Differentiation of intestinal epithelial stem cells from parthenogenetic embryonic stem cells

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Article

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Abstract

Intestinal epithelial stem cells (IESCs) have great potential for the repair of intestinal epithelial injury. However, the amplification of primarily cultured IESCs is relatively difficult. This study aims to obtain IESCs by inducing the differentiation of human parthenogenetic embryonic stem cells (hPESCs). The differentiation of hPESCs into to the definitive endoderm (DE) was performed by using activin A and Wnt3a firstly. Then the differentiation of DE into IESCs was conducted in the presence of epidermal growth factor (EGF). Markers of IESCs, i.e., Musashi 1(Msil) and hairy and enhancer of split 1(Hes1), were monitored dynamically with double immunocytochemical staining and real-time quantitative PCR (qPCR) to identify the differentiation of IESCs. Results showed that $Msil^{+}Hesl^{+}$ IESCs culminated after a 5-day culture in the presence of EGF and the expression of Msil and Hes1 in cells treated by EGF was 51.3- and 45.38-fold times, respectively, higher than those not treated by EGF. There results demonstrate that IESCs can be generated by inducing the differentiation of hPESCs. Therefore, this study provides a potential source of IESCs for the regeneration of injured intestinal epithelia and the theoretical and experimental basis for the clinical application of hPESCs.

Introduction

Intestinal epithelium injury (i.e., Crohn’s disease, ulcerative colitis, short bowel syndrome and radiation enterocolitis) causes malabsorption and even endangers the patient's life in severe cases. This disease is accepted as an intractable problem and the repair of intestinal epithelial injury is challenging clinically.

Intestinal epithelial stem cells (IESCs), which are responsible for the renewal of intestinal epithelial cells and intestinal epithelium, are considered to be crucial in the repair of intestinal epithelial injury (Barker et al.2014);(Gehart Clevers et al.2019);(Moorefield Andres et al.2017);(Qi Chen et al.2015). IESCs are positioned at the crypt base of intestinal epithelium, from which they normally migrate to the villus tip and drive proliferation up the crypt-villus axis (Parasram Karpowicz et al.2020). The entire migration process lasts about 3–5 days. During the migration process, IESCs constantly proliferate and also differentiate into Paneth cells, goblet cells, enterocytes (the absorptive lineage) and enteroendocrine cells. IESCs also are reportedly involved in the regulation of tissue maintenance under pathological conditions, thus receiving attention in the research of the repair of intestinal epithelial injury.

IESCs can be obtained through primary culture. However, the amplification of primarily cultured IESCs is relatively difficult. Stem cells, able to divide asymmetrically for the maintenance of totipotency and multiple differentiation potential, reportedly can differentiate into IESCs (Seetharaman Mahmood et al.2019). For example, it has been published that Musashi 1-positive cells can be obtained by inducing the differentiation of mouse embryonic stem cells (Lan Tan et al.2019). The generated IESCs were further developed into intestinal epithelial-like tissues.

Human parthenogenetic embryonic stem cells (hPESCs) are derived from haploid cells that do not have the potential to develop into a viable organism. In addition, hPESCs are homozygous for common alleles

in genes for major histocompatibility complex, which decreases the graft rejection rate when they are used for cell transplantation (Yabuuchi Rehman et al.2012).

hPESCs are reported to have the multiple differentiation potential. For instance, functional hepatocyte-like cells, neurons and isletlike clusters generated from hPESCs has been reported (Li He et al.2014);(Liang Wang et al.2020);(Wang Zhu et al.2018). Herein, it was hypothesized in this study that IESCs can be differentiated from hPESCs. To fulfill the design, firstly, the differentiation of hPESCs into definitive endoderm (DE) was conducted by using Activin A and Wnt3a. Then epidermal growth factor (EGF) was used for the differentiation of DE towards IESCs. Resultant cells were characterized by double immunocytochemical staining and real-time quantitative polymerase chain reaction (qPCR).

Results

The identification of undifferentiated hPESCs

Under an inverted microscope, undifferentiated hPESCs cultured on the human hFF feeder were closely arranged, with round and oval colonies (Figure 1A). There was a clear boundary between the hPESCs colonies and the hFF feeder layers (Figure 1A). Karyotype analysis showed that after 40 passages, hPESCs maintained a normal diploid karyotype 46, XX after 40 passages (Figure 1B). The AKP staining confirmed the undifferentiated state of hPESCs cultured on the hFF feeder layers (Figure 1C and D).

The DE differentiation of hPESCs

The differentiation of hPESCs into DE was monitored by examining the expression of CXCR4 and ECD via flow cytometry. Results from the flow cytometry showed that after a 24-, 48-, 72-, 96-, and 120-hour culture of hPESCs, the percentages of $CXCR4^+$ and ECD^+ cells in the treatment group (cells were cultured in the presence of activin A and Wnt3a) was significantly increased compared with those of cells in the control group (cells were cultured in the absence of activin A and Wnt3a) ($P < 0.05$) (Figure 2, Table 5 and Table 6). These data suggested the DE differentiation of hPESCs. Moreover, flow cytometry also showed that the percentages of $CXCR4^+$ and ECD^+ cells peaked at 48-hour activin A- and Wnt3a-induced differentiation (Figure 2, Figure 2, Table 5 and Table 6).

For the further identification of the DE differentiation of hPESCs, mRNA expression of the Sox17 and Gsc, the DE markers, was detected by qPCR (Figure 3). Consistent to the results from flow cytometry, qPCR results also demonstrated that after a 24-, 48-, 72-, 96-, and 120-hour culture, the expression of Sox17 and Gsc in the treatment group (cells were cultured in the presence of activin A and Wnt3a) was significantly increased compared with those in the control group (cells were cultured in the absence of activin A and Wnt3a) ($P < 0.05$), confirming the DE differentiation of hPESCs. Furthermore, the proportion of DE also peaked after a 48-hour activin A- and Wnt3a-induced differentiation (Figure 3). Therefore, the cells experienced a 48-hour activin A- and Wnt3a–induced differentiation were collected for the generation of IESCs.

