High Temperature Inhibits The Differentiation of Spermatogonial Stem Cells

Wei-Jun Gao
Ningxia Medical University
Hui-Xia Li
Ningxia Medical University
Juan Feng
Ningxia Medical University
Hua Jia
Ningxia Medical University
Wen-Zhi Ma (ma.wenzhi@gmail.com)
Ningxia Medical University https://orcid.org/0000-0002-3811-0622

Research Article

Keywords: Male infertility, High temperature, Spermatogonial stem cell, Differentiation, Inhibition

Posted Date: January 27th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1173188/v1

Abstract

Background

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) is a kind of germline stem cells which maintain the spermatogenesis through self-renewal and differentiation. At present, however, the effect of high temperature on SSC differentiation in vitro has not been reported.

Methods

In the present study, we used in vitro differentiation model of SSCs to research the effect of heat stress treatment on SSC differentiation. Firstly, real-time PCR was used to detect the expression levels of self-renewal and differentiation marker genes in differentiation-cultured SSCs after heat stress treatment. Then, the effect of heat stress on the transcriptome of differentiation cultured SSCs was analyzed by RNA-seq. Enrichment of functions and signaling pathways analysis were performed based on Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Results

We found that 2 days of 37℃ or 43℃ (30min/d) heat stress treatment significantly inhibited SSC differentiation. The differentiation related genes *c-kit*, *stra8*, *Rec8*, *Sycp3* and *Ovol1* were down-regulated after 2 days and 4 days of heat stress at 37℃. The transcriptome of SSCs was significantly differentially expressed on the second day and fourth day after heat stress treatment at 37℃. In total, 1660 and 7252 differentially expressed genes (DEGs) were identified by RNA-Seq in SSCs treated with heat stress at 37℃ for 2 days and 4 days respectively, compared with those cultured at 34℃. 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℃.

Conclusion

These results indicate that high temperature at 37℃ significantly inhibits SSC differentiation and promotes enrichment of P53, ribosome and carbon metabolism signaling pathways in stem cell differentiation, providing a reference for the pathogenesis of heat-induced azoospermia.

Background

The World Health Organization predicts that infertility will become the third most intractable disease after cancer and cardio-cerebrovascular disease in the 21st century [1]. Infertility occurs in 10–15% couples of childbearing age, and male factors account for 50% of cases [2]. A Global Burden of Disease survey reported that from1990 to 2017, the age-standardised prevalence of infertility increased annually by 0.291% in men [3]. Heat is one of the causes of male infertility [4]. Cryptorchidism or increased scrotal temperature leads to non-obstructive azoospermia or asthenospermia.

SSCs are the source of spermatogenesis 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_{singal} spermatogonia (A_s). In rodents, A_s spermatogonia generate two A_s spermatogonia without an intercellular

bridge. Subsequent cell divisions of the A_{pr} spermatogonia generate $A_{aligned-4}$, $A_{aligned-8}$, and $A_{aligned-16}$ (A_{al}), which will 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, In, and B spermatogonia, which goes into meiosis to form primary spermatocyte, secondary spermatocyte, and eventually form sperm [5, 6]. Retinoic acid (RA) is the inducer of differentiation in SSCs and induces differentiating spermatogonia to express early markers of spermatogenic differentiation, including KIT and STRA8 [7, 8]. To become sensitive to the differentiation-inducing stimulus (RA), A_{undiff} need to exit the self-renewing state and undergo differentiation priming [8, 9]. This transition involves the activation of the mTORC1 pathway that plays a critical role in maintenance of SSCs, and aberrant mTORC1 activation promotes stem cell exhaustion [10–13]. WNT/β-catenin signaling plays an important role in the differentiation priming of A_{undiff} by promoting the transition from self-renewing to RA-responsive state [14, 15]. Interestingly, Tokue et al identified SHISA6 as a novel marker for a specific subset of GFRα1-expressing A_{undiff} [16]. SHISA6 is suggested to act as a WNT signaling inhibitor and thus confer resistance to the differentiation-priming program. Mir-322 regulates SSC differentiation through the WNT/β-catenin signaling pathway, and miR-322 overexpression decreased STRA8, C-KIT and BCL6 expression [17]. Luo et al. found that SSCs can be derived into ovarian organoids and produce offspring [18]. In the study, they transdifferentiated SSCs into oocytes by transduction of *H19*, *Stella*, and *Zfp57* and inactivation of Plzf in SSCs. The regulation of SSC differentiation process is very complex, and any failure of the regulation can lead to male infertility. The causes of male infertility are wide ranging and poorly understood in most cases [19–21].

Temperature is considered to be a key regulator of reproductive activity and testicular homeostasis. Spermatogenesis and sperm maturation are closely related to temperature and require a temperature slightly lower than the normal body temperature 2-7℃ [22, 23]. Severe or repetitive heat exposures often induce male subfertility or infertility due to reduced sperm output and qualities [24]. Therefore, high temperature is an important external factor affecting multiple stages of spermatogenesis and sperm function. In the heat stress condition, mammalian male germ cells show a variety of changes in cellular events including stress granule (SG) formation, DNA damage and apoptosis [25]. A study showed that adult male mice were exposed to an elevated ambient temperature of 35 for 24h and followed by recovery 1 day, identified elevated sperm mitochondrial ROS generation, increased sperm membrane fluidity, pachytene spermatocytes and round spermatids DNA damage [26]. The most relevant consequence of heat stress on the testis is death of germ cells via apoptosis. Previous research indicate that the testicular germ-cell loss by apoptosis after exposure to abdominal heat stress occurs [27]. Studies have shown that p38 MAPK pathway regulates both apoptosis and spermatocyte differentiation [28–30].

