Original Article

Identification and characterization of stearoyl-CoA desaturase in *Toxoplasma gondii*

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Abstract

Few information of the function of stearoyl-coenzyme A (CoA) desaturase (SCD) in apicomplexan parasite has been obtained. In this study, we retrieved a putative fatty acyl-CoA desaturase (TGGT1_238950) by a protein alignment with *Plasmodium falciparum* SCD in ToxoDB. A typical Δ^9 -desaturase domain was revealed in this protein. The putative desaturase was tagged with HA endogenously in *Toxoplasma gondii*, and the endoplasmic reticulum localization of the putative desaturase was revealed, which was consistent with the fatty acid desaturases in other organisms. Therefore, the TGGT1_238950 was designated *T. gondii* SCD. Based on CRISPR/Cas9 gene editing technology, SCD conditional knockout mutants in the *T. gondii* TATi strain were obtained. The growth *in vitro* and pathogenicity in mice of the mutants suggested that SCD might be dispensable for tachyzoite growth and proliferation. The SCD-overexpressing line was constructed to further explore SCD function. The portion of palmitoleic acid and oleic acid were increased in SCD-overexpressing parasites, compared with the RH parental strain, indicating that *T. gondii* indeed is competent for unsaturated fatty acid synthesis. The SCD-overexpressing tachyzoites propagated slower than the parental strain, with a decreased invasion capability and weaker pathogenicity in mice. The TgIF2 α phosphorylation and the expression changes of several genes demonstrated that ER stress was triggered in the SCD-overexpressing parasites, which were more apt toward autophagy and apoptosis. The function of unsaturated fatty acid synthesis of TgSCD was consistent with our hypothesis. On the other hand, SCD might also be involved in tachyzoite autophagy and apoptosis.

Key words: *Toxoplasma gondii*, stearoyl-coenzyme A desaturase, gene editing, autophagy, endoplasmic reticulum stress

Introduction

Toxoplasma gondii is a zoonotic pathogen and one of the most widespread parasites, which chronically infects ~30% of the global human population, as well as animal species [1]. *Toxoplasma gondii* is the aetiological agent of toxoplasmosis, which causes severe neurological deficits in immunosuppressed individuals and hydrocephalus, chorioretinitis, and blindness in congenitally infected newborns [2].

Toxoplasma gondii infection results in abortion in farm animals, which causes significant reproductive losses and, as a result, economic losses [3].

Fatty acid uptake and synthesis is important for *T. gondii* growth in host cells. It has been shown that *T. gondii* take up fatty acids from culture medium [4]. Meanwhile, fatty acid is synthesized *de novo* in the apicoplast by a Type II fatty acid synthesis (FASII) pathway, which is indispensable for its survival [5,6]. The parasite is also capable of

modifying both the ingested and *de novo* synthesized fatty acid in its endoplasmic reticulum (ER), generating very long unsaturated fatty acids (C26:1) that the host cannot synthesize itself [7,8].

In addition to the elongation system, as the importance of unsaturated fatty acids, we are curious to know whether *T. gondii* is capable of desaturating fatty acids with a certain desaturase in the parasite. A stearoyl-CoA desaturase (SCD) was identified in *Plasmodium falciparum*. It is located in the ER and is capable of synthesizing oleic acid. Sterculic acid analogues, known to be specific $\Delta 9$ -desaturase inhibitors, exhibit significant, rapid, and irreversible antimalarial activity against the asexual blood stage parasites [9]. Our previous experiments showed that these compounds also inhibited the growth of *T. gondii* [10], indicating that unsaturated fatty acid synthesis might be essential for *T. gondii*.

In the present study, we identified a putative *T. gondii* SCD and confirmed its role as a fatty acid desaturase. Besides its function in unsaturated fatty acid synthesis, TgSCD was also involved in the autophagy of tachyzoites.

Materials and Methods

Parasite culture

Toxoplasma gondii tachyzoites of RH, Δ Ku80, and TATi strains were cultured and genetically manipulated as described previously [11]. The RNA, DNA, and total protein were extracted using TRIzol® Reagent (Invitrogen, Carlsbad, USA), genomic DNA fast extraction kit (Aidlab Biotechnologies, Beijing, China) and RIPA Lysis Buffer (P0013B; Beyotime, Shanghai, China), respectively, according to the manufacturers' instructions.