3.3 The differentiation of DE into IESCs

In the presence of EGF, both the number and the cloning volume of cells in the treatment group gradually increased, with no significant changes in the morphology observed. Meanwhile, the cloning volume of cells in the control group (cells were cultured in the absence of EGF) also gradually increased. However, irregular, epithelioid, spindled-shaped cells in the control group were observed after a 5-day culture (Figure 4).

The differentiation of IESCs from DE was evaluated by double immunocytochemical staining for Msi1 and Hes1 staining. The results were shown in Figure 5 and Figure 6.

As shown in Figure 6, the proportion of $Msi1^{+}Hes1^{+}$ cells was significantly increased after the 1-, 3-, 5-, 7-, and 9-day culture in the presence of EGF ($P < 0.05$). Figure 6 also showed that after the 1-, 3-, 5-, 7-, and 9-day culture, the proportion of $Msi1^{+}Hes1^{+}$ cells in the treatment group was significantly higher than that in the control group ($P < 0.05$). These findings suggested the differentiation of DE into $Msi1^{+}Hes1^{+}$ IESCs by using EGF. Besides, the proportion of $Msi1^{+}Hes1^{+}$ cells was found to peak after 5-day culture in the presence of EGF and reached a proportion of 59.7%.

The differentiation of DE into IESCs was also determined by qPCR through the examination of mRNA expression. As shown in Figure 7, the expression of Msil and Hes1 was significantly increased after the 1-, 3-, 5-, 7-, and 9-day culture in the presence of EGF ($P < 0.05$). Consistent to the results from immunochemical staining, after the 1-, 3-, 5-, 7-, and 9-day culture, both the expression of Msil and Hes1 in the treatment group was significantly higher than that in the control group ($P < 0.05$), confirming the differentiation of DE into IESCs by using EGF. Besides, after a 5-day culture in the presence of EGF, the expression of Msi1 and Hes1 was also found to peak and the expression of Msil and Hes1 in the treatment group was 51.3- and 45.38-fold times, respectively, higher than those in the control group. ($P < 0.05$) (Figure 7 and 8). Meanwhile, both the increase and decrease in expression of Msi1 are synchronous with the increase and decrease in expression of Hes1 in the treatment group, respectively (Figure 8).

Discussion

In this study, hPESCs were induced to differentiate into DE, followed by their differentiation into IESCs. The proportion of DE derived from hPESCs peaked after a 48-hour culture in the presence of activin A and Wnt3a. Therefore, the cells experienced a 48-hour activin A- and Wnt3a–induced differentiation were collected for the generation of IESCs. EGF signaling pathway plays a key role at the early stage of intestinal development. Many studies have demonstrated that EGF receptor-mediated signal transduction is involved in the proliferation and maturation of IESCs as well as intestinal epithelial development (Chen Yang et al.2019);(Kim Akhtar et al.2020);(Suzuki Sekiya et al.2010);(Turner George et al.2020);(Wang Zhu et al.2020). These findings suggest that EGF can be used to induce the differentiation of hPESCs into IESCs. And mouse embryonic stem cells have been found to undergo differentiation into IESCs in the

presence of EGF (Yu Chen et al.2010). Herein, our results showed that EGF maintained the cell morphology and promoted the differentiation of DE into IESCs.

Currently, Msil (George Sturmoski et al.2009);(Montgomery Breault et al.2008), Hesl(Yu Lan et al.2011), and leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) (Guiu Hannezo et al.2019);(Zhang Huang et al.2013) were accepted as the IESCs markers. However, the expression of Msi1, Hes1 and Lgr5 is also reported in other tissues and cells (Kozachenko Dzhamalutdinova et al.2017);(Matsuzaki Yoshihara et al.2019);(Padial-Molina de Buitrago et al.2019);(Sueda Imayoshi et al.2019);(Zhang Cai et al.2018). Therefore, it is not reliable to determine the IESCs in vitro according to the expression of only one markers. The crypt base columnar cells co-expressing Msi1 and Hes1 in mouse small intestine has been found to show the characteristics of IESCs (Kayahara Sawada et al.2003). Besides, few cells expressing Msi1 and Hes1 have been observed in adult stem cells or tissues other than IESCs .Therefore, the expression of Msi1 and Hes1 are accepted to identify IESCs. The expression of Msi1 and Hes1 was also investigated to identify the IESCs in the present study. The expression of Msi1 and Hes1 examined by double immunocytochemical staining and qPCR was significantly increased after the 1-, 3-, 5-, 7-, and 9-day culture in the presence of EGF ($P < 0.05$). These findings suggest the successful generation of IESCs and the promotion effect of EGF on differentiation of IESCs from DE. Meanwhile, it was found that after 5-day culture in the presence of EGF, the expression of Msi1 and Hes1 peaked and the expression of Msil and Hes1 in the treatment group was 51.3- and 45.38-fold times, respectively, higher than those in the control group. ($P < 0.05$).

Materials And Methods

Culture of hPESCs and the stepwise differentiation of hPESCs into IESCs

hPESCs (cell line chHES-32) were provided by National Engineering & Research Center of Human Stem Cells, Changsha, China. Human foreskin fibroblasts (hFFs) were isolated from foreskin tissues obtained from child circumcision as we previously reported (Lu Zhu et al.2010). All procedures were performed in accordance with guidelines set forth by Declaration of Helsinki. All experimental protocols were approval from the Ethics Committee of the Second Hospital of Tianjin Medical University. Informed consent was obtained from all legal guardians of the subjects.

hPESCs were cultured with human foreskin fibroblasts (hFFs) used as the feeder cells as previously reported.(Wang Li et al.2015) Briefly, hPESCs were cultured in the medium containing 50 ng/ml activin A (R&D, Emeryville, CA, USA) and 25 ng/ml Wnt3a (R&D, Emeryville, CA, USA) for 0, 24, 48, 72, 96, and 120 hours. hPESCs cultured in the medium without activin A and Wnt3a for 0, 24, 48, 72, 96, and 120 hours were used as the control group. After the DE differentiation of hPESCs, the culture medium was replaced with medium containing 40 ng/ml EGF and cells were cultured for 0 day, 1 day, 3 days, 5 days, 7 days, and 9 days. DE cultured in the medium without EGF for 0 day, 1 day, 3 days, 5 days, 7 days, and 9 days was used as the control group. The differentiation of hPESCs into DE was performed as previously

reported.(Liang Xiao et al.2020) Briefly, cell morphology was observed under an inverted microscope. Karyotype analysis was conducted to detect the karyotype of cultured cells. Alkaline phosphatase (AKP) staining was also performed to detect the undifferentiated hPESCs as previously reported (Wang et al.2015).