However, the role and mechanism of heat stress in regulating SSC development is unclear, due to the small number of SSCs in the mouse testis (only 0.02–0.03% of total testis cells) [31]. Our previous study shown that heat shock treatment at 43℃ for 45 min significantly inhibited SSC self-renewal through S phase cell cycle arrest but not apoptosis [32]. But there are few reports on the effect of high temperature on the differentiation of SSCs. In this study, we first successful used in vitro differentiation model of SSCs studied the effect of heat stress treatment on SSC differentiation.

Materials And Methods

SSCs self-renewal and differentiation culture

SSCs self-renewal culture medium was prepared according to our previously published paper [33]. In brief, the medium was based on Minimum Essential Medium α (MEM-α, 12571-063, Gibco, Grand Island, NY, USA), containing 2mM glutamine (G7012, Sigma, MO, USA), 10% foetal bovine serum (FBS) (16000-36, Gibco), 0.5× pen/strep (15240-062, Invitrogen, Grand Island, NY, USA), 1× nonessential amino acid (NEAA, 11140-050, Gibco) solution, 1× β-mercaptoethanol (β-ME, M3148, Sigma), 25μg/ml insulin(I1882, Sigma), 100μg/ml transferrin (T1428, Sigma), 60μM

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℃ in the presence of 5% CO2. The culture medium for SSCs differentiation was prepared according to the literature published by Zhou Q et al. [34]. In brief, on the basis of SSCs self-renewal medium, the differentiation culture medium was established by adding cytokine SCF (100ng/ml, R&D Systems), BMP4 (20ng/ml, R&D Systems), RA (10-6M Sigma) and activin A (100ng/ml, R&D Systems). The SSCs used for differentiation were incubated at 34℃ in the presence of 5% CO2.

Heat stress treatment of differentiated culture SSCs

The SSCs used in differentiation culture were subjected to heat stress of 37℃ and 43℃, respectively. For the 37℃ heat stress treatment, the culture conditions were the same as the differentiation culture except that the culture temperature was increased from 34℃ to 37℃. For the 43℃ heat stress treatment, SSCs were cultured in a 43℃ CO_2 incubator for 30 minutes every day and then returned to a 34℃ CO_2 incubator for further culture.

Quantitative real-time PCR

Some SSCs differentiation marker genes were detected by quantitative real-time (qRT) PCR. The following primers were used. *ID4,* Forward: TGCAGTGCGATATGA ACGAC, Reverse: GCAGGATCTCCACTTTGCTG; *Thy-1,* Forward: GCTCTCC TGCTCTCAGTCTT, Reverse: GCTGAACTCATGCTGGATGG; *c-kit,* Forward: GGGACACATTTACGGTGGTG, Reverse: GCTTTACCTGGGCTATGTGC; *Stra8,* Forward: TTGACGTGGCAAGTTTCCTG , Reverse: GGGCTCTGGTTCCTGGT TTA; *Rec8,* Forward: CCCGCTTCTCCCTCTATCTC , Reverse: CGATGTAGGT GCTCCAGGAT; *Sycp3,* Forward: CCAATCAGCAGAGAGCTTGG, Reverse: CC TCGAAGCATCTGAGGAAA; *Ovol1,* Forward: TGTCTTACAGGCAGAGCACA, Reverse: GGCCTGTCTCTGTAAGTGGT; *GAPDH* , Forward: AACGGATTTGG CCGTATTGG, Reverse: CATTCTCGGCCTTGACTGTG. We used the Tip Green qPCR SuperMix (Q311-02, Vazyme Biotech, Nanjing, China) in a 20μl reaction volume on a 7500 Fast Real-Time PCR System, and the reaction conditions were set at 95 ℃ for 30s followed by 42 cycles of 95 ℃ for 10s and 60℃ for 30s. The qRT-PCR primers were synthesized by Sangon Biotech (Shanghai) Co, Ltd. The data analysis was performed using the $2^{-\triangle\triangle CT}$ method.

Total RNA-sequence and bioinformatics

We used RNA-Sequence to analyze gene expression for functional enrichment analysis [35], all DEGs were mapped to terms in the GO databases, and then significantly enriched GO terms were searched for among the DEGs using $P < 0.05$ as the threshold. GO term analysis was classified into three subgroups, namely biological process (BP), cellular component (CC) and molecular function (MF). All DEGs were mapped to the KEGG database, and searched for significantly enriched KEGG pathways at $P < 0.05$ level.

Statistical analysis

The dates are presented as the mean ± standard error of mean. The data were analyzed using one-way 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 SSCs differentiation system

We successfully established the in vitro SSC differentiation culture system. We added SCF, BMP4, RA and Activing A to the SSC culture medium to induce SSCs differentiation and used real-time PCR to detect SSCs self-renewal and differentiation marker genes expression. The result showed compared with self-renewing SSCs cultured at 37°C, SSCs cultured for differentiation at 34°C showed obvious colony-like growth on the 4th and 6th day after differentiation culture (Fig. 1A). SSC marker genes *ID4* and *Thy-1* were significantly ($P \leq 0.05$) decreased at day 6 after differentiation culture. SSC differentiation marker gene *c-kit*, meiosis related genes *Stra8* and *Rec8*, and spermatocyte related gene *Sycp3* were significantly increased at day 6 after differentiation culture (Fig. 1B). These results showed that the SSCs differentiation system was successfully established.

