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

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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 TM4^{SVCT2-/-} cells and by profiling TM4^{SVCT2-/-} cells with a constitutively activated HIF-1 α 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-1 α 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-1 α stability.

Key Words

- ▶ Sertoli cells (SCs)
- ▶ lactate
- ▶ SVCT2
- ▶ HIF-1 α
- ▶ ubiquitination

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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 an array of oxidative stress-related factors with potent 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 ($22 \pm 1^\circ\text{C}$), 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 1× 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. TM4^{SVCT2-/-}/HA-HIF1α^{P402A/P564A} cells were generated by transfection of HA-HIF1α^{P402A/P564A}-pBabe-puro followed by selection with 50 ng/mL puromycin (Sigma-Aldrich). HA-HIF1α^{P402A/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 $1.0 \times 10^4 g$ 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 *Svct2* (NM_018824.2), 5'-GTGCGATCGGCGGGAC-3' and 5'-GTTGGCCTGGAGTGGTTACA-3'; *18S* (M10098.1), 5'-CTCGCCGCGCTCTACCTACCTA-3' and 5'-ATGAGCCATTCGCAGTTTCACTGTA-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 $\Delta\Delta C_t$ 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-1 α 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, TM4^{SVCT2-/-}, TM4 or TM4^{SVCT2-/-}/HA-HIF1 α ^{P402A/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)

TM4^{SVCT2-/-} or TM4 cells were transfected with pCMV6-Myc-Hif1a 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 $\pm$ 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

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 μ m. (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.

Figure 2

In vivo knockdown of SVCT2 expression causes defective spermatogenesis in mouse testis. (A) Schematic representation of the experimental procedure used in the *in vivo* SVCT2 siRNA treatment. (B) At the end of 79 days following the first injection with SVCT2 siRNA, the efficiency of SVCT2 knockdown was verified at the mRNA level using real-time PCR analysis in mouse testis ($n = 7$). Different superscript letters denote groups that are statistically different ($P < 0.05$). (C) At the end of 79 days following the first injection with SVCT2 siRNA, efficiency of SVCT2 knockdown was verified at the protein level using immunoblotting assay in mouse testis ($n = 7$). (D) Immunohistochemical analysis of testicular SVCT2 expression 79 days after the *in vivo* siRNA treatment. (E) Primary SCs were isolated from mouse testis at the end of 79 days following the first injection with SVCT2 siRNA, followed by immunoblotting assay. (F) ELISA analysis of serum testosterone levels 79 days after the *in vivo* siRNA treatment ($n = 7$). Different superscript letters denote groups that are statistically different ($P < 0.05$). (G) Effects of *in vivo* SVCT2 knockdown on testicular morphology were determined using H&E staining 79 days after the *in vivo* siRNA treatment. Bar, 25 µm. (H) ELISA analysis of testicular apoptosis after the *in vivo* SVCT2 siRNA treatment. Different superscript letters denote groups that are statistically different ($n = 7$, $P < 0.05$). (I) The deleterious effects of *in vivo* SVCT2 knockdown on the spermatogenic differentiation was evaluated by real-time PCR analyses using specific primers for *Rhox5* (SCs), *Cyp11a1* (Leydig cells), *Zbtb16* (undifferentiated spermatogonia), *Sohlh2* (differentiating spermatogonia), *Ldh-c4* (preleptotene spermatocytes), *Sycp3* (pachytene spermatocytes) and for *Acrv1* and *Dbil5* (haploid spermatids), respectively. Results presented as mean $\pm$ S.E.M. of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ when compared to the values in naïve testis. (J) After two cycles of SVCT2 siRNA injection as described in 'Materials and methods' section, mice were sacrificed and intratesticular levels of g lactate were determined using biochemical assays. Different superscript letters denote groups that are statistically different ($P < 0.05$).

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

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

Experimental groups	Reproductive capacity		Number of males mated	Characteristics of epididymal sperms	
	Pregnancies/females mated	Litter size		Number of sperm (10 ⁶ /epididymis)	Progressive motility (%)
Naïve	22/27 (81.5%) ^a	8.2 ± 2.4 ^a	7	32.6 ± 2.3 ^a	41.5 ± 1.9 ^a
Ctrl siRNA	20/24 (83.3%) ^a	7.9 ± 1.3 ^a	7	30.9 ± 1.1 ^a	40.2 ± 2.2 ^a
SVCT2 siRNA	4/26 (15.4%) ^b	2.7 ± 0.8 ^b	7	17.4 ± 0.7 ^b	10.6 ± 3.2 ^b

Different superscript letters denote groups that are statistically different in the same category ($P < 0.05$).

