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Sodium vitamin C transporter 2 orchestrates lactate metabolism in mouse Sertoli cells

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*(G Gao and Y Zhao contributed equally to this work)

Abstract

Transportation of vitamin C (also called ascorbic acid (AA)), an important water-soluble antioxidant and cofactor in testis, requires glucose transporter family (GLUTs) and sodium/vitamin C cotransporter family (SVCT1 and SVCT2). There is so far scant information vis-à-vis the functional roles of SVCTs in testis, although they possess higher affinity for transportation of AA compared to GLUTs. To analyze the biological effects of SVCT2 in testis, we assessed testicular expression of SVCT2 in different experimental settings and the effect of SVCT2 ablation on spermatogenesis. Persistent expression of SVCT2 was shown in the mouse testis at different stages of postnatal development, demonstrated on day 14 of testicular development in mice consistent with the appearance of pachytene spermatocytes during the first wave of spermatogenesis. Testicular expression of SVCT2 was enriched in the cytoplasm of murine Sertoli cells (SCs). We then showed that in vivo inhibition of SVCT2 in mouse testis significantly impaired male fertility by causing oligozoospermia and asthenospermia, which mainly stemmed from a deficiency in lactate production. By generating the TM4SVCT2−/− cells and by profiling TM4SVCT2−/− cells with a constitutively activated HIF-1α mutant, we demonstrated that SVCT2 deficiency led to impaired lactate synthesis and reduced expression of Ldha mRNA in SCs. Mechanistically, ablation of SVCT2 resulted in ubiquitination and subsequent degradation of HIF-1α protein in the FSH-stimulated SCs. Collectively, our data document a novel testicular site of action of SVCT2 in the control of lactate synthesis by SCs, probably via ubiquitination-dependent regulation of HIF-1α stability.

Introduction

Vitamin C (also called ascorbic acid/AA), acting as a major antioxidant inside testis (Angulo et al. 2011), is such a striking example. In guinea pig, it has been shown that the deficient diet causes massive degeneration of the seminiferous epithelium (Angulo et al. 2008). Using in vitro approaches, Dawson et al. have revealed that agglutination of normal spermatozoa occurs in the absence of vitamin C can be reversed by supplement with exogenous vitamin C (Dawson et al. 1992). Therefore, vitamin C is an essential micronutrient required for normal spermatogenesis. Transportation of vitamin C inside cells is dependent on two transport systems, namely glucose transporter family (GLUT1–GLUT14) and sodium/vitamin C cotransporter family (SVCT1 and SVCT2).
As compared to the low-affinity, high-capacity sodium-independent GLUTs, SVCTs exhibit a high specificity profile for reduced vitamin C and transport AA down the electrochemical sodium gradient (Liang et al. 2001). Both SVCT1 and SVCT2 have been shown to be expressed in testis (Angulo et al. 2011). From a functional standpoint, SVCT1 is involved in whole-body homeostasis of vitamin C, while SVCT2 protects metabolically active cells against oxidative stress. More importantly, only SVCT2 is reported to be functionally linked to concerted glucose transport inhibition and lactate transport stimulation in non-neuronal cells (Castro et al. 2008). To this end, it is tempting to hypothesize that SVCT2 may play a more important role in energy metabolism in Sertoli cells (SCs). Nonetheless, the functional details and corresponding molecular basis of testicular SVCT2 remain largely unknown.

During mammalian spermatogenesis, development and differentiation of meiotic spermatocytes and postmeiotic spermatids are solely dependent on the lactate produced by adjacent SCs, due to the fact that several enzymes in the glycolytic pathway are spermatogenic cell-specific (e.g. hexokinase, phosphoglycerate kinase-2 and glyceraldehyde 3-phosphate dehydrogenase) (Boussouar & Benahmed 2004). In fact, it has been recognized for nearly 20 years that SCs convert glucose to lactate to influence the survival of germ cells (GCs) (Boussouar & Benahmed 2004). Previous evidence has shown that disruption of lactate production by SCs contributes essentially to male infertility by causing defects or developmental arrest in germ cells (GCs) of specific cellular type (Jain & Halder 2012). In addition, during the last decades, it has become more apparent that oxidative stress, induced by high rates of cell division and mitochondrial oxygen consumption during spermatogenesis, are involved in the auto-/paracrine control of lactate metabolism and Sertoli–germ cell interaction under various pathophysiological conditions (Aitken & Roman 2008, Li et al. 2015, Dong et al. 2017, Shen et al. 2018, Sun et al. 2019, Jin et al. 2020, Uriostegui-Acosta et al. 2020). In this regard, emerging results have been recently reported for the testicular expression and/or direct biological actions of a range of oxidative stress-related factors with potential roles in modulating lactate metabolism, such as prostaglandin D2 (Rossi et al. 2016), GATA4 (Schrade et al. 2016) and nuclear factor (erythroid-derived 2)-like 2 (NRF2) (Regueira et al. 2015). Conceivably, these results together leave the open possibility that energy balance and redox homeostasis might be jointly regulated in testis, and clearly point out the functional diversity of a number of novel molecules whose biological functions largely exceed nutritional supply and redox metabolism.