Heat stress inhibited SSCs differentiation

In order to clarify the effect of high temperature on the SSC differentiation, the heat stress treatment on differentiated SSCs was carried out at 37°Cand 43°C, respectively (Fig. 2A). Firstly, we examined the effect of heat stress on SSCs marker genes expression during SSC differentiation. The results showed that 4 days after heat stress treatment, the expression of stem cell marker genes *ID4* and *Thy-1* in the 37°Cand 43°C heat shock-treated differentiation culture groups were significantly higher than those in the 34°C differentiation culture group. Then, we examined the effects of heat stress on the expression of SSCs differentiation related genes. The results showed that 2 and 4 days after heat stress treatment, the expression of *c-kit*, *Stra8*, *Rec8*, *Sycp3* and spermatocyte related gene *Ovol1* in 37°C and 43°C differentiation groups were significantly lower than that in 34°C differentiation group ($P \leq 0.05$). Finally, we compared the inhibitory effects of heat stress treatment at 37°Cand 43°C on SSC differentiation, and found that long-term heat stress treatment at 37°C inhibited the differentiation of SSCs more significantly than heat stress treatment at 43°C for 30min per day. Two and four days after heat stress treatment, the expression of differentiation related genes *c-kit*, *Stra8* and *Rec8* in 37°C treatment group was significantly lower than that in 43°C treatment group, and the expression of stem cell marker genes *Thy-1* was higher than that in 43°C treatment group (Fig. 2B). These results showed that heat stress inhibited SSCs differentiation, and long-term 37°C heat stress treatment inhibited SSC differentiation more significantly than 30 min 43°C short-term heat stress treatment. In the subsequent experiments, we adopted long-term 37°C heat stress to treat SSCs.

Heat stress changed gene expression of differentiation cultured SSCs

To reveal the molecular mechanism associated with the effect of heat stress treatment on SSCs differentiation, a DEG analysis was performed to identify gene expression changes between normal temperature (34°C) and heat stress temperature (37°C) during SSCs differentiation culture. The results showed that compared with SSCs cultured at 34°C, 765 genes were up-regulated and 895 genes were down-regulated in SSC cultured at 37°C on day 2; On day 4, 3892 genes were up-regulated and 3360 genes down-regulated (Fig. 3A and B). With the extension of heat stress treatment time from the second day to the fourth day, although the number of total expressed genes did not change significantly (29401 and 24713 respectively), the number of differentially expressed genes increased significantly (from 1160 to 7252) (Fig. 3C).

Gene ontology analysis of the differentially expressed genes

Gene Ontology (GO) analysis was used to characterize the functions of the DEGs obtained from RNA-Seq. Three different aspects of DEGs called biological processes (BP), cellular component (CC) and molecular function (MF) reflected the effects of thermal stress on cell differentiation (Fig. 4A and B). The top 30 enriched terms on the second and fourth day after 37°C heat stress treatment are shown in Fig. 4C and Fig. 4D. We compared the top 30 enriched terms on the second and fourth day after 37°C heat stress treatment and found 11 common GO Terms (Fig. 4E). These 11 GO terms are 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 (Fig. 4C 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 the effect of SSCs differentiation after heat stress treatment, we tested whether the DEGs were enriched in certain KEGG pathways. The top 33 enriched KEGG pathways on the second and fourth after 37℃ heat stress treatment are shown in Fig. 5A and Fig. 5B. We compared the top 33 enriched KEGG pathway on the second and fourth day after 37℃ heat stress treatment and found 6 common KEGG pathways (Fig. 5C). These 6 common KEGG pathways are Ribosome, Carbon metabolism, Citrate cycle (TAC cycle), P53 signaling pathway, Bacterial invasion of epithelial cells and Apoptosis. In these 6 common KEGG pathway, only the Ribosome, Carbon metabolism and Citrate cycle (TAC cycle) were significantly enrich on the second day after 37℃ heat stress treatment (Fig. 5A and B, Table 2).

Discussion

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

Previous studies have shown that heat stress treatment induces the spermatogenic cell apoptosis in mice and rats, and leads to infertility gradually [36]. However, the effects of high temperature on SSCs development are still poorly understood due to the low number in testes. In this study, we added cytokines RA, BMP4, SCF and activin A into the SSC differentiation medium and established an in vitro SSC differentiation system [33]. Previous studies indicated that RA was sufficient to induce the entry of SSCs into meiosis, and the expression of Stra8 was significantly increased 24h after induction of differentiation in combination with BMP4 [37, 38]. As a ligand of c-kit, SCF binds to it and plays an important role in regulating the balance between self-renewal and differentiation of SSCs [39]. Activin A plays an crucial role in germ cell maturation during the period when gonocytes resume mitosis to form the SSCs and differentiating germ cell populations [40], and Activin A is widely used as a cytokine in stem cell differentiation cultured in vitro [41–43]. Through this culture system, we are able to study SSC differentiation process.

We evaluated the stem cell differentiation culture system by detecting the expression of spermatogenic stage-specific marker genes [34]. The expression of the helix-loop-helix protein ID4 is selective for a subset of A_{single} in mouse testes and plays a role in maintenance of the SSC pool [44]. The level of ID4 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 [45]. Flow cytometric cell sorting and the SSC transplantation assay demonstrated that Thy-1 is a unique surface marker of SSCs in neonatal pup, and adult testes of the mouse [46]. c-kit has been a marker for SSCs pluripotency lost. In early studies, c-kit expression is detected in type A (A_1–A_4), intermediate, type B spermatogonia, as well as preleptotene spermatocytes, but not in the undifferentiated spermatogonia [47, 48]. 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 [49]. Rec8 is a key component of the meiotic cohesin complex. Rec8 has an essential role in mammalian meiosis, and both male and female Rec8 null mice have germ cell failure and sterility [50]. 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 [51, 52]. Ovol1 (previously known as movo1), encoding a member of the Ovo family of zinc-

finger transcription factors, regulate 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 [53].

We successfully established SSC in vitro differentiation culture system. The results of this study showed that after we added RA, BMP4, SCF and activing A in 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.