Gene identification and gene tagging

The protein sequence of *P. falciparum* SCD (PlasmaDB gene ID PF3D7_0511200) was used as a query sequence for Basic Local Alignment Search Tool (BLAST) against the protein sequences of *T. gondii* in the ToxoDB database. A putative fatty acyl-CoA desaturase was retrieved in the database. Then, the gene was designated TgSCD. The prediction for the *T. gondii* SCD was confirmed by real-time-polymerase chain reaction (RT-PCR) using RNA from *T. gondii* RH tachyzoites as a template. The full-length coding sequence was amplified from RH cDNA using High Fidelity DNA Polymerase (TransGen, Beijing, China), subsequently subcloned into plasmid pEASY T1 Simple (TransGen) and sequenced (Ruibiotech, Beijing, China). To identify the expression of SCD in *T. gondii* tachyzoites by western blot analysis, the recombinant truncated SCD-His was expressed in *Escherichia coli*, and polyclonal mouse antibodies were prepared. The sequence containing the desaturase domain of *T. gondii* SCD (1270–1942 bp) was amplified using DesF and DesD primers (Supplementary Table S1) and then subcloned into plasmid pET-28a(+) (Novagen, Madison, USA). The recombinant protein expression was induced, and the protein was purified according to Novagen's pET System Manual. Six-week-old BALB/c mice (Beijing Vital River Laboratory Animal Technology Co., Beijing, China) were immunized with the purified protein in complete Freund's adjuvant and boosted with the protein in incomplete Freund's adjuvant twice. The highly immunized sera were collected for the immunoblot assay.

C-terminal tagging was also applied in the Δ Ku80 tachyzoites for SCD localization and later experiments. The upstream 1045 bp sequence of the SCD stop codon was amplified from the genomic DNA using the TagF and TagR primers (Supplementary Table S1)

and subsequently subcloned into the plasmid pLIC-3×HA-dhfr (provided by Dr Silvia N.J. Moreno, University of Georgia, USA) using ligation-independent cloning. The recombinant plasmid pLIC-SCD-3×HA-dhfr was linearized with *Bsp*HI and transfected into the Δ Ku80 parasite line. Transgenic parasites were selected in the presence of 1 μ M pyrimethamine and isolated by limiting dilution in 96-well plates. Mutants were identified by PCR for integration, and expression of SCD-3×HA was confirmed by western blot analysis. The plasmid pDer1-GFP (provided by Dr Boris Striepen, University of Georgia) was transiently transfected into Δ Ku80-SCD-3×HA parasites, and a subsequent indirect immunofluorescence assay (IFA) was conducted to confirm whether SCD-3×HA was co-localized with Der1-GFP in the ER. Rabbit anti-*T. gondii* sera and mouse anti-*Neospora caninum* acyl carrier protein (ACP) antibodies were also used in the IFA to show the relative localization of SCD in the tachyzoites.

Construction of the plasmid and generation of mutants

The knockout of SCD in the TATi parasite line was conducted with a two-step strategy as previously described [12]. The gene targeting in the two steps was mediated by CRISPR/Cas9 gene-editing technology. First, the tetracycline regulated promoter and 3×HA tag with the HXGPRT 3' UTR (untranslated region) were amplified from the pDT7S4 vector [12] and pLIC-3×HA-dhfr, respectively. A pUC19-TetO7S4-SCD-3×HA plasmid was constructed by linking the SCD open reading frame (ORF) sequence between the TetO7S4 promoter and the 3×HA tag using the ClonExpress MultiS One Step Cloning Kit (Vazyme Biotech, Nanjing, China). Then, the TetO7S4-SCD-3×HA-HXGPRT 3' UTR sequence was inserted into the pUPRT plasmid between the 5' UPRT and 3' UPRT by the same method [13]. Next, The sequence from the 5' UPRT flank to the 3' UPRT flank was amplified by PCR, and 50 μ g of it was transfected along with 10 μ g of pSAG1-U6::sgUPRT plasmid into TATi tachyzoites. The Δ UPRT::SCD-3×HA mutants were selected under 10 μ M FUDR (Sigma, St Louis, USA), and isolates were obtained by limiting dilutions in 96-well plates. The 5' and 3' crossover was identified by PCR. This is the first step that introduced a regulated SCD-3×HA into the UPRT locus, and its expression in the absence or presence of 1 μ M Atc was identified by western blotting. In the second step, the plasmid pSAG1-U6::sgSCD1-sgSCD2 was constructed. The gRNA of sgSCD1 and sgSCD2 targeted just upstream of the SCD start codon and in the last exon, respectively. The DHFR-TS cassette was amplified from pDMG (provided by Xuenan Xuan, Obihrio University of Agriculture and Veterinary Medicine, Japan), and 50 μ g of it was transfected along with 10 μ g of pSAG1-U6::sgSCD1-sgSCD2 into Δ UPRT::SCD-3×HA mutants and selected in the presence of 1 μ M pyrimethamine. The deletion of the endogenous SCD was identified by PCR and western blotting.

The SCD overexpression plasmid pDMG-SCD-GFP was constructed by introducing the full-length SCD ORF sequence into the pDMG plasmid between the *Eco*RV and *Nsi*I sites. The original *Nsi*I site in the SCD sequence was mutated by overlapping PCR, replacing the cytosine with thymine. Approximately 50 μ g of recombinant plasmid was transfected into RH tachyzoites. With the strong promoter of *T. gondii* GRA1, SCD would be highly expressed with the GFP fused at the C terminal in the transgenic tachyzoites. Transgenic parasites were selected in the presence of 1 μ M pyrimethamine, and stable transformant was isolated by cell sorting and were designated SCD OE.