Because of the presence of sodium-dependent vitamin C transporters, AA is highly concentrated in brain, testis and adrenal glands (Castro et al. 2008). More importantly, AA has been shown to participate in a general mechanism for concerted glucose transport inhibition and lactate transport stimulation in both neuronal and non-neuronal cells (Castro et al. 2009). These reports thus raise the possibility that the factors mediating the vitamin C signaling might regulate lactate metabolism in certain cell types (e.g. SCs and GCs).

On the aforementioned basis, we sought: (1) to determine the expression profile of SVCT2 in mouse testis, (2) to illustrate any biological effects upon ablation of endogenous SVCT2 expression, and (3) to elucidate the potential mechanisms which might underlie the SVCT2 action in distinct testicular cell types. Overall, our systematic analysis should pave the way for a better understanding of the SVCT2 function during complicated spermatogenesis.

**Materials and methods**

**Animal model**

C57BL/6J male mice bred in the animal facility of Luoyang Central Hospital Affiliated to Zhengzhou University were used. Mice were maintained under a constant 12 h light:12 h darkness cycle (lights on at 08:00 h), and controlled conditions of humidity (between 70 and 80%) and temperature (22 ± 1°C), with free access to tap water and standard pellet chow. Mice were allowed to acclimatize for 7 days before experiments. To achieve the *in vivo* inhibition of SVCT2 expression, mice were injected intraperitoneally (i.p.) with SVCT2 MISSION® esiRNA (3 μg/mouse in 100 μL isotonic saline solution, *n* = 15, Sigma-Aldrich) daily for 10 days. Mice injected with Ctrl esiRNA (the sequence of the Ctrl esiRNA has no significant homology with any sequence in the databases) were used as negative controls. Mice received in total two cycles of esiRNA treatment (Dong et al. 2016). The protocols involved in animal work, strictly conformed to the National Institutes of Health guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978), were approved by Institutional Animal Care and Use Committee of Luoyang Central Hospital Affiliated to Zhengzhou University (Approval #: LYCH-2016-0354Y).
Assessment of male fertility and epididymal parameters

Effects of SVCT2 ablation on male fertility and epididymal parameters were determined at 70 days after siRNA treatment, according to a previous report (Zhang et al. 2018). One male mouse was mated with two naïve females. Once vaginal plugs were identified on the morning check, the female mouse with vaginal plugs was immediately replaced with another new one. A total of seven males in each experimental group were subjected to mating test within 21 days. Females with copulation plugs were finally checked for pregnancy and litter size. Characterization of the density and motility of caudal epididymal sperms was performed as described in detail in a previous report (Ma et al. 2010).

Measurement of serum testosterone and testicular apoptosis

Upon sodium pentobarbital anesthesia (0.05 mg/g body weight, i.p.), blood samples were collected from mouse orbital sinus, and serum testosterone levels were assessed using the Testosterone Parameter Assay Kit (R&D Systems), as per the manufacturer’s instructions. Testicular apoptosis was determined in testicular homogenates using an apoptosis ELISA kit (Roche Diagnostics), following the manufacturer’s instructions. Final spectrophotometry was performed at 405 nm on a Bio-Rad microplate reader.

Cell treatment

Primary SCs were isolated and purified from adult mice (10-week-old), using collagenase and DNase digestion combined with hypotonic treatment, as described elsewhere (Dong et al. 2016). Briefly, testes were decapsulated and seminiferous tubules were incubated with 0.1% (w/v), type IX collagenase (Sigma-Aldrich) at room temperature (RT) for 15 min. The resultant mixture was digested in culture medium containing 0.04% DNase I (Sigma-Aldrich) at 34°C for 30 min. The seminiferous tubules were subsequently incubated in 1x Hanks fluid (pH 7.4) containing 0.04% DNase I, 0.05% hyaluronidase, and 0.5% trypsin for at least 10 min at 34°C with agitation. After being centrifuged at 100 g and at 60 g for 3 min, respectively, cells in the supernatant were harvested and cultured for another 48 h. Cells were then hypotonically treated with 20 mM Tris (pH 7.4) at 22°C for 2.5 min to lyse residual germ cells (Xiao et al. 2011). The purity of primary SCs was confirmed using a real-time PCR.