High temperature inhibited in vitro cultured SSC differentiation. In most male mammals, the temperature in the scrotum is usually 2-7 ℃ lower than the core body temperature, and the temperature of the testes is strictly regulated by a heat exchange system [54]. Therefore, we used 34℃ as the temperature for SSC in vitro differentiation culture. In previous studies, the 32-34.5℃was widely used for function SSC culture in vitro [55–58]. In this study, we used 37℃ or 43℃ as the heat stress temperature. 37℃ is the core body temperature, which is equivalent to the testicular temperature in patients with cryptorchidism. In many studies, 43℃ has been widely used as a heat stress treatment temperature to study the effects of high temperature on male germ cells in vivo [59]. In our previous study, 43℃was used as a heat stress temperature to treat self-renewal cultured SSCs in vitro, and it was found that heat stress treatment at 43℃ inhibited the differentiation of SSCs and did not induce SSC apoptosis [32]. The results of this study showed that both 37℃ and 43℃ heat stress inhibited SSC differentiation. The expression of SSC marker genes ID4 and Thy-1 increased significantly in differentiation cultured SSCs after heat stress treatment, and untill now no similar reports have been reported. After heat stress treatment, the expression of differentiation related genes c-kit, Stra8, Rec8, Sycp3 and Ovol1 in differentiation cultured SSCs was significantly decreased. Previous studies have shown that the expression of SYCP3 in testis of C57 adult mice was significantly decreased 1d and 7d after 43℃ 15min heat stress treatment [60, 61]. Stra8, which was periodically expressed in germ cells in seminiferous tubule, was significantly decreased after heat stress [62]. In our study, we also found that long-term heat stress treatment at 37℃ had a more obvious inhibitory effect on germ cell differentiation-related gene expression than the 30 min heat stress treatment at 43℃, which provided ideas for the pathogenesis of azoospermia caused by SSC differentiation disorder in cryptorchidism.

In this study, RNA-Seq analysis was performed on the in vitro differentiation cultured SSCs after heat shock treatment, and many DEGs were found. GO and KEGG analysis found that after heat stress treatment at 37℃, there were significant inhibition of some DEGs in p53 signal pathway, carbon metabolism and ribosome signal pathway. Previous studies suggest that p53 signaling pathway relate to cell differentiation closely [63, 64]. Jain AK's study show that in response to the differentiation stimuli such as RA, p53 is activated after being acetylated by CBP/p300 histone acetyl transferases to induce embryonic stem cells (ESCs) differentiation [65]. In our RNA-seq results, the *Thrombospondins1* (*Thbs1*) gene in p53 signaling pathway was down-regulated. *Thbs1* is a member of the extracellular matrix (ECM) protein family. *Thbs1* is associated with angiogenic activity, endothelial cell migration and proliferation, and tumor angiogenesis [66]. Studies have shown that lung stem cell differentiation in mice directed by endothelial cells via a BMP4-NFATc1-Thbs1 axis [67]. Thbs1 was activated by TGF-β, as an intermediate factor plays an important role in the differentiation of mesenchymal stem cells [68]. The results of our study indicate that p53 signaling pathway may play an important role in inhibiting the differentiation of SSCs at high temperature.

Previous studies have shown that ribosome signaling pathway associated with cell differentiation. Sankaran's study found that ribosome levels selectively regulate translation and lineage commitment in human hematopoiesis [69]. The researchers note that a reduction in ribosome numbers leads 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 [69]. The results of our study showed that 7 ribosome related GO terms were found in the 11 GO terms that were enriched on the second and fourth day of heat stress treatment at 37℃ in differentiation cultured SSCs. We found that Rpl13a, Rpl17, Rpl34, Rps28 and Rps2

genes were enriched in the 6 ribosomal related GO terms. KEGG results indicated that Rpl13a, Mrps18c, and Rps28 genes were enriched in ribosome signaling pathway. The results of our study indicate that ribosome signaling pathway may play an important role in inhibiting the differentiation of SSCs at high temperature.

The Carbon metabolism signaling pathways enriched in this study may play an important role in inhibiting SSCs differentiation at high temperature. For many years, stem cell metabolism was viewed as a byproduct of cell fate status rather than an active regulatory mechanism [70]. Carbon metabolism is a crucial aspect of cell life. Many studies have found that carbon metabolism 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 [71]. And the folic acid is an important vitamin of the one-carbon metabolism pathway that provides carbon units for numerous cellular processes [72, 73]. Due to its essential role in nucleic acid synthesis, inhibition of folate metabolism blocks cellular proliferation [74]. 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 [75]. 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 [76]. Mitochondria dynamically regulate stem cell identity, self-renewal, and differentiation by orchestrating a transcriptional program [77].

Conclusion

These results indicate that high temperature at 37℃ significantly inhibits SSC differentiation and promotes enrichment of P53, ribosome and carbon metabolism signaling pathways in stem cell differentiation, providing a reference for the pathogenesis of heat-induced azoospermia.

Abbreviations

SSCs
Spermatogonial stem cells
As
A single
Aal
A aligned
Apr
A paired
DEGs
Differentially expressed genes
GO
Gene Ontology
KEGG
Kyoto Encyclopedia of Genes and Genomes
FBS
Foetal 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
HSP
Heat shock proteins
HSF
Heat shock factor
BP
Biological process
CC
Cellular component
MF
Molecular function.

Declarations

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 (81860266).

Availability of data and materials

Please contact the corresponding authors for data requests.

Data availability statement

The datasets presented in this study can be found in online repositories.

Author contributions

GWJ, LHX and FJ 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.

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 that they have no conflict of interest.

