Reproductive Sciences (2023) 30:1938–1951
https://doi.org/10.1007/s43032-022-01133-4

INFERTILITY: ORIGINAL ARTICLE

Transcriptome Analysis in High Temperature Inhibiting Spermatogonial Stem Cell Differentiation In Vitro

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Received: 9 May 2022 / Accepted: 14 November 2022 / Published online: 20 December 2022

Abstract

As one of the factors of male infertility, high temperature induces apoptosis of differentiated spermatogenic cells, sperm DNA oxidative damage, and changes in morphology and function of Sertoli cells. Spermatogonial stem cells (SSCs) are a type of germline stem cells that maintain spermatogenesis through self-renewal and differentiation. At present, however, the effect of high temperature on SSC differentiation remains unknown. In this study, an in vitro SSC differentiation model was used to investigate the effect of heat stress treatment on SSC differentiation, and RNA sequencing (RNA-seq) was used to enrich the key genes and pathways in high temperature inhibiting SSC differentiation. Results show that 2 days of 37 °C or 43 °C (30 min per day) heat stress treatment significantly inhibited SSC differentiation. The differentiation-related genes *c-kit*, *stra8*, *Rec8*, *Sycp3*, and *Ovol1* were down-regulated after 2 and 4 days of heat stress at 37 °C. The transcriptome of SSCs was significantly differentially expressed on days 2 and 4 after heat stress treatment at 37 °C. In total, 1660 and 7252 differentially expressed genes (DEGs) were identified by RNA-seq in SSCs treated with heat stress at 37 °C for 2 and 4 days, respectively. KEGG pathway analysis showed that p53, ribosome, and carbon metabolism signaling pathways promoting stem cell differentiation were significantly enriched after heat stress treatment at 37 °C. In conclusion, 37 °C significantly inhibited SSC differentiation, and p53, ribosome, and carbon metabolism signaling pathways were involved in this differentiation inhibition process. The results of this study provide a reference for further investigation into the mechanism by which high temperature inhibits SSC differentiation.

Keywords Male infertility · High temperature · Spermatogonial stem cell · Stem cell differentiation

Abbreviations

SSCs	Spermatogonial stem cells
A_s	*A* Single
A_{al}	*A* Aligned
A_{pr}	*A* Paired
DEGs	Differentially expressed genes
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
FBS	Fetal calf serum
bFGF	Basic fibroblast growth factor
SCF	Stem cell factor
BMP4	Bone morphogenetic protein 4
RA	Retinoic acid
NOA	Non-obstructive azoospermia
SNPs	Single nucleotide polymorphisms
HSPs	Heat shock proteins
HSF	Heat shock factor
BP	Biological process
CC	Cellular component
MF	Molecular function

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Introduction

The World Health Organization predicts that infertility will become the third most intractable disease after cancer and cardio-cerebrovascular disease in the twenty-first century [1]. Infertility occurs in 10–15% of couples of childbearing age, with male factors accounting for 50% of cases [2]. A Global Burden of Disease survey reported that the

age-standardized prevalence of infertility between 1990 and 2017 increased annually by 0.291% in men [3]. Heat is one of the causes of male infertility [2], as increased scrotal temperatures can lead to non-obstructive azoospermia or asthenozoospermia.

Occupational or lifestyle exposure to high temperatures can cause male infertility or subfertility. Artificial increases in scrotum or testicle temperature in fertile male bus drivers [4], bakers, and mechanics who took regular hot baths [5, 6] were found to reduce both sperm output and quality. In a study by Garolla et al. [7], a transient decrease in sperm count and motility, as well as impaired mitochondrial function and sperm DNA packaging, was observed in normozoospermic men who underwent two sauna sessions per week for 3 months. Shefi et al. [8] evaluated semen parameters in men with a known history of Jacuzzi, hot tub, and whirlpool bath use, and found similar results as those of Garolla et al. In another study, Bujan et al. [9] demonstrated that scrotal temperatures increased by 1.7–2.2 °C after sitting in a car for 2 h, which increased the risk factor for sperm parameter alterations. In a clinical trial, Jung et al. [10] found that sitting on a heated car seat for up to 60 min caused a 0.5–0.6 °C scrotal temperature increase compared to that of unheated seats, possibly impairing spermatogenesis. Sheynkin et al. [11] found that scrotal temperatures increased by 1 °C among people with laptops placed on their laps in a sitting position. Furthermore, exertional heat stroke (EHS) and testicular morphological changes were found to negatively affect sperm quality [11]. Additionally, rats have been found to display testicular temperature disruption, poorly differentiated seminiferous tubules, impaired sperm quality, and atrophy of interstitial Leydig cells, Sertoli cells, and peri-tubular cells in the testicular tissues, accompanied by a lack of spermatozoa [12].

Testicular thermoregulation plays an important role in normal spermatogenesis and sperm function. Spermatogenesis and sperm maturation require temperatures 2–7 °C lower than the normal body temperature [13, 14], and increased testicular temperatures hinder spermatocyte differentiation and maturation, leading to alteration of sperm parameters and apoptosis [9, 15, 16]. During heat stress conditions, mammalian male germ cells display a variety of changes in cellular events, including stress granule formation, DNA damage, and apoptosis [17]. A study in which adult male mice were exposed to an elevated ambient temperature of 35 °C for 24 h, followed by a 24-h recovery period, identified elevated sperm mitochondrial reactive oxygen species (ROS) generation, increased sperm membrane fluidity, pachytene spermatocytes, and round spermatid DNA damage [18]. The most relevant consequence of heat stress on the testis is death of germ cells via apoptosis, which occurs after exposure to abdominal heat stress [19]. Studies have shown that the p38 mitogen-activated protein kinase (MAPK) pathway regulates both apoptosis and spermatocyte differentiation [20–22]. However, the role and mechanism of heat stress in regulating spermatogonial stem cell (SSC) development is unclear due to the small number of SSCs in the mouse testis (only 0.02–0.03% of total testis cells) [23].

SSCs are the source of spermatozoa, and their differentiation is tightly regulated. SSCs are widely considered to be single undifferentiated spermatogonia cells existing on the basement membrane in seminiferous tubules. SSCs belong to A_{single} spermatogonia (A_s). In rodents, A_s spermatogonia generate two A_s spermatogonia without an intercellular bridge. Subsequent cell divisions of the A_{paired} (A_{pr}) spermatogonia generate $A_{aligned\text{-}4}$, $A_{aligned\text{-}8}$, and $A_{aligned\text{-}16}$ (A_{al}), which differentiate to type A_1 spermatogonia. The A_s, A_{pr}, and A_{al} spermatogonia are called undifferentiated spermatogonia (A_{undiff}), retaining the potential to differentiate into A_1, A_2, A_3, A_4, intermediate, and B spermatogonia, which go into meiosis to form primary spermatocytes, secondary spermatocytes, and eventually sperm [24, 25]. The regulation of the SSC differentiation process is very complex, and any failure of the regulation can lead to infertility. In most cases, however, the causes of male infertility are wide ranging and poorly understood [26–28].

Our previous study showed that heat shock treatment at 43 °C for 45 min significantly inhibited SSC self-renewal through S-phase cell cycle arrest but not apoptosis [29]; however, few other reports on the effect of high temperature on SSC differentiation exist. In this study, we examined the effect of heat stress on SSC differentiation using an SSC in vitro differentiation model and analyzed the gene expression pattern after heat stress using RNA sequencing (RNA-seq).

