozoa.

ACKNOWLEDGMENT

We thank Dr. Lynn Doglio and the Northwestern University Transgenic Core Facility for generation of the transgenic mice. We are grateful to the High Throughput Analysis laboratory, for the ATP measurements, and to Genomics Core, for DNA sequencing, in Northwestern University.

REFERENCES

1. Everse J, Kaplan NO. Lactate dehydrogenases: structure and function. In: Meister A (ed.), Advances in Enzymology and Related Areas Molecular Biology, vol. 37. Hoboken, NJ: John Wiley & Sons, Inc.; 1973:61–133.
2. Odet F, Duan C, Willis WD, Goulding EH, Kung A, Eddy EM, Goldberg E. Expression of the gene for mouse lactate dehydrogenase C (Ldhc) is required for male fertility. Biol Reprod 2008; 79:26–34.
3. Miki K, Qu W, Goulding EH, Willis WD, Bunch DO, Strader LF, Perreault SD, Eddy EM, O'Brien DA. Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. Proc Natl Acad Sci U S A 2004; 101: 16501–16506.
4. Krisfalusi M, Miki K, Magyar PL, O'Brien DA. Multiple glycolytic enzymes are tightly bound to the fibrous sheath of mouse spermatozoa. Biol Reprod 2006; 75:270–278.
5. Goldberg E. Immunochemical Specificity of Lactate Dehydrogenase-X. National Academy of Sciences Proceedings 1971; 68:349–352.
6. Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P, Kopf GS. Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation.Development 1995; 121: 1129–1137.
7. Urner F, Sakkas D. Protein phosphorylation in mammalian spermatozoa. Reproduction 2003; 125:17–26.
8. Emiliozzi C, Fenichel P. Protein tyrosine phosphorylation is associated with capacitation of human sperm in vitro but is not sufficient for its completion. Biol Reprod 1997; 56:674–679.
9. Li S, Zhou W, Doglio L, Goldberg E. Transgenic mice demonstrate a testis-specific promoter for lactate dehydrogenase, LDHC. J Biol Chem 1998; 273:31191–31194.
10. Tang H, Kung A, Goldberg E. Regulation of murine lactate dehydrogenase C (Ldhc) gene expression. Biol Reprod 2008; 78:455–461.
11. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. 'Green mice' as a source of ubiquitous green cells. FEBS Lett 407:313–319.
12. Sawicki JA, Morris RJ, Monks B, Sakai K, Miyazaki J. A composite CMV-IE enhancer/beta-actin promoter is ubiquitously expressed in mouse cutaneous epithelium. Exp Cell Res 1998; 244:367–369.
13. Kato M, Yamanouchi K, Ikawa M, Okabe M, Naito K, Tojo H. Efficient selection of transgenic mouse embryos using EGFP as a marker gene. Mol Reprod Dev 1999; 54:43–48.
14. Sleight SB, Miranda PV, Plaskett NW, Maier B, Lysiak J, Scrable H, Herr JC, Visconti PE. Isolation and proteomic analysis of mouse sperm detergent-resistant membrane fractions: evidence for dissociation of lipid rafts during capacitation. Biol Reprod 2005; 73:721–729.
15. Hiraoka BY, Li SS. Lactate dehydrogenase-A mRNA in mouse testis and somatic tissues contain different 5′ noncoding sequences. J Genet Mol Biol 1990; 1:1–6.
16. Maekawa M, Inomata M, Sasaki MS, Kaneko A, Ushiama M, Sugano K, Takayama J, Kanno T. Electrophoretic variant of a lactate dehydrogenase isoenzyme and selective promoter methylation of the LDHA gene in a human retinoblastoma cell line. Clin Chem 2002; 48:1938–1945.
17. Holmes RS, Goldberg E. Computational analyses of mammalian lactate dehydrogenases: human, mouse, opossum and platypus LDHs. Comput Biol Chem 2009; 33:379–385.
18. Narisawa S, Hecht NB, Goldberg E, Boatright KM, Reed JC, Millán JL. Testis-specific cytochrome c-null mice produce functional sperm but undergo early testicular atrophy. Mol Cell Biol 2002; 22:5554–5562.
19. Travis AJ, Jorgez CJ, Merdiushev T, Jones BH, Dess DM, Diaz-Cueto L, Storey BT, Kopf GS, Moss SB. Functional relationships between capacitation-dependent cell signaling and compartmentalized metabolic pathways in murine spermatozoa. J Biol Chem 2001; 276:7630–7636.
20. Duncan AE, Fraser LR. Cyclic AMP-dependent phosphorylation of epididymal mouse sperm proteins during capacitation in vitro: identification of an M(r) 95,000 phosphotyrosine-containing protein. J Reprod Fertil 1993; 97:287–299.
21. Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, Pan D, Olds-Clarke P, Kopf GS. Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development 1995; 121:1139–1150.
22. Mukai C, Okuno M. Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. Biol Reprod 2004; 71:540–547.
23. Baker MA, Hetherington L, Ecroyd H, Roman SD, Aitken RJ. Analysis of the mechanism by which calcium negatively regulates the tyrosine phosphorylation cascade associated with sperm capacitation. J Cell Sci 2004; 117:211–222.
24. Aitken RJ, Harkiss D, Knox W, Paterson M, Irvine DS. A novel signal transduction cascade in capacitating human spermatozoa characterized by a redox-regulated, cAMP-mediated induction of tyrosine phosphorylation. J Cell Sci 1998; 111:645–656.
25. Allen JM. Multiple forms of lactic dehydrogenase in tissues of the mouse: their specificity, cellular localization, and response to altered physiological conditions. Ann N Y Acad Sci 1961; 94:937–951.
26. Goldberg E. Lactate dehydrogenase in spermatozoa: subunit interactions in vitro. Arch Biochem Biophys 1965; 109:134–141.
27. Goldberg E. Amino acid composition and properties of crystalline lactate dehydrogenase X from mouse testes. J Biol Chem 1972; 247:2044–2048.
28. Li SS, Feldmann RJ, Okabe M, Pan YC. Molecular features and immunological properties of lactate dehydrogenase C4 isozymes from mouse and rat testes. J Biol Chem 1983; 258(11):7017–7028.
29. Read JA, Winter VJ, Eszes CM, Sessions RB, Brady RL. Structural basis for altered activity of M- and H-isozyme forms of human lactate dehydrogenase. Proteins 2001; 43:175–185.
30. Odet F, Gabel SA, Williams J, London RE, Goldberg E, Eddy EM. Lactate dehydrogenase C and energy metabolism in mouse sperm. Biol Reprod 2011; 85:556–564.