The murine Sertoli cell line TM4, obtained from American Type Culture Collection, was maintained in a 1:1 mixture of Ham’s F12 medium and DMEM with 1.2 g/L sodium bicarbonate and 15 mM HEPES, 92.5% horse serum and 5% fetal bovine serum. On day 3 of cell culture, cells were incubated for 48 h with FSH (100 ng/mL) or bFGF (30 ng/mL) to stimulate lactate production. Both FSH and bFGF were purchased from Sigma–Aldrich. Primary Leydig cells (LCs) and germ cells (GCs) were prepared based on the methods described in previous reports (Zhao et al. 2008, Li et al. 2011). To stably knockdown the SVCT2 expression, TM4 cells were transfected with SVCT2 pCMV-Neo-shRNA or with Ctrl shRNA (Sigma–Aldrich) using Lipofectamine™ 3000 (Thermo Fisher Scientific), followed by selection with 0.5 µg/mL G418 (Thermo Fisher Scientific), following the manufacturer’s instructions. TM4SVCT2−/−/HA-HIF1αP402A/P564A cells were generated by transfection of HA-HIF1αP402A/P564A-pBabe-puro followed by selection with 50 ng/mL puromycin (Sigma–Aldrich). HA-HIF1αP402A/P564A-pBabe-puro was a gift from William Kaelin (Addgene plasmid # 19005; http://n2t.net/addgene:19005; RRID:Addgene_19005). TM4 cells, which are established cell line cultures derived from immature not adult mice, possess major properties of normal SCs (Luo et al. 2018).

Determination of lactate and ammonium levels

In this study, 10% (w/v) testicular homogenates were prepared in pre-cold 0.5 M monophosphoric acid, followed by incubation at 4°C for 20 min with 5 M potassium carbonate solution to eliminate the acid. After being centrifuged at 1.0 × 10^4 g for 15 min, the supernatants were collected and subjected to measurement of intratesticular lactate levels using a Lactate Colorimetric/Fluorometric Assay Kit (BioVision, Milpitas, CA, USA).

In another experimental setting, following the stimulation with FSH (100 ng/mL) or bFGF (30 ng/mL) for 48 h, the conditioned cell culture media were harvested and subjected to measurement of lactate and ammonium levels using the BioVision Lactate Colorimetric/Fluorometric Assay Kit and an Ammonia Assay Kit (Abcam), respectively.

Real-time PCR

Total RNA was isolated with the aid of E.Z.N.A.® HP Total RNA Kit (Omega Bio-tek, Norcross, GA, USA). After a DNase Digestion for RNA purification using RNeasy Kit (Qiagen), RNA samples were subjected to
RT using QuantiTect RT Kit (Qiagen). Subsequent real-time PCR was performed on a Step-One Real-Time PCR System (Applied Biosystems). Amplification of 18S was used as an internal control. Primer used were Svt2 (NM_018824.2), 5’-GTGCCATCGGCGGAC-3’ and 5’-GGCCTGGAGTTGATCA-3’; 18S (M10098.1), 5’-CTCGCCGCCTCCTACCTACCTA-3’ and 5’-ATGAGCCATTGCAGTTTCG-3’. Primers for other target genes have been reported elsewhere (Zhang et al. 2018). The relative expression levels of target genes were determined using the comparative ΔΔCt method (Li et al. 2011).

Immunoblotting

Protein samples were prepared from cultured cells and testicular tissues using Minute™ Protein Extraction Kit (Invent Biotechnologies, Plymouth, MN, USA). After protein identification on a NanoDrop™ spectrophotometer (Thermo Fisher Scientific), ~30 μg of protein samples were subjected to SDS–PAGE, and were then transferred onto a PVDF membrane (Thermo Fisher Scientific). Membranes were incubated at 4°C overnight with different primary antibodies (Supplementary Table 1, see section on supplementary materials given at the end of this article). Subsequent chemiluminescent detection of the target proteins was performed on a Freedom Rocker™ BlotBot® automated blot processor, following the manufacturer’s instructions, with β-actin serving as the loading control.

Morphological examination

Bouin’s fluid-fixed paraffin-embedded testicular tissues were subjected to a 5-μm-sectioning, followed by staining with a Hematoxylin and Eosin Staining Kit (Beyotime, Shanghai, China).

To reveal the localization of SVCT2 protein in situ, 4% paraformaldehyde-fixed paraffin-embedded testicular sections were routinely deparaffinized and rehydrated, and were subjected to citrate buffer antigen retrieval at 95°C for 20 min. After blocking of endogenous peroxidase by incubating slides in 0.3% H2O2 for 20 min at room temperature (RT), sections were incubated at 4°C overnight with an SVCT2 antibody (Supplementary Table 1), followed by sequential incubation at RT with biotinylated second antibodies and streptavidin-peroxidase complex (Vector Lab, Burlingame, CA, USA) for 1 h, respectively. Final immunoreaction was developed at RT for 10 min using 0.7 mg/ml 3,3’-diaminobenzidine tetrahydrochloride in 1.6 mg/ml urea hydrogen peroxide (Sigma-Aldrich).