Materials and Methods

SSC Self-renewal and Differentiation Culture

The CD1 SSC cell line from mice was donated by Professor Wu Ji's laboratory from Shanghai Jiao Tong University. The SSC self-renewal culture medium was prepared according to our previously published paper [29]. The medium was based on Minimum Essential Medium α (MEM-α, 12,571–063, Gibco, Grand Island, NY, USA), containing 2 mM glutamine (G7012, Sigma, MO, USA), 10% fetal bovine serum (FBS) (16,000–36, Gibco), pen/strep (15,240–062, Invitrogen, Grand Island, NY, USA), nonessential amino acid (NEAA, 11,140–050, Gibco) solution, β-mercaptoethanol (β-ME, M3148, Sigma), 25 μg/ml insulin (I1882, Sigma), 100 μg/ml transferrin (T1428, Sigma), 60 μM putrescine (P5780, Sigma), 60 ng/ml progesterone (P8783, Sigma), and 8 ng/ml basic fibroblast growth factor (bFGF, F0291, Sigma). The feeder layer cells were STO cells treated with mitomycin

(M0503, Sigma). The SSCs were incubated at 37 °C in the presence of 5% CO_2. The culture medium for SSC differentiation was prepared according to the method published by Zhou et al. [30]. On the basis of the SSC self-renewal medium, the differentiation culture medium was established by adding cytokine stem cell factor (SCF) (100 ng/ml, R&D Systems), BMP4 (20 ng/ml, R&D Systems), RA (10-6 M Sigma), and activin A (100 ng/ml, R&D Systems). The SSCs used for differentiation were incubated at 34 °C in the presence of 5% CO_2 (Fig. 1A).

Heat Stress Treatment of Differentiation Cultured SSCs

Based on the different temperatures of heat stress treatment, SSCs used in differentiation culture were divided into two groups, which were subjected to heat stress at 37 °C and 43 °C, respectively. For the 37 °C heat stress treatment, the culture conditions were the same as the differentiation culture except that the culture temperature was increased from 34 to 37 °C. For the 43 °C heat stress treatment, SSCs were cultured in a 43 °C CO_2 incubator for 30 min daily and then returned to a 34 °C CO_2 incubator for further culture (Fig. 1B).

Quantitative Real-Time PCR

Some SSC differentiation marker genes were detected by quantitative real-time (qRT) polymerase chain reaction (PCR) analysis. The following primers were used: *Id4*, Forward: TGCAGTGCGATATGA ACGAC, Reverse: GCA GGATCTCCACTTTGC TG; *Thy-1*, Forward: GCTCTCC TGCTCTCAGTCTT, Reverse: GCTGAACTCATG CTG GATGG; *c-kit*, Forward: GGGACACATTTACGGTGGTG, Reverse: GCTTTA CCTGGGCTATGTGC; *Stra8*, Forward: TTGACGTGGCAAGTTTCCTG, Reverse: GGGCTCTGG TTCCTGGT TTA; *Rec8*, Forward: CCCGCTTCTCCCTCT ATCTC, Reverse: CGATGTAGGT GCTCCAGGAT; *Sycp3*, Forward: CCAATCAGCAGA GAGCTTGG, Reverse: CCT CGAAGCATCTGAGGAAA; *Ovol1*, Forward: TGTCT TACAGGCAGAGCACA, Reverse: GGCCTGTCTCTGTAA GTGGT; and *GAPDH*, Forward: AACGGATTTGG CCG TATTGG, Reverse: CATTCTCGGCCTTGACTG TG. We used the Tip Green qPCR SuperMix (Q311-02, Vazyme Biotech, Nanjing, China) in a 20 µl reaction volume on a 7500 Fast Real-Time PCR System, and the reaction conditions were set to 95 °C for 30 s followed by 42 cycles of 95 °C for 10 s and 60 °C for 30 s. The qRT-PCR primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. The data analysis was performed using the $2^{-\Delta\Delta CT}$ method, with three replicates in each group.

Western Blot Analysis

The cells were grown in 24-well plates, and hydrolyzed at 4 °C for 30 min using a protein extraction kit (Keygentec, Nanjing, China) to collect lysates. Cell lysates were separated using 12% SDS-PAGE and then transferred to PVDF membranes. The membrane was placed in TBST containing 5% skim milk powder and incubated at room temperature for 1 h. Subsequently, the PDVF membranes were incubated with primary antibodies, β-actin (1:1000, sc-58673, Santa Cruz Biotechnology, USA), Id4 (1:500, sc-365656, Santa Cruz Biotechnology, USA), PLZF (1:500, sc-22893, Santa Cruz Biotechnology, USA), Stra8 (1:1000, ab49602, Abcam, UK), and Sycp3 (1:1000, 23,024–1-AP, Proteintech, Wuhan, China). After washing with TBST three times, the PDVF membrane was incubated with horseradish peroxidase conjugated goat anti-mouse or anti-rabbit secondary antibody (1:20,000 diluted) at room temperature for 1 h. Finally, the membranes were incubated in ECL reagents (RM00021, ABclonal), and the signals were detected with a ChemiDoc™ XRS + (Bio-Rad, USA).

Total RNA-Sequence and Bioinformatics

We performed functional enrichment analysis of gene expression in 34 °C and 37 °C differentiation culture groups by RNA-seq [31]. All differentially expressed genes (DEGs) were mapped to terms in the Gene Ontology (GO) databases, and significantly enriched GO terms were then searched for in all DEGs with $P < 0.05$ as the significance threshold. GO term analysis was classified into three subgroups: biological process (BP), cellular component (CC), and molecular function (MF). All DEGs were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and we searched for significantly enriched KEGG pathways at the $P < 0.05$ level. Each group had three replication samples (Fig. 1C).

Statistical Analysis

The dates are presented as the mean ± standard error of mean. The data were analyzed using one-way analysis of variance (ANOVA). $P \leq 0.05$ was considered to indicate a statistically significant difference, and $P \leq 0.01$ was considered to indicate a highly significant difference among the different treatment groups.

Results

Establishment of In Vitro SSC Differentiation System

To overcome the limitation of small numbers of SSCs in vivo, we established an in vitro differentiation system

