Knockdown of Sucla2 decreases the viability of mouse spermatocytes by inducing apoptosis through injury of the mitochondrial function of cells

Shaoping Huang¹,², Jing Wang³, Lei Wang²

¹Department of Human Anatomy and Neuroscience, Medical School, Southeast University, Nanjing, China
²State Key Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing, China
³MingDe Hospital affiliated with Nanjing Medical University, Nanjing, China

Abstract
Introduction. Sucla2, a β subunit of succinyl coenzyme A synthase, is located in the mitochondrial matrix. Sucla2 catalyzes the reversible synthesis of succinate and adenosine triphosphate (ATP) in the tricarboxylic acid cycle. Sucla2 expression was found to be correlated with the capacitation of boar spermatozoa. We have previously reported that Sucla2 was decreased in the testes of rats with spermatogenesis failure after exposure to endocrine disruptor BDE47. Yet, the expression model of Sucla2 in spermatogenesis and the function of Sucla2 in spermatogenic cells are still unclear. Our objective was to explore the localization of Sucla2 during mouse spermatogenesis and its function in the mouse spermatocyte line (GC2).

Material and methods. The localization of Sucla2 in the mouse testis was explored through immunohistochemistry (IHC). Sucla2 was knocked down in GC2 cells and its expression was detected by Western blot (WB) to verify the efficiency of the siRNA transfection. Mitochondrial membrane potential (MMP), apoptosis and ROS of GC2 were detected by flow cytometry. ATP production was measured by the luminometric method and the presence of Bcl2 of GC2 was detected by WB.

Results. Sucla2 is highly expressed in all germ cells but not in interstitial cells. Coarse Sucla2 signals are found in spermatocytes in stages VII–XII of mouse spermatogenesis. In GC2 cells, knockdown of Sucla2 decreased cell viability and MMP, induced apoptosis of GC2 cells, decreased ATP production, and Bcl2 expression, and increased ROS levels.

Conclusions. Sucla2 is related to the developmental stages of mouse spermatogenesis. Knockdown of Sucla2 decreases the viability of mouse spermatocytes by inducing apoptosis via decreased mitochondrial function of the cells. (Folia Histochemica et Cytobiologica 2016, Vol. 54, No. 3, 134–142)

Key words: mouse; Sucla2 knockdown; spermatogenesis; spermatocyte; mitochondria; apoptosis; Bcl2; ROS; ATP

Introduction

Sucla2, a β subunit of Succinyl coenzyme A synthase, is located in the mitochondria and catalyzes the reversible synthesis of succinate and adenosine triphosphate (ATP) in the tricarboxylic acid cycle. Sucla2 is encoded by the nuclear gene SUCLA2 [1], and is associated with the maintenance of mitochondrial DNA (mtDNA) and function of mitochondria [2]. Defects in the SUCLA2 gene result in myopathic mtDNA depletion syndrome, characterized by a significant reduction in mtDNA levels and mitochondrial dysfunction [3–5]. In patients with SUCLA2 mutation, impaired production of ATP and high levels of reactive oxygen species (ROS) were found in fibroblasts [6]. Only a few authors have studied Sucla2 expression in males. Recently, proteomic analysis showed that Sucla2 is related to capacitation of boar spermatozoa [7].
In a previous study, we found that the protein level of Sucla2 was decreased in testes of rats with spermatogenesis failure, and decreased in mouse spermatogonia cell line (GC1) with increased apoptosis after exposure to the endocrine disruptor BDE47; Sucla2 is located in germ cells and is highly expressed in VII–XII stages of rat spermatogenesis [8]. This previous study indicates that Sucla2 is important during spermatogenesis, and probably plays an important role in the proliferation and apoptosis of germ cells. Nonetheless, the function of Sucla2 in mammalian spermatogenic cells has not yet been investigated and the precise localization of the Sucla2 protein in mouse testis is still unclear. The aim of our study was thus to explore the function and expression of Sucla2 during mouse spermatogenesis through knockdown of its mRNA in the mouse spermatocyte cell line (GC2).