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Tables

Table 1
The 11 common GO terms enriched on the second and fourth day 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: 0022626	cytosolic ribosome	*Rps28, Rpl13a*	*Uba52, Rps2*	3.18E-14	*Rpl17, Rpl10, Rpl34*	*Rps10, Repin1, Rp121*	6.78E-21	CC
GO: 0044391	ribosomal subunit	*Rpl13a, Rps28*	*Rbm3, Uba52, Rps2*	3.34E-10	*Rpl13, Rpl17, Npm1*	*mt-Co1, mt-Nd5*	1.54E-28	CC
GO: 0003735	Structural constituent of ribosome	*Rpl13a, Rps28*	*Rps2, Rps25, Rpl10*	1.14E-09	*Rpl17, Rpl10, Rpl34*	*Rps10, Rpl21*	9.69E-27	MF
GO: 0005840	ribosome	*Rpl13a, Nufip2, Rps28*	*Rbm3, Uba52, Rps2*	1.57E-08	*Rbm3, Rpl17, Npm1*	*Rps10, Repin1, Rrbp1*	2.56E-28	CC
GO: 0044445	Cytosolic part	*Rpl13a, Ahr, Pfkl*	*Uba52, Rps2, Gm11808*	4.41E-08	*Rpl17, Rpl10, Rpl34*	*Eno2, Adcy6, Ahr*	1.02E-14	CC
GO: 0022625	cytosolic large ribosomal subunit	*Rpl13a*	*Uba52, Gm11808, Rpl10*	2.21E-06	*Rpl17, Rpl10, Rpl34*	*Rpl21*	1.96E-14	CC
GO: 0005198	structural molecule activity	*Mapk8ip3,Rpl13a, Col5a2*	*Col18a1, Sept9, Rps2*	5.14E-06	*Rpl13, Rpl17, Rpl10, Mrpl14*	*Mapk8ip3, Col1a1, Col3a1*	3.34E-11	MF
GO: 0042254	ribosome biogenesis	*Ddx17, Skiv2l2, Utp14b*	*Rps2, Rps25, Rpl10*	3.03E-05	*Rpl13, Fb1, Rpl17, Rpl10,*	*Mapk8ip3, Col1a1, Col3a1*	2.43E-25	BP
GO: 0019843	rRNA binding	-	*Cirbp, Rps13, Ncl*	1.23E-04	*Rpl17, Npm1,Ncl*	*Cavin1, Mdm2,Lipe*	1.22E-07	MF
GO: 0015934	large ribosomal subunit	*Rpl13a*	*Rbm3, Uba52, Gm11808*	1.42E-04	*Rbm3, Rpl17, Npm1*	*Rpl21*	5.00E-19	CC
GO: 0050839	cell adhesion molecule binding	*Nisch, Flnb, Jaml*	*Vwf, Tgfbi, Efhd2*	1.27E-03	*Rps2,Bzw1, Serbp1, Rps26*	*Thbs1, Cyr61*	3.82E-12	MF

Table 2
The 6 common KEGG pathways enriched on the second and fourth day 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