Flow cytometry

After the 0-, 24-, 48-, 72-, 96-, and 120-hour culture of hPESCs in the medium with and without activin A and Wnt3a, flow cytometry was performed to examine the expression of DE markers, i.e., C-X-C motif chemokine receptor 4 (CXCR4) and E cadherin (ECD). Briefly, a single-cell suspension was collected and washed twice with PBS. Next, 10 μl PE-conjugated anti-human CXCR4 monoclonal antibodies (R&D) and APC-conjugated anti-human ECD monoclonal antibodies (R&D) was added into a 200 μl single-cell suspension (10^6 cells/ml in PBS), respectively. After 30-min incubation with antibodies at 4°C, unconjugated antibodies were removed by washing with PBS and samples were analyzed using a FACSC Canto II cytosorter (BD Bioscience Pharmingen Inc., San Diego, CA, USA). Data were recorded and analyzed by using FACSDiva V6.1.3 (BD Bioscience).

Real time quantitative PCR

After the 0-, 24-, 48-, 72-, 96-, and 120-hour culture of hPESCs in the medium with and without activin A and Wnt3a, qPCR was performed to examine the expression of DE markers, i.e., SRY-box 17 (Sox17) and Goosecoid(GSC). Briefly, total RNA was extracted from cultured cells using the Trizol reagent (Invitrogen). Then cDNA was synthesized by reverse transcription. The DE markers Sox17 and Gsc were then measured by qPCR. All primers were synthesized by TaKaRa Biotechnology Co., Ltd (Dalian, China) and the sequences of primer are listed in Table 1. The cycling conditions and annealing temperature are shown in Table 2.
Similarly, after the 0-day, 1-day, 3-day, 5-day, 7-das, and 9-day culture of DE in the medium with and without EGF, qPCR was performed to examine the expression of IESCs markers, i.e., hairy and enhancer of split 1 (Hes1) and Musashi-1 (Msi1). Briefly, total RNA was extracted from cultured cells using the Trizol reagent (Invitrogen). Then cDNA was synthesized by reverse transcription. The IESCs markers Hes1 and Msi1 were measured by qPCR. The primers were synthesized by TaKaRa Biotechnology Co., Ltd (Dalian, China) and the sequences of primer are listed in Table 3. The PCR cycling conditions and annealing temperature are shown in Table 4.

Double immunocytochemical staining

The expression of IESCs markers, i.e., Msi1 and Hes1, was also measured by double immunochemical staining to identify the IESCs. Cultured cells were collected and suspended in PBS at a final density of $1x10^5$ cells/ml. Two drops of the cell suspension were placed on a clean glass slide, followed by 10-min fixation with absolute ethanol at room temperature. The slides were then air-dried at room temperature and stored at 4 °C. Normal adult small intestine was used as positive control tissue samples. The tissues were processed routinely with formalin fixation and paraffin embedding. Next, 3μm-thick sections were

mounted on poly-L-lysine coated slides. For immunochemical staining, endogenous peroxidase activity was blocked with peroxidase blocking reagent for 5 min. Sections were subsequently treated with normal nonimmunone serum for 10 min. The sections were incubated with Msi1 antibody at room temperature for 1 hour, followed by the biotin-labeled secondary antibody for 10 min. After a 5-min wash in PBS for 3 times, Streptavidin-Alkalinephosphatase was applied for 10 min. After a 5-min wash in PBS for 3 times, sections were incubated in 5-bromo-4-chloro-3-inodlyl-phosphat / Nitro-Blue-Tetrazolium chromogenic solution. After a 5-min wash in PBS for 3 times, double dye enhancer was applied for 10 min. After a 5-min wash in PBS for 3 times, sections were subsequently treated with normal nonimmunone serum for 10 min. Next, sections were treated with Hes1 antibodies for 60 min at room temperature, followed by biotin-labeled secondary antibody for 10 min. After a 5-min wash in PBS for 3 times, Streptavidin-Alkalinephosphatase was applied for 10 min. After a 5-min wash in PBS for 3 times, sections were treated by fresh 3-amino-9-ethylcarbazole solution. Sections were finally stained by hematoxylin and observed under microscope. The positive expression of Msil was determined when purple black were found within the cell nucleus, and the positive expression of Hesl was determined when red cytoplasm was observed. 1000 cells were randomly selected and the percentages of $Msil^{+}Hesl^{-}$ cells, $Msil^{-}Hesl^{+}$ cells and $Msil^{+}Hesl^{+}$ cells were calculated, respectively.

Statistical analysis

All analyses were performed with the Statistical Package for Social Science (SPSS19.0 for Windows, Stanford University, CA, USA) and data were analyzed using the *t*-test and ANOVA. A value of $P<0.05$ was considered statistically significant.

Conclusions

In summary, the current study demonstrated a stepwise method to induce differentiation for the generation of hPESCs-derived IESCs by using activin A- and Wnt3a followed by EGF. Besides, EGF was found to significantly promote the differentiation of IESCs *in vitro* in the present study. And the proportion of $Msi1^{+}$ and $Hes1^{+}$ IESCs peaked after a 5-day culture in the presence of EGF. Therefore, this study provides a potential source of IESCs for the regeneration of injured intestinal epithelia. Our findings also provide the theoretical and experimental basis for the clinical application of hPESCs.

Declarations

Declaration of conflicting interest

The authors declare no competing financial interest.

