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Siran Tian April 9, 2019

Effects of Atrazine on Rhesus Monkey & Human Pluripotent Stem Cells and Differentiating Male Germ Cells *in Vitro*

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An abstract of
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Abstract

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Each year, there are approximately five million people exposed to herbicide atrazine (ATZ) via contaminated drinking water. Unfortunately, current research mostly focused on high-dose and relatively short-term exposure of ATZ to examine acute effects on animals and cells. Therefore, these studies might not fully recapitulate the effects of chronic ATZ exposure on human health. Additionally, there was only a limited number of studies examining the impact of ATZ on early development and the association between individuals with pre-existing genetic conditions and their susceptibility to the environmental toxicants. We proposed to examine the developmental toxicity of ATZ by exposing rhesus monkey embryonic stem cells (rESCs) and human induced pluripotent stem cells (hiPSCs) with Huntington's disease (HD) or without HD (wild-type; WT) to various ATZ concentrations (0.3 and/or 30 μM) and exposure times (15 and/or 30 days). In addition to assessing the impact of ATZ on cell properties including pluripotency, apoptosis, and cell cycle, rESCs were also differentiated into spermatogonial stem cells (rSSCs) to examine the effects of ATZ on male germ cell development *in vitro*. Our findings suggested that 30 μM ATZ impacted cell cycle progression of WT-hiPSCs evidenced by the downregulation of cell cycle promoter genes *CCNB1* and *CDK1,* despite no significant impact on pluripotency or apoptosis in rESCs and hiPSCs. Additionally, we found dysregulation of apoptotic markers before and after differentiation under ATZ exposure with no major effect on spermatogenesis *in vitro.* Lastly, we noticed that WT-hiPSCs were susceptible to 30 days treatment of higher dose of ATZ (30 μM), whereas HD-hiPSCs with larger trinucleotide expansion were susceptible to lower dose of ATZ (0.3 μM) in 15 days. Our study shed new light on the importance of toxicant concentrations, exposure times, the effector cell types, and individuals who had pre-existing health conditions in response to environmental toxicants. We laid the groundwork of developing a stem cell model to investigate the impact of environmental toxicant exposure on human health for the future studies on ATZ.

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Abstract

Each year, there are approximately five million people exposed to herbicide atrazine (ATZ) via contaminated drinking water. Unfortunately, current research mostly focused on high-dose and relatively short-term exposure of ATZ to examine acute effects on animals and cells. Therefore, these studies might not fully recapitulate the effects of chronic ATZ exposure on human health. Additionally, there was only a limited number of studies examining the impact of ATZ on early development and the association between individuals with pre-existing genetic conditions and their susceptibility to the environmental toxicants. We proposed to examine the developmental impact of ATZ by exposing rhesus monkey embryonic stem cells (rESCs) and human induced pluripotent stem cells (hiPSCs) with Huntington's disease (HD) or without HD (wild-type; WT) to various ATZ concentrations (0.3 and/or 30 μM) and exposure times (15 and/or 30 days). In addition to assessing the impact of ATZ on cell properties including pluripotency, apoptosis, and cell cycle, rESCs were also differentiated into spermatogonial stem cells (rSSCs) to examine the effects of ATZ on male germ cell development *in vitro*. Our findings suggested that 30 μM ATZ impacted cell cycle progression of WT-hiPSCs evidenced by the downregulation of cell cycle promoter genes *CCNB1* and *CDK1,* despite no significant impact on pluripotency or apoptosis in rESCs and hiPSCs. Additionally, we found dysregulation of apoptotic markers before and after differentiation with no major effect on spermatogenesis *in vitro.* Lastly, we noticed that WT-hiPSCs were susceptible to 30 days treatment of higher dose of ATZ (30 μM), whereas HD-hiPSCs with larger trinucleotide expansion were susceptible to lower dose of ATZ (0.3 μM) in 15 days. Our study shed new light on the importance of toxicant concentrations, exposure times, the effector cell types, and individuals who had pre-existing

health conditions in response to environmental toxicants. We laid the groundwork of developing a stem cell model to investigate the impact of environmental toxicant exposure on human health for the future studies on ATZ.

Background

Prevalence of Atrazine in U.S.

Atrazine (ATZ) is an herbicide commonly used in U.S. agriculture to control broadleaf and weeds with the annual usage of 70 million pounds in recent years [1]. Majority of ATZ is applied in the "Corn Belt" region of the mid-western U.S. for soil pre-planting or foliage post-emergence; therefore, farmers and herbicide applicators are expected to have much higher ATZ exposure than the general population [2]. ATZ is resistant to hydrolysis and photolysis, and its half-life in the water is 578 days on average [3]. For the general population, the primary route of exposure is via the consumption of the contaminated drinking water [4]. In 1974, U.S. Environmental Protection Agency (E.P.A.) signed the Safe Water Drinking Act that set the Maximum Contaminant Level (M.C.L.) of ATZ at 3 μg/L (~0.01 μM) [1]. In 2017, ATZ remained detectable in over 200 community water systems, especially in Missouri where six systems had concentrations over the M.C.L. [5]. Today, at least five million people in the U.S. are exposed to ATZ by drinking contaminated water [5].

How ATZ Became a Human Health Concern

ATZ is classified as an endocrine disrupting chemical (E.D.C.), which affects the neuroendocrine system, interferes hormonal regulation, and impacts human and wildlife reproduction [6-11]. Chronic ATZ exposure resulted in prolonged estrogenic-like signaling and had been associated with the development of cancer [12] and reproductive dysfunction [7, 13]. Specifically, ATZ led to male infertility, caused feminization, and reduced sperm viability in *Drosophila*, *Xenopus* and *Danio* [14-16]. In addition, ATZ targeted hypothalamus-pituitary axis

and resulted in increased serum estrogen and estradiol, but inhibited the release of gonadotropin releasing hormone, luteinizing hormone, or testosterone in both male and female rodent models [7, 17, 18]. Despite the challenges in examining the direct effect of ATZ on humans, an epidemiological study showed that pregnant women who lived in the area with high ATZ exposure may have higher risk to give preterm birth than those who lived in the area with lower ATZ exposure [19].

Cellular Response to ATZ

Currently, the underlying mechanism on how ATZ exerts its effect on the cells remains largely unknown. However, multiple studies indicated that ATZ might behave like xenoestrogen or as a genotoxicant to cause DNA damage [11, 20-22]. For example, by using human cancer cell lines, Albanito et al. found that ATZ specifically interacted with seven-transmembrane estrogen receptor GPR30 (GPER) but not Estrogen Receptor α or β to activate extracellular-signal-regulated kinase 1/2 (ERK 1/2) pathway, and induced the expression of estrogen target genes [20]. Their findings were supported by prior studies that active ERK pathway promoted cell proliferation, inhibited apoptosis, and was a strong candidate for carcinogenesis [23-28]. In addition, by using a human epithelial cell line, Huang et al. demonstrated the activation of the ataxia telangiectasia and Rad3-related protein-checkpoint kinase 1 (ATR-Chk1) pathway after ATZ induced double-strand breaks (DSBs) [21]. However, no follow-up study examining how ATZ induced DNA damages or the downstream effects of ATR-Chk1 pathway was reported. Nevertheless, Huang et al.'s study supported the contention that ATZ dysregulated cell cycle, induced apoptosis, and impacted mitochondrial functions as reported by other studies [29-31]. Furthermore, ATZ induced

inheritable epigenetic changes through the alteration of DNA methylation and histone modification in rat germ cells, while the underlying mechanism remained still unclear [32, 33].

Association between Environmental Toxicants and Neurological Disorders

There is an increased interest in investigating the associations between environmental toxicants, such as pesticides and herbicides, and the development of neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD) [34-41]. Epidemiological studies demonstrated positive correlations between the occupational or residential exposure to pesticides and the increased risk of developing PD [37, 38, 42].

Despite the extensive effort in studying the impact of environmental toxicants in neurodegenerative disorders, the association between herbicide ATZ and HD was unclear. Unlike PD, HD is an autosomal monogenic neurodegenerative disease caused by the expansion of the polyglutamine (polyQ; CAGs) tract in the exon 1 of the huntingtin (*IT15; HTT*) gene [43]. Therefore, HD is an ideal disease model to study the impact of the interaction between genes and environment on individuals with inherited genetic conditions. In fact, the size of the polyQ tract is negatively correlated to the age of onset while positively related to the severity. Moreover, the variations in clinical measures and disease progression suggest an additional layer of complexity beyond the contributions of genetic factors to the pathogenesis and the development of HD [44-47]. Previous study found that pesticides and manganese could accelerate the aggregation of mutant HTT (mHTT) protein and altered striatal protein expression respectively [48, 49]. In addition, bisphenol A (BPA), another xenoestrogen similar to ATZ, was reported to impact hippocampal neurogenesis, increase the number of microglia, and induce mitochondrial

fragmentation within the central nervous system [34, 50, 51]. Therefore, it is important to examine whether environmental toxicant ATZ may influence HD pathogenesis and alter disease onset as well as brain development.

Objectives

Most of the prior studies had focused on organisms that were distant from humans, such as fruit flies, zebrafish or rats, to study the effects of ATZ on embryogenesis and reproduction [7, 9, 11, 16, 33, 52, 53]. A few studies used human cell lines that were often exposed to high concentrations of ATZ for relatively short times within 48 hours to investigate cellular responses to ATZ [21, 29, 54]. These exposure paradigms were more likely to mimic acute exposure condition rather than long-term, low-dose exposure that naturally occurred in general human populations.

Pluripotent stem cells (PSCs) including embryonic stem cells (ESCs) and induced pluripotency stem cells (iPSCs) are capable to differentiate into different cell lineages including germ cells [55-58]. Such differentiation flexibility can provide a unique opportunity to investigate the long-term developmental impacts of ATZ exposure. Recently, Midic et al. reported the effects of a relatively long-term (30 days) high-dose (30 μM) ATZ exposure in rhesus macaque ESCs (rESCs) using transcriptomic approach to evaluate the impact of ATZ on pluripotency, differentiation ability, cell-cycle etc. [8]. Although Midic et al.'s study suggested the importance of long-term effects of ATZ in PSCs, the team did not take advantage of the pluripotent capacity of PSCs to assess the impact of ATZ on differentiating cell lineages. Moreover, most of the human

populations were exposed to ATZ at a relatively low concentration by consuming contaminated drinking water [5]. Thus, a high concentration and a single end-point assessment described by Midic et al. were unlikely to recapitulate ATZ exposure conditions of the affected human populations. Ideally, a long-term continuous exposure for months or even years at low concentrations in non-human primate (NHP) or human PCSs followed by *in vitro* differentiation into different cell lineages and cell types could provide new insights on the extent of developmental impact. Furthermore, such longer exposure paradigm could be useful to determine whether different biological consequences in response to ATZ were triggered in different tissues and cells of our body.

Besides the limitations in model systems and exposure methods used in ATZ research, epidemiological studies on environmental toxicants and neurological disorders are also limited by the inaccuracy of self-reported data, the validity of the model, and specificity of the toxicants [35]. Therefore, experimental studies with controlled variables could complement and confirm epidemiological findings for better understanding of the underlying mechanisms of environmental toxicants on the development of neurodegenerative diseases as well as human health.

In order to study the effect of ATZ exposure on human development and to determine whether HD provoked different molecular responses to ATZ, we followed Midic et al.'s paradigm and chose 30 days exposure as our end-point with an additional mid-point at day 15 to examine the effect of exposure time. In this study, we used a wild-type (WT) rESC cell line (rWT), a transgenic rESC cell line (rTG), two WT human iPSCs (hWTs; hWT1 & hWT2), and two human hiPSCs with HD (hHDs; hHD1 & hHD2). While exposure duration of 30 days and 30 μM were aimed to replicate Midic et al.'s study, we also used a lower concentration of 0.3 μM to determine if different response in gene expression was triggered. To investigate the effects of ATZ on male

germ cell development, rESCs after 30 days 30 μM ATZ exposure continued to be differentiated into male germ cells with continuous ATZ exposure during the ten-day differentiation *in vitro*. We hypothesized that different exposure times and dosages of ATZ might affect rESCs, hiPSCs, and male germ cell development by disrupting cell cycle, inducing apoptosis, and affecting spermatogenesis. We also hypothesized that hHDs respond to ATZ differently from hWTs.