Figures

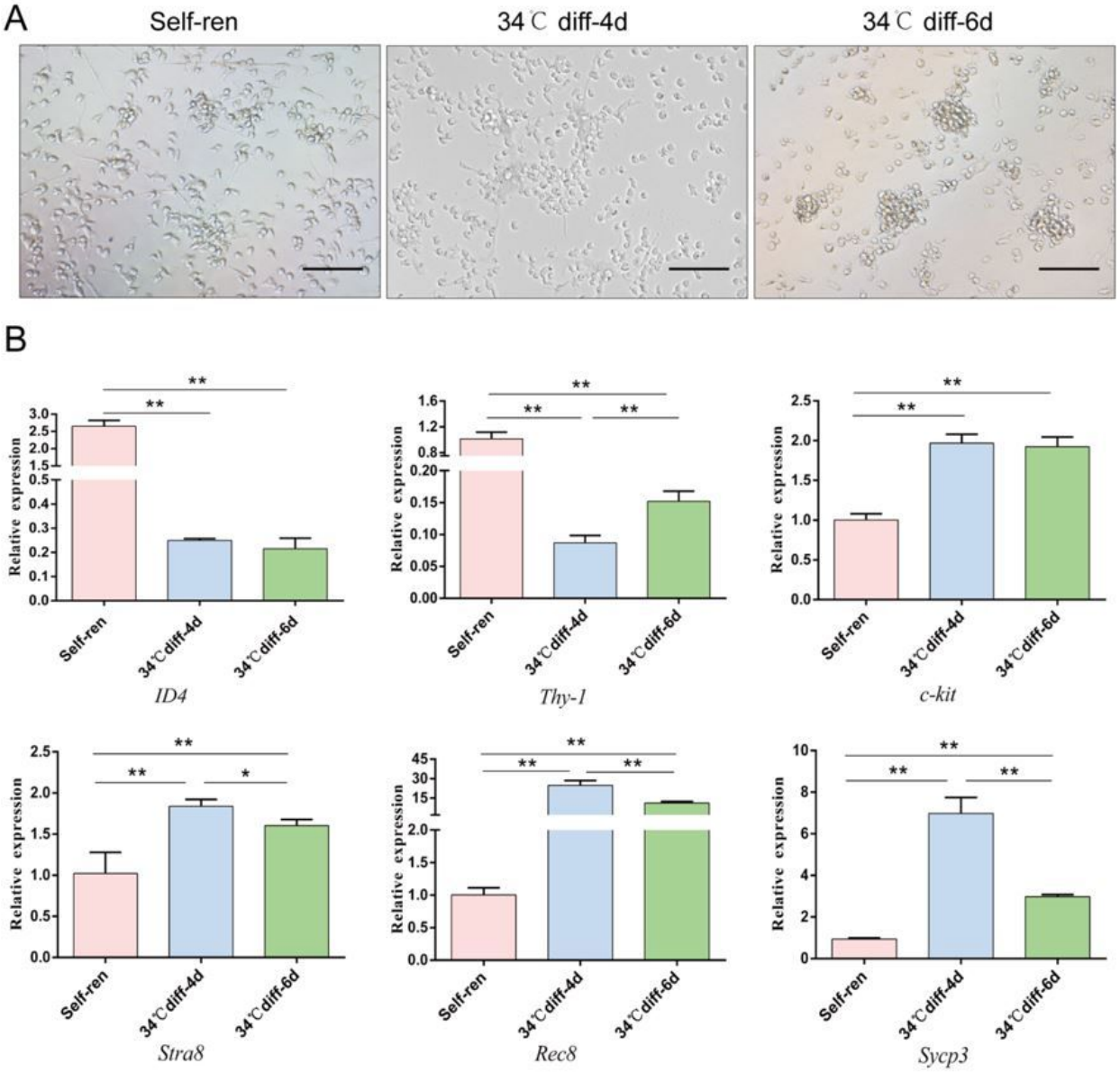

Figure 1

Establishment of in vitro SSC differentiation system. **A**. SSCs grew well in 37°C self-renewal culture group and 34°C differentiation culture group. Bar=100μm. **B**. SSC marker genes *ID4* and *Thy-1* were significantly decreased at day 4 and 6 after differentiation culture, and 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, the fourth day of differentiation culture at 34°C; 34°Cdiff-6d, the sixth day of differentiation culture at 34°C. *$P \leq 0.05$, **$P \leq 0.01$.