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Acknowledgements

Not applicable.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

Authors' contributions

Zhiqiang Wang is responsible for the design of the studies, interpretation of the data and writing of the manuscript. Qingyang Meng and Wanpu Wang cultured cells, conducted flow cytometry analysis, and prepared and edited the manuscript. Lilin Luo and Leilei Li performed quantitative PCR studies and analysis. Shuai Dong performed the double immunocytochemical staining. Menglong Li and Tianxing Chen: conducted the differentiation of cells. Rui Liang conceived, designed and oversaw all of the studies, reviewed the manuscript, and is responsible for the primary undertaking, completion, and supervision of all experiments.

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Tables

Table 1 Primer sequences

Gene	Forward (5 -3)	Reverse (5 -3)	Length (bp)
β-actin	TGGCACCCAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA	186
Sox17	CTGCAGGCCAGAAGCAGTGTTA	CCCAAACTGTTCAAGTGGCAGA	153
Gsc	ACCTCCGCGAGGAGAAAGTG	GACGACGACGTCTTGTTCCA	121

Table 2 qPCR cycling conditions and annealing temperature

Gene	Cycling conditions	Annealing temperature(°C)
β-actin	94°C, 30 s —[94°C, 30 s —64°C, 30 s]×40 cycles	64
Sox17	94°C, 5 min —[94°C, 30 s —59°C, 30 s —72°C, l min]×40 cycles	59
Gsc	94°C, 5 min —[94°C, 30 s —61°C, 30 s —72°C, l min]×40 cycles	61

Table 3 Primer sequences

Gene	Forward (5 -3)	Reverse (5 -3)	Length (bp)
β-actin	TGGCACCCAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA	186
Msil	CGTTTGAGCTGAGTCCTGAGACAC	GCTGGCTTCGAAACACCATGTA	164
Hesl	GGACATTCTGGAAATGACAGTGA	AGCACACTTGGGTCTGTGCTC	87

Table 4 qPCR cycling conditions and annealing temperature

Gene	Cycling conditions	Annealing temperature (°C)
β-actin	94°C, 30 s —[94°C, 30 s —64°C, 30 s]×40 cycles	64
Msil	94°C, 5 min —[94°C, 30 s —61°C, 30 s —72°C, 40 s]×40 cycles	61
Hesl	94°C, 5 min —[94°C, 30 s —62°C, 30 s]×40 cycles	62

Table 5 Percentage of $CXCR4^+$ cells after a 0-, 24-, 48-, 72-, 96-, and 120-hour culture of hPESCs

Group	0 hour (%)	24 hours (%)	48 hours (%)	72 hours (%)	96 hours (%)	120 hours (%)
Treatment Group	0.51±0.04	35.7±1.55	61.4±2.01	36.8±1.94	25.5±1.04	24.7±0.97
Control Group	0.51±0.04	19±0.92	16.1±1.31	20.4±0.93	16.2±0.93	19.9±0.59

Table 6 Percentage of ECD^+ cells after a 0-, 24-, 48-, 72-, 96-, and 120-hour culture of hPESCs

Group	0 hour (%)	24 hours (%)	48 hours (%)	72 hours (%)	96 hours (%)	120 hours (%)
Treatment Group	0.47±0.02	6.4±0.28	18.7±0.62	11.7±0.41	7.9±0.37	4.9±0.22
Control Group	0.47±0.02	3.2±0.13	4.6±0.27	4.3±0.16	3.7±0.17	4.7±0.21