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 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 ~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 restoration 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 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-1 α -elicited transactivation of *Ldha* gene by inducing ubiquitination and instability of HIF-1 α 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-1 α 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-1 α protein in FSH-challenged TM4 cells (Fig. 5E). To further establish whether HIF-1 α contributes to SVCT2's function in lactate production, we performed rescue experiments using a HA-tagged constitutively activated HIF-1 α mutant construct (HA-HIF1 α ^{P402A/P564A}) (Yan *et al.* 2007). As expected, enforced overexpression of HA-HIF1 α ^{P402A/P564A} restored the nuclear expression of HIF-1 α , even in the absence of FSH stimulation, in TM4^{SVCT2-/-} cells (Fig. 5F and G). Consequently, constitutive activation of

Figure 3

Supplement with exogenous lactate ameliorates spermatogenic defects in SVCT2 siRNA-treated testis. (A) Schematic representation of the experimental procedure for *in vivo* supplement with sodium L-lactate in our mouse model. (B) At the end of 79 days following the first injection with SVCT2 siRNA, efficiency of SVCT2 knockdown was verified at the protein level using immunoblotting assay in mouse testis ($n = 7$). (C) At the end of 79 days following the first injection with SVCT2 siRNA, efficiency of SVCT2 knockdown was verified at the mRNA level using real-time PCR analysis in mouse testis ($n = 7$). Different superscript letters denote groups that are statistically different ($P < 0.05$). (D) Effects of *in vivo* SVCT2 knockdown plus lactate supplementation on testicular morphology were determined using H&E staining 79 days after the *in vivo* siRNA treatment. Bar, 25 µm. (E) ELISA analysis of testicular apoptosis after combined SVCT2 siRNA and lactate treatment. Different superscript letters denote groups that are statistically different ($n = 7$, $P < 0.05$). (F) Effects of *in vivo* SVCT2 knockdown plus lactate supplementation on the spermatogenic differentiation was evaluated by real-time PCR analyses, as described previously. Results presented as mean $\pm$ s.e.m. of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ when compared to the values in naive testis. Different superscript letters denote groups that are statistically different ($P < 0.05$).

HIF-1 α successfully restored the FSH-stimulated *Ldha* transactivation (Fig. 5H) and lactate production (Fig. 5I) in TM4^{SVCT2-/-} cells. Considering that HIF-1 α 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

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

Experimental groups	Reproductive capacity		Characteristics of epididymal sperms		
	Pregnancies/females mated	Litter size	Number of males mated	Number of sperm (10 ⁶ /epididymis)	Progressive motility (%)
Ctrl siRNA	20/25 (80.0%) ^a	8.8 ± 1.7 ^a	7	34.9 ± 4.8 ^a	46.3 ± 4.8 ^a
SVCT2 siRNA	5/27 (18.5%) ^b	3.1 ± 0.8 ^b	7	15.6 ± 1.2 ^b	12.4 ± 3.5 ^b
SVCT2 siRNA					
Saline	7/32 (21.8%) ^b	3.6 ± 0.4 ^b	7	14.8 ± 1.7 ^b	16.4 ± 2.2 ^b
Sodium L-lactate	16/28 (57.1%) ^c	7.2 ± 0.6 ^c	7	24.3 ± 2.4 ^c	39.8 ± 5.6 ^a

Different superscript letters denote groups that are statistically different in the same category ($P < 0.05$).

control of the HIF-1 α 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-1 α 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 HIF-1 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