Research Questions

- Did ATZ impact pluripotency of rESCs and hiPSCs?
- Did ATZ induce apoptosis and disrupt cell cycle by altering the expression of apoptotic and cell cycle related genes?
- Would different exposure times and dosages of ATZ trigger different molecular responses?
- Did ATZ affect spermatogenesis?
- Would HD-hiPSCs and WT-hiPSCs respond differently to ATZ?

Materials and Methods

Experimental Design

We used a WT rESC cell line (rWT), a transgenic rESC with green fluorescent protein (GFP)-tagged histone 2B (H2B) (rTG), two WT hiPSCs (hWTs; hWT1 and hWT2), and two HD hiPSCs (hHDs; hHD1 and hHD2) in our studies. hHD1 and hHD2 had 44 and 180 CAG repeats respectively. rWT and rTG were collectively called rESCs.

We first examine the effects of 30 μM ATZ on the pluripotency of rESCs and their ability to *in vitro* differentiate into rhesus spermatogonial stem cells (rWT-SSCs and rTG-SSCs; collectively called rSSCs). rESCs were treated 30 μM ATZ for 30 days or without ATZ treatment (Figure 1A). At day 15 and day 30 of ATZ treatments, rESC samples were collected for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Immunocytochemistry (ICC) was performed on day 30 to determine the expression of pluripotency markers. After 30 days of ATZ treatment, rESCs were continued for *in vitro* differentiation into rSSCs based on a 10-day SSC differentiation protocol with continued exposure of ATZ [59]. At day 10 of SSC differentiation, rSSC samples were collected for qRT-PCR. ICC was also performed on day 5 of SSC differentiation for SSC markers (Figure 1A).

In addition to determine if we could replicate similar responses as described by Midic et al. [8], we also investigated the effect of ATZ concentrations (0.3 μM and 30 μM ATZ) on hWTs exposed for 15 and 30 days, and hHDs exposed for 15 days. Similarly, cells were fixed for ICC and cell samples were collected for qRT-PCR on day 15 and/or day 30 of treatment to determine the impact on stem cell properties (Figure 1B).

rESC & hiPSC Culture

rESCs were cultured on mouse embryonic fibroblast feeders (MEFs) in 35-mm culture dishes with rESC medium (80% knockout DMEM (Gibco), 20% KSR (Gibco), 1X NEAA (non-essential amino acids, Invitrogen), 1X L-glutamine (Invitrogen), 1X P/S (Penicillin/Streptomycin, Invitrogen), and 4 ng/mL hbFGF (human basic fibroblast growth factor, BD Biosciences)) [42, 60]. Fresh media was replaced daily, and the cells were passaged mechanically at approximately every 4-7 days as described previously [36, 60]. All hiPSC lines (hWTs & hHDs) were cultured on the Matrigel (Corning) coated dish with mTeSR™ (STEMCELL Technologies) based on the G103-0179 Guidelines for Handling RUCDR iPSC Lines. All cells were cultured at 37°C with 5% CO_2.

Preparation of Atrazine

ATZ (250 mg) from PESTENAL® (Sigma: 45330) was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution of 60 mM, protected from light and stored at room temperature. The stock solution was used to prepare culture media with final ATZ concentrations of 0.3 μM or 30 μM prior to the replacement of fresh media.

***In Vitro* Differentiation of rESCs into rSSCs**

A ten-day human SSC differentiation protocol as described by Easley et al. was used to differentiate rESCs into rSSCs [59]. Briefly, rESC colonies were seeded onto mitomycin C-inactivated mouse STO feeders and cultured for 48 hours in rESC medium prior to the induction

of SSC differentiation. After 48 hours, the rESC medium was replaced by SSC medium that was composed of MEM alpha (Invitrogen), 0.2% Bovine Serum Albumin, 5 µg/ml insulin, 10 µg/ml transferrin, 60 µM putrescine, 2 mM L-glutamine (Invitrogen), 50 µM β-mercaptoethanol, 1 ng/ml hbFGF (human basic fibroblast growth factor, BD Biosciences), 20 ng/ml GDNF (glial-derived neurotrophic factor, R&D Systems), 30 nM sodium selenite, 2.36 µM palmitic acid, 0.21 µM palmitoleic acid, 0.88 µM stearic acid, 1.02 µM oleic acid, 2.71 µM linoleic acid, 0.43 µM linolenic acid, 10 mM HEPES, and 0.5X penicillin/streptomycin (Invitrogen). SSC medium was replaced every two days within 10-day period.

Immunocytochemistry (ICC)

On day 30 of the ATZ exposure, all rESCs (0 & 30 µM ATZ) were fixed and stained for OCT4. At day 15 and day 30 after ATZ treatment, all hiPSCs (0, 0.3 & 30 µM ATZ) were fixed and stained for OCT4 and SOX2. On day 5 of SSC differentiation, all rSSCs were fixed and stained for PLZF. All rESCs and hiPSCs were fixed in 4% paraformaldehyde (PFA). Primary antibodies and concentrations used in this study were OCT4 (1:400) (Santa Cruz Biotechnology), SOX2 (1:250) (Santa Cruz Biotechnology), and PLZF (1:200) (R&D Systems). OCT4 and SOX2 are pluripotency markers [61], and PLZF is a SSC marker [62, 63]. Fixed cells were incubated with primary antibodies overnight at 4°C. Secondary antibodies Alexa Fluor® 488 (green) (1:1000) (Molecular Probes) or Alexa Fluor® 594 (red) (1:1000) (Molecular Probes) were incubated for 45 minutes at room temperature. DNA was stained with Hoechst 33342 (1:1000) (Molecular Probes). Olympus BX51 Fluorescence microscope was used to examine the staining, and the images were captured by using CellSens software.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA was extracted by using Trizol (Invitrogen). cDNA was synthesized with 500 ng of total RNA per reaction with TURBO DNA-free™ Kit (Invitrogen). qRT-PCR was performed *using* iQ™ SYBR® Green Supermix (Bio-Rad) on CFX96™ Real-Time System (Bio-Rad). The gene expression was calculated using the $2^{-\Delta CT}$ method normalized with GAPDH or $2^{-\Delta\Delta CT}$ normalized to control as previously described [64]. Primers are listed in Table S1.

Statistical Analysis

qRT-PCR using 2 samples were analyzed by non-parametric t-test, whereas those using 3 samples were analyzed by Kruskal-Wallis followed by Dunn's multiple comparison tests. The results were calculated by GraphPad InStat. For all data, asterisks represent statistical significance, * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Highlights

- Effects of ATZ on pluripotency were inconclusive based on *NANOG* and *OCT4* expression patterns so it merits further investigation.
- ATZ exhibited inhibitory effect on *SOX2* expression in monkey and human PSCs.
- 30 days 30 μM ATZ altered cell cycle related gene expressions, but no significant effect of inducing apoptosis in rESCs and hWTs was observed.
- ATZ exposure during SSC differentiation resulted in dysregulation of apoptotic gene expressions.
- hWTs did not respond to 15 and 30 days 30 μM ATZ differently based on gene expressions related to pluripotency, cell cycle and apoptosis**.**
- hHD2 with larger polyQ tract was more susceptible to lower concentration and shorter exposure time of ATZ than hHD1 with shorter polyQ tract and hWTs.

Results

Effects of ATZ on Pluripotency of rESCs and hiPSCs

Previous studies had shown that exposure to EDCs can lead to dysregulation in pluripotency markers, including *OCT4* and *SOX2*, that impact differentiation capacity [36, 58, 65]. To examine whether ATZ has similar effects on pluripotency, we exposed rESCs and hiPSCs to ATZ as illustrated in Figure 1.

We first determined if ATZ affected the pluripotent gene expression in rESCs or hiPSCs. In 30 days exposure to ATZ , both rESC lines expressed OCT4 (Figures 2B and 2C) and hiPSCs expressed OCT4 and SOX2 (Figures 4A, 4B, 5A and 5B) based on ICC. Thus, ATZ exposure did not affect the expression of OCT4 or SOX2 after 30 days treatments in rESCs (Figure 2) and hWTs (Figure 4), or 15 days treatments in hHDs (Figure 5).

To quantify the levels of expression of pluripotent stem cell markers *NANOG, OCT4* and *SOX2*, we ran qRT-PCR on all cell lines. Overall, all six cell lines did not have significantly consistent changes in *NANOG, OCT4* and *SOX2* expressions with ATZ treatments (Figures 3, 6 & 7). However, rTG showed a significant increase in *NANOG* after 15 days 30 μM ATZ treatment but returned to a level similar to the control with no ATZ treatment by day 30 (Figure 3B). Similar to rTG, at day 15 of 30 μM ATZ treatment, hWTs showed lower *OCT4* and *SOX2* expression than those without ATZ. However, hHD1 (44Q) showed significantly higher *OCT4* expression at day 15 of 30 μM ATZ, while hHD2 (180Q) showed significantly lower *SOX2* expression at day 15 of 0.3 μM ATZ treatment (Figure 7). While not significant, rESCs, hWTs and hHD2 tended to have lower *SOX2* expression in 30μM ATZ treatment (Figures 3, 6 & 7B). rESCs and hHD1 treated with 30μM ATZ had higher expression of *OCT4* (Figures 3 & 7A).

In summary, although there were some significant changes in the expression of *NANOG, OCT4* and *SOX2* (Figures 3B, 6 & 7), the overall levels of the pluripotent stem cell markers were not consistently affected by ATZ treatments across the six cell lines.

Effects of Dosages and Cell Types on Apoptosis and Cell Cycle in Response to ATZ

To evaluate if ATZ negatively impacted the cell cycle and induced apoptosis in PSCs as reported by Midic et al. [8], we examined the expression level of anti-apoptotic markers *ALKBH5* and *BCL2*, pro-apoptotic markers *CASP3* and *CASP9*, the cell cycle promoters *CAV1, CCNB1, CDK1* and *EGR1,* and the cell cycle inhibitor *FOXO4* [66-73]. We found that 15 and 30 days 30 μM ATZ did not alter the expression of the aforementioned markers in rWTs (Figure 8A). There was no significant change in *CASP3* expression in rWT even a higher *CASP3* expression was observed in 30 days treatment compared to 15 days treatment (Figure 8A). Similarly, rTG had a much lower *CASP3* expression for both 15 and 30 days treatments compared to the group without ATZ treatment even it was not significantly different ($p = 0.0619$) (Figure 8B). Therefore, neither rWT nor rTG under 30 μM ATZ treatment for 30 days strongly suggested ATZ induced apoptosis in rESCs.

We then investigated whether ATZ might induce apoptosis and dysregulate cell cycle in hWTs. Despite there were differences from individual cell lines in response to ATZ (Figure S1), we found that 30 μM ATZ resulted in reduced expressions of *ALKBH5, CASP3, CDK1, EGR1* and *FOXO4* compared to 0.3 μM ATZ treatment (Figure 9). Specifically, the cell cycle promoter genes *CCNB1, CDK1* and *EGR1* showed significant downregulation on both 15 days and 30 days 30 μM ATZ treatments (Figure 9). Unlike 30 μM, 0.3 μM ATZ showed less prominent effects on these genes, except for *CCNB1*, which was significantly reduced after 30 days treatment (Figure 9). Similar to rTG, hWTs showed a significantly lower expression of *CASP3* after 30 days of 30 μM ATZ treatment (Figure 9).

In order to examine if the responses to ATZ treatment were influenced by the duration of exposure, hWTs were treated with 0.3 and 30 μM ATZ for 15 and 30 days. Except for the significantly higher *ALKBH5* expression at 30 μM ATZ treatment for 30 days compared to that of 15 days, there was no significant difference in gene expressions between 15 and 30 days of 0.3 or 30 μM ATZ treatments in hWTs (Figure 10).