Figures

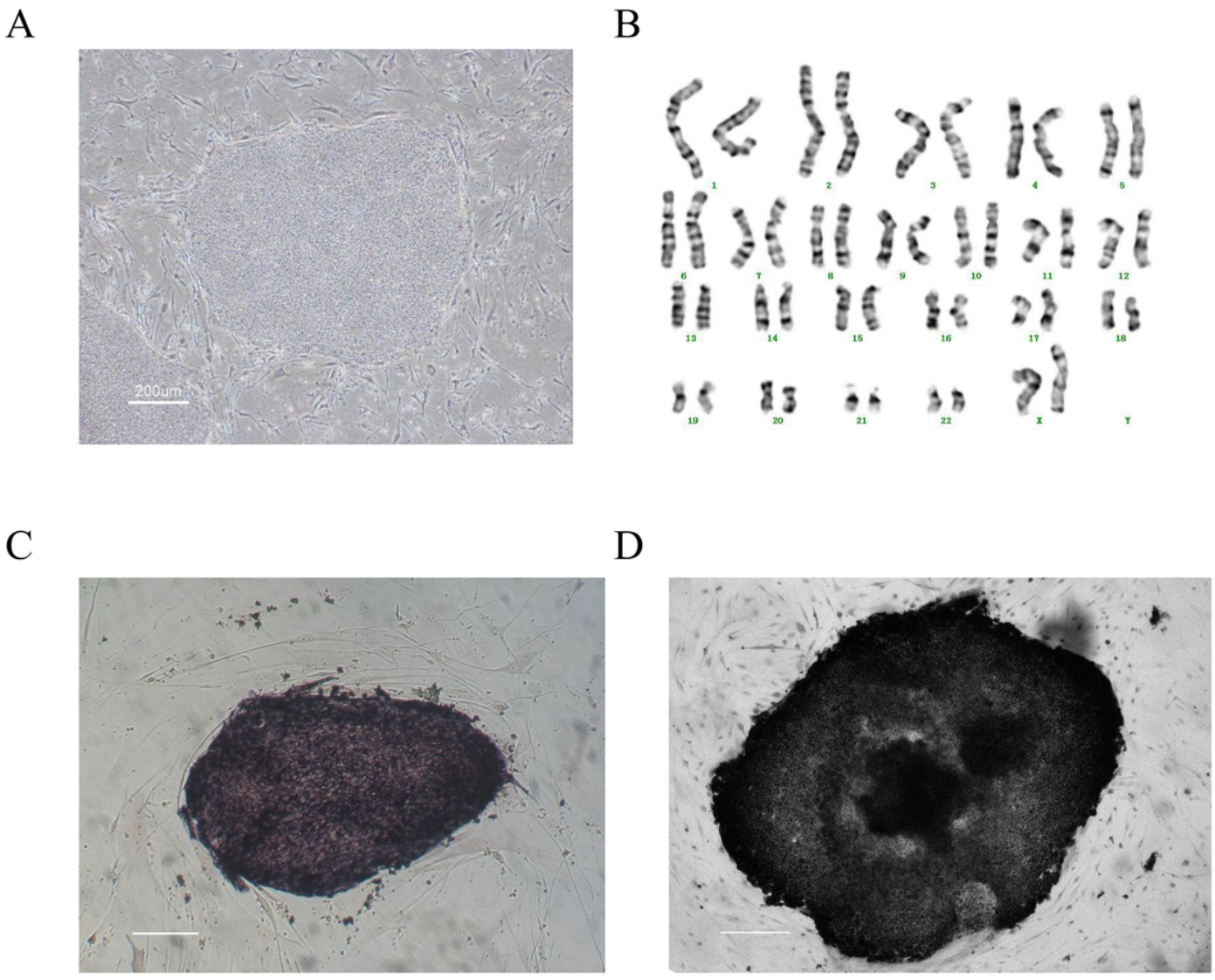

Figure 1

(A) The morphology of hPESCs cultured on the hFF feeder layer (scale bar=200 μm); (B) the maintained normal 46,XX diploid of hPESCs cultured on the hFF feeder layer (100× magnification); (C) AKP staining showing the undifferentiated hPESCs cultured on the hFF feeder layer (scale bar=200 μm); (D) AKP staining of differentiated hPESCs (scale bar=200 μm).

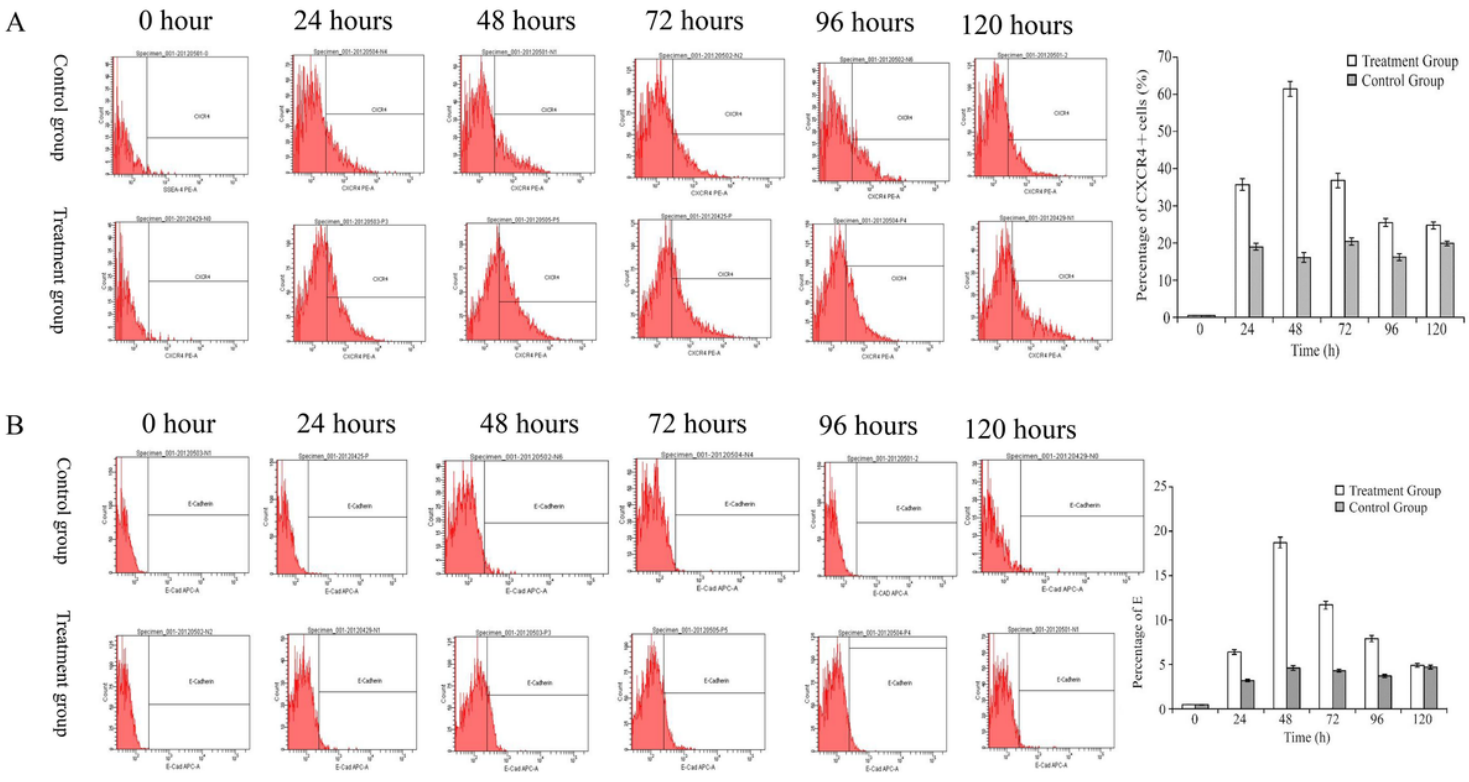

Figure 2

Representative flow cytometry histogram showing the differentiation of hPESCs into DE. (A) The percentage of $CXCR4^+$ cells before and after a 24-, 48-, 72-, 96-, and 120-hour culture of hPESCs in the treatment and control group. (B) The percentage of ECD^+ cells before and after a 24-, 48-, 72-, 96-, and 120-hour culture of hPESCs in the treatment and control group.