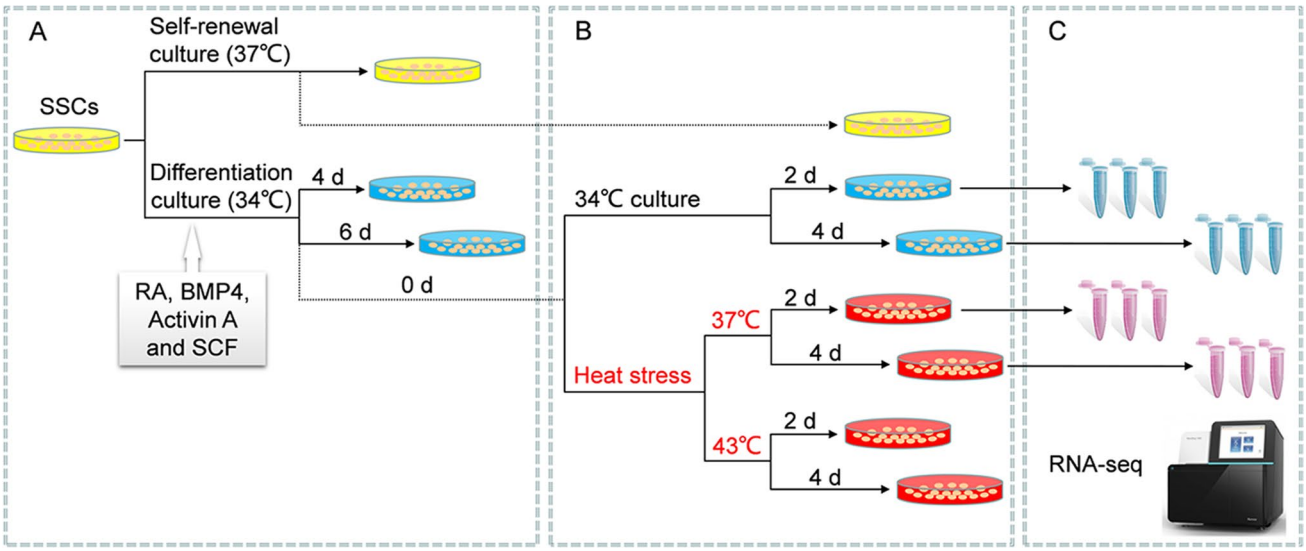


Fig. 1 Graphic illustration of the experimental schedule: **A** RA, BMP4, activin A, and SCF were added to the culture medium to establish the in vitro differentiation culture system of the SSCs. The differentiation culture temperature was 34 °C. The mRNA and protein expression levels of differentiation marker genes in spermatogenic cells were detected on days four and six after differentiation culture ($n=3$). **B** To investigate the effect of heat stress treatment on SSC differentiation, in vitro cultured SSCs were subjected to heat stress at 37 °C and 43 °C, respectively. The expression levels of differentiation marker genes in spermatogenic cells were detected on days two and four after differentiation culture ($n=3$). **C** RNA-seq was used to analyze differentially expressed genes and key pathways of differentiation cultured SSCs on days two and four after heat stress treatment at 37 °C ($n=3$)

of SSCs. We added SCF, BMP4, RA, and activin A to the SSC culture medium to induce SSC differentiation, and used real-time PCR to detect SSC self-renewal and differentiation marker gene expression. Compared with self-renewing SSCs cultured at 37 °C, SSCs cultured for differentiation at 34 °C had apparent colony-like growth on days four and six after differentiation culture (Fig. 2A). Undifferentiating spermatogonia marker genes *Id4* and *Thy-1* were significantly ($P \leq 0.05$) reduced on day six after differentiation culture initiation. Conversely, SSC differentiation marker gene *c-kit*, meiosis-related genes *Stra8* and *Rec8*, and spermatocyte-related gene *Sycp3* were significantly increased on day six after differentiation culture initiation (Fig. 2B). These results show that the SSC differentiation system was successfully established.

Heat Stress Inhibited SSC Differentiation

To determine the effect of high temperature on SSC differentiation, SSCs in the differentiation culture were subjected to heat stress treatment at 37 °C and 43 °C, respectively (Fig. 3A). The results indicate that heat stress inhibited SSC differentiation. First, we examined the effect of heat stress on undifferentiating spermatogonia marker gene expression during SSC differentiation. The results showed that the expressions of the stem cell marker genes *Id4* and *Thy-1* in the 37 °C and 43 °C heat shock-treated differentiation culture groups were significantly higher 4 days after heat stress treatment than those in the 34 °C differentiation culture group. We then examined the effects of heat stress on the expression of SSC differentiation-related genes. The results showed that the expression of *c-kit*, *Stra8*, *Rec8*, *Sycp3*, and the spermatocyte-related gene *Ovol1* in the 37 °C and 43 °C differentiation groups were significantly lower 2 and 4 days after heat stress treatment initiation than that in the 34 °C differentiation group ($P \leq 0.05$). We compared the inhibitory effects of heat stress treatment at 37 °C and 43 °C on SSC differentiation, and found that heat stress treatment at 37 °C inhibited the differentiation of SSCs more significantly than short-term (30 min per day) heat stress at 43 °C. Two and 4 days after heat stress treatment initiation, the expression of the differentiation-related genes *c-kit*, *Stra8*, and *Rec8* in the 37 °C treatment group was significantly lower than that in the 43 °C treatment group, and the expression of the stem cell marker gene *Thy-1* was higher than that in the 43 °C treatment group (Fig. 3B). In the subsequent experiments, we subjected SSCs to 37 °C heat stress treatment.

Heat Stress Altered Gene Expression in Differentiation Cultured SSCs

To reveal the molecular mechanism associated with the effect of heat stress treatment on SSC differentiation, gene expression changes between normal (34 °C) and heat stress (37 °C) temperatures were identified using DEG analysis. In SSCs cultured at 37 °C, 765 genes were up-regulated

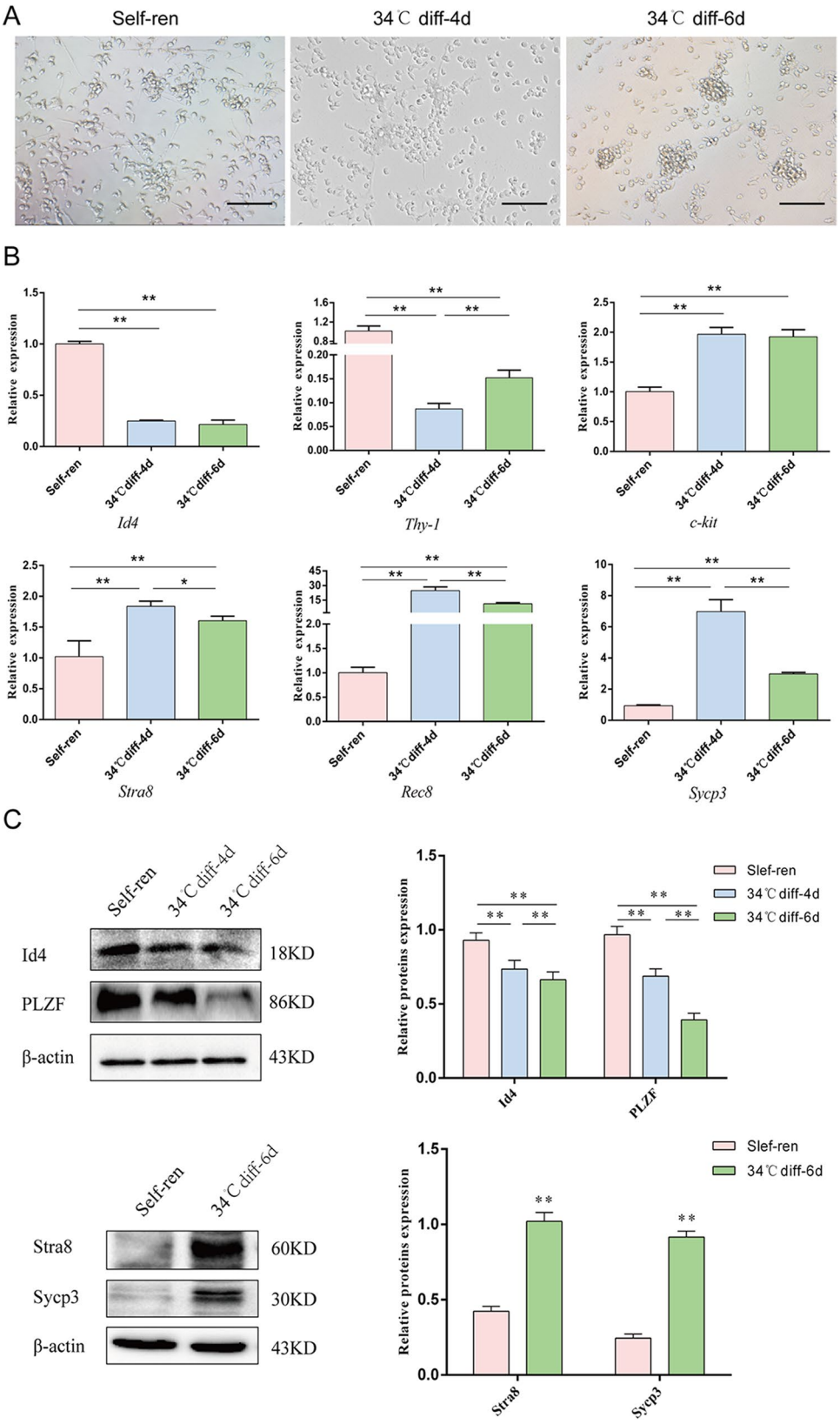


Fig. 2 Establishment of in vitro SSC differentiation system. **A** SSCs grew well in the 37 °C self-renewal culture group and in the 34 °C differentiation culture group. Bar = 100 μm. **B** Undifferentiating spermatogonia marker genes *Id4* and *Thy-1* were significantly decreased at days four and six after differentiation culture, and the SSC differentiation marker gene *c-kit* and meiosis-related genes *Stra8*, *Rec8*, and *Sycp3* were significantly increased. Self-ren, self-renewal; 34°Cdiff, differentiation culture at 34 °C; 34°Cdiff-4d, day four of differentiation culture at 34 °C; 34°Cdiff-6d, day six of differentiation culture at 34 °C. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$