**Material and methods**

**Animals and treatment.** Adult male ICR mice (28 ± 2 g) were obtained from the Laboratory Animal Center of Nanjing Medical University (Nanjing, China) and maintained in a 12/12-h light/dark cycle at 20–22°C and 50–70% humidity, with food and water provided *ad libitum*. The animals were treated according to the rules of the Nanjing Medical University and sacrificed with carbon dioxide. Testes were fixed in Bouin’s solution. The study was approved by The Animal Ethical Committee of Nanjing Medical University, Nanjing, China; study number: 20090598). The fixed tissues were embedded in paraffin, sectioned, and stained as described before [9].

**Immunohistochemistry (IHC).** IHC was used to explore the localization of Sucla2 in mouse testes. Paraffin sections of mouse testes were immunostained as described previously [9]. In brief, deparaffinized sections of mouse testes were incubated in 2% H2O2 to quench the endogenous peroxidase activity, washed in phosphate-buffered saline (PBS), blocked with a blocking serum and incubated with primary antibody against Sucla2 (1:50, 12627-1-AP, ProteinTech Group Inc., Chicago, IL, USA) overnight at 4°C. The next day, sections were washed with PBS, and incubated with HRP-conjugated secondary antibody (1:1500, ab97080, abcam, Cambridge, MA, USA) overnight at 4°C for 1 h, and the insoluble matter was removed by centrifugation at 40,000 g and 4°C for 1 hour. Protein concentrations were determined by the Bradford method using BSA as the standard [8].

**Protein extraction.** After siRNA transfection, the GC2 cells were collected and homogenized in a lysis buffer containing 1% (v/w) protease inhibitor cocktail (78415, Pierce Biotechnology, Rockford, IL, USA). The mixture was shaken at 4°C for 1 h, and the insoluble matter was removed by centrifugation at 40,000 g and 4°C for 1 hour. Protein concentrations were determined by the Bradford method using BSA as the standard [8].

**Western blot analysis.** Protein levels of Sucla2 and Bcl2 in GC2 after siRNA transfection were analyzed by Western blot, as previously described [12]. Glycerinaldehyde-3-phosphate dehydrogenase (Gapdh) was used as an internal reference. Antibodies against Sucla2 (1:1500, 12627-1-AP, ProteinTech Group, Inc., Chicago, IL, USA), Bcl-2 (1:50, sc-7382, Santa Cruz Biotech), and Gapdh (1:1000, Kc-5G5, KangChen Bio-tech Inc., Shanghai, China) were purchased. Protein samples were electrophoresed on 12% SDS poly-
acrylamide gels and transferred to nitrocellulose membranes (Amersham Biosciences AB, Uppsala, Sweden). Membranes were then blocked in phosphate-buffered saline (PBS) containing 5% nonfat milk powder for 1 h, and incubated overnight with anti-Sucla2, anti-Bcl2, or anti-GAPDH primary antibody in PBS containing 5% nonfat milk powder. The next day, membranes were washed three times (10 min each) with PBS and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Beijing ZhongShan Biototechnology Co. Ltd., Beijing, China) at 37°C for 1 h. Specific proteins were detected using an ECL kit (Amersham Biosciences, Buckinghamshire, England) and AlphaImager (FluorChem5500; Alpha Innotech, San Leandro, CA, USA).

**Detection of mitochondrial membrane potential (MMP).** MMP was detected by flow cytometry using JC-1 mitochondrial potential detection kit (C2006, Beyotime Biotech, Shanghai, China) as previously indicated [8]. In brief, GC2 cells after Sucla2-targeting siRNA transfection were incubated with JC-1 dye for 15 min in DMEM at 37°C, and then subjected to flow cytometry. Healthy cells emitted red fluorescence, while cells with disrupted MMP emitted green fluorescence. The red/green fluorescence ratio (Y/X ratio) is an indicator of MMP: a lower ratio indicates mitochondrial depolarization. The data were analyzed with Cell Quest software (Becton Dickinson, Franklin Lakes, NJ, USA).