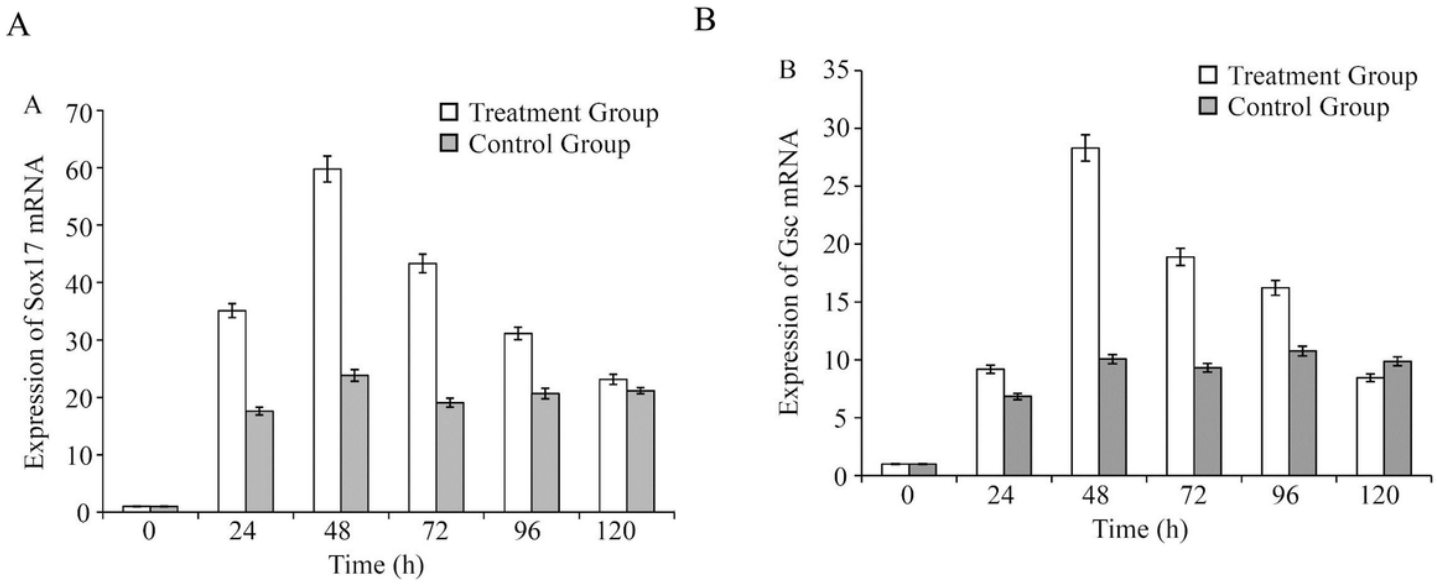

Figure 3

Expression of Sox17 (A) and Gsc (B).

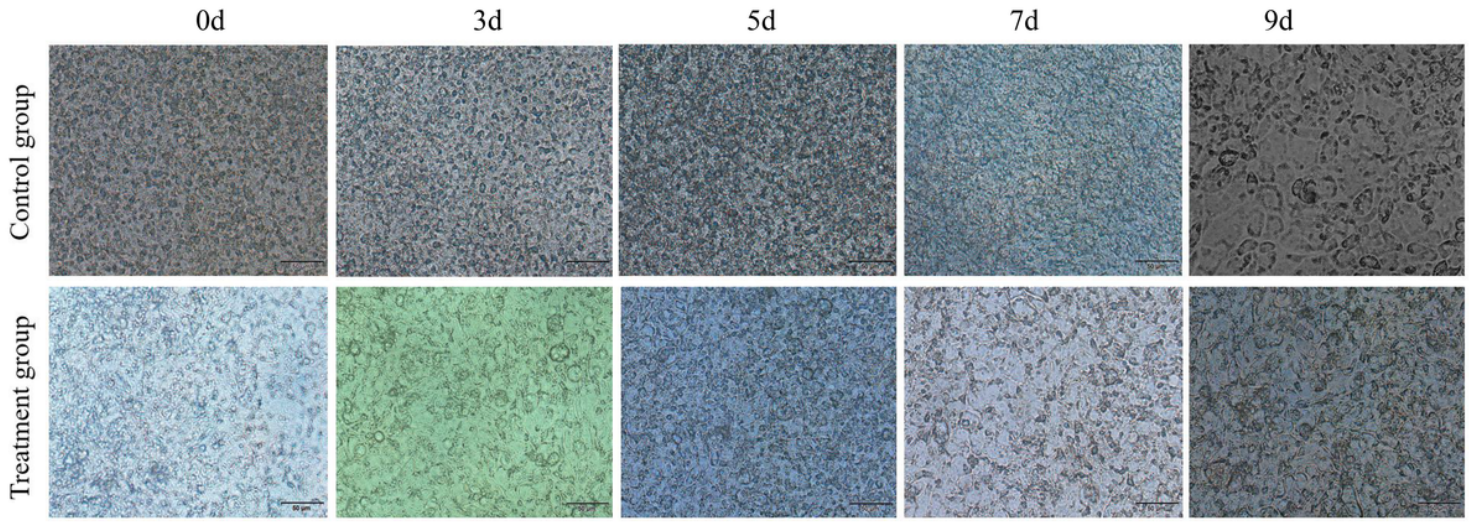

Figure 4

The morphology of cells in the treatment and control groups (scale bar=50 μm).