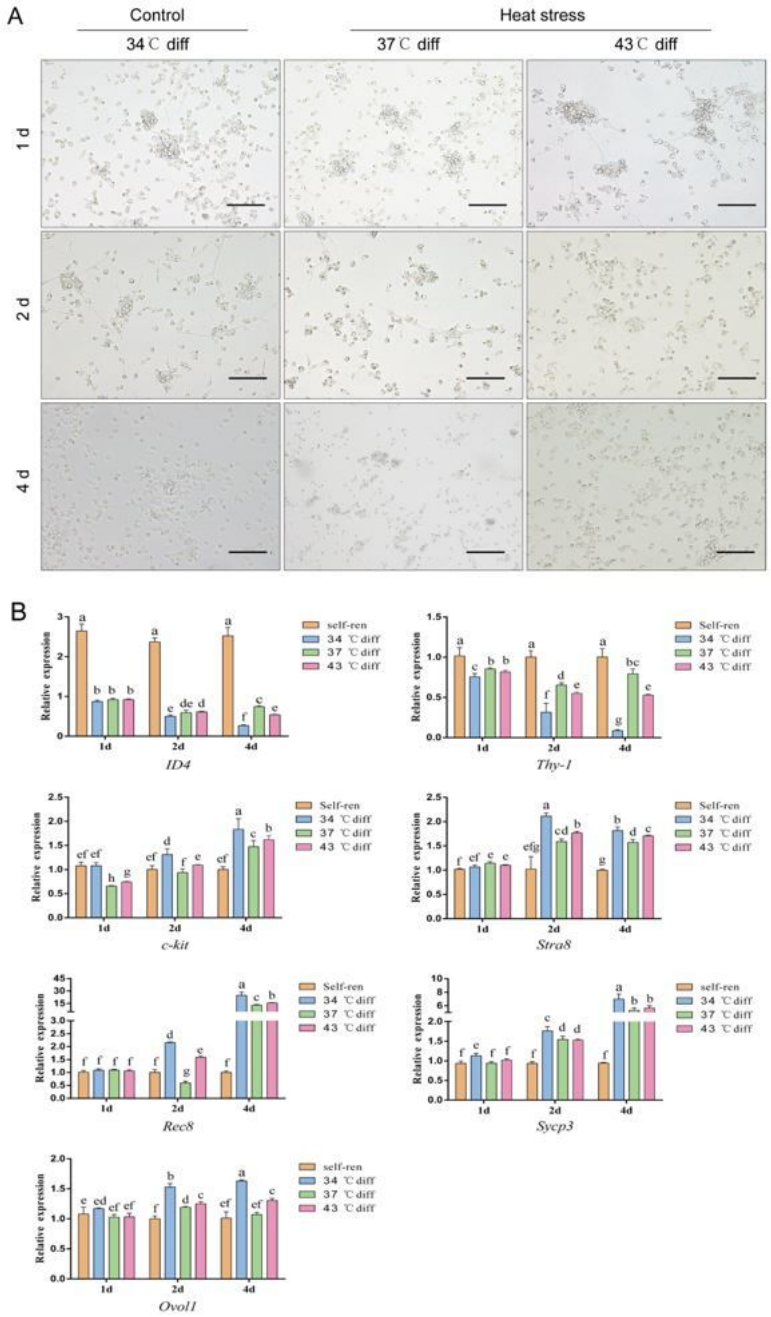

Figure 2

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

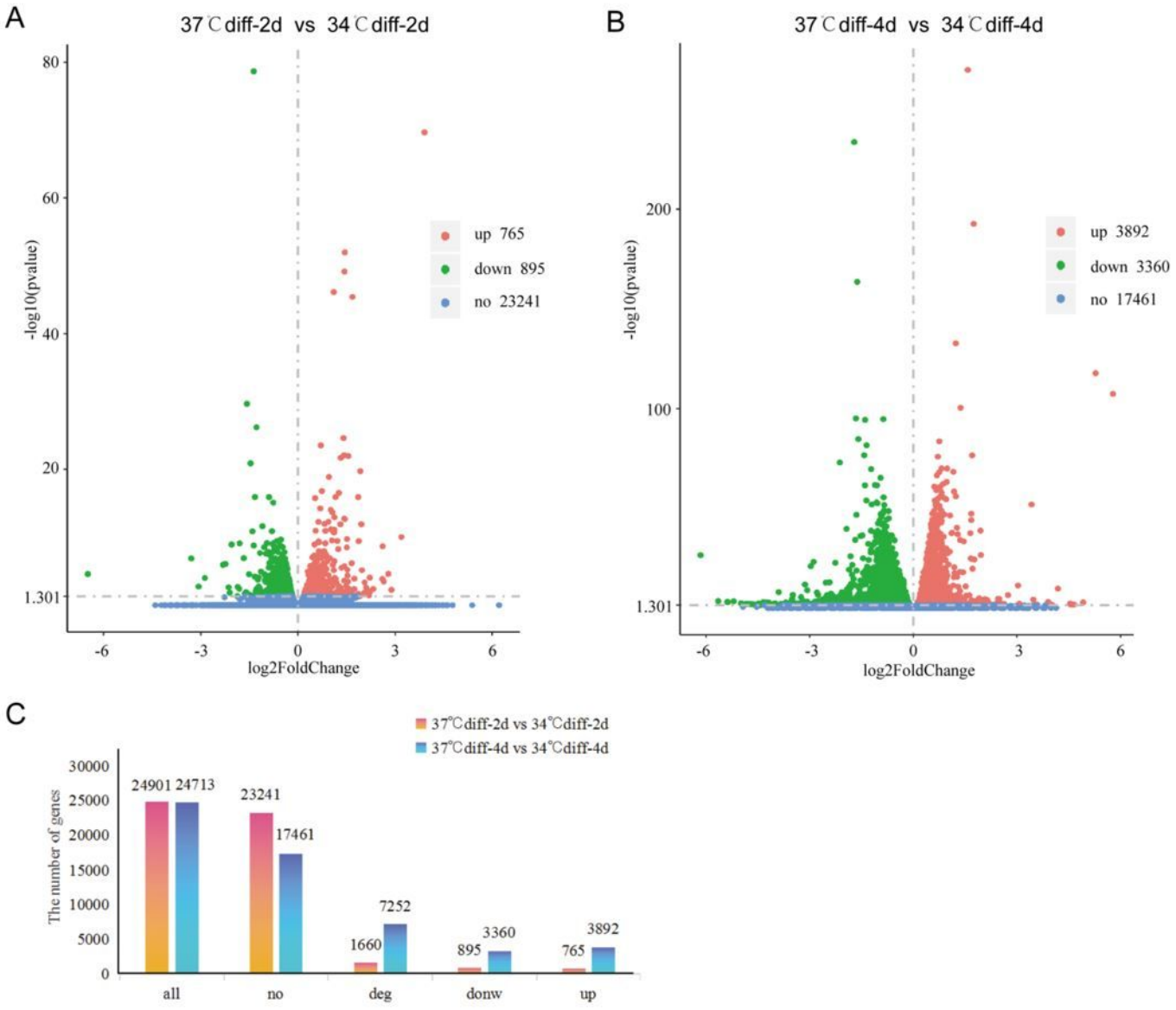

Figure 3

Heat stress changed gene expression of differention cultured SSCs. **A**. The volcano figure of 37°Cdiff-2d vs 34°Cdiff-2d. **B.** The volcano figure of 37°C diff-4d vs 34°Cdiff-4d. **C**. The number of differentially expressed genes in 37 °C heat stress group and control group.