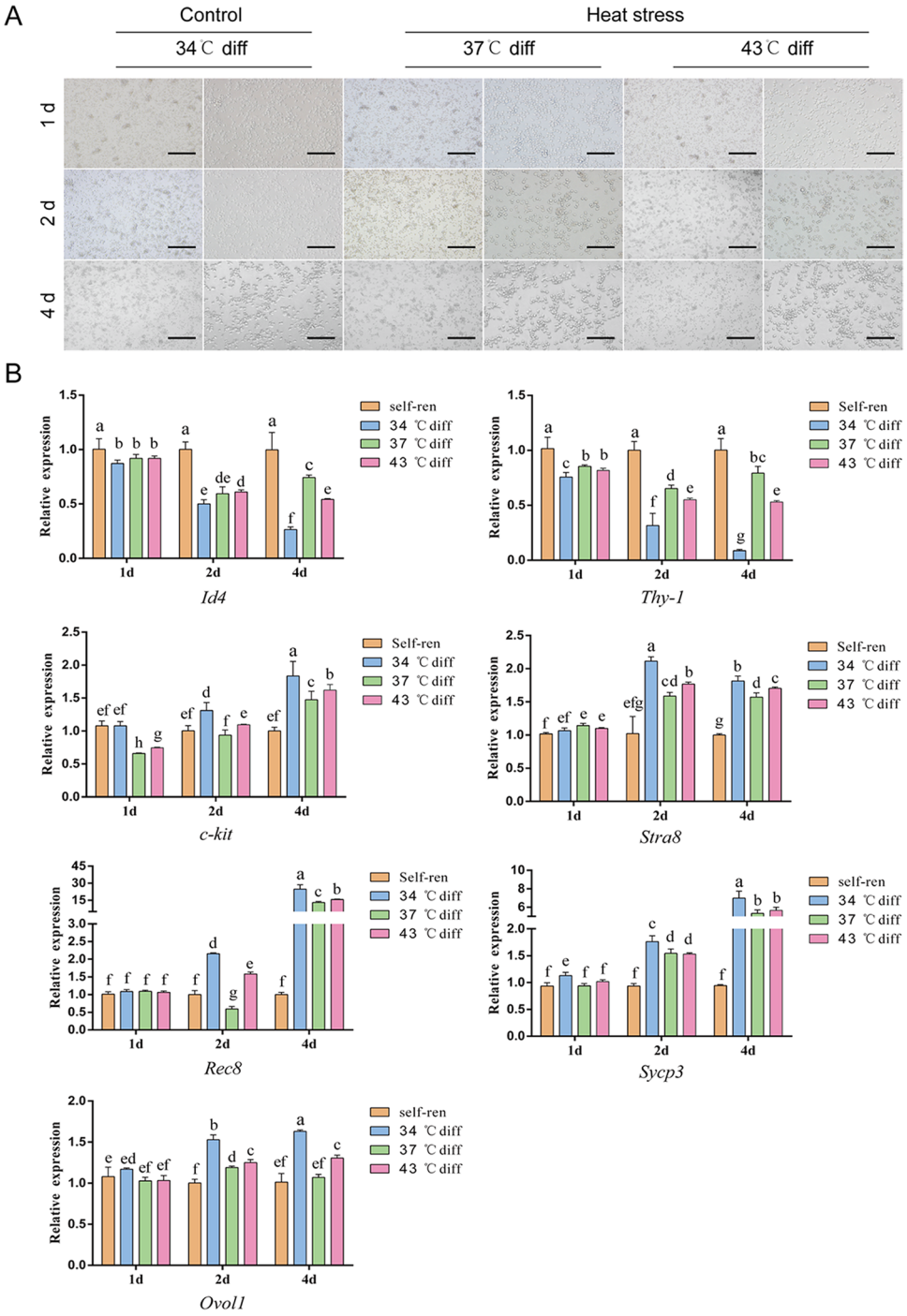


Fig. 3 Heat stress inhibited the SSC differentiation. **A** In the control group, SSC was cultured at 34 °C. SSCs in the heat stress treatment groups were cultured at 37 °C and 43 °C, respectively. Bar = 100 μm. **B** The expression of undifferentiating spermatogonia marker genes *Id4* and *Thy-1* in the 37 °C and 43 °C differentiation culture groups were significantly higher than those in the 34 °C differentiation culture group. The expression of the SSC differentiation marker gene *c-kit*, meiosis-related genes *Stra8* and *Rec8*, and the spermatocyte leptotene- and pachytene-related genes *Sycp3* and *Ovol1* in the 34 °C differentiation groups was significantly higher than those in the 37 °C and 43 °C differentiation groups

and 895 genes were down-regulated on day two, while 3892 genes were up-regulated and 3360 genes were down-regulated on day four (Fig. 4A and B). With the extension of heat stress treatment time from day two to day four, despite the total number of expressed genes not changing significantly (29,401 and 24,713 respectively), the number of differentially expressed genes increased significantly (from 1160 to 7252) (Fig. 4C).

GO Analysis of the Differentially Expressed Genes

GO analysis was used to characterize the functions of the DEGs obtained from RNA-seq. Three different aspects of DEGs, i.e., BPs, CC, and MF, reflected the effects of thermal stress on cell differentiation (Fig. 5A and B). We compared the 30 most enriched terms on days two and four after the 37 °C heat stress treatment (Fig. 5C and D) and found the