These results suggested that 15 and 30 days ATZ treatments did not significantly induce apoptosis in rESCs and hWTs as indicated by the expression of apoptosis-related markers (Figures 8 & 9). However, 30 μM ATZ treatment may affect cell cycle progression as indicated by the reduced expressions of *CCNB1, CDK1* and *EGR1*, while lower dose of ATZ (0.3 μM) may not exert the same inhibitory effect on cell cycle progression (Figure 9). Moreover, there was no difference in response between 15 days and 30 days exposure at the same concentration of ATZ in hWTs in general (Figure 10).

Effects of ATZ on Spermatogenesis *in Vitro*

Spermatogenesis is a complicated process that required a seamless regulation of cell type specific gene expression arrangement, as male germ cells progress toward functional gametes [63, 74]. Prior studies had shown that ATZ delayed meiosis that resulted in reduced sperm count and impacted the quality of sperm in rodents [18, 75, 76]. We aimed to determine if ATZ affected monkey spermatogenesis *in vitro*. We measured the expression levels of genes that have been widely used to study male germ cell development because of their distinct expression patterns during spermatogenesis. These markers were *NANOS3, PLZF, GFRα1, C-KIT, TOP2B, PIWIL1, VASA, TNP1,* and *TNP2* (Table S2) [59, 62, 63, 74].

We performed immunostaining on differentiating rSSCs on day 5 of the ten-day differentiation protocol using antibody specifically against PLZF to confirm $PLZF^+$ rSSCs. After 30-day 30 μM ATZ treatment during rESC culture and continuous exposure of the same treatment during *in vitro* spermatogenesis, rESCs with ATZ remained competent in differentiating into $PLZF^+$ rSSCs (Figure 11). Similarly, the expression levels of *PLZF* were not significantly different in *in vitro* derived rSSCs regardless of ATZ treatments, even *PLZF* of rTG-SSC exposed to ATZ had a higher expression level (Figure 12).

To examine whether ATZ induced apoptosis, we measured the levels of *ALKBH5, BCL2, CASP3* and *CASP9* expression before and after SSC differentiation. Most of the apoptotic markers *ALKBH5, BCL2, CASP3* and *CASP9* were downregulated in ATZ treated rWT-SSC and rTG-SSC (Figure 13). Specially, rTG-SSC had significant decreases in these markers when compared to rTG-ESC exposed to 30 days 30 μM ATZ prior to *in vitro* differentiation (Figure 13B). In contrary, the levels of apoptotic markers in rWT-SSC and rTG-SSC with no ATZ exposure were all upregulated in rSSCs compared to respective rESCs without ATZ exposure (Figure 13).

In summary, at day 10 of SSC differentiation we did not observe significant effect on spermatogenesis with the exposure of ATZ (Figure 12). However, ATZ had major effects on the expression of apoptotic genes in differentiating rSSCs. For instance, rSSCs with ATZ had reduced levels of apoptotic markers *ALKBH5, BCL2, CASP3* and *CASP9*, but rSSCs without ATZ had increased levels of the aforementioned genes (Figure 13).

Differential Reponses to ATZ between HD and WT hiPSCs

Because of the well-documented associations between environmental toxicants and the development of neurological disorders [36-38], we hypothesized that hHDs might respond to herbicide ATZ differently from hWTs. We focused on hHDs exposed to 0.3 and 3.0 μM ATZ for 15 days followed by quantitative measurement of the expression of apoptotic and cell cycle markers *ALKBH5, CASP3, CAV1, CCNB1, CDK1, EGR1,* and *FOXO4* as described previously for hWTs.

In general, hHDs responded differently to ATZ compared to hWTs in most of the apoptotic and cell cycle related genes (Figure 14). Specifically, the treatment of 0.3 μM ATZ resulted in significant changes in *ALKBH5, CAV1, CCNB1* and *EGR1* for hHD2 and significant increase in *EGR1* for hHD1 (Figure 14B), whereas hWTs showed either no effect or downregulation (Figure 14A) on these genes. Interestingly, distinct expression patterns were observed between 0.3 and 30 μM ATZ treatments in hWT2 and hHD2 (Figure 14). More significant decreases in cell cycle related gene expression were shown in hWTs exposed to 30 μM ATZ than those exposed to 0.3 μM ATZ (Figure 14A). However, unlike hWTs, hHD2 is more susceptible to 0.3 μM than 30 μM ATZ in 15 days exposure (Figure 14B).

To further determine the dose responses of ATZ, we grouped the data of hWTs, hHD1 and hHD2 by 0 μM (untreated), 0.3μM and 30 μM ATZ. 30 μM ATZ resulted in more significantly different expression levels of cell cycle related genes among hWTs, hHD1 and hHD2 (Figure 15C) when compared to untreated and 0.3 μM ATZ treatments (Figure 15A and B).

In conclusion, hHD2 with a higher number of CAG repeats (Q180) might be more susceptible to the lower dosage of ATZ than hHD1 which had a lower number of CAG repeats (Q44) within a short-term exposure (15 days). Most interestingly, hHDs responded inversely to ATZ when compared to hWTs (Figure 14: red vs. blue) that merits further investigation.

Discussion

This study aimed to investigate the effects of ATZ with relatively longer exposure time (30 days) and different concentrations on rESCs, hiPSCs and differentiating rhesus male germ cell *in vitro*. By applying two time-point assessments (15 days and 30 days) and two different concentrations of ATZ (0.3 μM and 30 μM), we did not observe consistent impacts of ATZ on stem cell pluripotency or found the evidence of inducing apoptosis based on the expression levels of pluripotent and apoptotic genes (Figures 3, 6, 7, 8, 9 & 10). However, a higher dosage of ATZ might affect cell cycle progression in hWTs (Figure 9). Most importantly, hHDs exhibited inverse responses to ATZ in terms of apoptosis and cell cycle related gene expression compared to hWTs (Figure 14). Moreover, hHDs with a larger number of CAG repeats (hHD2) were more susceptible to ATZ at lower dose and shorter exposure time (Figure 14B). This further suggested the crucial role of the gene-environment interaction that could impact human health via the synergistic effect between environmental exposure and individuals with pre-existing genetic conditions.

Although neither 0.3 μM nor 30 μM ATZ treatment can recapitulate the environmental human exposure of M.C.L. level at 0.01 μM according to the Safe Water Drinking Act [1], it is important to replicate Midic et al.'s paradigm and to compare our results with their study. Midic

et al. exposed one WT rESC cell line to 30 μM ATZ for four weeks and the RNA was processed for RNAseq analysis [8]. According to Midic et al.'s Ingenuity Pathway Analysis data, the canonical pathways which were significantly affected by ATZ were not directly correlated with pluripotency [8]. Similarly, we did not observe consistent negative impact of ATZ on the expressions of *OCT4* and *NANOG* in our monkey and human PSCs (Figures 3 & 6). However, although the data were not all significant, the expression of *SOX2* was lower in ATZ treated cells (Figures 3, 6 & 7). In addition, we also picked up *EGR1*, which significantly increased in Midic et al.'s study, *FOXO4, CAV1* and *ALKBH5*, which significantly decreased in their study, for qRT-PCR analysis in our hWTs [8]. In our experiments, we observed significant decrease in *EGR1*, no effect on *CAV1,* but similar significant decreases in *FOXO4* and *ALKBH5* in hWTs (Figure 9). Despite these slight discrepancies, like Midic et al., we did not observe strong evidence of apoptosis based on the expressions of *BCL2, CASP3* and *CASP9* in our rESCs and hWTs (Figures 8 & 9). However, although the cell cycle related genes *CCNB1* and *CDK1* did not significantly change in Midic et al.'s experiment, we observed significant decreases in these genes at 30 μM ATZ for both 15 and 30 days exposure in hWTs (Figure 9). Therefore, our results in terms of ATZ effects on pluripotency and apoptosis of PSCs generally agreed with Midic et al.'s study. However, whether ATZ can inhibit cell cycle progression may require more experimental analyses with an extended panel of cell cycle markers to confirm our current data.

We also added an additional mid-point assessment (15 days) and a lower ATZ concentration (0.3 μM) in this study. In addition, we used multiple cell lines including two rESC lines and four hiPSC lines to examine if ATZ concentrations (0.3 μM and 30 μM) provoked different cellular responses such as inducing apoptosis and disrupting pluripotency as well as cell cycle progression. In addition to the effects of doses, variations among cell lines and cell types

were also examined. In this study, we observed that rTG, a transgenic rESC line expressing histone 2B tagged with GFP, was more susceptible to ATZ with altered expression of *NANOG* and apoptosis-related genes when compared to rWT (Figures 3B & 8B). However, the discrepancy in the expression of pluripotency markers could be due to the quality of rESC culture. For example, the levels of *NANOG* and *OCT4* of rTG without ATZ were lower than rWT without ATZ (Figure 3B). Another reason could be that the expression of H2B-GFP transgene might interfere histone dynamics and affect gene expression, which cannot be excluded.

The benefits of using NHP and human PSCs are their biological relevance to human development and their differentiation capability that provides a unique opportunity to study the developmental impact of environmental toxicants such as ATZ and how individuals with pre-existing genetic conditions such as HD responded to chronic exposure of herbicides. Although our goal is to assess the impacts of dosages and exposure times of environmental toxicants on human development, our study is limited by the number of cell lines and heterogeneity among NHP and human PSCs. Prior study had shown that human ESCs or iPSCs had distinct subpopulations within each cell line which differed in pluripotent gene expression profiles and more importantly, responded differently to E.D.C. [65]. Therefore, the inconsistent differences in pluripotency after ATZ treatment in our models could be due to the inherent differences among our cell lines, resulting in inconsistent responses to ATZ. Pluripotency is a complicated network that interplayed by many genes besides *NANOG, OCT4* and *SOX2* [77]. Nonetheless, we only used three representative pluripotent markers *NANOG, OCT4* and *SOX2* to provide evidence of pluripotency. In future study, a full assessment by an expanded panel of markers or RNA-seq could better support the status of pluripotency before and after ATZ exposure. The well-assessed

genetic profiles of cell lines could also serve as a selection criterion for choosing cell lines with similar baselines before ATZ exposure for the future experiments.

There was a strong preference to use acute exposure conditions of high ATZ concentration and short exposure times on human cell lines or model systems that are distant to humans that elicit robust physiological responses to ATZ [7, 11, 16, 21, 29, 33, 54]. However, human exposure to ATZ is primarily via the consumption of contaminated water [2]. Thus, continuous long-term exposure with low ATZ concentration is more relevant in mimicking human environmental exposure, and would be helpful for scientists to better understand the underlying mechanisms of ATZ biological impact on human health. In the future experiments, we would like to scale down our ATZ concentrations to nM and pM besides the concentrations closer to the maximum contaminant level and also expose our monkey and human PSCs to ATZ beyond 30 days.

Although we hypothesized that ATZ would affect spermatogenesis based on previous reports [75, 76], significant impact on the expression of a selected panel of spermatogenesis markers was not observed (Figure 12). Several explanations might contribute to these findings and would direct our future studies. First, our continuous effort in improving our ten-day male germ cell differentiation protocol [59] would promote the production of later stage male germ cells beyond secondary spermatocytes and early round spermatids as we currently achieved. Due to the nature of progressive development of male germ cells, one approach is to develop a reliable method such as fluorescence-activated cell sorting (FACS) to isolate specific cell populations of interest and to determine whether ATZ might impact specific male germ cell populations. To further improve our SSC differentiation method, we may co-culture testicular supporting cells such as Leydig and Sertoli cells with our differentiating SSCs. A recent study

suggested that the main pathology of ATZ to testicular tissue first started from Leydig cell degeneration with a significant reduction in testosterone production, leading to Sertoli cell dysfunction and ultimately disrupting spermatogenesis [76]. Therefore, one of our ongoing efforts is to co-culture Leydig and Sertoli cells with SSCs during differentiation in order to improve male germ cell differentiation *in vitro* and to study the impacts of ATZ on spermatogenesis relying on testosterone signaling pathways. On the other hand, feeder free SSC differentiation from hiPSCs based on our ten-day differentiation protocol also showed great improvement in male germ cell production [74]. Thus, the other possibility is to adapt the feeder free system to improve SSC differentiation efficiency and study if ATZ may directly target male germ cells. Since our current *in vitro* SSC differentiation protocol did not rely on co-culture of Leydig or Sertoli cells with SSCs or feeder free system but STO feeders, different cascades of responses could be induced based on different differentiation protocols which needs further investigation.