Immunoblot and immunofluorescence assays

For western blot analysis of the endogenous TgSCD, α -SCD antibodies against the truncated SCD were prepared from the mouse immunized with recombinant SCD-His protein and the titre was diluted 1:400 for the primary antibody. We also used rabbit α -TgActin (1:2000) and mouse α -HA (1:1000) (Santa Cruz Biotech, Santa Cruz, USA) as primary antibodies. The secondary antibodies were HRP-conjugated goat anti-mouse IgG (1:5000) or goat anti-rabbit IgG (1:10,000) (Abcam, Shanghai, China). The phosphorylation detection of *Toxoplasma* eukaryotic initiation factor-2 α (TgIF2 α) was performed as previously described [14]. The primary and secondary antibodies were incubated at 4°C overnight and 37°C for 1 h for ECL detection, respectively. Immunofluorescence assays were performed as previously described [15] using mouse anti-HA monoclonal antibody (1:50; NEB, Ipswich, USA), rabbit anti-HA polyclonal antibody (1:50; NEB), α -N. *caninum* ACP (1:25; home-made), and α -T. *gondii* (1:100; home-made) antibodies. Fluorescein isothiocyanate (FITC) or Cy3-conjugated secondary antibodies were used for detection. The home-made anti-T. *gondii* ATG8 (autophagy-associated gene 8) mouse serum (1:500) was used in autophagy detection. The TUNEL BrightRed Apoptosis Detection Kit (Vazyme Biotech) was used in the detection of tachyzoite apoptosis according to the manufacturer's instruction. All samples were Hoechst stained, and fluorescence images were captured using a Leica TCS SP5 II confocal microscope (Leica, Buffalo Grove, USA).

Quantitative RT-PCR for related genes

Aliquots of 1 μ g of total RNA were extracted from RH and SCD OE tachyzoites, which were used in separate, parallel reverse transcription reactions using Easyscript reverse transcriptase (TransGen) according to the manufacturer's instructions. Several genes of which expression levels changed significantly during ER stress were chosen for quantitative PCR (qPCR) detection using primers listed in **Supplementary Table S1** [16]. For the detection of the SCD transcription level in TATi conditional knockdown line, parasites were cultured in the presence or absence of 1.5 μ g/ml ATc for 48 h. Total RNA was extracted, and the cDNAs were synthesized as described above. The SCD RT F and SCD RT R primer pairs were used to amplify the TgSCD segment from the cDNA. TgACT1 was amplified as an endogenous control (**Supplementary Table S1**) in all the reactions. qPCR was performed using ABI 7500 real-time PCR (Applied Biosystems Inc., Foster City, USA) with a reaction mixture volume of 20 μ l, containing SYBR[®] Premix Ex Taq[™] II (Tli RNaseH Plus) (Takara, Dalian, China), according to the manufacturer's recommended conditions. Data analysis was conducted using 7500 software (ABI). The relative TgSCD expression levels were calculated as previously described [17].

Stable isotope labeling and metabolomics analyses

SCD OE- and RH-infected human foreskin fibroblasts (HFFs) were grown in Dulbecco's modified Eagle's medium (DMEM; MAC-GENE, Beijing, China) in T175 flasks. The experiment was performed as previously described [8]. Briefly, the medium was supplemented with 16-mM [13 C]glucose for 48 h prior to egress. Free parasites were separated from the host cells by filtration through a membrane with a 3- μ m pore size. Parasites were quenched by rapid chilling of the cell suspension in a dry ice/ethanol bath and recovered by centrifugation (4000 g, 25 min, 0°C). Cell pellets were washed three times with ice-cold phosphate buffered saline, and cell

aliquots (2×10^8 cells) were centrifuged (10,000 g, 30 s, 0°C) and resuspended in 1 ml of 80% acetonitrile water, followed by freezing and thawing at -80°C three times. Then, the supernatants were collected after centrifugation. The samples were concentrated by blowing nitrogen before loading in the LC-30A liquid phase system (Shimadzu, Kyoto, Japan) and AB 5600+ Q TOF mass spectrometer (Applied Biosystems, Waltham, USA) for parasite fatty acid detection. Data shown are the three technical replicates.

Replication assay

Growth between different parasite lines was compared using an intracellular replication assay. Freshly egressed RH and SCD OE tachyzoites were used to infect the HFF cell monolayers grown on coverslips. Following 20-h culture, the monolayers were fixed, permeabilized, and stained with anti-T. *gondii* rabbit sera (1:50) and then with FITC-conjugated goat anti-rabbit IgG as described above. Monolayers were mounted in Vectashield containing DAPI and examined by epifluorescence microscopy (IX71; Olympus, Tokyo, Japan). TATi SCD conditional knockout tachyzoites were cultured in the presence or absence of 1.5 μ g/ml ATc for 48 h before challenging the HFF cells. The parasites were cultured for another 24 h in the presence or absence of 1.5 μ g/ml ATc. Staining and examination of the TATi SCD conditional knockout tachyzoites were performed as above. The number of parasites per vacuole was determined by counting 100 or more cells from each of the three coverslips in two or more experiments.