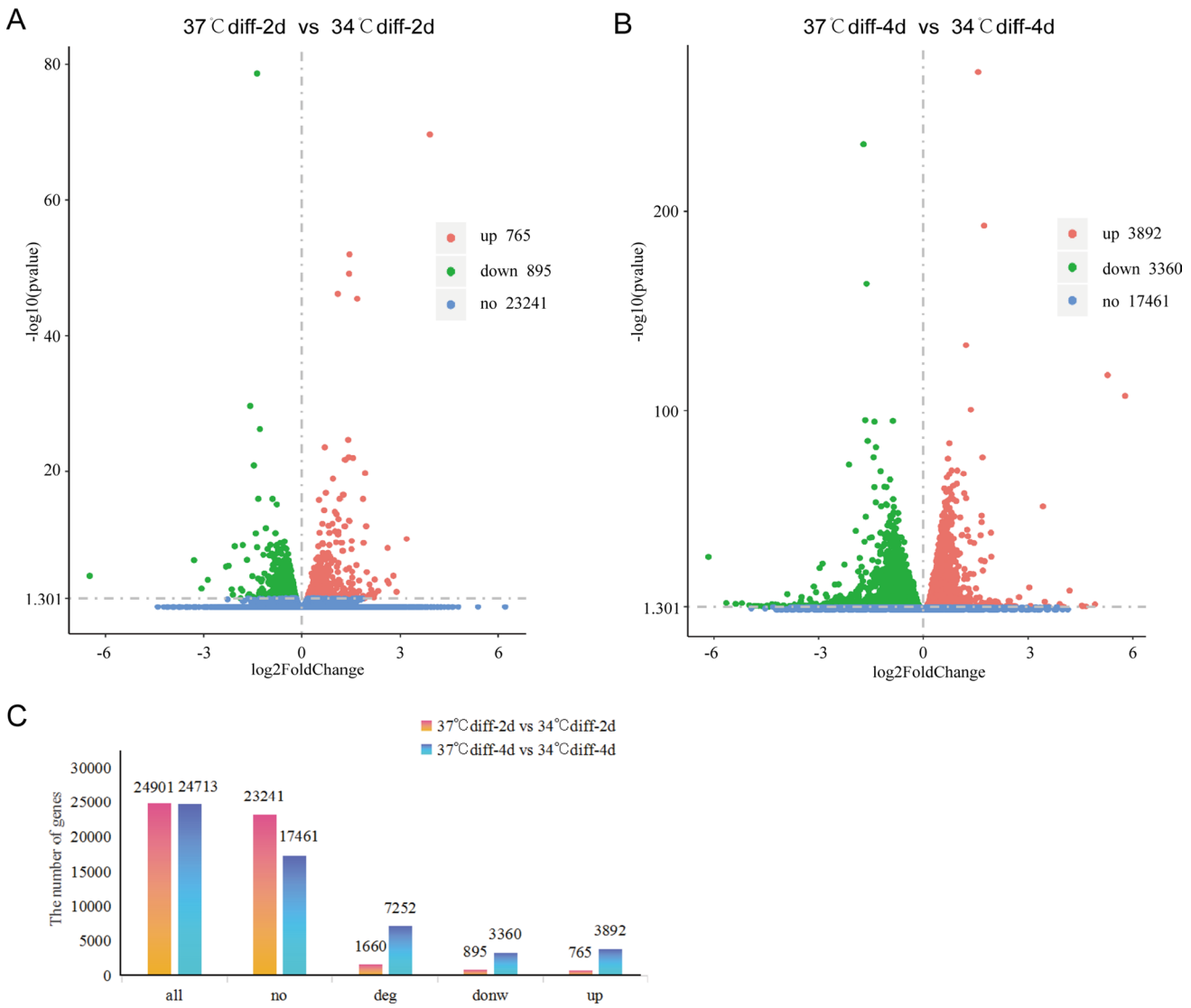
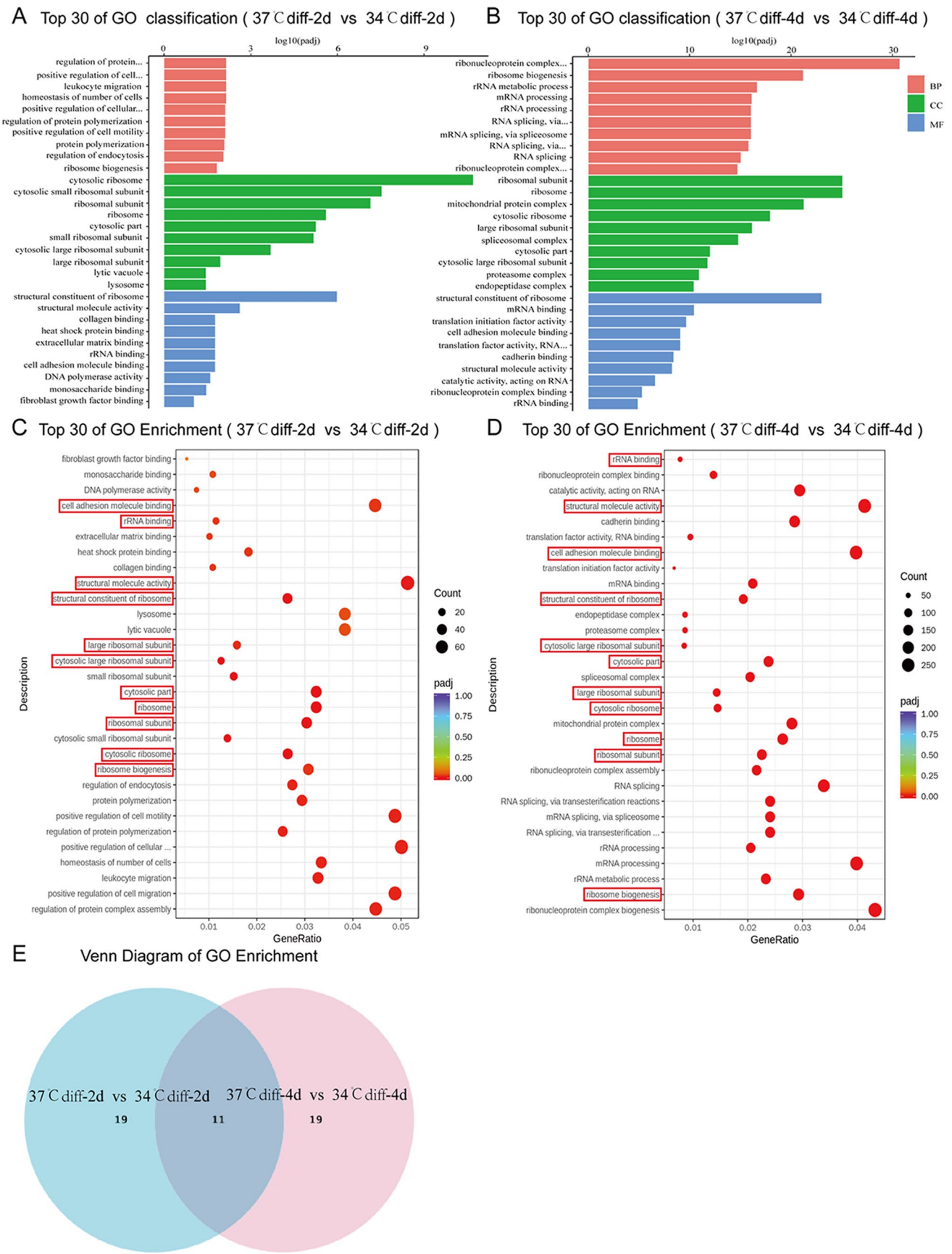


Fig. 4 Heat stress changed the gene expression of differentiation cultured SSCs. **A** Volcano figure of 37°Cdiff-2d vs 34°Cdiff-2d. **B** Volcano figure of 37°Cdiff-4d vs 34°Cdiff-4d. **C** The number of differentially expressed genes in the 37 °C heat stress and control groups

following eleven common GO terms (Fig. 5E): cell adhesion molecule binding, rRNA binding, structural molecule activity, structural constituent of ribosome, large ribosomal subunit, cytosolic large ribosomal subunit, cytosolic part, ribosome, ribosomal subunit, cytosolic ribosome, and ribosome biogenesis. In addition, the heat shock protein binding GO term was enriched on day 2 after heat stress at 37 °C, but not on day 4 (Fig. 5C and D, Table 1).

KEGG Analysis of the Differentially Expressed Genes

KEGG enrichment analysis of DEGs can reveal pathways with significant enrichment, which is helpful for finding significantly altered biological regulatory pathways. To further explore the roles of DEGs in SSC differentiation after heat stress treatment, we tested whether the DEGs were enriched in certain KEGG pathways. We compared the 33 most enriched KEGG pathway on days two and four after 37 °C heat stress treatment (Fig. 6A and B) and found the following six common KEGG pathways (Fig. 6C): ribosome, carbon metabolism, citrate cycle (TAC cycle), p53 signaling pathway, bacterial invasion of epithelial cells, and apoptosis. Out of these KEGG pathways, only ribosome, carbon metabolism, and citrate cycle (TAC cycle) were significantly enriched on day two after 37 °C heat stress treatment (Fig. 6A and B, Table 2).