We were excited to observe that hHD2 which had 180 ployQ was more susceptible to 0.3 μM ATZ compared to hWTs which responded to a much higher dosage of ATZ (30 μM). In future experiment, it will be interesting to use isogenic HD-iPSC models to study the effects of ATZ on HD pathogenesis. It also will be interesting to examine if different cell types such as neurons, astrocytes or male germ cells derived from the same PSCs respond differently to ATZ with conditions that mimic human environmental exposure with concentrations similar or lower than 0.01 μM with a prolonged exposure time more than 30 days.

Due to the limited amount of time, we are unable to extend exposure time beyond 30 days and perform in-depth examinations on the impacts of ATZ in areas including DNA damage,

mitochondrial dysfunction, epigenetic changes and chromatin architectures. However, all cell lines are carried on by other lab members and continue to be exposed to ATZ for future studies.

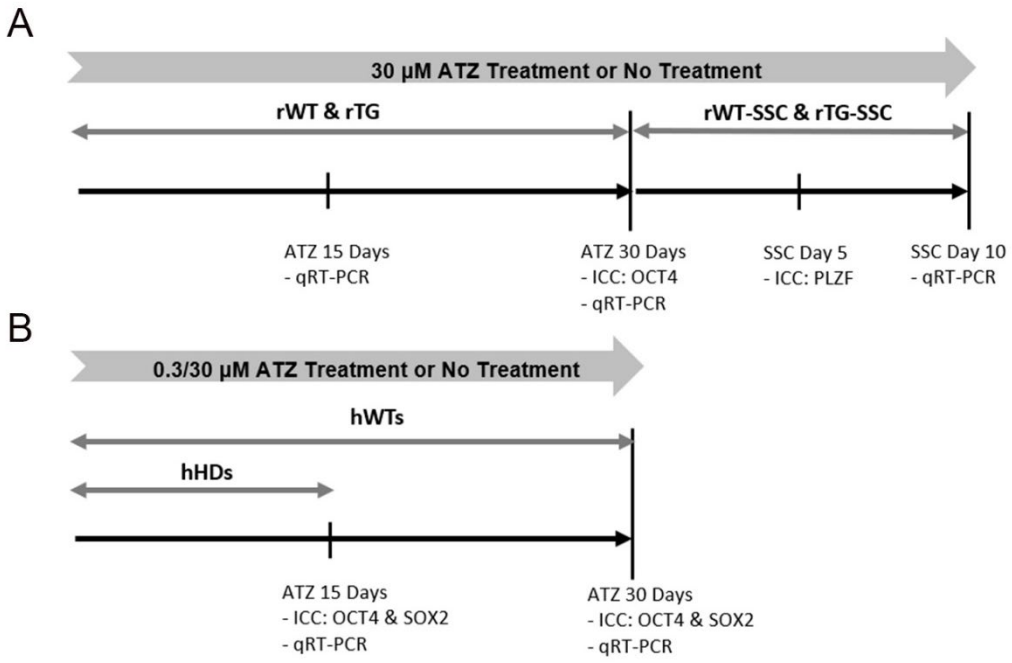

Figure 1. Schematics of Experimental Design.

(A) To determine the effects of 30 days 30 µM ATZ on rESCs (rWT and rTG) and their derivative spermatogonial stem cells rSSCs (rWT-SSC and rTG-SSC) *in vitro.* Samples of rESCs with ATZ for 15 and 30 days as well as those without ATZ (untreated) were collected. Similarity, samples of rSSCs on day 10 of male germ cell differentiation with and without ATZ were also collected. All these samples were run by qRT-PCR to assess the levels of expression of pluripotency and apoptosis genes. In addition, ICC was performed on rESCs at day 30 of ATZ treatment and rSSCs at day 5 of male germ cell differentiation using antibodies OCT4 and PLZF specifically for pluripotency and SSCs respectively.

(B) To determine the effects of doses and exposure times of ATZ on hWTs (hWT1 and hWT2) and hHDs (hHD1 and hHD2). Similar to (A), samples of hHDs were collected on day 15 of ATZ

treatments. Samples of hWTs were collected at both day 15 and day 30 ATZ treatments. qRT-PCR was run to quantify the levels of expression of pluripotency, apoptosis and cell cycle markers. ICC was performed to determine the expression of pluripotency at day 15 on hHDs and at both day 15 as well as 30 on hWTs.

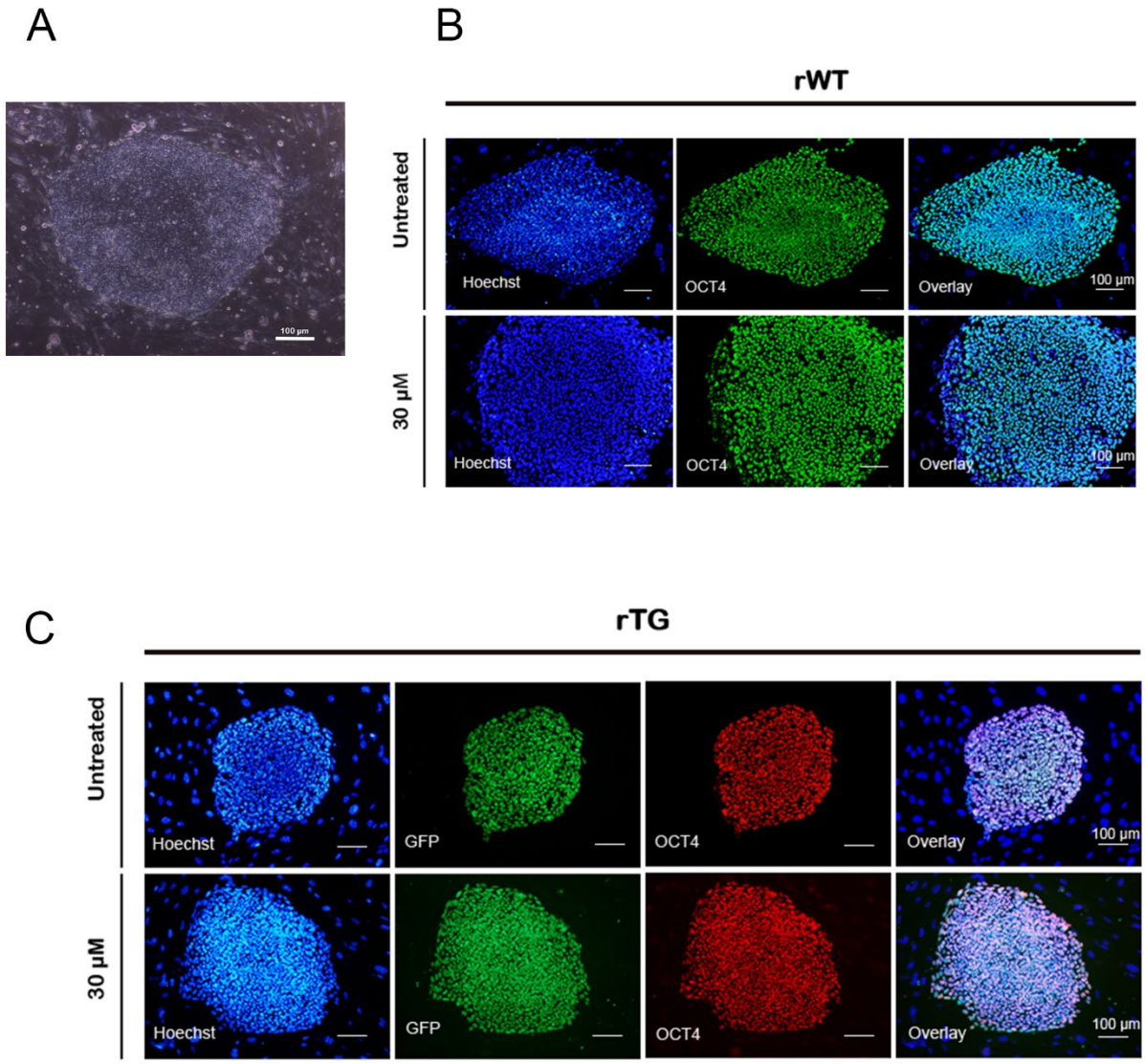

Figure 2. Immunocytochemistry of rESCs after 30 Days 30 μM ATZ Treatment.

(A) A rWT colony with small cell size and cobblestone morphology was cultured on the mouse embryonic fibroblast (MEF) feeder cells. (B) rWT expressed OCT4 (Green), and (C) rTG expressed OCT4 (Red) and GFP-H2B (Green) with or without ATZ treatment. DNA was stained by Hoechst 33342 (Blue).

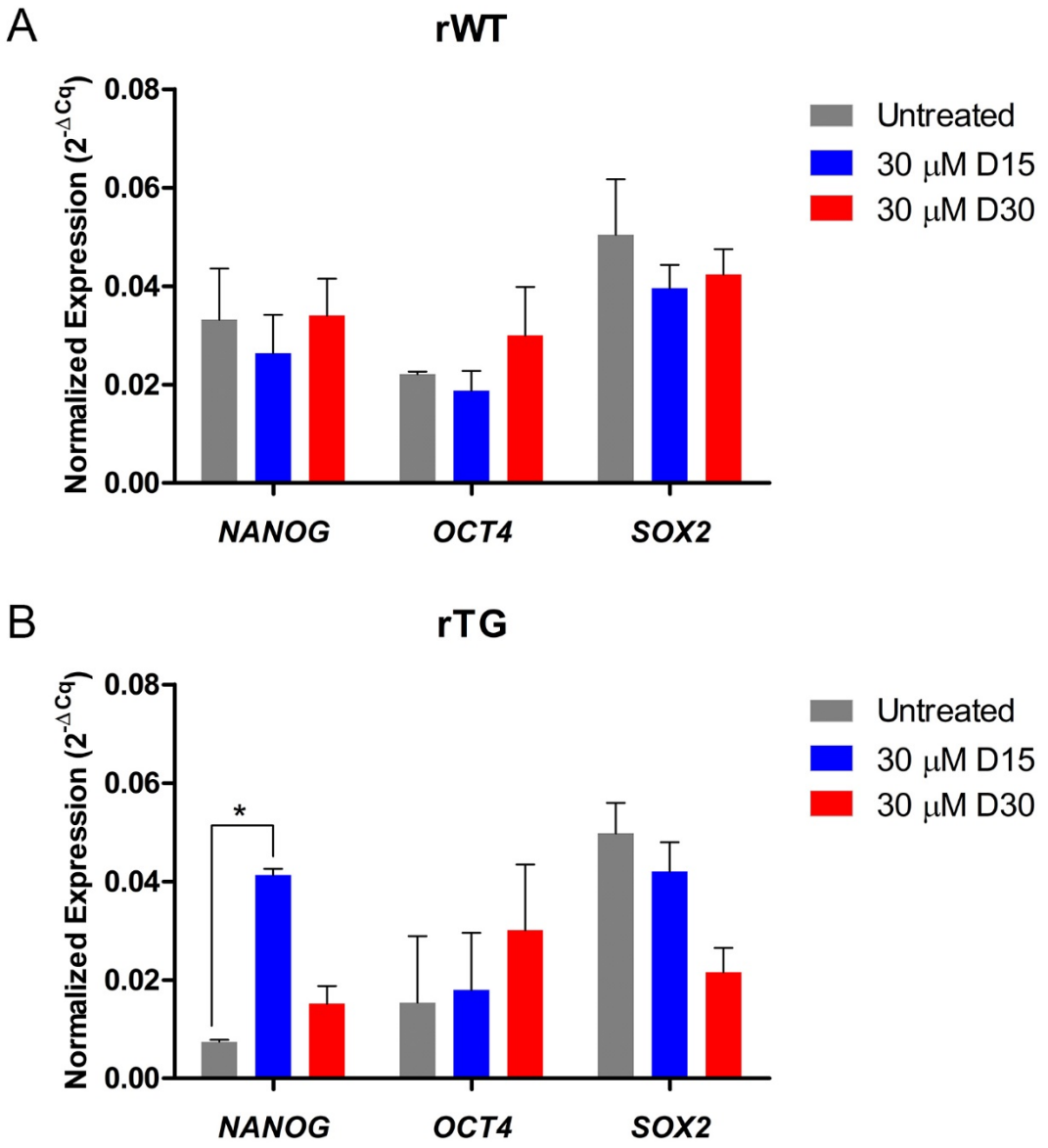

Figure 3. Expression of Pluripotent Stem Cell Markers after ATZ Treatment in rESCs. The expression of pluripotency stem cell markers *NANOG, OCT4* and *SOX2* in rWT (A) and rTG (B) after exposed to 30 μM ATZ for 15 and 30 days. qRT-PCR was performed and data were represented as mean +1 SEM with three technical replicates normalized to *GAPDH*. * $p < 0.05$.