Plaque assay

In total, 200 parasites were inoculated into six-well plates per well and cultivating for 7 days, followed by 4% paraformaldehyde fixation and crystal violet staining. The area of at least 50-well-separated plaques per assay was manually measured in ImageJ and normalized to the same assay performed on a parental cell line.

Invasion assay

The invasion ability of RH and SCD OE tachyzoites was compared using an invasion assay as described previously [18]. Freshly released RH and SCD OE tachyzoites were incubated in DMEM at 37°C for 0, 1, 2, and 4 h before infecting HFF monolayers during a short invasion pulse (20 min). Extracellular parasites were incubated with anti-SAG1 antibodies and then Cy3-conjugated antibodies. Monolayers were then permeabilized with 0.5% saponin. Both intracellular and extracellular parasites were incubated again with anti-SAG1 antibodies and then FITC-conjugated antibodies. Monolayers were washed and mounted in Vectashield containing Hoechst. The percentage of intracellular parasites was determined as the inverse of the ratio of red extracellular parasites versus the entire green population. Four independent experiments were performed. More than 100 parasites were counted for each condition.

Mouse survival and physiology

Female 6-week-old BALB/c mice were purchased from Beijing HFK Bioscience Co., Ltd (Beijing, China). They were housed under specific pathogen-free conditions for 7 days before manipulation. Food and water were freely available throughout the experiments. The method of parasite inoculation and animal treatment was described previously [19]. The RH and SCD OE tachyzoites grown in HFF cell monolayers were purified from freshly lysed HFF cells.

The tachyzoites were injected intraperitoneally (IP) into the mice at 100 or 200 parasites per animal. The TATi, Δ UPRT::iSCD and i Δ SCD tachyzoites were also injected IP into the mice at 1000 per animal. The mice in each group were subdivided into two groups, each containing five mice. Normal drinking water was supplied to one group and water with 0.2 mg/ml ATc was supplied to the other. All animals were monitored three times a day for clinical signs and mortality for 30 days post injection. The mice were humanely euthanized when they were unable to reach food or water for >24 h and lost 20% normal body weight. The mice were humanely euthanized by cervical dislocation after anaesthetization.

All experiments with animals in this study were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China. All experimental procedures were approved by the Institutional Animal Care and Use Committee of China Agricultural University (The certificate of Beijing Laboratory Animal employee, ID: 18049). All efforts were made to minimize animal suffering.

Statistical analysis

All experiments and measurements were performed in triplicate. PASW Statistics 18 and GraphPad Prism 5 were used for statistical analysis. $P < 0.05$ indicated significant difference.

Results

Identification of a putative *T. gondii* SCD

Using BLAST in the ToxoDB database (www.toxodb.org) with *P. falciparum* SCD (PfSCD) as the query sequence (PF3D7_0511200), a putative fatty acyl-CoA desaturase was retrieved in *T. gondii*. The 13-exon gene encodes a protein consisting of 1042 amino acid residues (117 kDa, predicted pI of 8.8). We obtained the full-length coding sequence by overlap extension PCR using RH cDNA as the template (Fig. 1). Sequencing results showed that it was identical to that of GT1 (TGGT1_238950). The protein sequence of this putative *T. gondii* acyl-CoA desaturase was analyzed in National Center for Biotechnology Information's conserved domain database and a central conserved domain (aa 398 to 638) was revealed. This gene was then designated as TgSCD. Alignments of TgSCD with the putative Δ 9-desaturases from the apicomplexan parasites *N. caninum*, *P. falciparum* and *Eimeria tenella* and other SCDs from *Arabidopsis thaliana*, *Drosophila melanogaster*, *Mus musculus*, and humans indicate that the sequence is well conserved in the central domain (Supplementary Fig. S1). The identity rates between the central domain of TgSCD and the four eukaryotic orthologues are 27%, 47%, 45%, and 46%, respectively. Although the identity rate is obviously lower in TgSCD versus *A. thaliana*, these SCDs contain the classic catalytic domain, comprised of three histidine boxes and eight conserved His residues (two HXXHH motifs and one HX₄H motif) in the three boxes, which are essential for the SCF activity [20]. The ninth histidine residue (His568), which had not been previously identified, is also conserved in TgSCD. Asn564 and His568 comprise a NX₃H motif that is symmetrically equivalent to the HX₄H motif [21]. The Δ 9 desaturation reaction also requires the cytochrome b5 and NADPH cytochrome b5-reductase as co-enzymes [9]. The co-enzymes were found to be present in the *T. gondii* genome with the accession numbers TGGT1_276110 and TGGT1_262910 (ToxoDB, release 26).