A Top 30 of GO classification (37℃diff-2d vs 34℃diff-2d)
log10(padj)
regulation of protein...
positive regulation of cell...
leukocyte migration
homeostasis of number of cells
positive regulation of cellular...
regulation of protein polymerization
positive regulation of cell motility
protein polymerization
regulation of endocytosis
ribosome biogenesis
cytosolic ribosome
cytosolic small ribosomal subunit
ribosomal subunit
ribosome
cytosolic part
small ribosomal subunit
cytosolic large ribosomal subunit
large ribosomal subunit
lytic vacuole
lysosome
structural constituent of ribosome
structural molecule activity
collagen binding
heat shock protein binding
extracellular matrix binding
rRNA binding
cell adhesion molecule binding
DNA polymerase activity
monosaccharide binding
fibroblast growth factor binding
B Top 30 of GO classification (37℃diff-4d vs 34℃diff-4d)
log10(padj)
ribonucleoprotein complex...
ribosome biogenesis
rRNA metabolic process
mRNA processing
rRNA processing
RNA splicing, via...
mRNA splicing, via spliceosome
RNA splicing, via...
RNA splicing
ribonucleoprotein complex...
ribosomal subunit
ribosome
mitochondrial protein complex
cytosolic ribosome
large ribosomal subunit
spliceosomal complex
cytosolic part
cytosolic large ribosomal subunit
proteasome complex
endopeptidase complex
structural constituent of ribosome
mRNA binding
translation initiation factor activity
cell adhesion molecule binding
translation factor activity, RNA...
cadherin binding
structural molecule activity
catalytic activity, acting on RNA
ribonucleoprotein complex binding
rRNA binding
BP
CC
MF
C Top 30 of GO Enrichment (37℃diff-2d vs 34℃diff-2d)
Description
GeneRatio
Count
padj
D Top 30 of GO Enrichment (37℃diff-4d vs 34℃diff-4d)
Description
GeneRatio
Count
padj
E Venn Diagram of GO Enrichment
37℃diff-2d vs 34℃diff-2d
37℃diff-4d vs 34℃diff-4d
19
11
19

◂**Fig. 5** GO analysis of the differentially expressed genes ($n=3$). **A** GO classification in the 37 °C and 34 °C differentiation cultured groups on day two. **B** GO classification in the 37 °C and 34 °C differentiation cultured groups on day four. **C** The 30 most enriched GO terms in the 37 °C and 34 °C differentiation cultured groups on day two. **D** The 30 most enriched GO terms in the 37 °C and 34 °C differentiation cultured groups on day four. The red boxes represent the common GO terms enriched on days two and four of heat stress treatment at 37 °C. **E** Venn diagrams show that eleven of the 30 most enriched GO terms were the same on days two and four after heat stress treatment

Discussion

In this study, we successfully investigated the effect of heat stress on SSC differentiation by using in vitro differentiation cultured SSCs. The results show that high temperatures inhibit SSC in vitro differentiation and alter the expression of SSC transcriptome. RNA-seq analyses identified significantly inhibited pathways in DEGs after heat stress treatment, including p53 signaling pathways, carbon metabolism, and ribosome signaling pathways. These results provide new insights for the diagnosis and treatment of human oligospermia associated with high temperature.

We successfully established a SSC in vitro differentiation culture system. When we added RA, BMP4, SCF, and activin A in the SSC differentiation medium for differentiation culture, the expressions of *Id4* and *Thy-1* were down-regulated, while the expressions of *c-kit*, *Stra8*, *Rec8*, *Sycp3*, and *Ovol1* were up-regulated, indicating that we successfully established the differentiation culture system of SSCs. The expression of the helix-loop-helix protein *Id4* is selective for a subset of A_s in mouse testes and plays a role in maintaining the SSC pool [32]. The *Id4* level is predictive of stem cell or progenitor capacity in spermatogonia and dictates the interface of transition from the stem cell to the immediate progenitor state [33]. Flow cytometric cell sorting and the SSC transplantation assay demonstrated that *Thy-1* is a unique surface marker of SSCs in neonatal pups, and adult testes of mice [34]. *c-kit* is considered a marker for SSC pluripotency loss. In early studies, *c-kit* expression was detected in type A (A1–A4), intermediate, and type B spermatogonia, as well as in preleptotene spermatocytes, but not in undifferentiated spermatogonia [35, 36]. *Stra8*, as a response gene to RA, plays an important role in the initiation of meiosis during spermatogenesis and is a marker for germ cells to enter meiosis [37]. *Rec8* is a key component of the meiotic cohesin complex, and has an essential role in mammalian meiosis, with both male and female *Rec8*-null mice exhibiting germ cell failure and sterility [38]. *Sycp3* (or *Scp3*) is a DNA-binding protein that forms a structural component of the ligand complex, which mediates chromosome binding or homologous pairing during meiosis in germ cells [39, 40]. *Ovol1*, encoding a member of the Ovo family of zinc-finger transcription factors, regulates meiotic pachytene progression during spermatogenesis by repressing *Id2* expression, and the targeted deletion of *Ovol1* leads to germ cell degeneration and defective sperm production in adult mice [41].

We found that high temperatures inhibited in vitro cultured SSC differentiation. In most male mammals, the temperature in the scrotum is usually 2–7 °C lower than the core body temperature, and is strictly regulated by a heat exchange system [14]. Therefore, we used 34 °C as the temperature for SSC in in vitro differentiation culture. In previous studies, a temperature range of 32–34.5 °C has been widely used for SSC culture function in vitro [42–45], but in this study, we used 37 °C or 43 °C as heat stress temperature. 37 °C is the core body temperature, which is equivalent to the testicular temperature in patients with cryptorchidism. In many studies, 43 °C has been widely used as a heat stress treatment temperature to study the effects of high temperature on male germ cells in vivo [46]. In our previous study, 43 °C was used as heat stress temperature to treat self-renewal cultured SSCs in vitro, and we found that it inhibited SSC self-renewal and did not induce SSC apoptosis [29]. This study indicates that both 37 °C and 43 °C heat stress inhibit SSC differentiation. The expression of the undifferentiating spermatogonia marker genes *Id4* and *Thy-1* increased significantly in differentiation cultured SSCs after heat stress treatment, and the expression of the differentiation-related genes *c-kit*, *Stra8*, *Rec8*, *Sycp3*, and *Ovol1* in differentiation cultured SSCs significantly decreased after heat stress treatment. Previous in vivo studies have shown that 43 °C scrotal hyperthermia for 30 min caused a reduction in the expression of the *stra8* and *c-kit* genes in mice [47], and the expression of *SYCP3* in testes of C57 adult mice significantly decreased 1 and 7 days after 15 min 43 °C heat stress treatment [17, 48]. The results of these in vivo experiments are similar to those of our in vitro experiments. In our study, we also found that heat stress treatment at 37 °C had a more apparent inhibitory effect on germ cell differentiation-related gene expression than the 30 min heat stress treatment at 43 °C, which provided ideas for the pathogenesis of azoospermia caused by SSC differentiation disorders in cryptorchidism.