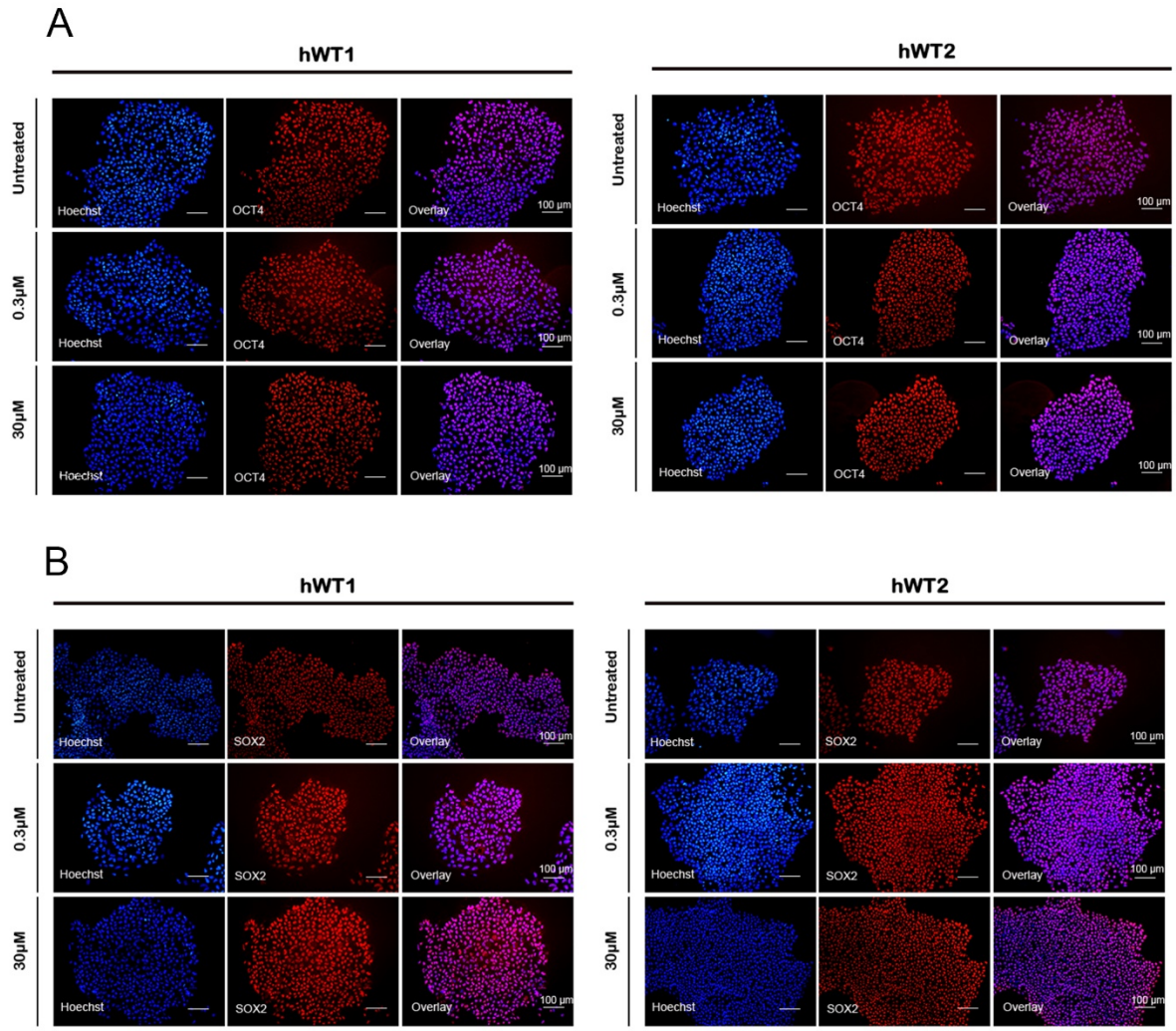

Figure 4. Immunocytochemistry of hWT1 and hWT2 after 30 Days 0.3 or 30 μM ATZ Treatments.

Expression of (A) OCT4 (Red) and (B) SOX2 (Red) in hWT1 (left) and hWT2 (right) after 0.3 and 30 μM ATZ treatments for 30 days. DNA was stained by Hoechst 33342 (Blue).

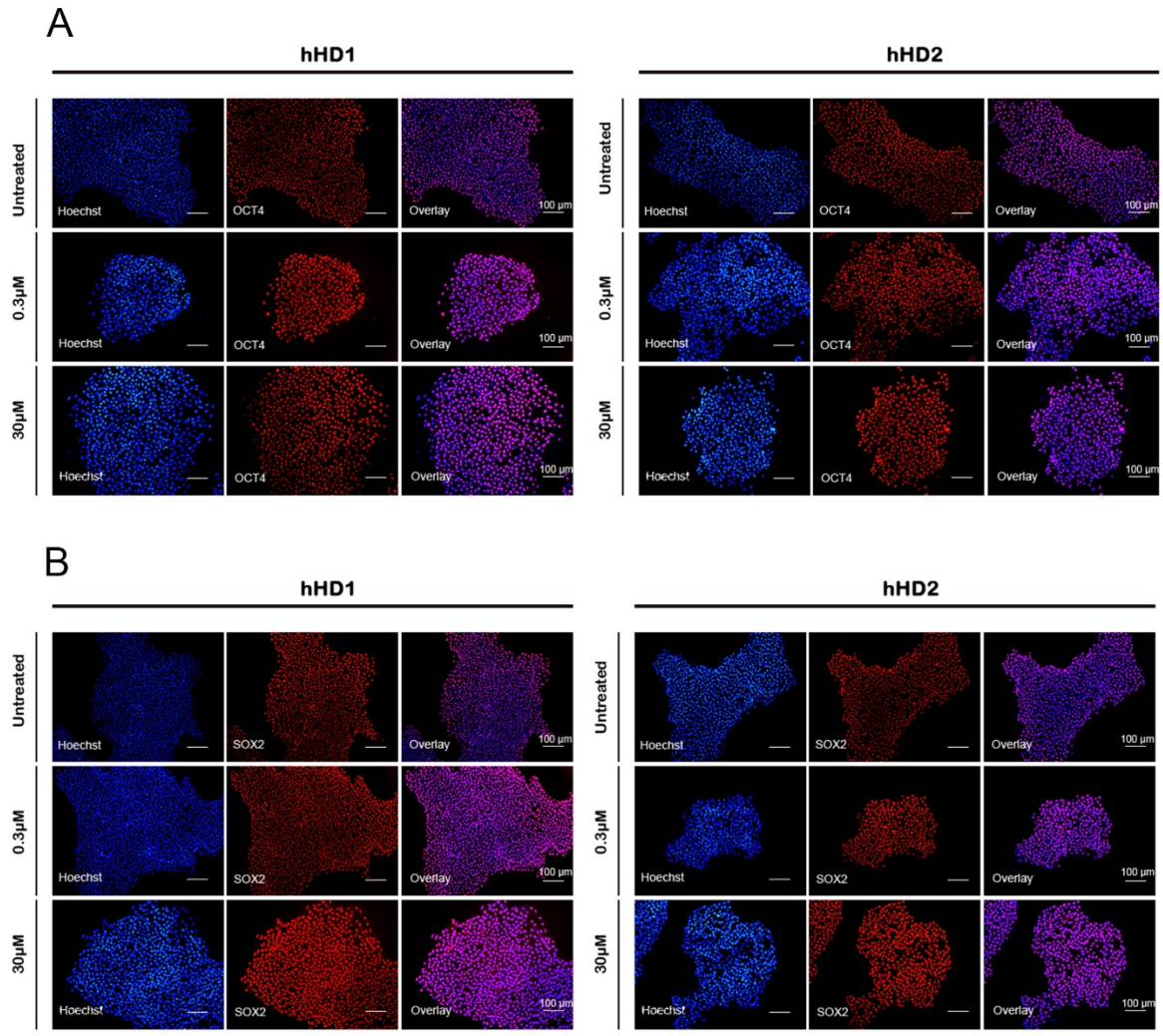

Figure 5. Immunocytochemistry of hHD1 and hHD2 after 15 Days 0.3 or 30 μM ATZ Treatments.

Expression of (A) OCT4 (Red) and (B) SOX2 (Red) in hHD1 (left) and hHDT2 (right) after the 0.3 and 30 μM ATZ treatments for 15 days. DNA was stained by Hoechst 33342 (Blue).

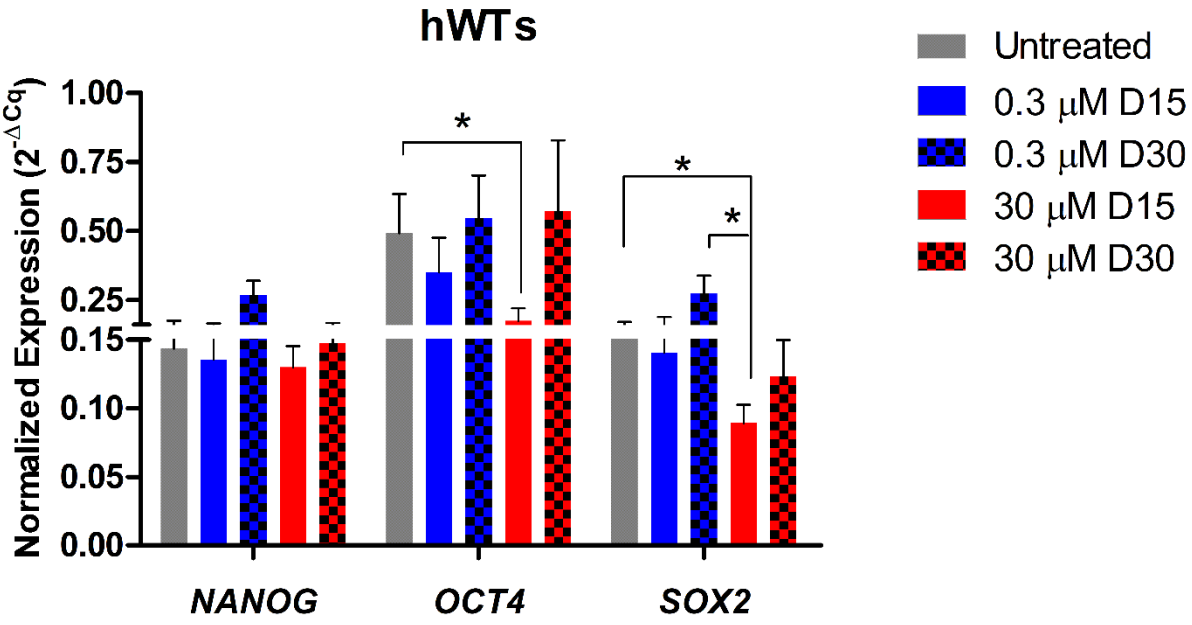

Figure 6. Expression of Pluripotent Stem Cell Markers after ATZ Treatments in hWTs. The levels of expression of pluripotent stem cell markers *NANOG, OCT4* and *SOX2* in hWTs (hWT1 and hWT2) after 0.3 or 30 μM ATZ treatments for 15 and 30 days. qRT-PCR was performed and the data were represented as mean +1 SEM by three technical replicates normalized to *GAPDH*. * $p < 0.05$.