The genes of the putative SCD and the two co-enzymes are present in all three *Toxoplasma* strains. Only five amino acids in

Figure 1. RT-PCR for the full-length coding sequence of *T. gondii* SCD. (A) The 5' and 3' TgSCD coding sequences were obtained by PCR, and the lengths were 1442 bp and 1795 bp, respectively. (B) The full length TgSCD coding sequence was amplified by overlap PCR, and the full length was 3129 bp. (C) The overlap region was from base 1335 to 1442, including 108 bp.

SCD are different among the three strains (aa 175, 295, 296, 358, and 922), and these amino acids are beyond the central domain, suggesting that the Δ 9 desaturation reaction is ubiquitous in *T. gondii*.

For subcellular localization of TgSCD in *T. gondii*, the 3'-terminal tagging was performed in Δ Ku80 tachyzoites as nonhomologous DNA end joining is disrupted in this line, and the probability of homologous recombination was higher than that in the other wild types [22]. With a homologous fragment of 1045 bp just upstream of the stop codon of SCD in the *T. gondii* genome sequences, the 3 $\times$ HA tag was added to the C-terminus of SCD, which was confirmed by western blotting and IFA (Fig. 2A,B). Then, the localization of SCD in *T. gondii* was examined by IFA and observed under the confocal microscope. Obviously, TgSCD with the HA signal was located neither on the surface of the tachyzoites nor in the nuclei (Fig. 3). Instead, the signal distributed in the cytoplasm and around

Figure 2. SCD was tagged endogenously with HA at the C-terminus in Δ Ku80. (A) The HA tag could be detected by western blotting in mixed tachyzoites before monoclonal screening. The molecular weight of the detected protein was as expected with SCD-HA (~120 kDa). TgActin was used as a reference gene. (B) The HA fused protein could be detected by IFA only in tachyzoites with the expected recombination. Bars, 5 μ m.

Figure 3. SCD-HA localized in the cytoplasm of the parasite SCD-HA mainly co-localized with the ER protein Der1 and partly co-localized with the apicoplast protein ACP. For the color uniformity of SCD-HA, pseudo colors were applied for the co-localization of SCD-HA and Der1-GFP. The actual color of SCD-HA was red while Der1-GFP was green under the fluorescence microscope. Bars, 5 μ m.

Figure 4. The i Δ SCD-HA line was constructed by a two-step stratagem (A) Scheme of the construction of i Δ SCD-HA line. (B) The punctual green fluorescence of Cas9-eGFP in the parasites' nucleus could be observed 60 h after electroporation. Bars, 20 μ m. (C) Three primer pairs were used for PCR identification: UPRT out primer pair for PCR1, Up UPRT for PCR2 and Down UPRT for PCR3. Due to the replacement of the UPRT by iSCD-HA in the parasites where the expected recombination occurred, specific fragments could not be amplified by PCR1, while PCR2 and PCR3 could amplify expected fragments. In the parental TATI strain, PCR1 could amplify the UPRT fragment, while no fragments could be amplified by PCR2 and PCR3.

the nuclei and was co-localized with Der1-GFP (an ER marker) (Fig. 3, upper two lines), which validated our presumption that TgSCD localized in the ER of the tachyzoite, similar to that in other eukaryotic organisms [23]. Interestingly, we found that some TgSCD accumulated in the cytoplasm. Given the hypothesis that TgSCD was associated with fatty acid metabolism, we investigated

whether the location of accumulated TgSCD overlapped with ACP, a crucial protein for growing fatty acid chains as a central part of the FASII in *T. gondii*. The partial colocalization of TgSCD with ACP (Fig. 3, lower two lines) indicated that TgSCD might be involved in the further modification of fatty acids in both the ER and apicoplast.

Figure 5. Identification of the conditional knockout i Δ SCD-HA tachyzoites Two copies of SCD existed in the Δ UPRT::iSCD-HA line. One was the endogenous SCD, and the other was HA tagged SCD, which replaced UPRT. Therefore, two fragments were amplified by PCR4 in this line. The endogenous SCD was replaced by DHFR-TS in i Δ SCD. PCR4 could only amplify SCD without introns at the UPRT loci. PCR5 could not amplify any fragments because SCD contained no introns in this line (A,B). In IFA, the expression of SCD-HA could not be observed in 24 h after ATc was added in the medium (C). The expression of SCD-HA could be detected by anti-HA monoclonal antibodies in the two mutants but not in the parental TATI. The expression of SCD-HA could not be detected by the western blotting 48 h after ATc was added in the medium. An unspecific band was below TgActin because of over incubation (D).