**Determination of apoptotic GC2 cells.** Sucla2 siRNA transfection apoptotic GC2 cells were detected by flow cytometry (Becton Dickinson, FACS Canto, Franklin Lakes, NJ, USA) using the Annexin V–FITC/PI Apoptosis Detection Kit (BI-AP0103, Bioinquirer Technology, Hongzhou, China). FITC-labeled Annexin V binds phosphatidylserine on the surface of apoptotic cells, but of not living cells, whereas PI can be excluded by living cells. The Annexin V-positive/PI-negative cells (lower right quadrant, LR) were early apoptotic cells, the double-positive (upper right quadrant, UR) were late apoptotic cells, the Annexin V-negative/PI-positive (upper left quadrant, UL) were dead/necrotic cells, and the double-negative cells (lower left quadrant, LL) were viable cells. The sum of LR and UR is the percentage of apoptotic GC2 cells [13].

**Detection of reactive oxygen species (ROS) production.** ROS detection kit (s0033, Beyotime Biotech, Shanghai, China) and flow cytometry were used to detect the reactive oxygen species (ROS) in the cells, as described in a previous paper [14]. 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich, Saint Louis, MO, USA) is a fluorescent dye with no fluorescence. When DCFH-DA enters into cytoplasm of cells, it can be oxidized by ROS to form 2',7'-dichlorodihydrofluorescein (DCF) emits fluorescence allowing ROS levels to be determined through measuring the DCF fluorescence intensity. The cells were collected and incubated with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich) at 37°C for 30 min, washed with PBS, and analyzed by flow cytometry.

**Statistical analysis.** The numerical data were generated from three independent experiments and expressed as means ± SDs. ANOVA (with the LSD post-hoc statistical test) was used to evaluate the significant differences between different groups (SSPS 20.0, IBM Co., Armonk, NY, USA). There results were considered to be statistically significant at P < 0.05.

**Results**

**Immunohistochemistry (IHC) of Sucla2 in mouse testes**

The IHC of Sucla2 was performed because the localization of Sucla2 in the mouse testis is still unclear. We found that Sucla2 is located specifically in the cytoplasm of the Sertoli cells (Ser) and all the spermatogenic cells such as leptotene spermatocytes (Le), pachytene spermatocytes (Pc), round spermatids (rSt), and elongated spermatids (eSt) (Figure 1). Coarse Sucla2 signals can be observed in the cytoplasm of Pc in stages VII–XII, but there are very faint signals in Leydig cells and other cells present in the interstitium of the testes. The expression of Sucla2 protein in mouse testes is related to the developmental stages of spermatogenesis (Figure 1).

**Efficiency of Sucla2-targeting siRNA transfection and viability of GC2 cells under Sucla2 knockdown**

The Western blot technique was used to verify the efficiency of Sucla2-targeting siRNA transfection. Sucla2 expression was reduced through Sucla2 knockdown in GC2 cells; also, Sucla2 was decreased by increasing the Sucla2-targeting siRNA reagent from 6 μL in the Si1 group to 12 μL in the Si2 group (Figure 2A).

In the GC2 cells transfected by Sucla2-targeting siRNA, cell viability in the Neg, Si1 group (treated with 6 μL Sucla2-targeting siRNA), and Si2 group (treated with 12 μL Sucla2-targeting siRNA) was 100% ± 3.4%, 76.1% ± 5.3% and 59.1% ± 9.9%.
respectively. Compared with the control, the decrease in cell viability after Sucla2 knockdown was statistically significant in the Si1 and Si2 groups (Figure 2B).

**Sucla2 knockdown affects MMP of GC2 cells**

In the Neg (control) group, cells grown in higher density emitted orange fluorescence after JC-1 staining, indicating a higher MMP (orange arrow),
in the Si1 and Si2 groups, cells were smaller in size and grew with lower density, indicating lower MMP (green arrow) and lower viability (Figure 3A). The red/green fluorescence ratio was 1.09 ± 0.07, 0.92 ± 0.02, 0.87 ± 0.01 in the control (Neg), Si1 and Si2 groups, respectively, and the differences vs. control were significant (Figure 3B). Thus, the knockdown of Sucla2 decreased MMP in the GC2 cells.