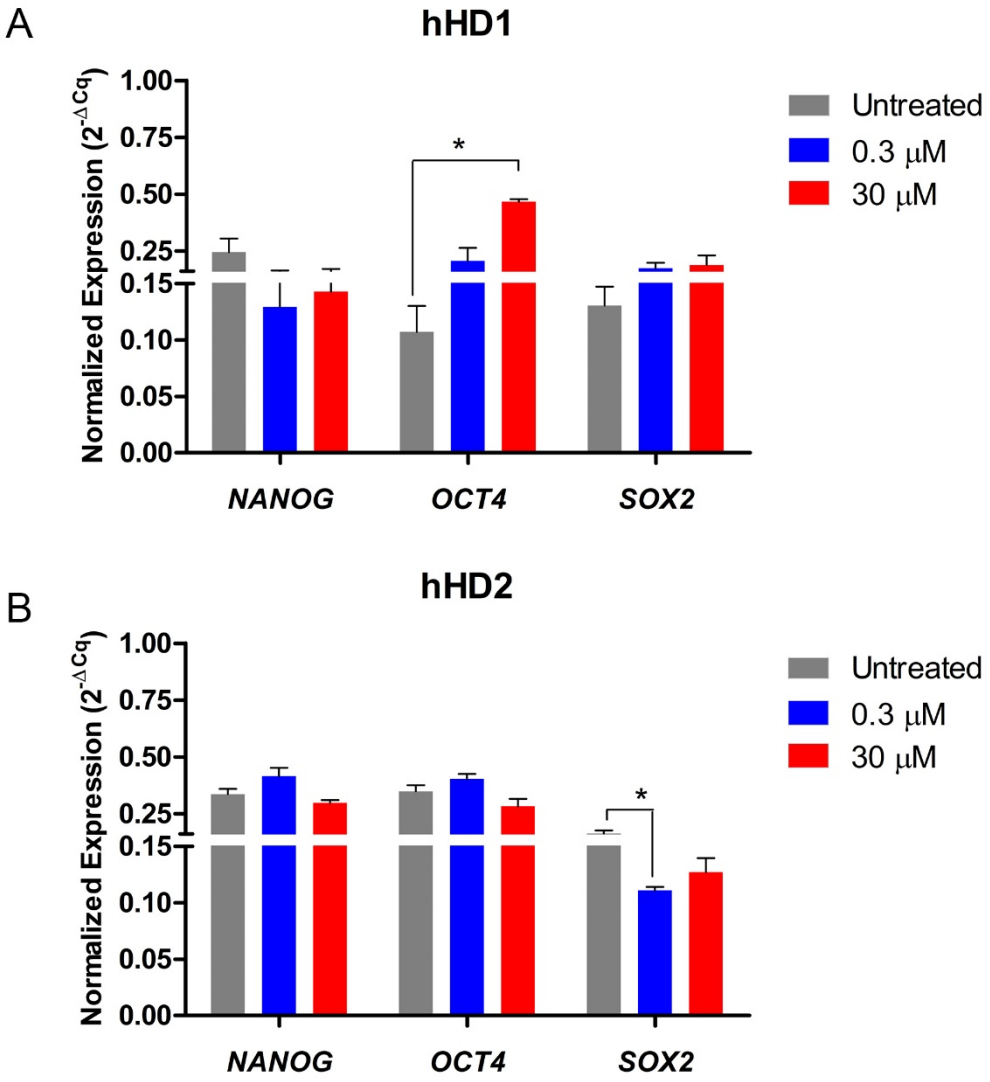

Figure 7. Expression of Pluripotent Stem Cell Markers after ATZ Treatments in hHDs. The expressions of pluripotency stem cell markers *NANOG, OCT4* and *SOX2* in hHD1 (A) and hHD2 (B) treated with 0.3 or 30 μM ATZ for 15 days or without ATZ treatment (untreated). qRT-PCR was performed and the data were represented as mean +1 SEM by three technical replicates normalized to *GAPDH*. * $p < 0.05$.

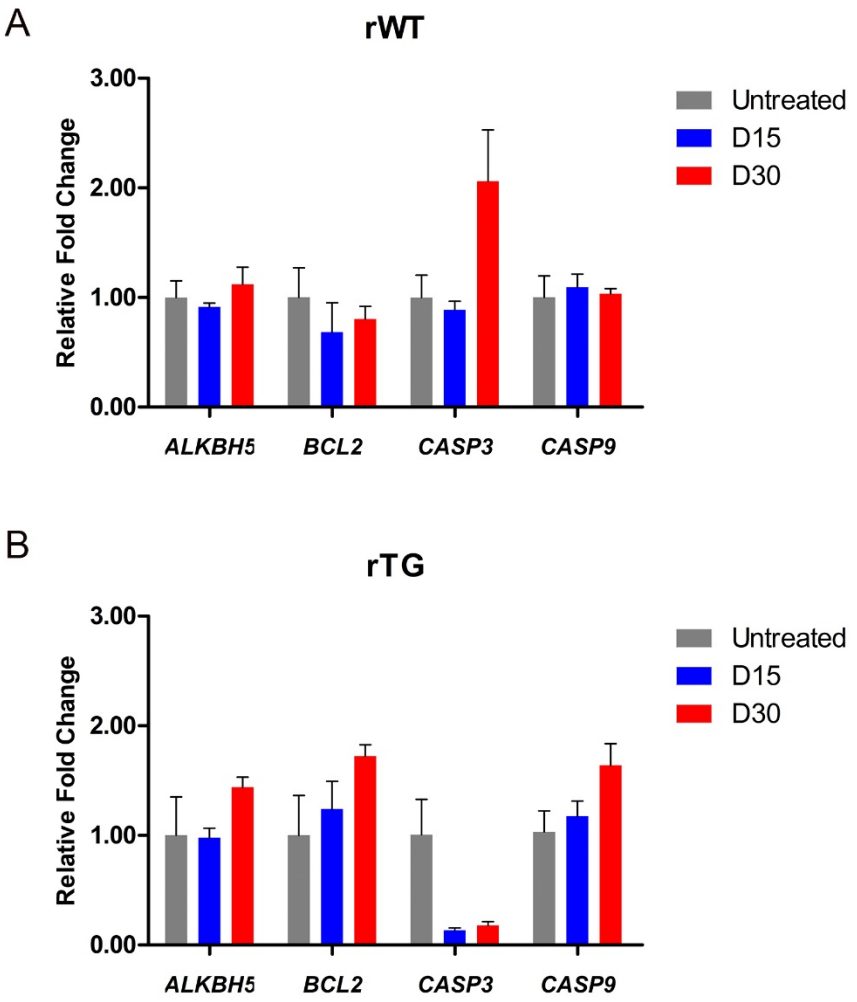

Figure 8. Expression of Apoptotic Markers after ATZ Exposure in rWT and rTG.

The expression levels of apoptotic markers *ALKBH5, BCL2, CASP3* and *CASP9* were quantitatively measured by qRT-PCR on rWT (A) and rTG (B) after 30 μM ATZ for 15 and 30 days. Data were represented as mean +1 SEM and normalized to the untreated groups.

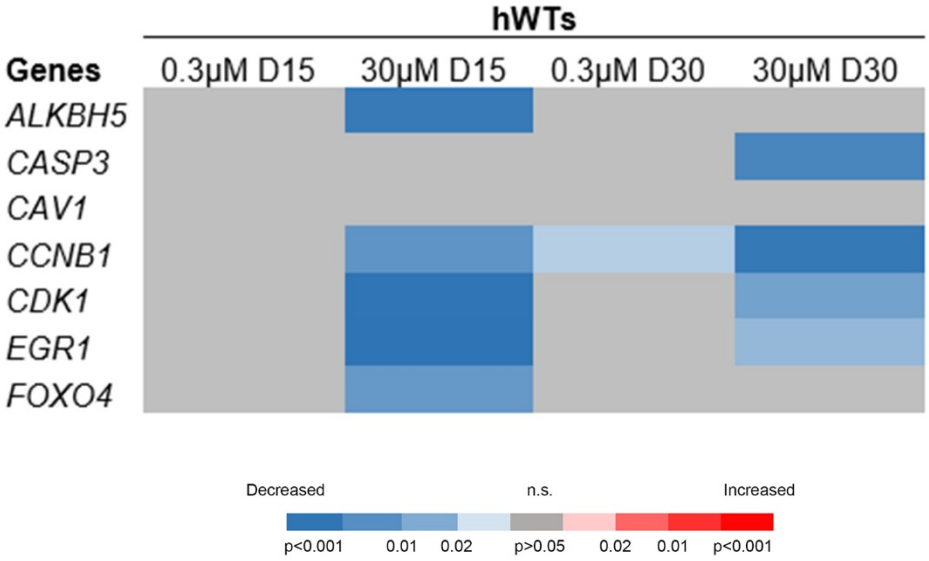

Figure 9. Differential Expression of Apoptotic and Cell Cycle Markers in Response to Different Concentrations of ATZ Exposed for 15 and 30 days in hWTs.

The expression levels of apoptotic markers *ALKBH5* and *CASP3* and cell cycle markers *CAV1, CCNB1, CDK1, EGR1* and *FOXO4* of hWTs with 0.3 and 30 μM ATZ for 15 and 30 days were quantified by qRT-PCR via three technical replicates. hWT1 and hWT2 were combined as hWTs. The levels of expression of the genes were normalized to the untreated group.

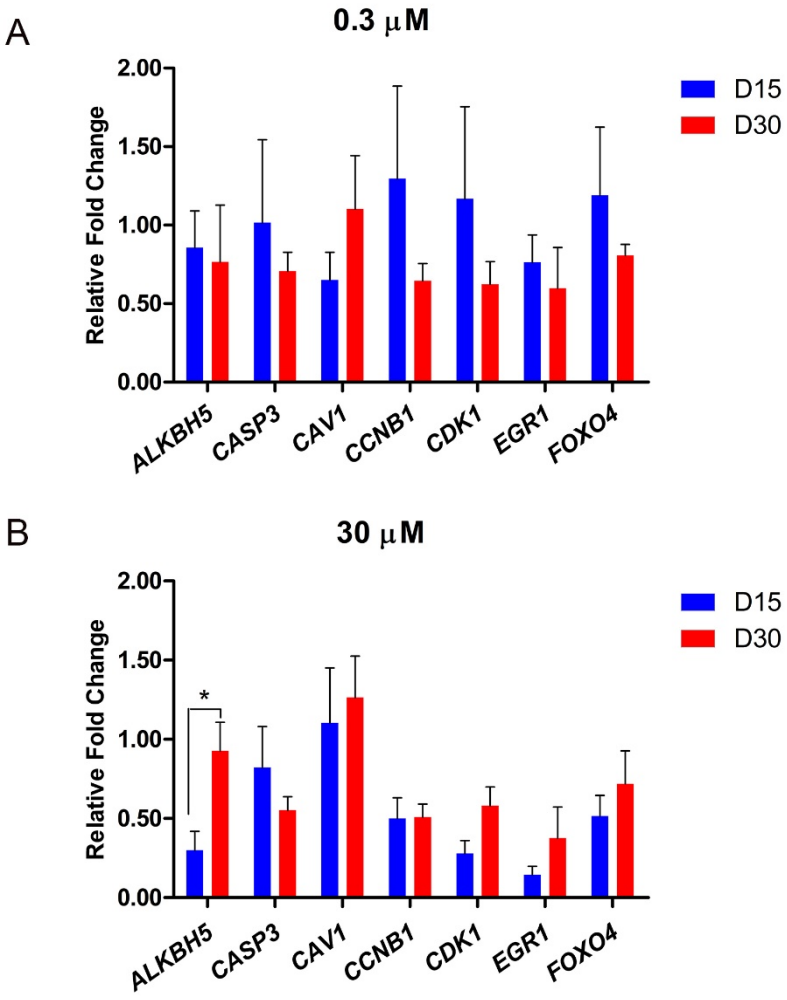

Figure 10. Expression Levels of Apoptotic and Cell Cycle Related Genes in Response to Different Exposure Times with 0.3 and 30 µM ATZ in hWTs.