Deletion of SCD in tachyzoites did not affect the phenotype *in vitro* and *in vivo*

The two-step conditional knockout in TATI was mediated by CRISPR/Cas9 editing technology. In the first step, UPRT was replaced by SCD-3 $\times$ HA, which could be regulated under the TetO7S4 promoter in the presence or absence of ATc (Fig. 4A). The punctate fluorescence of Cas9-eGFP could be observed in tachyzoites of a parasitophorous vacuole (Fig. 4B), demonstrating transfected parasites proliferated normally. After screening in FUDR medium, monoclonal tachyzoites were obtained by limiting dilution and identified by PCR (Fig. 4C). Then, endogenous SCD was replaced by DHFR-TS and screened in pyrimethamine medium (designated as i Δ SCD, Fig. 5A). The deletion of endogenous SCD

was identified by PCR (Fig. 5B). SCD expression could not be detected by IFA after treatment with ATc for 24 h (Fig. 5C) or western blot analysis after 48 h of treatment (Fig. 5D). SCD transcription decreased to 9.2% in the presence of ATc (data not shown).

The i Δ SCD tachyzoites were capable of forming plaques *in vitro* in the presence of ATc, and the size of plaques was not significantly different regardless of whether the SCD was knocked out (Fig. 6A). The proliferation rate of i Δ SCD decreased with the addition of ATc. However, the growth of the parental tachyzoites also slowed when ATc was added, implying that the negative effect on proliferation was not caused by the knockout of SCD (Fig. 6B,C). Similar to the results of *in vitro* experiments, whether or not SCD

Figure 6. Plaque assay of iΔSCD tachyzoites in HFFs The three parasites lines were inoculated in HFF cells and cultured in the presence or absence of ATc for 7 d before fixation and staining. The two random mutants (iΔSCD #1 and #2) had similar performance (A) and the data showed in B and C represented the results of iΔSCD #1. The plaque area was calculated as pixels using photoshop software (B) and the ratio of the different growth stage tachyzoites were calculated from 100 random parasitophorous vacuoles in every proliferation assay. All the data were collected from three independent experiments and the results are shown as the mean $\pm$ SD. The significant difference was judged by the Student's *t*-test.

Figure 7. Overexpressed SCD was co-localized with endogenous SCD-HA (A) The localization of SCD-GFP in the overexpressed parasites remained in the cytoplasm during the different growth stages. (B) The IFA results demonstrated the identical localization of the overexpressed SCD-GFP with endogenous SCD-HA in the ER. Bars, 5 μ m.

was conditionally knocked out, the infected mice all died within 10 days (data not shown), without significant difference in survival time.

More C18:1 was synthesized in TgSCD overexpression tachyzoites

The TgSCD overexpression parasite line (SCD OE) was obtained by electroporation of pDMG-SCD-GFP plasmid. The fluorescence of the fused SCD-GFP protein was observed in the tachyzoites that were electroporated with pDMG-SCD-GFP plasmid (Fig. 7A). To confirm the location of the overexpressed SCD-GFP in the parasites, the protein was also expressed in the Δ Ku80-SCD-3 $\times$ HA. The co-localization of the SCD-GFP and endogenous SCD-HA demonstrated that the overexpressed SCD-GFP indeed localized in the ER (Fig. 7B). The expression of SCD-GFP could be detected by western blot analysis using anti-GFP antibodies (Fig. 8A). Compared with the parental RH tachyzoites, SCD expression was up-regulated by approximately 40 folds in the SCD OE parasites (Fig. 8B).

As SCD is capable of catalyzing the synthesis of palmitoleic acid and oleic acid using palmitic acid and stearic acid, respectively, as substrates, we detected the amount of the above fatty acids labeled with 13 C in SCD OE and parental tachyzoites. The unsaturation index of the C18 fatty acid was augmented significantly (Fig. 8C), implying that TgSCD indeed functioned as a fatty acid desaturase in *T. gondii* tachyzoites.

Invasion and proliferation were affected in SCD overexpression tachyzoites

SCD overexpression monoclonal tachyzoites were obtained by flow cytometry. Although SCD OE parasites could be normally passaged in Vero and HFF cells, they could not form typical and obvious plaques compared with parental RH parasites (Fig. 9A). In contrast, hyperchromatic areas were observed because released SCD OE tachyzoites gathered instead of invaded near cells (Fig. 9B). As the plaque assay could reflect the comprehensive performance, the invasion and proliferation of SCD OE tachyzoites were

Figure 8. Identification of SCD-OE lines (A) An expected band emerged in the mixed tachyzoites before cell sorting and a strong SCD-GFP band reacted with GFP antibodies in the overexpressed parasites with the expected molecular weight. (B) The expression of SCD in two SCD-OE monoclonal lines (1C9 and 2E3) was up-regulated by 42.6 and 40.7 folds, respectively. (C) TgActin was used as a reference gene in both experiments. Unsaturation index of C16 and C18 fatty acid was calculated in RH and SCD-OE. Data shown are the three technical replicates from four biological replicates.

Figure 9. Plaque assay of SCD-OE lines (A) After 7 days of culture, obvious plaques were formed by the RH parasites but not the SCD-overexpressing parasites. (B) Although no obvious plaques were formed by the SCD-overexpressing parasites, the deeply stained area indicated the propagation of the SCD-overexpressing parasites.

compared with RH. Although the invasion capability of freshly released SCD OE tachyzoites was comparable to that of RH, the invasion rate decreased more in SCD OE parasites over the extracellular time (Fig. 10A). A proliferation assay was performed in

HFF cells. SCD OE parasites divided slower than RH, with fewer parasitophorous vacuoles that contained up to eight tachyzoites in 20 h (Fig. 10B).