Figure 4

Identification of *Ldha* as the most affected target gene upon SVCT2 depletion in FSH/bFGF-stimulated TM4 cells. (A) Generation of the TM4^{SVCT2-/-} 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 TM4^{SVCT2-/-} 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) TM4^{SVCT2-/-} 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 TM4^{SVCT2-/-} cells were treated for 48 h with 100 ng/mL FSH, followed by immunoblotting assay ($n = 4$). (C) TM4 or TM4^{SVCT2-/-} 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 TM4^{SVCT2-/-} 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 ($n = 4$, $P < 0.05$). (E) TM4^{SVCT2-/-} or TM4 cells were transfected with pCMV6-Myc-Hif1a using Lipofectamine™ 3000. Forty-eight hours later, cells were stimulated with 100 ng/mL FSH for another 48 h, followed by Co-IP assay using the antibody as indicated. (F) Establishment of the TM4^{SVCT2-/-}/HA-HIF1α^{P402A/P564A} cells were verified using immunoblotting assay. (G) The TM4^{SVCT2-/-}/HA-HIF1α^{P402A/P564A} 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. (H) TM4^{SVCT2-/-}/HA-HIF1α^{P402A/P564A} 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 ($n = 5$, $P < 0.05$). (I) TM4^{SVCT2-/-}/HA-HIF1α^{P402A/P564A} cells were incubated for 48 h with FSH (100 ng/mL) 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. 2J). (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 TM4^{SVCT2-/-} 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 TM4^{SVCT2-/-} 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-1 α is significantly increased under hypoxic conditions (Fanale *et al.*

2013). However, emerging data evidence that *Hif1a* 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-1 α signaling in the modulation of SCs lactate synthesis. FSH regulates *Hif1a* subunits expression and HIF-1 α transcriptional activity, at the same time of stimulating lactate production in SCs. Moreover, a higher HIF-1 α 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-1 α , and HIF-1 α positively regulated the transactivation of the *Ldha* gene in FSH/bFGF-challenged SCs (Supplementary Fig. 3). Thus, it is very likely that the HIF-1 α -mediated transcriptional regulatory program may represent a novel modulator of the function of lactate metabolism in SCs. By profiling TM4^{SVCT2-/-} cells in the setting of ectopic Myc-Hif1a or HA-HIF1 α ^{P402A/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-1 α 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 (PDLSCs) (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

Figure 6

Model for involvement of the posttranslational modification of the HIF-1 α signaling pathway in SVCT2 deficiency-mediated lactate disruption.

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-1 α 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-1 α protein and the inevitable HIF-1 α 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-1 α 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

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-20-0101>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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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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References

- Aitken RJ & Roman SD 2008 Antioxidant systems and oxidative stress in the testes. *Advances in Experimental Medicine and Biology* **636** 154–171. (https://doi.org/10.1007/978-0-387-09597-4_9)
- Angulo C, Castro MA, Rivas CI, Segretain D, Maldonado R, Yanez AJ, Slebe JC, Vera JC & Concha II 2008 Molecular identification and functional characterization of the vitamin C transporters expressed by Sertoli cells. *Journal of Cellular Physiology* **217** 708–716. (<https://doi.org/10.1002/jcp.21545>)
- Angulo C, Maldonado R, Pulgar E, Mancilla H, Cordova A, Villarreal F, Castro MA & Concha II 2011 Vitamin C and oxidative stress in the seminiferous epithelium. *Biological Research* **44** 169–180. (<https://doi.org/S0716-97602011000200009>)
- Boussouar F & Benahmed M 2004 Lactate and energy metabolism in male germ cells. *Trends in Endocrinology and Metabolism* **15** 345–350. (<https://doi.org/10.1016/j.tem.2004.07.003>)