The expression levels of apoptotic markers *ALKBH5* and CASP3, and cell cycle markers *CAV1, CCNB1, EGR1, CDK1, FOXO4* were quantitatively measured by qRT-PCR on hWTs in response to 0.3 µM (A) and 30 µM (B) ATZ treatments for 15 and 30 days. Data were represented as mean +1 SEM and normalized to the untreated groups. The expression level of each gene was compared between the treatments. * $p < 0.05$.

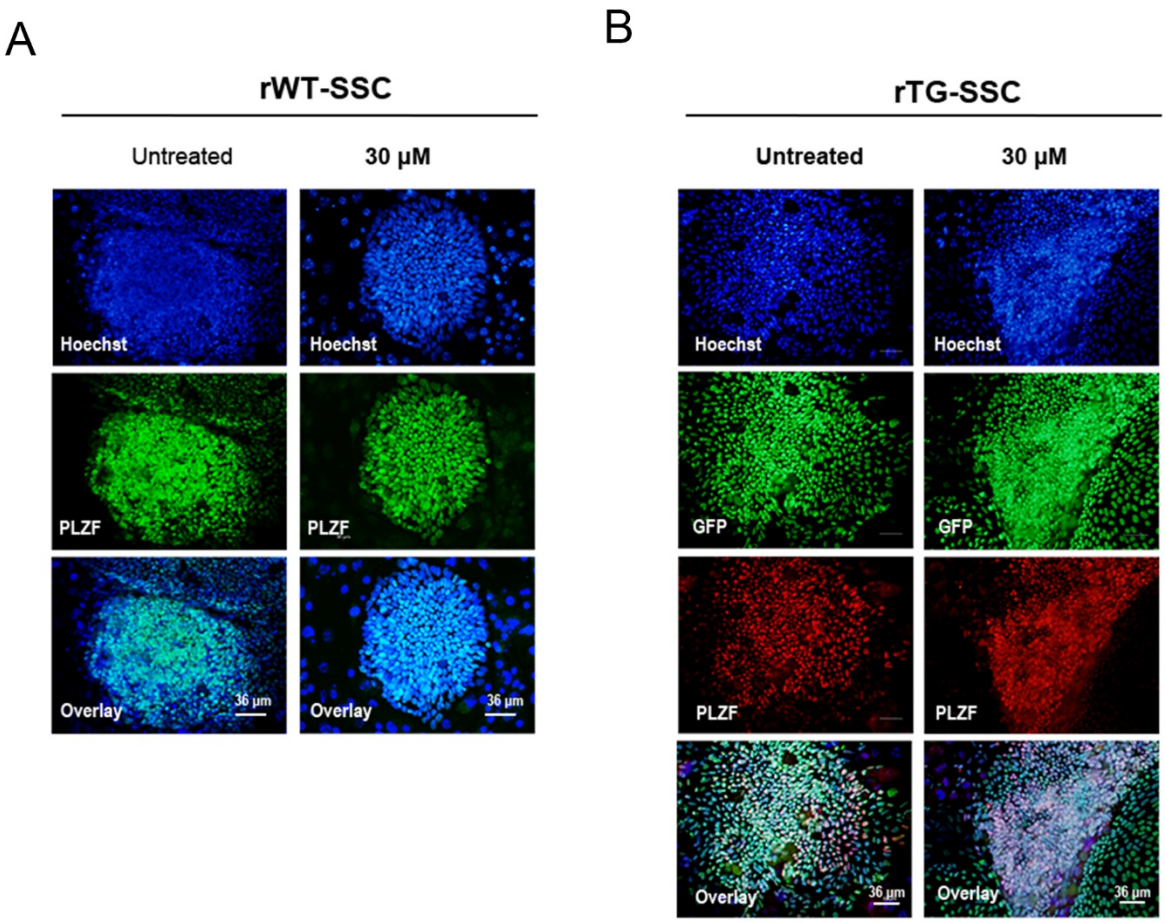

Figure 11. Immunocytochemistry of rWT-SSC and rTG-SSC after ATZ treatment.

rWT-SSC (A) and rTG-SSC (B) were derived from rWT and rTG respectively after 30 μM ATZ treatment for 30 days and continued to be exposed to ATZ during *in vitro* SSC differentiation. On day 5 of SSC differentiation, both rSSCs with and without ATZ treatment were stained against PLZF, an SSC marker. (A) rWT-SSC: PLZF (Green). (B) rTG-SSC: GFP tagged H2B (Green) and PLZF (Red). DNA was stained by Hoechst 33342 (Blue).

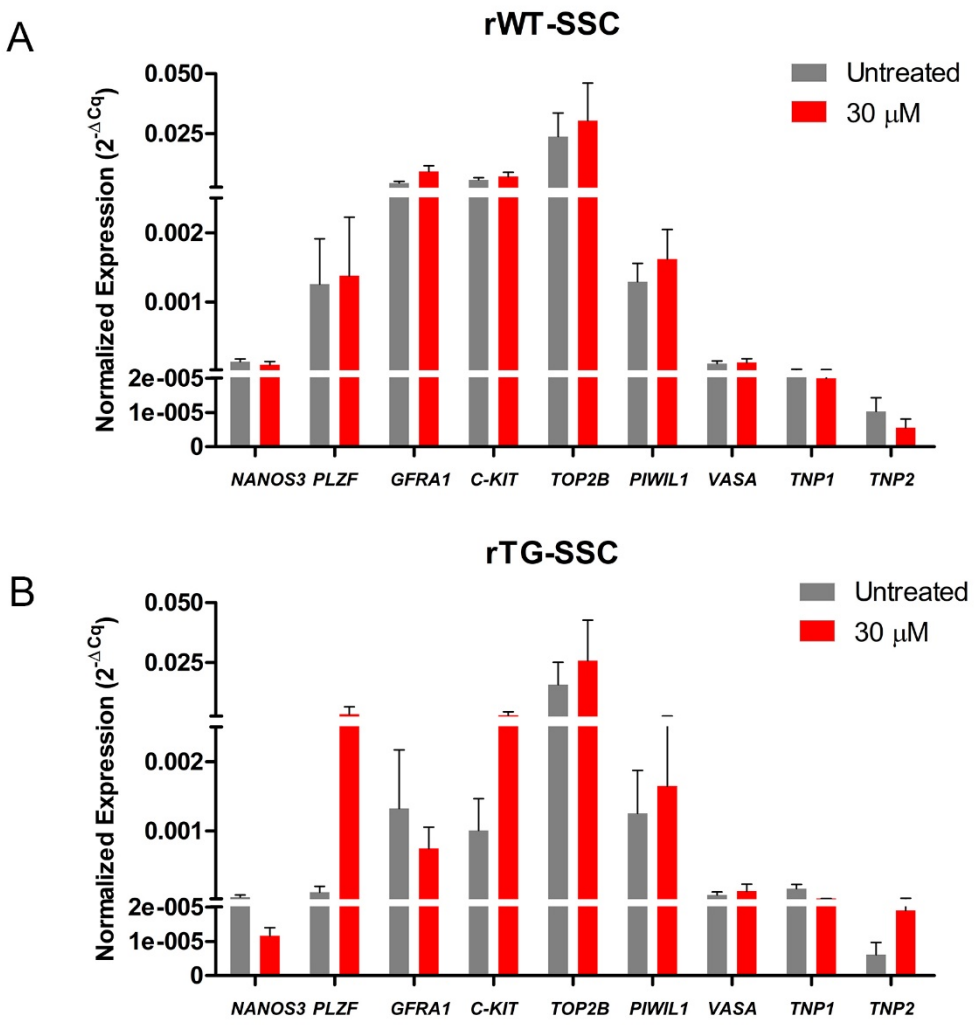

Figure 12. Effect of ATZ on Monkey SSC Differentiation *in Vitro*.

The expression levels of SSC markers were measured at day 10 of SSC differentiation by qRT-PCR on rWT-SSC (A) and rTG-SSC (B). The data was represented as mean +1 SEM. The markers from left to right represented earlier to later stages of SSC differentiation (see Table S2).

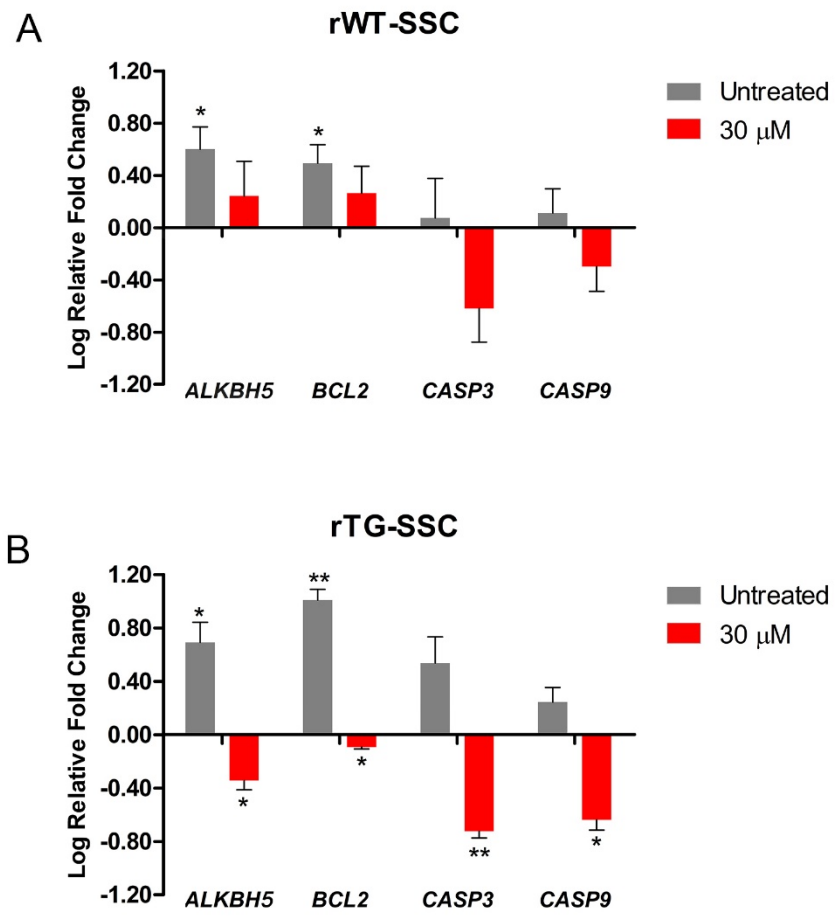

Figure 13. Differential Expression of Apoptosis-Related Markers in Response to ATZ Treatment during SSC Differentiation.

The expression levels of apoptosis markers were quantified by qRT-PCR with three technical replicates in (A) rWT-SSC and (B) rTG-SSC on day 10 of SSC differentiation. The data were represented as mean +1 SEM and the significance was calculated by comparing the rSSCs to the rESCs of the respective cell lines and the treatment. The negative value indicated the gene expression of rSSCs was lower than the respective rESCs prior to differentiation and *vice versa.* * $p < 0.05$; ** $p < 0.01$.