Lower mortality rate in SCD OE-infected mice

SCD OE parasites showed subdued invasion rate and proliferated slower *in vitro* as described above. We then observed the pathogenicity of SCD OE parasites in mice. As Type I *T. gondii*, wild-type RH resulted in death in 100% of the mice; by contrast, infection of 100 or 200 SCD OE tachyzoites still resulted in a survival rate of >40% (Fig. 11). Immunoconversion of mice was tested by western blot analysis (data not shown).

TgSCD was involved in tachyzoite autophagy and apoptosis

The phenotype changes both *in vitro* and *in vivo* suggested that the viability of SCD OE parasites might be affected. Tachyzoite autophagy was compared between the SCD OE parasites and the parental RH strain because the SCD activity was reported to be involved in the formation of the autophagosome [24]. Autophagy could be induced in both SCD OE and RH parasites in HBSS (Hank's Balanced Salt Solution) (Fig. 12A). The autophagy rate, however, was higher in SCD OE tachyzoites as the induction time increased (Fig. 12B). Another programmed cell death pathway, apoptosis, was also detected in the two *T. gondii* lines. The apoptosis rate was also higher in SCD OE tachyzoites with the increase of extracellular time (Fig. 12C,D). These results indicated that the overexpression of SCD made the tachyzoites more apt to undergo autophagy and apoptosis.

Figure 10. The invasion and proliferation were slow in SCD OE compared to parental RH (A) The decrease in the invasion capability of SCD OE increased with extended extracellular time. (B) After the proliferation of HFF cells for 20 h, 20% of the RH parasitophorous vacuoles contained eight tachyzoites, while the percentage in the SCD OE parasites was only approximately 5%.

Figure 11. The survival curve of the mice infected with RH or SCD-overexpressing parasites

ER stress was induced in SCD overexpression tachyzoites

The overexpressed SCD localized in the ER. In view of the close relationship between apoptosis/autophagy and ER stress [25,26], we hypothesized that apoptosis and autophagy of SCD OE parasites may be attributed to the occurrence of ER stress. The high level of SCD protein accumulation in the ER might induce the unfolded protein response (UPR), and then the ER stress might be triggered. First, the phosphorylation of TgIF2 α was shown by western blot analysis, implying that the parasites were under stress [27] (Fig. 13A). Several genes were reported to be up-regulated when ER stress occurred [16]. The transcription levels of these genes were compared between SCD OE and RH parasites by RT-PCR. The significantly higher transcription level of these genes in SCD OE tachyzoites supported the hypothesis that ER stress was triggered in the SCD OE parasites (Fig. 13B).

Discussion

The study of *T. gondii* fatty acid desaturase is very limited. Lu *et al.* [28] investigated codon usage bias, base composition variations and protein sequence in ten available protozoan complete stearoyl-CoA desaturase gene sequences from *T. gondii*, *N. caninum*, etc.

The cited *T. gondii* SCD sequence was 95% identical to the amino acid sequence in the present study; however, the claimed *T. gondii* SCD was annotated as a putative fatty acyl-CoA desaturase in GenBank. Ramakrishnan *et al.* [7] mentioned a putative SCD was present in *T. gondii* (TGGT1_053030, a former accession number of TGGT1_238950 in earlier version of ToxoDB) but did not confirm its function.

In apicomplexan parasites, SCD was only identified in *P. falciparum*. It converts C18:0 to C18:1 oleic acid and is important for blood-stage survival [9]. The high similarity of a putative fatty acyl-CoA desaturase (TGGT1_238950) to that of *P. falciparum* and the existence of two coenzymes for SCD, cytochrome b5 and NADPH cytochrome b5-reductase, in *T. gondii* strongly suggested a Δ^9 -desaturase is active in the parasite.

In contrast, with most eukaryotic SCDs that contain 2 to 4 transmembrane regions [23,29,30], 14 transmembrane domains were predicted in TgSCD, which was comparable to PfSCD that contains 18. These high number of transmembrane domains, high isoelectric point (IP = 8.81), and high hydrophobicity explain why it is difficult to produce the full-length TgSCD in *E. coli*. The acquisition of a soluble central domain of TgSCD protein failed despite repeated attempts.

As we did not obtain high quality SCD antibodies because of immunization with denatured recombinant SCD, the localization of SCD was then confirmed by endogenous 3' tagging. The ER localization of TgSCD was consistent with SCDs previously described in mice, rats, and *P. falciparum* [9,23]. The partial co-localization of TgSCD with ACP implied that the desaturation of fatty acids may be related to FASII in the apicoplast. Interestingly, the ER location of TgSCD seemed to depend neither on the typical ER KDEL retention signal, as was that of *P. falciparum* [9,31], nor on the double lysine motif -KKXX or -KXKXX and aromatic amino acid-rich motif -YXXL- or -W/YX₂₋₃W/Y/F- at the C-terminus. However, the aa 15–18 (-LLRR-) at the N-terminal end of TgSCD conformed another ER retention motif, which was composed of a double arginine motif -XXRR- [32].