ATP production and ROS level in GC2 cells under Sucla2 knockdown

The ATP production of GC2 cells in the Neg, Si1, and Si2 groups was 58.39 ± 3.76, 45.48 ± 2.28, 25.25 ± 6.11 nmol/(L·mg) respectively (Figure 4A). Compared with the control (Neg) cells, Sucla2 knockdown induced a statistically significant decrease in ATP production in Si1 and Si2 cells, respectively.

The average fluorescence intensities of DCF in cells from the Neg, Si1, and Si2 groups were 41.72 ± 1.13, 20.53 ± 0.47, 17.77 ± 0.3, respectively. Relative to the Neg (control cells), the increase in ROS production in the Si1 and Si2 groups was statistically significant (Figure 4B).

Effects of Sucla2 knockdown on apoptosis and Bcl2 level of GC2 cells

The percentage of apoptotic GC2 cells in the Neg, Si1, and Si2 groups was 6.19 ± 0.68%, 6.78 ± 1.07%, and 9.75 ± 1.3%, respectively. Compared with the control, the increase of apoptotic cells in the Si2...
Sucla2 affects viability and apoptosis of mouse spermatocytes

The level of anti-apoptotic factor Bcl2, as determined by Western blotting, was significantly decreased by Sucla2 knockdown of GC2 cells (p < 0.01, Figure 5B). Thus, Sucla2 knockdown induced apoptosis of GC2 cells through the mitochondrial apoptotic pathway.

Discussion

Spermatogenesis involves the self-renewal of spermatogonial stem cells, differentiation, meiosis of spermatocytes and spermiogenesis [15]. This process is characterized by a delicate balance between cell proliferation and germ cells apoptosis [16]. Mitochondria are complex organelles that not only participate in ATP synthesis and ROS production, but also play important roles in proliferation, differentiation, and apoptosis of germ cells [17, 18]. Mitochondrial function depends not only on mitochondrial molecules encoded by nuclear DNA (nDNA), but also depends on molecules encoded by mitochondria DNA (mtDNA) [19, 20]. The literature shows that many kinds of abnormal expression of mitochondrial proteins are associated with mitochondrial dysfunction, resulting in mitochondrial diseases such as neurodegeneration, cancer, infertility, and ageing [21–23].

Recently, it has been shown that the abnormal forms of some proteins encoded by nDNA are related to the maintenance of mtDNA. For example, nuclear genetic mutations in EFTu (Tu translation elongation factor, mitochondrial) may result in a disorder of the...
mitochondrial respiratory chain [24]; nuclear genetic mutation in DGUOK (mitochondrial deoxyguanosine kinase) [25] or POLG (mitochondrial DNA polymerase gamma) [24] is associated with mtDNA depletion and disorder of the mitochondria.

Sucla2, encoded by a nuclear gene, is related to mtDNA depletion and mitochondrial dysfunction [3, 26]. Sucla2 is the β subunit of succinyl coenzyme A synthase present in the mitochondrial matrix, and catalyzes the reversible synthesis of ATP in tricarboxylic acid cycle. The defects in the SUCLA2 gene can result in myopathic mitochondrial DNA depletion syndrome, which is characterized by a significant reduction in mtDNA content [1]. In a culture medium of fibroblasts from a patient with SUCLA2 mutation, Western blotting showed a significant decrease in Sucla2 protein and mitochondrial ATP contents and enhanced levels of ROS [6].