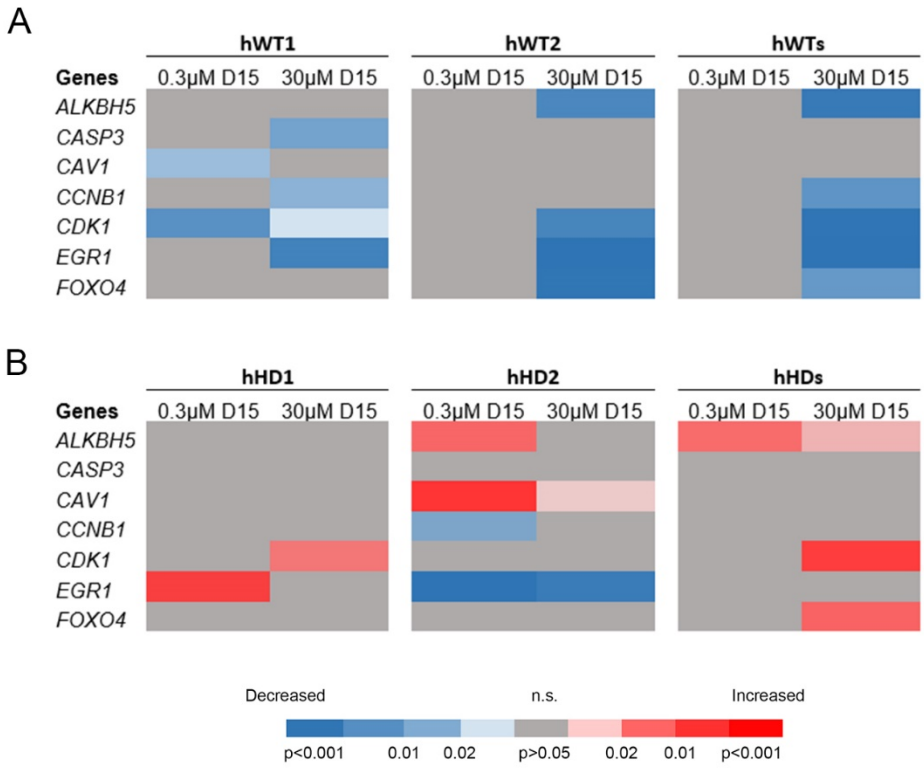

Figure 14. Differential Responses to ATZ at 15 Days Exposure between hWTs and hHDs. At day 15 of 0.3 or 30 μM ATZ treatment, the expression levels of apoptosis and cell cycle related markers were quantitatively measured by qRT-PCR via three technical replicates in hWTs (A) and hHDs (B). hWT1 & 2 and hHD1 & 2 were combined as hWTs and hHDs respectively. The data were normalized to untreated groups. The significance ($p < 0.05$) was calculated between the treated and untreated groups.

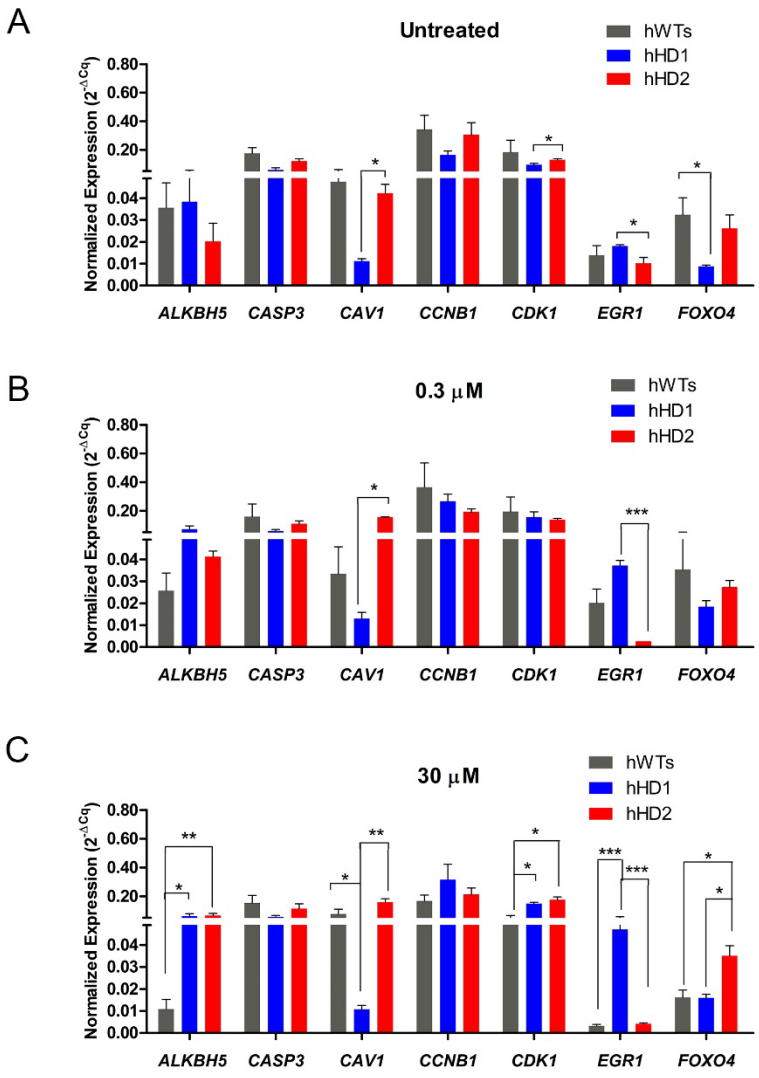

Figure 15. Differential Responses to 15 Days Treatments of 0, 0.3 and 30 μM ATZ in hWTs, hHD1 and hHD2.

Differential expression of apoptosis and cell cycle related genes were quantitatively measured by qRT-PCR via three technical replicates in hWTs and hHDs in response to untreated / 0 μM ATZ (A), 0.3 μM ATZ for 15 days (B) and 30 μM ATZ for 15 days (C). hWTs represented the combined data of hWT1 and hWT2. The data were represented as mean +1 SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

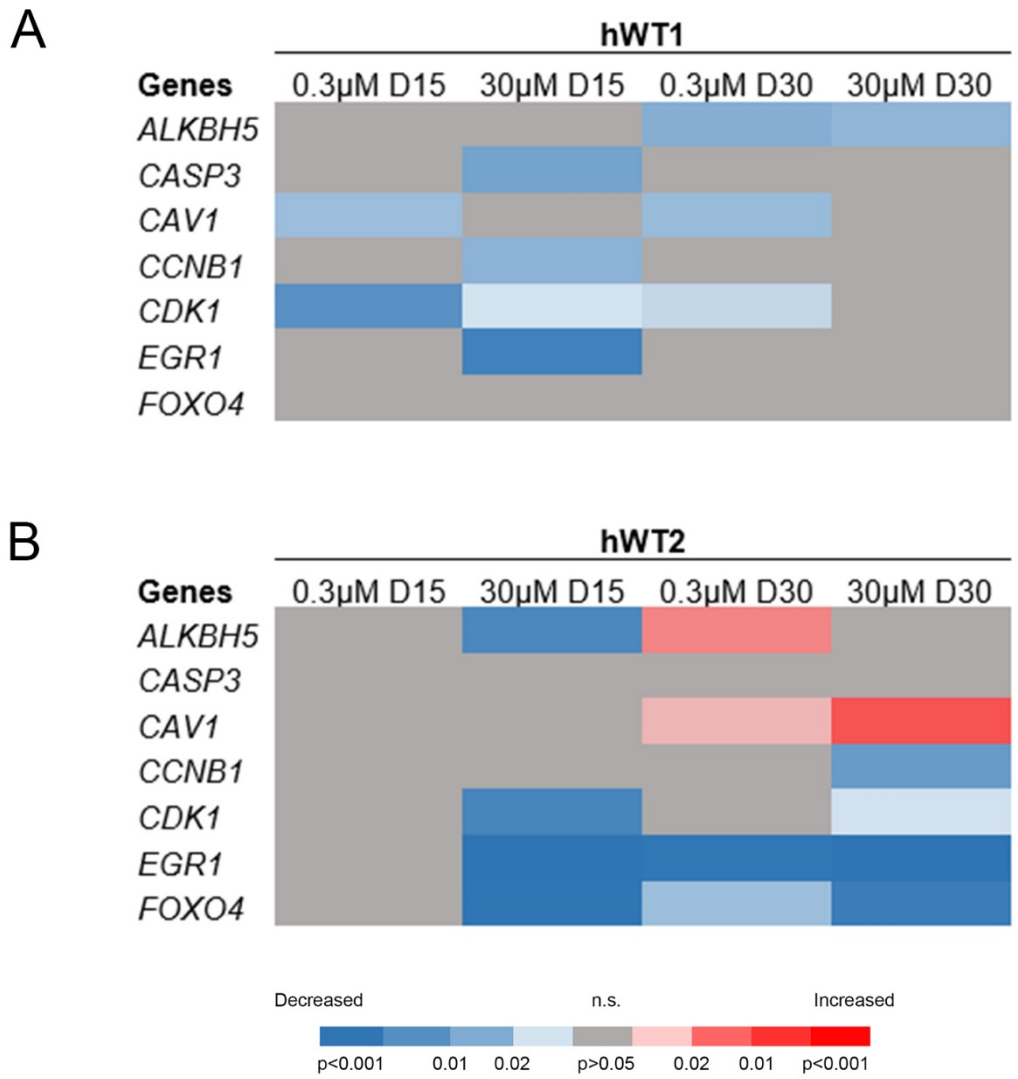

Figure S1. Differential Response to ATZ between hWT1 and hWT2.

At 15 day and 30 day 0.3 or 30 μM ATZ exposure, the expression levels of apoptosis and cell cycle related markers were measured by qRT-PCR on hWT1 (A) and hWT2 (B) with three technical replicates.

Table S1. The Primers Used for qRT-PCR

Name*	Forward (5'-3')	Reverse (5'-3')
hALKBH5	ATCCTCAGGAAGACAAGATTAG	TTCTCTTCCTTGTCCATCTC
hCAV1	CGACCCTAAACACCTCCACGA	TAAATGCCCCAGATGAGTGC
hCCNB1	AATAAGGCGAAGATCAACATGGC	TTTGTTACCAATGTCCCCAAGAG
hCDK1	AAACTACAGGTCAAGTGGTAGCC	TCCTGCATAAGCACATCCTGA
hEGR1	CTCTCCAGCCTGCTCGTC	AGCAGCATCATCTCCTCCAG
hFOXO4	GCCTCGTTGTGAACCTTGATG	ACTGACACTTGCCCAGATTTACG
hGAPDH	CCCACTGCCAACGTGTCA	AAGTCAGAGGAGACCACCT
rALKBH5	TTCGGCTGCAAGTTCCAGTT	CAGCAGCATATCCACTGAGCA
rBCL2	CGGGATGGGGTAAACTGG	AGGTGGTCATTCGTGG
rCASP9	GGTGGGGAGCAGAAAGACC	AGCTGGTCGAAGGTCCTCAA
rGAPDH	GAAGGTGAAGGTCGGAGT	CATTGACAACAATATCC
rNANOG	CCTATGCCTGTGATTTGTGGG	AGGTTGTTTGCCTTTGGGAC
rOCT4	GGAATGAGGGACAGGGGGAG	ACTCCCCTGCCCCCACCCT
rC-KIT	ACACGTGCACCAACAAACAC	CAAGGAGCGGTCAACAAGGA
rGFRA1	GGGAGAAGCCCAACTGTTTG	GACAGCTGCTGACAGACCTTGA
rNANOS3	CCTGCACAGGTTTCAGAGGT	TGGGAGTGGTCCTCATAGGG
rPIWIL1	ATAACTGGCCAGGTGTCATTCGT	AGGTAGTAAAGGCGGTTTGACA
rPLZF	AGCGGTTCCTGGATAGTTTGC	TTCGAAAACTGTGCACCACACT

rTNP1	AATTACCGCTCCCACTTGTGA	TGATCCACATTCCATAGGCTCC
rTNP2	GAGCTCAGGACGGAAATCCAA	CCTGCAAGAAGATTGACTTCG
rTOP2B	GCACTGACCTGGGTGAACAA	CCCACATGAACTGCGTCAAT
rVASA	GAAGCTGATCGCATGTTGGATA	TGCAGCCAACCTTTGAATTTC
CASP3	TCGCTTTGTGCCATGCTGAAAC	TGTTGCCACCTTTCGGTTAACC
SOX2	GCCGAGTGGAAACTTTTGTCG	GGCAGCGTGTACTTATCCTTCT

*names which start with “h” mean primers used for hiPSCs only, whereas “r” for rhesus monkey cells. Names without “h” or “m” mean used for both species in the experiment.

Table S2. Expression of Male Germ Cell Specific Markers During Spermatogenesis.

	Spermatogenesis Related Markers								
Cell Types	NANOS3	PLZF	GRFα1	C-KIT	TOP2B	PIWIL1	VASA	TNP1	TNP2
Early Germ Cells									
Spermatogonia									
Early Spermatocytes									
Late Spermatocytes									
Round Spermatids									

*Note: the expression profile was based on previous research [59, 63, 74]

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