- Castro MA, Angulo C, Brauchi S, Nualart F & Concha II 2008 Ascorbic acid participates in a general mechanism for concerted glucose transport inhibition and lactate transport stimulation. *Pflugers Archiv* **457** 519–528. (<https://doi.org/10.1007/s00424-008-0526-1>)
- Castro MA, Beltran FA, Brauchi S & Concha II 2009 A metabolic switch in brain: glucose and lactate metabolism modulation by ascorbic acid. *Journal of Neurochemistry* **110** 423–440. (<https://doi.org/10.1111/j.1471-4159.2009.06151.x>)
- Crisostomo L, Alves MG, Gorga A, Sousa M, Riera MF, Galardo MN, Meroni SB & Oliveira PF 2018 Molecular mechanisms and signaling pathways involved in the nutritional support of spermatogenesis by Sertoli cells. *Methods in Molecular Biology* **1748** 129–155. (https://doi.org/10.1007/978-1-4939-7698-0_11)
- Dawson EB, Harris WA, Teter MC & Powell LC 1992 Effect of ascorbic acid supplementation on the sperm quality of smokers. *Fertility and Sterility* **58** 1034–1039. ([https://doi.org/10.1016/S0015-0282\(16\)55456-9](https://doi.org/10.1016/S0015-0282(16)55456-9))
- Dong YS, Hou WG, Li Y, Liu DB, Hao GZ, Zhang HF, Li JC, Zhao J, Zhang S, Liang GB, *et al.* 2016 Unexpected requirement for a binding partner of the syntaxin family in phagocytosis by murine testicular Sertoli cells. *Cell Death and Differentiation* **23** 787–800. (<https://doi.org/10.1038/cdd.2015.139>)
- Dong BW, Jin XH, Yan CY, Yang T, Cai GQ & Lu J 2017 Synergistic upregulation of NONO and PSPC1 regulates Sertoli cell response to MEHP via modulation of ALDH1A1 signaling. *FEBS Letters* **591** 914–923. (<https://doi.org/10.1002/1873-3468.12568>)
- Fanale D, Bazan V, Corsini LR, Caruso S, Insalaco L, Castiglia M, Cicero G, Bronte G & Russo A 2013 HIF-1 is involved in the negative regulation of AURKA expression in breast cancer cell lines under hypoxic conditions. *Breast Cancer Research and Treatment* **140** 505–517. (<https://doi.org/10.1007/s10549-013-2649-0>)
- Galardo MN, Gorga A, Merlo JP, Regueira M, Pellizzari EH, Cigorraga SB, Riera MF & Meroni SB 2017 Participation of HIFs in the regulation of Sertoli cell lactate production. *Biochimie* **132** 9–18. (<https://doi.org/10.1016/j.biochi.2016.10.006>)
- Gonzalez-Herrera IG, Prado-Lourenco L, Pileur F, Conte C, Morin A, Cabon F, Prats H, Vagner S, Bayard F, Audigier S, *et al.* 2006 Testosterone regulates FGF-2 expression during testis maturation by an IRES-dependent translational mechanism. *FASEB Journal* **20** 476–478. (<https://doi.org/10.1096/fj.04-3314fje>)
- Haase VH 2006 Hypoxia-inducible factors in the kidney. *American Journal of Physiology: Renal Physiology* **291** F271–F281. (<https://doi.org/10.1152/ajprenal.00071.2006>)
- Huang L, Lin Y, Jin S, Liu W, Xu Y & Zheng Y 2012 Alternative splicing of testis-specific lactate dehydrogenase C gene in mammals and pigeon. *Animal Biotechnology* **23** 114–123. (<https://doi.org/10.1080/10495398.2011.652325>)
- Jain M & Halder A 2012 Sertoli cell only syndrome: status of Sertoli cell maturation and function. *Indian Journal of Endocrinology and Metabolism* **16** S512–S513. (<https://doi.org/10.4103/2230-8210.104154>)
- Jin X, Zhang S, Ding T, Zhao P, Zhang C, Zhang Y & Li W 2020 Testicular Lmcd1 regulates phagocytosis by Sertoli cells through modulation of NFAT1/Txlna signaling pathway. *Aging Cell* **19** e13217. (<https://doi.org/10.1111/acer.13217>)
- Jungmann RA, Huang D & Tian D 1998 Regulation of LDH-A gene expression by transcriptional and posttranscriptional signal transduction mechanisms. *Journal of Experimental Zoology* **282** 188–195. ([https://doi.org/10.1002/\(SICI\)1097-010X\(199809/10\)282:1/2<188::AID-JEZ21>3.0.CO;2-P](https://doi.org/10.1002/(SICI)1097-010X(199809/10)282:1/2<188::AID-JEZ21>3.0.CO;2-P))
- Jurek S, Sandhu MA, Trappe S, Bermudez-Pena MC, Kolisek M, Sponder G & Aschenbach JR 2020 Optimizing adipogenic transdifferentiation of bovine mesenchymal stem cells: a prominent role of ascorbic acid in FABP4 induction. *Adipocyte* **9** 35–50. (<https://doi.org/10.1080/21623945.2020.1720480>)