Owing to the importance of Sucla2 in mitochondrial function, we focused on its function in the male reproductive system. Our previous study showed that Sucla2 expression decreased in rat testes with failure of spermatogenesis, and decreased in mouse spermatogonia cell line (GC1 cells) with an increase in apoptotic cells; moreover, Sucla2 was strongly expressed in spermatocytes of stages VII–XII in rat spermatogenesis [8]. Yet, whether the expression of Sucla2 in mouse testes is similar to that in rat testes is unknown; also, the function of Sucla2 in the spermatocyte is still unclear. As shown in present study,
Sucla2 affects viability and apoptosis of mouse spermatocytes

141

Sucla2 in mouse testis, Sucla2 is located specifically in the cytoplasm of all spermatogenic cells, with coarse signals in the pachytene spermatocytes (Pc) in stages VII–XII and very faint signal in interstitial cells, such as Leydig cells (Lc) (Figure 1). The expression of Sucla2 in mouse testis is similar to that in rat testis; also, Sucla2 is associated with the developmental stages of spermatogenesis. Based on these results, we suspect that Sucla2 is one of the important markers that is decreased by some stresses, and related to mitochondrial dysfunction and the development of germ cells. In present study, Sucla2 was knocked down by Sucla2-targeting siRNA transfection in GC2 cells resulting in decreased cell viability. This finding agrees with the results of our previous study, in which Sucla2 expression was decreased in impaired rat spermatogenesis with an increased ratio of apoptotic germ cells [8].

Mitochondrial membrane potential, a sensitive indicator of mitochondrial function, was determined by flow cytometry. We found that MMP in GC2 cells was reduced significantly by Sucla2 knockdown; moreover, other indicators of mitochondrial status showed that Sucla2 knockdown decreased ATP and increased ROS contents. An increasing ratio of apoptotic GC2 cells was induced gradually by increasing the Sucla2-targeting siRNA reagent, and Bcl2, a marker of the mitochondrial apoptotic pathway, was also reduced in cells after Sucla2-targeting siRNA transfection. These results suggest that the knockdown of Sucla2 decreased the viability of spermatocytes by inducing mitochondrial dysfunction and apoptosis of germ cells. Although Sucla2 has been referred to in studies of patients with mitochondrial depletion syndrome (MDS) including progressive external ophthalmoplegia (PEO), ataxia-neuropathy, and mitochondrial neurogastrointestinal encephalomyopathy [26, 27], yet its function in germ cells has rarely been investigated. The present results shed a light on the relationship between Sucla2 and apoptosis or degeneration of male germ cells and encourage further research in this area of study.

Mitochondria are multitasking organelles [28], and many environmental and stress stimuli can cause mitochondrial dysfunction [29, 30], thereby affecting spermatogenesis [31–34]. The present study is a follow-up to our previous works, which showed that Sucla2 expression decreased in testes and GC1 cells with increasing apoptotic cells after exposure to endocrine disruptor BDE47 [8]. Our present data indicate that Sucla2 most likely serves as a link between mitochondrial function and apoptosis of germ cells, and may be one of the targeting markers affected by some stress stimulators. Most studies on Sucla2 focused on the mutation in the SUCLA2 nuclear gene and its relationship with the clinical syndromes of patients [1, 3, 6, 26]. On the other hand, the majority of studies on the apoptosis of germ cells were focused on the detection of markers of mitochondrial dysfunction or apoptosis, such as ATP production, ROS level, Bcl2, or caspase 3/9 [8, 18, 35, 36]. Our studies provide a new way of understanding the mechanism of mitochondrial dysfunction and the apoptosis of germ cells affected by mitochondrial proteins. Based on our findings, Sucla2 is specifically expressed in germ cells and is related to developmental stages of mouse spermatogenesis; knockdown of Sucla2 decreases the viability of mouse spermatocytes, probably by inducing apoptosis through injuring the mitochondrial function of cells.

Acknowledgements

This study was supported by the Chinese 973 program (2009CB941703) and the Basic Scientific Research Fund, Southeast University of China (2242015K42118; 3224006414).

Conflict of interest

The authors declare that they have no conflicts of interest.

References


Submitted: 31 May, 2016
Accepted after reviews: 26 September, 2016
Available as Aop: 3 October, 2016