We found significant inhibition of some DEGs in p53 signal pathways, carbon metabolism, and ribosome signal pathways by transcriptome sequencing analysis. Previous studies suggest that p53 signaling pathways relate closely with cell differentiation [49, 50]. A study by Jain et al. [51] showed that, in response to differentiation stimuli such as RA, p53 is activated after being acetylated by CBP/p300 histone acetyl transferases to induce embryonic stem cell (ESC) differentiation. In our RNA-seq results, the *Thrombospondins1* (*Thbs1*) gene in the p53 signaling pathway was down-regulated. *Thbs1* is a member of the extracellular matrix (ECM) protein family, and is associated with

Table 1 The eleven common GO terms enriched on days two and four after 37 °C heat stress treatment

GO ID	GO term	37 °C diff-2d vs 34 °C diff-2d			37 °C diff-4d vs 34 °C diff-4d			Type
		Up-genes	Down-genes	*P*-value	Up-genes	Down-genes	*P*-value	
GO: 0,022,626	Cytosolic ribosome	*Rps28*, *Rpl13a*	*Uba52*, *Rps2*	3.18E-14	*Rpl17*, *Rpl10*, *Rpl34*	*Rps10*, *Repin1*, *Rp121*	6.78E-21	CC
GO: 0,044,391	Ribosomal subunit	*Rpl13a*, *Rps28*	*Rbm3*, *Uba52*, *Rps2*	3.34E-10	*Rpl13*, *Rpl17*, *Npm1*	*mt-Co1*, *mt-Nd5*	1.54E-28	CC
GO: 0,003,735	Structural constituent of ribosome	*Rpl13a*, *Rps28*	*Rps2*, *Rps25*, *Rpl10*	1.14E-09	*Rpl17*, *Rpl10*, *Rpl34*	*Rps10*, *Rpl21*	9.69E-27	MF
GO: 0,005,840	Ribosome	*Rpl13a*, *Nufip2*, *Rps28*	*Rbm3*, *Uba52*, *Rps2*	1.57E-08	*Rbm3*, *Rpl17*, *Npm1*	*Rps10*, *Repin1*, *Rrbp1*	2.56E-28	CC
GO: 0,044,445	Cytosolic part	*Rpl13a*, *Ahr*, *Pfkl*	*Uba52*, *Rps2*, *Gm11808*	4.41E-08	*Rpl17*, *Rpl10*, *Rpl34*	*Eno2*, *Adcy6*, *Ahr*	1.02E-14	CC
GO: 0,022,625	Cytosolic large ribosomal subunit	*Rpl13a*	*Uba52*, *Gm11808*, *Rpl10*	2.21E-06	*Rpl17*, *Rpl10*, *Rpl34*	*Rpl21*	1.96E-14	CC
GO: 0,005,198	Structural molecule activity	*Mapk8ip3*, *Rpl13a*, *Col5a2*	*Col18a1*, *Sept9*, *Rps2*	5.14E-06	*Rpl13*, *Rpl17*, *Rpl10*, *Mrpl14*	*Mapk8ip3*, *Col1a1*, *Col3a1*	3.34E-11	MF
GO: 0,042,254	Ribosome biogenesis	*Ddx17*, *Skiv2l2*, *Utp14b*	*Rps2*, *Rps25*, *Rpl10*	3.03E-05	*Rpl13*, *Fb1*, *Rpl17*, *Rpl10*	*Mapk8ip3*, *Col1a1*, *Col3a1*	2.43E-25	BP
GO: 0,019,843	rRNA binding	-	*Cirbp*, *Rps13*, *Ncl*	1.23E-04	*Rpl17*, *Npm1*, *Ncl*	*Cavin1*, *Mdm2*, *Lipe*	1.22E-07	MF
GO: 0,015,934	Large ribosomal subunit	*Rpl13a*	*Rbm3*, *Uba52*, *Gm11808*	1.42E-04	*Rbm3*, *Rpl17*, *Npm1*	*Rpl21*	5.00E-19	CC
GO: 0,050,839	Cell adhesion molecule binding	*Nisch*, *Flnb*, *Jaml*	*Vwf*, *Tgfbi*, *Efhd2*	1.27E-03	*Rps2*, *Bzw1*, *Serbp1*, *Rps26*	*Thbs1*, *Cyr61*	3.82E-12	MF
GO: 0,031,072	Heat shock protein binding	*Hsph1*, *Ppid*, *Hspa1b*	*Hspa9*, *Arhgdia*	8.26E-05	-	-	-	MF

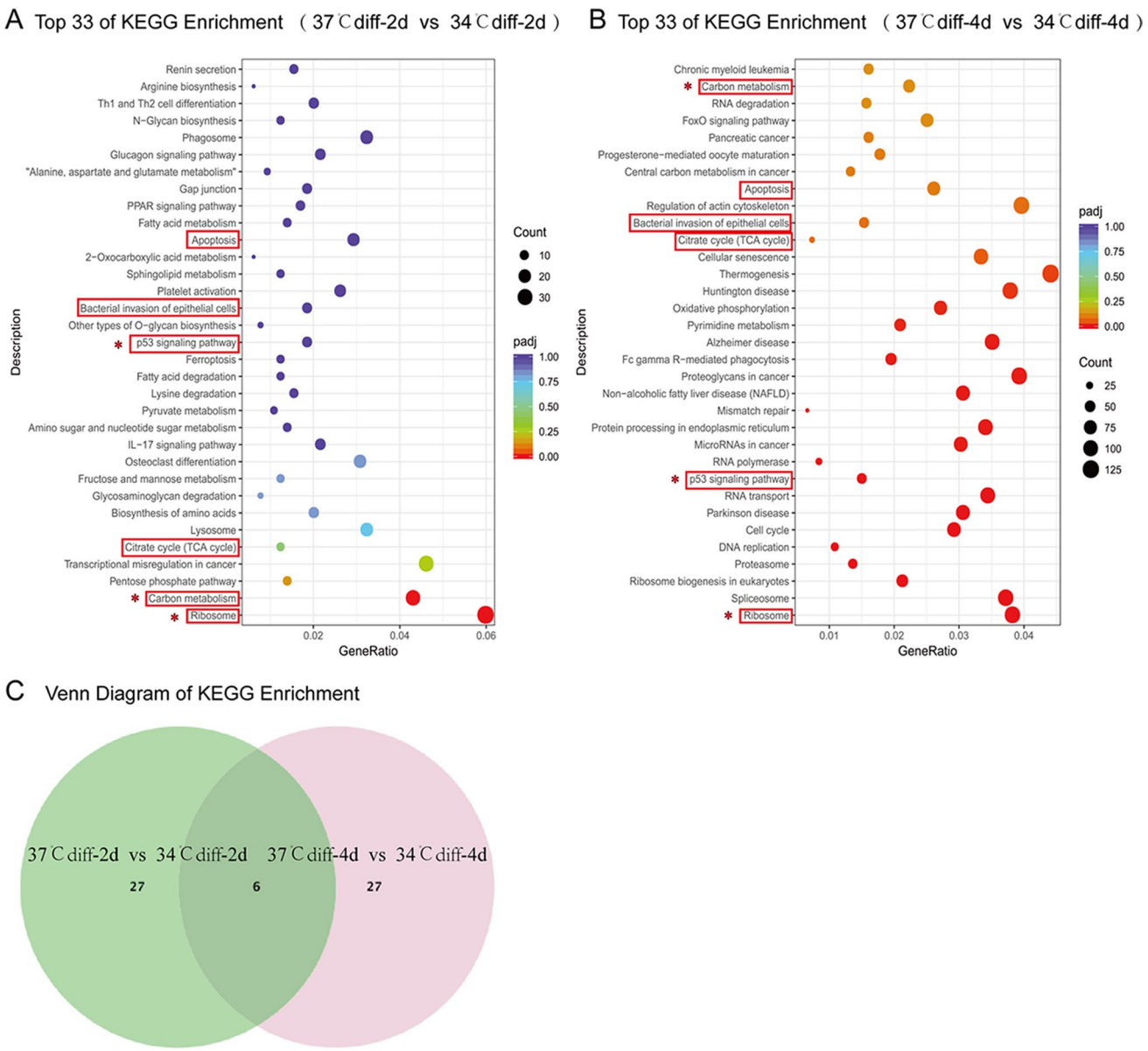


Fig. 6 KEGG analysis of the differentially expressed genes ($n=3$). **A** The top 33 KEGG enrichment pathways in the 37 °C and 34 °C differentiation cultured groups on day two. **B** The top 33 KEGG enrichment pathways in the 37 °C and 34 °C differentiation cultured groups on day four. The red boxes represent the common KEGG pathways enriched on days two and four of heat stress treatment at 37 °C. **C** Venn diagrams show that six of the top 33 KEGG enrichment pathways were the same on days two and four after heat stress treatment