To visualize the subcellular localization of SVCT2 protein, TM4 cells were treated for 48 h with 100 ng/mL FSH, and were then fixed in 4% paraformaldehyde at RT for 20 min. Upon completion of incubation with blocking solution (10% donkey serum, 0.5% BSA plus 0.3% triton X-100 in PBS) for 30 min, cells were incubated with the anti-HIF-1α antibody at 4°C overnight. Final immunoreactions were developed by incubation with FITC 488–conjugated second antibody at RT for 1 h, followed by observation under a Zeiss 510 confocal microscope.

Luciferase reporter assay

pLightSwitch-Luc-Ldha and the corresponding blank vector were obtained from SwitchGear Genomics (Shanghai, China). For reporter assay, TM4SVCT2−/−, TM4 or TM4SVCT2−/−/HA-HIF1αP402A/P564A cells were transfected with 100 ng reporter plasmids using Lipofectamine™ 3000. 48 h after transfection, cells were stimulated with 100 ng/mL FSH for another 12 h, followed by measurement of the relative luciferase activities using the LightSwitch reporter assay system, as instructed by the manufacturer.

Co-immunoprecipitation (Co-IP)

TM4SVCT2−/− or TM4 cells were transfected with pCMV6-Myc-Hif1α using Lipofectamine™ 3000. 48 h later, cells were stimulated with 100 ng/mL FSH for another 48 h, followed by Co-IP assay as described in detail in a previous report (Zhang et al. 2012).

Data presentation and statistical analysis

Representative results are presented from at least three independent experiments. All outlier data points have been included in the main statistical analysis. Quantitative data were expressed as the mean ± S.E.M. Results were analyzed for statistically significant differences using Student’s t-test or ANOVA plus Tukey’s test. P < 0.05 was considered statistically significant.

Results

SVCT2 is specifically expressed in SCs in murine testis

As a first step to explore the testicular action of SVCT2, we examined its expression pattern in 10-week-old mouse testis. Immunoblotting with the anti-SVCT2 antibody revealed a single band of the target protein in the whole blot. By contrast, immunoblotting with the preabsorbed
primary antibody failed to detect any positive signal, verifying the antibody specificity (Fig. 1A). Subsequent PCR and immunoblotting analyses in different testicular cells demonstrated that SVCT2 was exclusively expressed in SCs (Fig. 1B). In line with these results, our immunostaining showed that SVCT2 protein was enriched in the cytoplasm of SCs (arrows in Fig. 1C). Moreover, evaluation of SVCT2 expression by immunoblotting and real-time PCR analyses showed that SVCT2 expression began to emerge from postnatal day 14 (corresponding to the appearance of pachytene spermatocytes), and increased thereafter, with the highest levels being observed in adulthood (Fig. 1D and E). These findings suggest that SVCT2 expression is developmentally regulated in mouse testis.

**In vivo knockdown of SVCT2 expression significantly impairs male infertility by inducing oligozoospermia and asthenospermia**

To understand the biological effects of testicular SVCT2 expression, we knocked down the *in vivo* SVCT2 expression by employing a previously validated protocol (Fig. 2A) (Gonzalez-Herrera *et al.* 2006, Dong *et al.* 2016). Mice received i.p. injections with SVCT2 MISSION® esiRNA on daily basis for consecutive 10 days, followed by a 25-day break. At the end of 79 days after first injection, mice were sacrificed and testes were harvested. Real-time PCR revealed that esiRNA decreased the *Svct2* mRNA content in testes by ~57.3% within two cycles of siRNA treatment (Fig. 2B). The effectiveness of esiRNA on the inhibition of SVCT2 expression was further confirmed at the protein level by immunoblotting (Fig. 2C) and immunohistochemistry (Fig. 2D). Consistently, immunoblotting with the protein samples prepared from the primary SCs from esiRNA-treated testes also showed a significant reduction in the SVCT2 protein levels, compared to those from Ctrl siRNA-treated or naïve testes (Fig. 2E). Consequently, knockdown of SVCT2 expression had no effects on serum testosterone levels (Fig. 2F), but resulted in a noticeable change in testicular morphology, including desquamation of GCs, loss of lumen inside seminiferous tubules and decrease of testis weight/body weight ratio (Fig. 2G and

![Figure 1](https://jme.bioscientifica.com)