- Li W, Wu ZQ, Zhao J, Guo SJ, Li Z, Feng X, Ma L, Zhang JS, Liu XP & Zhang YQ 2011 Transient protection from heat-stress induced apoptotic stimulation by metastasis-associated protein 1 in pachytene spermatocytes. *PLoS ONE* **6** e26013 (<https://doi.org/10.1371/journal.pone.0026013>)
- Li W, Fu J, Zhang S, Zhao J, Xie N & Cai G 2015 The proteasome inhibitor bortezomib induces testicular toxicity by upregulation of oxidative stress, AMP-activated protein kinase (AMPK) activation and deregulation of germ cell development in adult murine testis. *Toxicology and Applied Pharmacology* **285** 98–109. (<https://doi.org/10.1016/j.taap.2015.04.001>)
- Liang WJ, Johnson D & Jarvis SM 2001 Vitamin C transport systems of mammalian cells. *Molecular Membrane Biology* **18** 87–95. (<https://doi.org/10.1080/09687680110033774>)
- Loboda A, Jozkowicz A & Dulak J 2012 HIF-1 versus HIF-2 – is one more important than the other? *Vascular Pharmacology* **56** 245–251. (<https://doi.org/10.1016/j.vph.2012.02.006>)
- Luo Y, Mohsin A, Xu C, Wang Q, Hang H, Zhuang Y, Chu J & Guo M 2018 Co-culture with TM4 cells enhances the proliferation and migration of rat adipose-derived mesenchymal stem cells with high stemness. *Cytotechnology* **70** 1409–1422. (<https://doi.org/10.1007/s10616-018-0235-3>)
- Ma L, Li W, Zhu HP, Li Z, Sun ZJ, Liu XP, Zhao J, Zhang JS & Zhang YQ 2010 Localization and androgen regulation of metastasis-associated protein 1 in mouse epididymis. *PLoS ONE* **5** e15439. (<https://doi.org/10.1371/journal.pone.0015439>)
- Nualart F, Mack L, Garcia A, Cisternas P, Bongarzone ER, Heitzer M, Jara N, Martinez F, Ferrada L, Espinoza F, *et al.* 2014 Vitamin C transporters, recycling and the bystander effect in the nervous system: SVCT2 versus GLUTs. *Journal of Stem Cell Research and Therapy* **4** 209. (<https://doi.org/10.4172/2157-7633.1000209>)
- Rato L, Alves MG, Socorro S, Duarte AI, Cavaco JE & Oliveira PF 2012 Metabolic regulation is important for spermatogenesis. *Nature Reviews: Urology* **9** 330–338. (<https://doi.org/10.1038/nrurol.2012.77>)
- Regueira M, Riera MF, Galardo MN, Camberos Mdel Mdel C, Pellizzari EH, Cigorraga SB & Meroni SB 2015 FSH and bFGF regulate the expression of genes involved in Sertoli cell energetic metabolism. *General and Comparative Endocrinology* **222** 124–133. (<https://doi.org/10.1016/j.ygcen.2015.08.011>)
- Rossi SP, Windschuttl S, Matzkin ME, Rey-Ares V, Terradas C, Ponzio R, Puigdomenech E, Levalle O, Calandra RS, Mayerhofer A, *et al.* 2016 Reactive oxygen species (ROS) production triggered by prostaglandin D2 (PGD2) regulates lactate dehydrogenase (LDH) expression/activity in TM4 Sertoli cells. *Molecular and Cellular Endocrinology* **434** 154–165. (<https://doi.org/10.1016/j.mce.2016.06.021>)
- Salazar K, Martinez M, Ulloa V, Bertinat R, Martinez F, Jara N, Espinoza F, Bongarzone ER & Nualart F 2016 SVCT2 overexpression in neuroblastoma cells induces cellular branching that is associated with ERK signaling. *Molecular Neurobiology* **53** 6668–6679. (<https://doi.org/10.1007/s12035-015-9553-z>)
- Schrade A, Kyronlahti A, Akinrinade O, Pihlajoki M, Fischer S, Rodriguez VM, Otte K, Velagapudi V, Toppari J, Wilson DB, *et al.* 2016 GATA4 regulates blood-testis barrier function and lactate metabolism in mouse Sertoli cells. *Endocrinology* **157** 2416–2431. (<https://doi.org/10.1210/en.2015-1927>)
- Semenza GL 2010 HIF-1: upstream and downstream of cancer metabolism. *Current Opinion in Genetics and Development* **20** 51–56. (<https://doi.org/10.1016/j.gde.2009.10.009>)
- Shen L, Tang X, Wei Y, Long C, Tan B, Wu S, Sun M, Zhou Y, Cao X & Wei G 2018 Vitamin E and vitamin C attenuate Di-(2-ethylhexyl) phthalate-induced blood-testis barrier disruption by p38 MAPK in immature SD rats. *Reproductive Toxicology* **81** 17–27. (<https://doi.org/10.1016/j.reprotox.2018.06.015>)
- Sun J, Yin B, Tang S, Zhang X, Xu J & Bao E 2019 Vitamin C mitigates heat damage by reducing oxidative stress, inducing HSP expression in TM4 Sertoli cells. *Molecular Reproduction and Development* **86** 673–685. (<https://doi.org/10.1002/mrd.23146>)