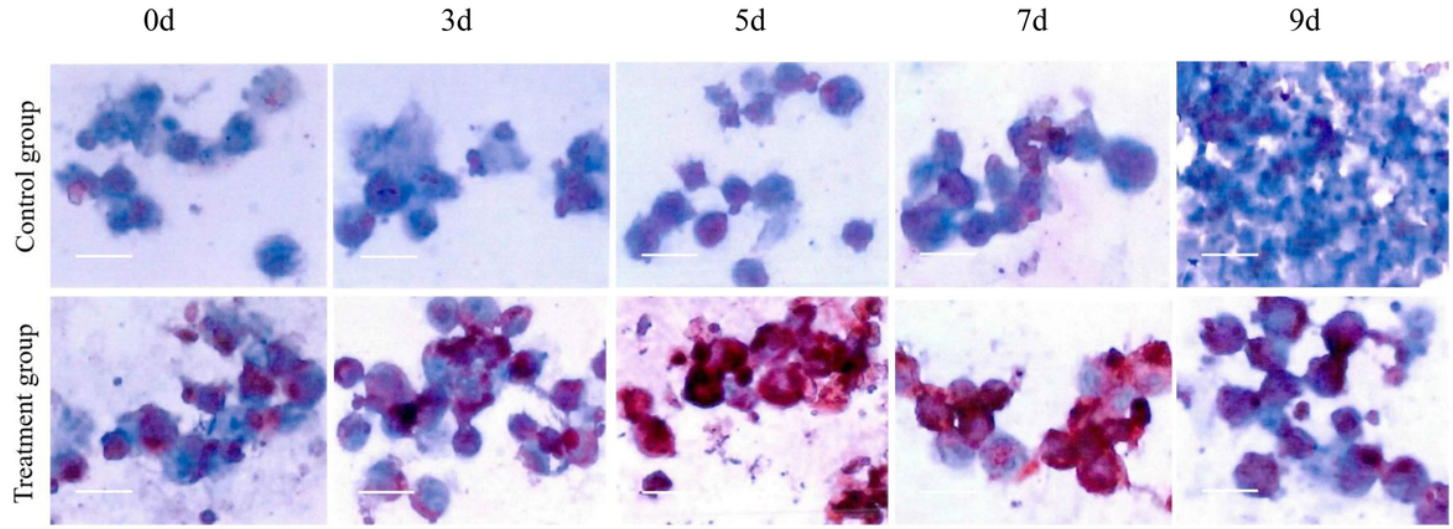

Figure 5

Results from double immunochemical staining for Msi1 and Hes1 (scale bar=20 μm).

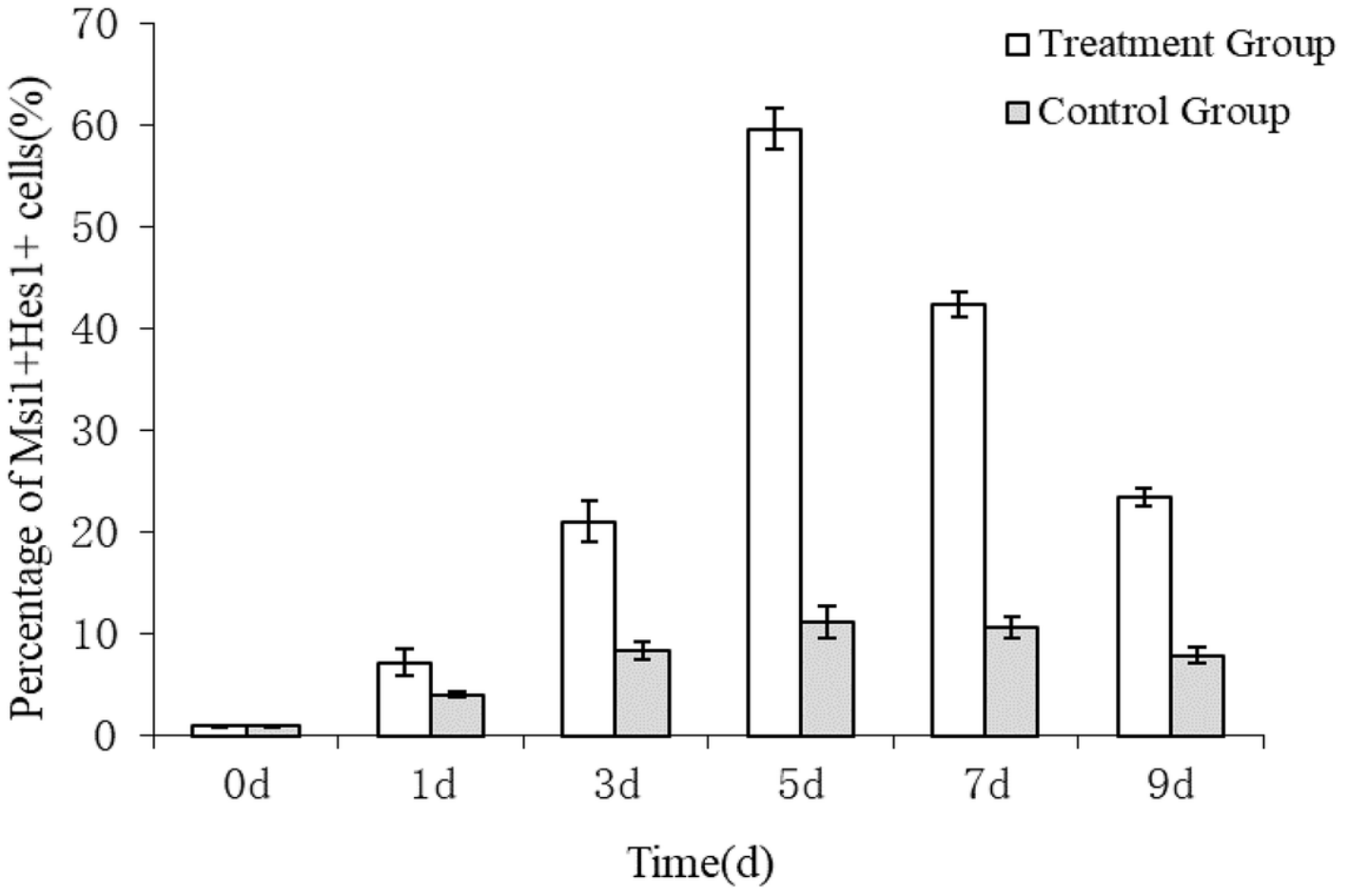

Figure 6

The proportion of $Msi1^{+}Hes1^{+}$ cells showed by double immunochemical staining.