Table 2 The six common KEGG pathways enriched on days two and four after 37 °C heat stress treatment

KEGG ID	KEGG pathway	37 °C diff-2d vs 34 °C diff-2d		37 °C diff-4d vs 34 °C diff-4d	
		Gene name	*P*-value	Gene name	*P*-value
mmu03010	Ribosome	*Rpl13a*, *Mrps18c*, *Rps3*, *Rps28*	1.59E-09	*Rpl17*, *Rpl10*, *Rplp1*, *Rpl7a*	1.44E-18
mmu01200	Carbon metabolism	*Aldoa*, *Fbp2*, *Pfkl*, *Ogdh*, *Rpe*	4.86E-06	*Phgdh*, *Gcsh*, *Sdhb*, *Me2*, *Eno2*	1.36E-02
mmu00020	Citrate cycle (TCA cycle)	*Sdhb*, *Ogdh*, *Idh1*, *Mdh2*, *Sucla2*	7.46E-03	*Sdhb*, *Idh3a*, *Pdha1*, *Idh3g*, *Sdhd*	1.36E-02
mmu04115	p53 signaling pathway	*Thbs1*, *Ccnd3*, *Perp*, *Fas*, *Bcl2l1*	6.16E-02	*Bbc3*, *Ccng2*, *Thbs1*, *Chek1*, *Pmaip1*	1.53E-04
mmu05100	Bacterial invasion of epithelial cells	*Sept9*, *Was*, *Cbl*, *Arpc2*, *Sept11*	7.36E-02	*Was*, *Fn1*, *Hcls1*, *Arpc2*, *Sept9*	6.11E-03
mmu04210	Apoptosis	*Fos*, *Ctsw*, *Fas*, *Tnfsf10*, *Itpr3*	8.89E-02	*Fos*, *Gzmb*, *Tuba8*, *Tuba1b*, *Cycs*	8.14E-03

angiogenic activity, endothelial cell migration and proliferation, and tumor angiogenesis [52]. Studies have shown that lung stem cell differentiation in mice directed by endothelial cells via a BMP4-NFATc1-Thbs1 axis [53]. *Thbs1* was activated by TGF-β, as an intermediate factor, which plays an important role in the differentiation of mesenchymal stem cells [54]. The results of our study indicate that the p53 signaling pathway may play an important role in inhibiting the differentiation of SSCs at high temperatures.

Previous studies have shown that ribosome signaling pathways are associated with cell differentiation. A study by Sankaran et al. [55] found that ribosome levels selectively regulate translation and lineage commitment in human hematopoiesis. The researchers noted that a reduction in ribosome numbers led to a reduction in the output of the GATA1 protein in blood stem cells, which in turn affects their differentiation into mature red blood cells [55]. The results of our study showed that seven ribosome-related GO terms were found in the eleven GO terms co-enriched in differentiation cultured SSCs on days two and four of heat stress treatment at 37 °C. We found that *Rpl13a*, *Rpl17*, *Rpl34*, and *Rps28* were up-regulated, but *Rps2* was down-regulated in the six ribosomal-related GO terms. KEGG results indicate that *Rpl13a*, *Mrps18c*, and *Rps28* genes were enriched in ribosome signaling pathways. The results of our study indicate that ribosome signaling pathways may play an important role in inhibiting the differentiation of SSCs at high temperatures.

The carbon metabolism signaling pathways enriched in this study may also play an important role in inhibiting SSC differentiation at high temperatures. For many years, stem cell metabolism was viewed as a byproduct of cell fate status rather than an active regulatory mechanism [56]. Carbon metabolism is a crucial aspect of cell life, and many studies have found that it is inseparable from cell differentiation. Both folate receptor 1 (folr1) overexpression and treatment with folinic acid stimulate β-cell differentiation in zebrafish and pig islets [57], and folic acid is an important vitamin of the one-carbon metabolism pathway that provides carbon units for numerous cellular processes [58, 59]. Due to its essential role in nucleic acid synthesis, inhibition of folate metabolism blocks cellular proliferation [60]. Mitochondria are bioenergetic organelles that produce ATP via oxidative phosphorylation (OXPHOS) and play an important role in mediating stem cell fate and function. In the pre-implantation stage of mammalian development, cellular energy in the form of adenosine triphosphate (ATP) is generated primarily through the oxidation of carbon sources [61]. Loss of the mitochondrial complex III subunit Rieske iron-sulfur protein (RISP) in fetal mouse hematopoietic stem cells allows them to proliferate but impairs their differentiation, leading to anemia and prenatal death [62]. Mitochondria dynamically regulate stem cell identity, self-renewal, and differentiation by orchestrating a transcriptional program [63].

In RNA-seq analysis, heat shock protein (HSP)-related genes, such as *Hspa9*, *Hsph1*, *Hsp90ab1*, *Hsp90aa1*, and *Hspa8*, were also enriched. Heat-stressed cells exhibit a robust HSP production. Cells exposed to heat-stressed respond by synthesizing heat shock proteins. This protein family is classified by their molecular size [64]. HSPs can stimulate active cellular processes resulting in thermotolerance. HSPs are common proteins essential to proteostasis, most being stress-inducible with multiple chaperone functions, such as protein complex disaggregation, protein trafficking, and folding and refolding [65, 66]. Furthermore, they play an essential role in spermatogenesis. Previous studies found that HSP90α-deficient male mice were sterile due to a complete failure to produce sperm, which is related to the first wave of spermatogenesis before puberty as well as the maintenance of adult testis spermatogenesis [67, 68]. A study by Liu et al. [69] demonstrated the occurrence of heat shock up-regulation of HSP production by inducing ROS expression and activation of p38/Akt signaling in human placenta-derived multipotent cells (hPDMCs), while the transcription activity of HSF1 increased, contributing to HSP production. Hsp90 has the function of regulating spermatogenesis, location of germ cells, and formation of sperm microtubes. Studies have shown significant changes in the location and expression of Hsp90 in the sperm of oligospermia and asthenospermia patients [70]. In our next study, we will also focus on the role of HSP in SSC differentiation in vitro after heat stress.

Conclusion

These results indicate that 37 °C significantly inhibited SSC differentiation, and p53, ribosome, and carbon metabolism signaling pathways were involved in this differentiation inhibition process. The results of this study provide a reference for further investigation into the mechanism by which high temperature inhibits SSC differentiation.

Author Contribution GWJ, LHX, FJ, LXR, and YPL were responsible for the experiments, data analysis, and editing of the manuscript. JH participated in the design of the study and edited the manuscript. MWZ was contributed to the conception, supervision, and editing of the manuscript. All authors read and approved the final manuscript.

Funding This work was supported by the Key Research and Development Program of Ningxia Hui Autonomous Region (2021BEG02029) and National Natural Science Foundation of China (82260634, 81860266).

Data Availability The datasets presented in this study can be found in online repositories. Please contact the corresponding authors for data requests.

Declarations

Ethics Approval The experiments using mice were approved by the ethics committee of Ningxia Medical University, and all animal care and experiments were carried out in accordance with the institutional ethical guidelines for animal experiments.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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