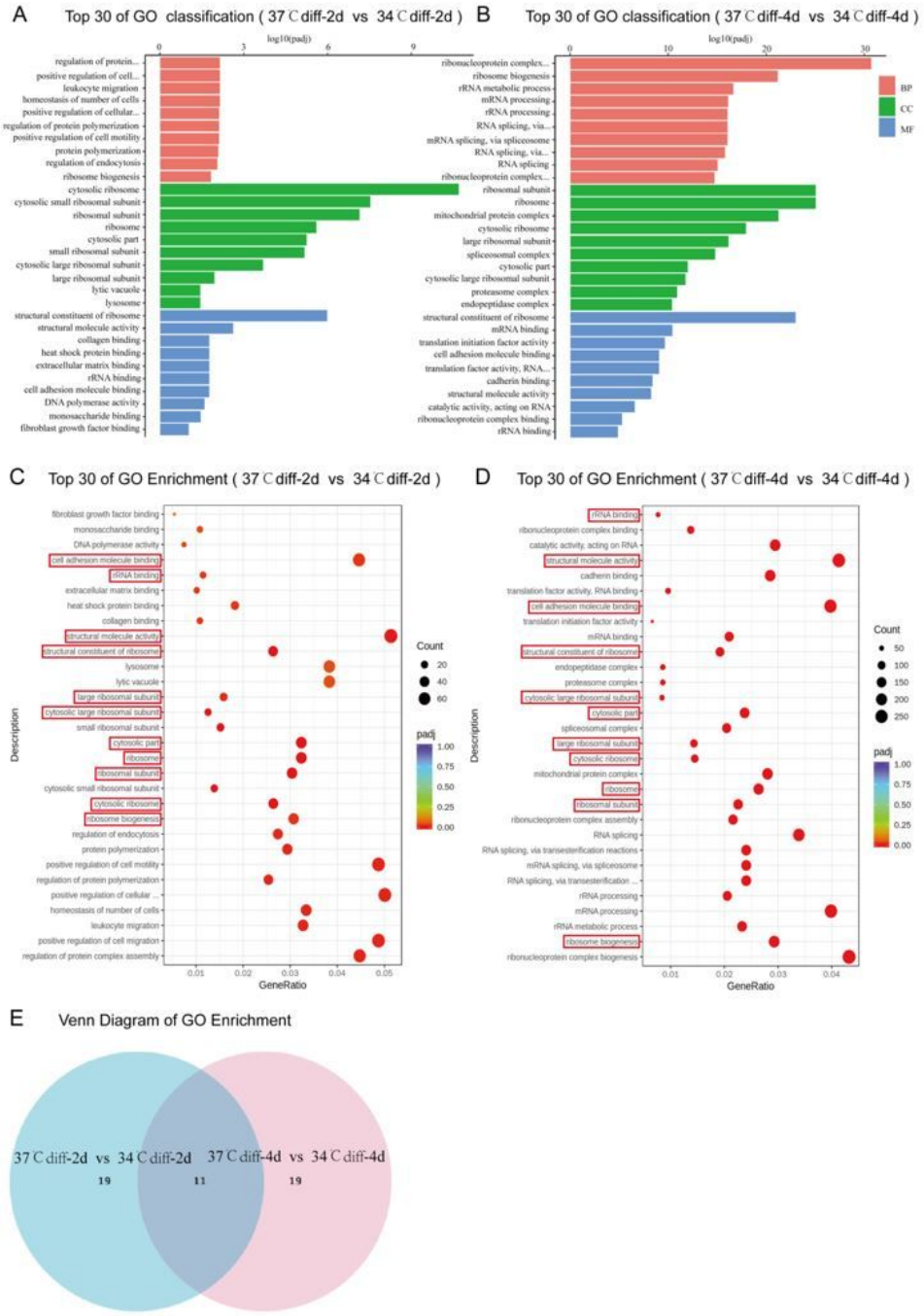

Figure 4

Gene Ontology analysis of the differentially expressed genes. **A**. GO classification in 37℃ and 34℃ differentiation cultured groups on the second day. **B**. GO classification in 37℃ and 34℃ differentiation cultured groups on the fourth day. **C**. The top 30 enrichment GO terms in 37℃ and 34℃ differentiation cultured groups on the second day. **D**. The top 30 enrichment GO terms in 37℃ and 34℃ differentiation cultured groups on the fourth day. The red boxes represent the common GO terms enriched on the second and fourth day of heat stress treatment at 37℃. **E**. Venn diagrams show that 11 of the top 30 enrichment GO terms were the same on day 2 and day 4 after heat stress treatment.

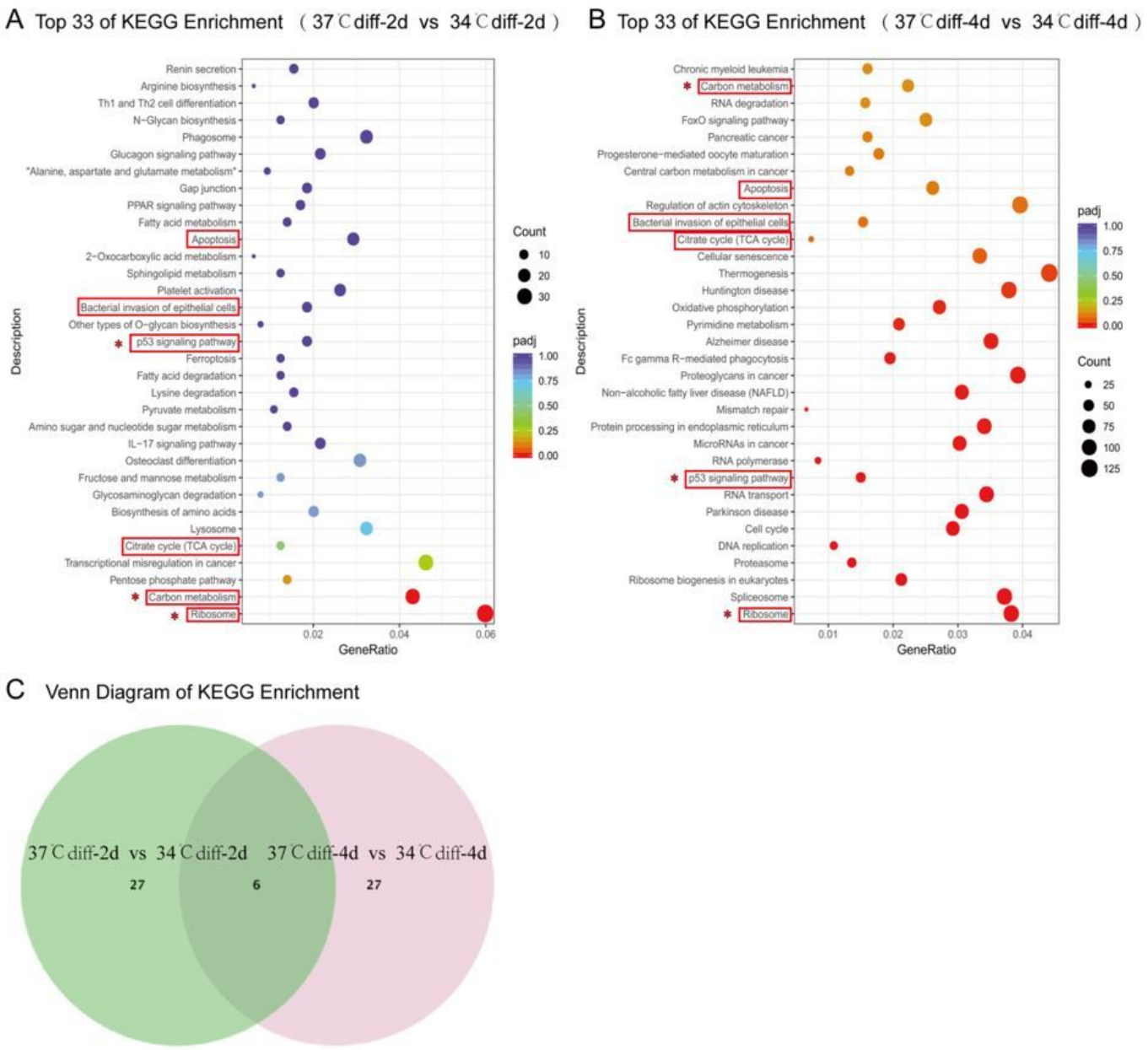

Figure 5

KEGG analysis of the differentially expressed genes. **A**. The top 33 KEGG enrichment pathways in 37°C and 34°C differentiation cultured groups on the second day. **B**. The top 33 KEGG enrichment pathways in 37°C and 34°C differentiation cultured groups on the fourth day. The red boxes represent the common KEGG pathways enriched on the second and fourth day of heat stress treatment at 37°C. E. Venn diagrams show that 6 of the top 33 KEGG enrichment pathways were the same on day 2 and day 4 after heat stress treatment.