Expression pattern of SVCT2 in mouse testis. (A) Immunoblot stained with an anti-SVCT2 antibody or a preabsorbed serum demonstrated the specificity of this antibody. The protein samples were harvested from testes of 10-week-old mice. (B) Expression profile of SVCT2 was evaluated in different testicular cells and in adult mouse testis using PCR and immunoblotting assays, respectively. 18S and β-actin served as internal controls. (C) Immunohistochemical analysis in mouse testis (T) revealed a distinct cytoplasmic localization of SVCT2 in SCs (arrows). Replacement of the primary antibody with preabsorbed primary antibody (P) abolished the positive immunostaining, confirming the specificity of the assay. Bar, 25 μm. (D) Diagram of the stages of murine spermatogenesis during postnatal development. (E) Immunoblotting analysis of testicular SVCT2 protein levels throughout postnatal development. (F) Real-time PCR analysis of testicular *Svct2* mRNA levels throughout postnatal development. Different superscript letters denote groups that are statistically different (*P* < 0.05). UD, undetectable.
Supplementary Fig. 1) and increase in germ cell apoptosis (~5.4-fold, Fig. 2H). In keeping with the histological changes, SVCT2 deficiency significantly impaired male fertility by causing oligozoospermia and asthenospermia (Table 1). Of note, SVCT2 was not expressed in mouse epididymis (Supplementary Fig. 2). To clarify at which stage the spermatogenesis was blocked by SVCT2 knockdown, we performed qPCR analyses on testis RNA from Ctrl siRNA or esiRNA-treated testes using specific primers for various testicular cell types (Zhang et al. 2018). SVCT2 deficiency caused more than ~62.8% reduction in the expression levels of Sycp3, Acrv1 and Dbil5 transcripts (Fig. 2I), indicating that the absolute numbers of haploid cells (round and elongated spermatids) and tetraploid cells (mainly pachytene spermatocytes) were significantly decreased in esiRNA-treated testes. Given that the differentiation of tetraploid spermatocytes and haploid spermatids is solely fueled by Sertoli cell-produced lactate, we tended to believe that the germ cell loss by SVCT2 deficiency might be ascribed to a disturbance in lactate metabolism. In favor of our hypothesis, biochemical analysis using the testicular homogenates uncovered a ~57.4% reduction in the intratesticular lactate levels in esiRNA-treated testes, compared to those from Ctrl.
siRNA-treated or naïve testes (Fig. 2J). Together, SVCT2 deficiency caused qualitative and quantitative defects during spermatogenesis.

Supplement with the exogenous lactate ameliorates SVCT2 deficiency-impaired spermatogenesis

To validate the biological effects of testicular SVCT2 on lactate production, we provided the mice with sodium \(\text{L-lactate} \) by i.p. injection at the same time of siRNA treatment (Fig. 3A). Apparently, supplement with the exogenous lactate had no effects on the expression levels of SVCT2 in mouse testis (Fig. 3B and C). However, replenishment of exogenous lactate successfully rescued the loss of testis weight (Supplementary Fig. 1), and improved SVCT2 deficiency-induced GCs desquamation and restored the lumen structure in the majority of seminiferous tubules (Fig. 3D). In line with these histological changes, replenishment of exogenous lactate prevented the SVCT2 deficiency-induced GCs apoptosis by \(\sim 56.4\%\) (Fig. 3E), and significantly ameliorated the male fertility and epididymal parameters in esiRNA-treated testis (Table 2). The latter was likely due to a partial restorement of the absolute numbers of tetraploid spermatocytes and haploid spermatids, as revealed by qPCR analyses (Fig. 3E).

Ablation of the endogenous SVCT2 expression attenuates FSH/bFGF-stimulated lactate synthesis in TM4 cells

To further explore the functional details of SVCT2 in lactate metabolism, we generated the TM4\(^{SVCT2\,-/-}\) cells using shRNA transfection. The successful establishment of TM4\(^{SVCT2\,-/-}\) cells was verified by real-time PCR (Fig. 4A) and immunoblotting assays (Fig. 4B). As a result, the FSH/bFGF-stimulated lactate production and the metabolic waste product ammonium during lactate synthesis were both was significantly decreased in conditioned media from TM4\(^{SVCT2\,-/-}\) cells, as compared to naïve or Ctrl shRNA-treated TM4 cells. Of note, SVCT2 depletion had no effects on the basal levels of lactate synthesis in TM4 cells (Fig. 4C and D). Spurred by the metabolic phenotype, we sought to determine the expression levels of several core genes known to be essential for lactate synthesis in SCs (Fig. 4E) (Zhang et al. 2018). Upon stimulation with FSH or bFGF, expression levels of all the target transcripts studied were significantly induced in naïve or Ctrl shRNA-treated TM4 cells. By contrast, FSH/bFGF failed to stimulate the expression of \(Ldha\) mRNA in the TM4\(^{SVCT2\,-/-}\) cells (Fig. 4F). Thus, SVCT2 depletion may cause defects at the last step of lactate synthesis in SCs.