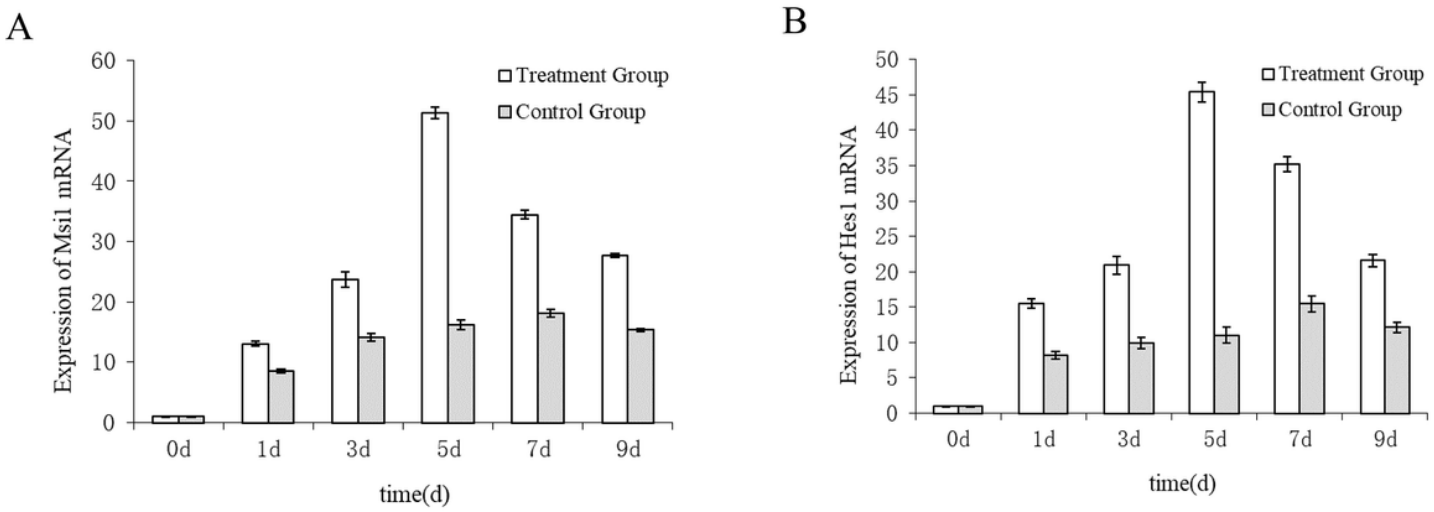

Figure 7

Expression of Msi1 (A) and Hes1 (B).

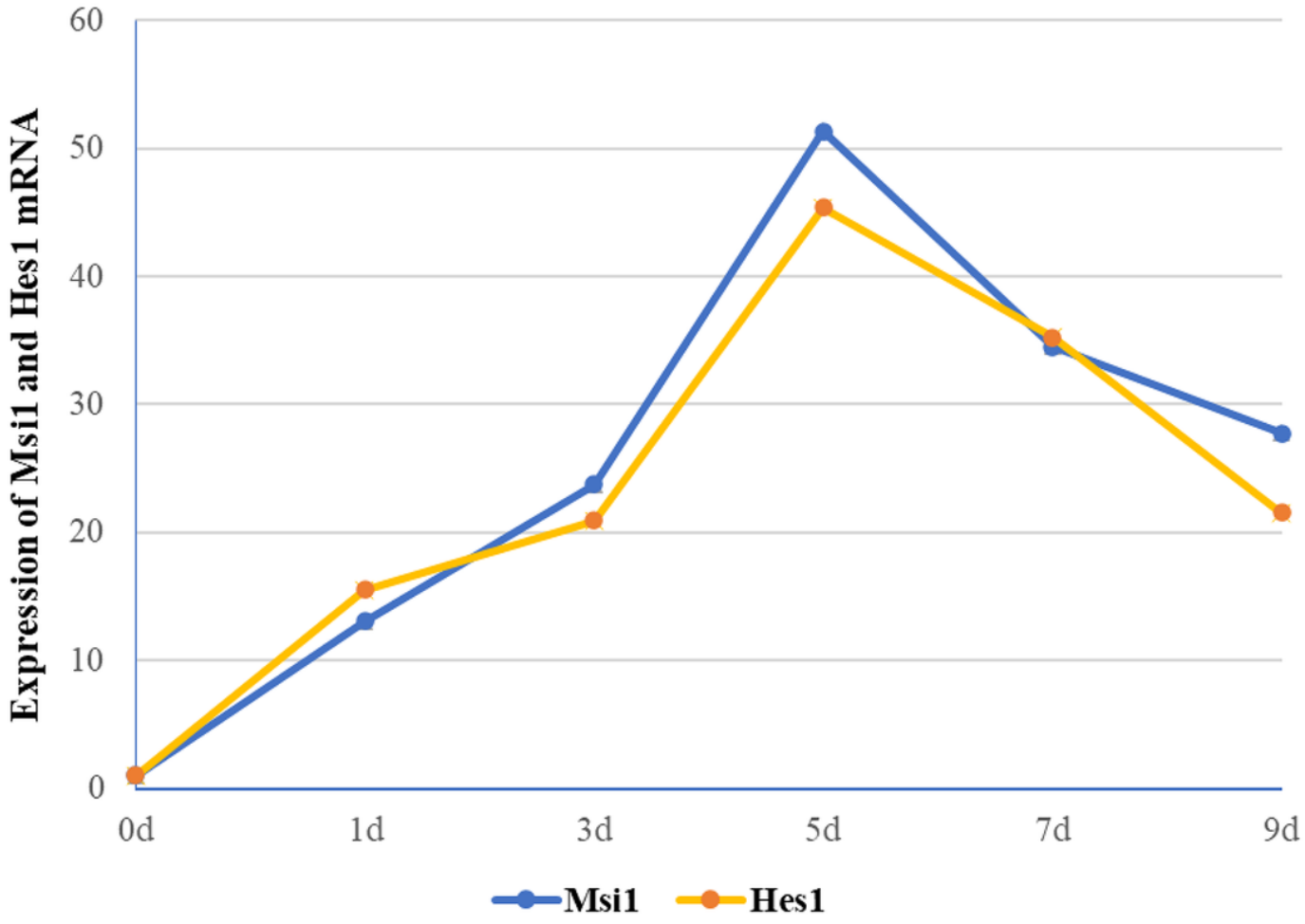

Figure 8

Alterations in the expression of Msi1 and Hes1.