In our previous study, SCD inhibitors, sterculic acid, and its analogues were competent at inhibiting *T. gondii* *in vitro* and *in vivo* [10]. Based on the results, we made the prediction that TgSCD may play a critical role in the synthesis of oleic acid and be indispensable for parasite growth. We also tried to get complete TgSCD-knockout parasites for the function research of the protein by traditional homologous recombination method. However, we did not obtain such parasite line despite of many attempts.

Figure 12. Autophagy and apoptosis detection in SCD OE tachyzoites (A,B) The Atg8 was found in puncta in the parasites when autophagy occurred, while the Atg8 in normal parasites were equally distributed in the cytoplasm (A). In HBSS, the percentage of parasites with Atg8 puncta was higher in the SCD-overexpressing parasites than in the RH (B). (C,D) Apoptotic parasites were marked with white arrows. The bright red fluorescence localized in the nucleus (C). The difference in the apoptosis ratio between the RH and SCD-overexpressing parasites was shown as early as 30 min. The apoptosis ratio of the RH parasites was <30%, while that of the SCD-overexpressing parasites were close to 50% after 270 min (D).

Figure 13. The occurrence of endoplasmic reticulum stress in SCD OE (A) A mobility shift was observed in the phosphorylated TgIF2 α . The observed molecular weight of TgIF2 α -P was higher than that of TgIF2 α . (B) The expression levels of the above genes were increased by 27.9, 7.0, 36.6, 17.0 and 4.2 folds, respectively.

In the present study, the conditional TgSCD-knockout parasite line was constructed. The desaturase activity of TgSCD was confirmed by fatty acid detection, with more oleic acid generated in the SCD OE tachyzoites. However, the deletion of TgSCD did not affect the viability of *T. gondii* in the present research. In our previous study, we found that sterculic and its analogues inhibited the growth of *T.*

gondii and inferred that the anti-*T. gondii* activity of sterculic acid and its analogues was most likely mediated by inhibiting TgSCD [10]. Combined with the present results, a deeper cause may be that tachyzoites utilizes sterculic acid and its analogues instead of oleic acid, resulting in synthesis of abnormal lipid, which does not support the growth of tachyzoites. On the other hand, disruption of TgSCD

only affects the synthesis of C18:1 fatty acid, while tachyzoites may salvage more C18:1 fatty acid from the host as a compensation for normal growth.

The presence of SCD has been reported in a wide variety of organisms. SCD is the key enzyme that catalyzes the introduction of the first double bond in the *cis*- $\Delta 9$ position of several saturated fatty acyl-CoAs, such as palmitic acid (C16:0) and stearic acid (C18:0), to generate palmitoleic acid (C16:1) and oleic acid (C18:1) [33]. However, TgSCD seemed to be highly specific for the generation of oleic acid, similar to the *P. falciparum* SCD [9]. If palmitoleic acid can be synthesized in *T. gondii*, there must be a specialized palmitoyl-CoA-specific $\Delta 9$ fatty acid desaturase that has not yet been identified in the parasite. Then, palmitoleic acid may be used as a substrate for the synthesis of oleic acid by ELO-A (one of three elongase in the fatty acid elongation system) [7] when TgSCD function is deficient. There is a specialized $\Delta 9$ -desaturase for the conversion from palmitic acid to palmitoleic acid in *Caenorhabditis elegans* [34]. This specialized $\Delta 9$ -desaturase might be important for *de novo* synthesis of unsaturated fatty acid in the parasite, as well as for lipids intake. Although the lack of SCD is tolerated in the tachyzoites, it does not mean that SCD is redundant through all the life cycle of *T. gondii* because SCD may be crucial in the tissue cyst or oocyst stage when exogenous fatty acid is limited.

In conclusion, in this study, the *T. gondii* SCD was identified by RT-PCR, western blot analysis, and gene targeting, which does play a role as a $\Delta 9$ desaturase in tachyzoites, although deficiency of this enzyme does not cause discernible change in phenotype during the tachyzoites stage. SCD in mammalian organisms, apart from its role in monounsaturated fatty acid synthesis, has recently been demonstrated to have a novel function in the modulation of metabolic and signaling processes related to cell proliferation, survival, and malignant transformation in cancer [35]. Overexpression of SCD had negative impacts on tachyzoites. This phenomenon may be attributed to autophagy, which is connected with SCD activity as described previously [24,36]. On the other hand, massive overexpressed SCD accumulated at the ER triggers ER stress and mediates apoptosis or autophagy [37,38]. Such programmed cell death pathways could affect the viability of *T. gondii* both *in vitro* and *in vivo*.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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