SVCT2 deficiency attenuates the HIF-1α-elicited transactivation of \(Ldha\) gene by inducing ubiquitination and instability of HIF-1α during lactate synthesis

During the complicated lactate synthesis in SCs, expression of \(Ldha\) gene is subjected to a delicate control at the transcriptional level (Jungmann et al. 1998). Indeed in our study, treatment with 100 ng/mL FSH for 12 h significantly stimulated the promoter activity of \(Ldha\) gene in TM4 cells, while this stimulatory effect was totally abolished in the TM4\(^{SVCT2\,-/-}\) cells (Fig. 5A). As further exploration of the molecular basis underlying the aforementioned phenotype, we unexpectedly observed that FSH-induced HIF-1α expression (Fig. 5B) and nuclear translocation (Fig. 5C) were totally lost in the TM4\(^{SVCT2\,-/-}\) cells. Moreover, SVCT2 depletion had no effects on the expression levels of \(Hif1a\) mRNA (Fig. 5D), but resulted in a dramatic increase in the ubiquitination of HIF-1α protein in FSH-challenged TM4 cells (Fig. 5E). To further establish whether HIF1-α contributes to SVCT2’s function in lactate production, we performed rescue experiments using a HA-tagged constitutively activated HIF-1α mutant construct (HA-HIF1αP402A/P564A) (Yan et al. 2007). As expected, enforced overexpression of HA-HIF1αP402A/P564A restored the nuclear expression of HIF1-α, even in the absence of FSH stimulation, in TM4\(^{SVCT2\,-/-}\) cells (Fig. 5F and G). Consequently, constitutive activation of

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**Table 1** Assessment of male fertility and epididymal parameters after in vivo transfection.

<table>
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<tr>
<th>Experimental groups</th>
<th>Reproductive capacity</th>
<th>Characteristics of epididymal sperms</th>
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<tr>
<td></td>
<td>Pregnancies/females mated</td>
<td>Litter size</td>
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<tr>
<td>Naïve</td>
<td>22/27 (81.5%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.2 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ctrl siRNA</td>
<td>20/24 (83.3%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.9 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SVCT2 siRNA</td>
<td>4/26 (15.4%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.7 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

Different superscript letters denote groups that are statistically different in the same category (\(P < 0.05\)).
HIF-1α successfully restored the FSH-stimulated Ldha transactivation (Fig. 5H) and lactate production (Fig. 5I) in TM4SVCT2−/− cells. Considering that HIF-1α pathway activation regulates fundamentally the lactate production in SCs (Galardo et al. 2017), and mouse Ldha promoter contained two putative binding sites of HIF1-α and HIF1-α positively regulated the transactivation of the Ldha gene (Supplementary Fig. 3), we have identified SVCT2's role in mouse testis.
control of the HIF-1α stability program in SCs as a major mechanism during lactate synthesis.

**Discussion**

To our knowledge, this is the first study to provide a comprehensive evaluation of the pattern of expression and functional implication of SVCT2 in mouse testis. Three main observations were obtained so far. First, testicular SVCT2 expression was exclusively enriched in SCs, and its expression was indispensable for normal spermatogenesis. Second, ablation of endogenous SVCT2 expression significantly impaired male fertility by disrupting the lactate production. Last, SVCT2 deficiency attenuated lactate synthesis by inducing the ubiquitination and degradation of HIF-1α protein in FSH-challenged TM4 cells. Overall, it is tempting to consider SVCT2 as a novel modulator of the energy metabolism through posttranslational regulation of HIF1-α singling pathway in mouse testis.

When meiosis begins from puberty, the differentiation of tetraploid spermatocytes and haploid spermatids becomes more specialized and their biochemical machinery is insufficient to fulfill their metabolic demands. To this end, lactate produced by the adjacent SCs remains the sole energy source fueling the continuous dividing of these unique germ cells (Crisostomo et al. 2018). Intriguingly, SVCT2 expression began to emerge right after the appearance of tetraploid pachytene spermatocytes, as revealed by our profiling analysis using testicular samples from different postnatal ages (Fig. 1D, E and F). These findings point to a close correlation between SVCT2 expression and energy requirement along postnatal testicular maturation.

Validation of the functional importance of SVCT2 expression in SCs was achieved using an in vivo siRNA approach. The effects of SVCT2 knockdown, along with the supplementation with l-lactate, on male fertility and epididymal parameters are shown in Table 2.

<table>
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<th>Characteristics of epididymal sperms</th>
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<td></td>
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</tr>
<tr>
<td>Ctrl siRNA</td>
<td>20/25 (80.0%)ᵃ</td>
<td>8.8 ± 1.7ᵃ</td>
</tr>
<tr>
<td>SVCT2 siRNA</td>
<td>5/27 (18.5%)ᵇ</td>
<td>3.1 ± 0.8ᵇ</td>
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<tr>
<td>SVCT2 siRNA saline</td>
<td>7/32 (21.8%)ᵇ</td>
<td>3.6 ± 0.4ᵇ</td>
</tr>
<tr>
<td>Sodium L-lactate</td>
<td>16/28 (57.1%)ᶜ</td>
<td>7.2 ± 0.6ᶜ</td>
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Different superscript letters denote groups that are statistically different in the same category (P < 0.05).

**Table 2** Effects of in vivo SVCT2 knockdown, along with the supplementation with l-lactate, on male fertility and epididymal parameters.