- Uriostegui-Acosta M, Tello-Mora P, Solis-Heredia MJ, Ortega-Olvera JM, Pina-Guzman B, Martin-Tapia D, Gonzalez-Mariscal L & Quintanilla-Vega B 2020 Methyl parathion causes genetic damage in sperm and disrupts the permeability of the blood-testis barrier by an oxidant mechanism in mice. *Toxicology* **438** 152463. (<https://doi.org/10.1016/j.tox.2020.152463>)
- Wu CY, Zhang B, Kim H, Anderson SK, Miller JS & Cichocki F 2020 Ascorbic acid promotes KIR demethylation during early NK cell differentiation. *Journal of Immunology* **205** 1513–1523. (<https://doi.org/10.4049/jimmunol.2000212>)
- Xiao X, Mruk DD, Lee WM & Cheng CY 2011 c-yes regulates cell adhesion at the blood-testis barrier and the apical ectoplasmic specialization in the seminiferous epithelium of rat testes. *International Journal of Biochemistry and Cell Biology* **43** 651–665. (<https://doi.org/10.1016/j.biocel.2011.01.008>)
- Xing W, Pourteymoor S & Mohan S 2011 Ascorbic acid regulates osterix expression in osteoblasts by activation of prolyl hydroxylase and ubiquitination-mediated proteosomal degradation pathway. *Physiological Genomics* **43** 749–757. (<https://doi.org/10.1152/physiolgenomics.00229.2010>)
- Yan Q, Bartz S, Mao M, Li L & Kaelin WG, Jr 2007 The hypoxia-inducible factor 2alpha N-terminal and C-terminal transactivation domains cooperate to promote renal tumorigenesis in vivo. *Molecular and Cellular Biology* **27** 2092–2102. (<https://doi.org/10.1128/MCB.01514-06>)
- Yang Y, Wang T, Zhang S, Jia S, Chen H, Duan Y, Wang S, Chen G & Tian W 2020 Vitamin C alleviates the senescence of periodontal ligament stem cells through inhibition of Notch3 during long-term culture. *Journal of Cellular Physiology* **236** 1237–1251. (<https://doi.org/10.1002/jcp.29930>)
- Zhang S, Li W, Zhu C, Wang X, Li Z, Zhang J, Zhao J, Hu J, Li T & Zhang Y 2012 Sertoli cell-specific expression of metastasis-associated protein 2 (MTA2) is required for transcriptional regulation of the follicle-stimulating hormone receptor (FSHR) gene during spermatogenesis. *Journal of Biological Chemistry* **287** 40471–40483. (<https://doi.org/10.1074/jbc.M112.383802>)
- Zhang LL, Ma J, Yang B, Zhao J, Yan BY, Zhang YQ & Li W 2018 Interference with lactate metabolism by mmu-miR-320-3p via negatively regulating GLUT3 signaling in mouse Sertoli cells. *Cell Death and Disease* **9** 964. (<https://doi.org/10.1038/s41419-018-0958-2>)
- Zhao Y, Hou WG, Zhu HP, Zhao J, Wang RA, Xu RJ & Zhang YQ 2008 Expression of thyrotropin-releasing hormone receptors in rat testis and their role in isolated Leydig cells. *Cell and Tissue Research* **334** 283–294. (<https://doi.org/10.1007/s00441-008-0680-y>)

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