**Figure 4** Identification of Ldha as the most affected target gene upon SVCT2 depletion in FSH/bFGF-stimulated TM4 cells. (A) Generation of the TM4SVCT2−/− cells was validated at the mRNA level using real-time PCR analysis. Different superscript letters denote groups that are statistically different (n = 5, P < 0.05). (B) Generation of the TM4SVCT2−/− cells was validated at the protein level using immunoblotting assay (n = 5). (C and D) Different TM4 cells were incubated for 48 h with FSH (100 ng/mL) or bFGF (30 ng/mL), followed by measurement of lactate and ammonium levels in culture media using a colorimetric assay kit. Different superscript letters denote groups that are statistically different (n = 5, P < 0.05). (E) Schematic presentation depicting the main metabolic pathways responsible for lactate production in SCs. (F) Altered expression of genes impacting lactate metabolism in SCs transfected with SVCT2 shRNA or Ctrl shRNA were quantified by real-time PCR analysis. Different superscript letters denote groups that are statistically different (n = 4, P < 0.05).
Figure 5
SVCT2 deficiency led to impaired lactate production and reduced Ldha mRNA expression, mainly through induction of the ubiquitination and subsequent degradation of HIF-1α protein in stimulated SCs. (A) TM4SVCT2−/− or TM4 cells were transfected with 100 ng reporter plasmids using Lipofectamine™ 3000. Forty-eight hours after transfection, cells were stimulated with 100 ng/mL FSH for another 12 h, followed by measurement of the relative luciferase activities using the LightSwitch reporter assay system. Different superscript letters denote groups that are statistically different (n = 5, P < 0.05). (B) TM4 or TM4SVCT2−/− cells were treated for 48 h with 100 ng/mL FSH, followed by immunoblotting assay (n = 4). (C) TM4 or TM4SVCT2−/− cells were treated for 48 h with 100 ng/mL FSH, followed by evaluation of the subcellular localization of HIF-1α using immunofluorescence plus observation under a confocal microscope. (D) TM4 or TM4SVCT2−/− cells were treated for 48 h with 100 ng/mL FSH, followed by real-time PCR analysis. Different superscript letters denote groups that are statistically different (n = 4, P < 0.05). Establishment of the TM4SVCT2−/−/HA-HIF1αP402A/P564A cells were verified using immunoblotting assay. (G) The TM4SVCT2−/−/HA-HIF1αP402A/P564A cells were treated for 48 h with 100 ng/mL FSH, followed by measurement of lactate levels in culture media using a colorimetric assay kit. Different superscript letters denote groups that are statistically different (n = 5, P < 0.05).
treatment. In this setting, inhibition of SVCT2 expression by ~57.3% within two cycles of mouse spermatogenesis significantly impaired male fertility by causing oligozoospermia and asthenospermia (Table 1), and this phenotype was probably ascribed to the disrupted lactate synthesis. The latter hypothesis is supported by three lines of evidence: (1) The most affected GCs by SVCT2 deficiency are pachytene spermatocytes and haploid spermatids, as revealed by the qPCR analysis in siRNA-treated testis (Fig. 2I). (2) Intratesticular lactate content was noticeably reduced in siRNA-treated testis (Fig. 2I). (3) Most importantly, replenishment of sodium l-lactate by i.p. injection successfully ameliorated histological changes (Fig. 3D), restored germ cell composition (Fig. 3F) and improved male fertility in SVCT2-depleted mice (Table 2). Thus, the available data strongly suggest that normal occurrence of lactate synthesis in SCs requires proper levels of SVCT2 expression.

By generating the TM4SVCT2−/− cells, we further identified Ldha as the most affected gene in SVCT2-depleted TM4 cells (Fig. 4F). The lactate dehydrogenase (LDH), mainly consisting of LDHA and/or LDHB subunits, catalyzes the final step of lactate synthesis, namely conversion of pyruvate to lactate (Huang et al. 2012). Concerning the testicular function of LDHA, two aspects should be emphasized. On one hand, despite the coexistence of LDHB subunit, LDHA is so far believed to be the most important LDH subunit in SCs (Rato et al. 2012, Schrade et al. 2016). Indeed in our study, repression of Ldha expression by SVCT2 depletion dramatically compromised FSH/bFGF-stimulated lactate production in TM4SVCT2−/− cells (Fig. 4C and D). On the other hand, the expression of Ldha gene is tightly controlled at the transcriptional level. Previous studies have identified multiple cis-acting elements including functional sites for Sp1, c-Myc, CREB and AP1 located in the promoter region of Ldha gene. Likewise, a 8-bp cAMP-responsive element (CRE) has been found to be located at the −48/−41-bp upstream region of Ldha promoter, and this site regulates fundamentally the Ldha transcription (Jungmann et al. 1998). Our results extend these understandings by identifying SVCT2 as an indispensable regulator of Ldha mRNA expression during lactate synthesis in SCs. Because SVCT2 does not function as a transcription factor (Nualart et al. 2014), it is tempting to hypothesize that SVCT2 may regulate Ldha mRNA expression in an indirect manner that requires cooperation of other key transcription factors.

It is well known that the transcriptional activity of the master transcription factor HIF-1α is significantly increased under hypoxic conditions (Fanale et al. 2013). However, emerging data evidence that Hif1α transactivation can also be induced under certain normoxic conditions, including glucose metabolism, angiogenesis and maintenance of iron homeostasis (Haase 2006, Semenza 2010, Loboda et al. 2012). In this context, fragmentary information had previously pointed to a potent involvement of HIF-1α signaling in the modulation of SCs lactate synthesis. FSH regulates Hif1α subunits expression and HIF-1α transcriptional activity, at the same time of stimulating lactate production in SCs. Moreover, a higher HIF-1α transcriptional activity is associated with an augmentation of Ldha mRNA levels in rat SCs (Galardo et al. 2017). In accordance with these pioneer findings, we have shown that mouse Ldha promoter contained two putative binding sites of HIF-1α, and HIF-1α positively regulated the transactivation of the Ldha gene in FSH/bFGF-challenged SCs (Supplementary Fig. 3). Thus, it is very likely that the HIF-1α-mediated transcriptional regulatory program may represent a novel modulator of the function of lactate metabolism in SCs. By profiling TM4SVCT2−/− cells in the setting of ectopic Myc-Hif1α or HA-HIF1αP402A/P564A expression, we further demonstrated that SVCT2 deficiency caused impaired lactate production and reduced Ldha mRNA expression, mainly through induction of the ubiquitination and subsequent degradation of HIF-1α protein in stimulated SCs (Fig. 5). In support of our observation, ascorbic acid has been shown to regulate osterix expression in osteoblasts by activation of prolyl hydroxylase and ubiquitination-mediated proteosomal degradation pathway (Xing et al. 2011). Therefore, regulation of the stability of the target protein via modulation of its ubiquitination status appears to be a general mechanism of ascorbic acid/SVCT2 system in energy metabolism. Furthermore, our findings support the idea that the lactate synthesis could utilize a mode of regulation that links the control of gene expression by key transcription factors and posttranslational modifications.

As the active component of vitamin C, AA serves as a vital protector of cells against oxidant stress. It regulates cell differentiation and is involved in signal transduction in many systems (Jurek et al. 2020). For example, AA treatment promotes osteogenic differentiation capability in periodontal ligament stem cells (PDLCs) (Yang et al. 2020). Supplement with AA helps to maintain early NK cell differentiation by facilitating the demethylation of killer Ig-like receptors (KIR) (Wu et al. 2020). Likewise, vitamin C/AA is involved in the differentiation of precursor cells throughout the whole brain (Salazar et al. 2016). From a mechanistic standpoint, the enhancement of intracellular concentrations of AA has been long...
considered to be the primary molecular mechanism underlying the physiological actions of SVCT2. In our study, however, SVCT2 is mainly considered as a signaling molecule, with no attempt to relate its expression to AA uptake and accumulation. In favor of our observation, Salazar et al. have reported that overexpression of SVCT2 induces neuronal branching and thereby activates the differentiation and maturation in neuroblastoma N2a cells, whereas increasing intracellular vitamin C content via the overexpression of SVCT1 or AA supplementation does not completely reproduce the differentiating changes induced by SVCT2 overexpression (Salazar et al. 2016). Combined with our findings, the available data suggest that SVCT2 per se can function as a regulatory molecule in both differentiating (e.g. neuronal stem cells) and differentiated (e.g. SCs).

Based on the available data, we propose a novel role of SCs-expressing SVCT2. Upon energy requirement for the differentiation of meiotic and postmeiotic GCs, levels of lactate production-promoting hormones (e.g. FSH and bFGF) surge inside the testis. These hormones elicit a significant increase in SVCT2 expression, which inhibits ubiquitination and degradation of HIF-1α via unidentified mechanism and then facilitates Ldha mRNA expression and subsequent lactate synthesis. Conversely, ablation of SVCT2 expression may result in the disruption of the ubiquitination of HIF-1α protein and the inevitable HIF-1α instability, followed by an unusual suppression of Ldha transcription and lactate production (Fig. 6). Limitations of the current study lie in two aspects: (1) We need to answer how SVCT2 regulates the ubiquitination of HIF-1α protein in stimulated SCs. (2) A Sertoli cell-specific knockout of SVCT2 will be of great value to disclose the full involvement of SVCT2 in the control of lactate synthesis in vivo, and is currently under progress in our group.

Supplementary materials
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Declaration of interest
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Author contribution statement
W F designed and supervised the experiments, contributed reagents and helped to analyzed the data and wrote the manuscript. G G, Z Y and W K performed the experiments and analyzed the data. G G helped to write the manuscript.

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