The study on the gender difference in the proliferation rate of human umbilical mesenchymal stem cells

Abstract

Human umbilical cod mesenchymal stem cells (hUC-MSCs) are more and more paid attention to by the medical doctor and the researcher, because of their specific advantages. But, the gender difference of hUC-MSCs is usually neglected, which is not beneficial to the delicate operations using hUC-MSCs. This research originated from the finding during the routine research involving for hUC-MSCs and aimed to investigate the proliferation rate difference between the male and the female hUC-MSCs systematically. In this research, under the same conditions, the consecutive 14 batches of the male and the female hUC-MSCs were prepared and identified. Then, the proliferation rates of these cells were measured and analyzed statistically. Finally, the transcriptional expression patterns for the 12 proliferation-related genes were representatively analyzed by quantitative PCR (qPCR). The results showed: under the same preparative conditions, the male hUC-MSCs proliferated faster that the female counterpart significantly; meanwhile, for the transcriptional expression levels for most of the 12 proliferation-related genes, the male was higher than the female. This research was the first report to investigate the proliferation rate difference between the male and female hUC-MSCs and preliminarily clarify the possible mechanism, which expanded the understanding for hUC-MSCs.

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Introduction

The stem cell therapy is being more and more focused on, because it may improve the human disease therapy revolutionarily. Human umbilical cord mesenchymal stem cells (hUC-MSCs), as a kind of adult stem cell, are greatly paid attention to, because they are easily prepared, low immunogenic and have no ethic problems. Many stem-cell-related therapy and research use hUC-MSCs as the source cell [1-6].

However, the gender of hUC-MSCs is usually neglected. In fact, if hUC-MSCs is from the male baby, its genotype is XY; if hUC-MSCs is from the female baby, its genotype is XX. During the routine hUC-MSCs related research, we noticed that there was difference about the proliferation rate between the male hUC-MSCs and the female hUC-MSCs and no relative report was published currently, so, in order to investigate this phenomenon systematically, we carried out this research abut the proliferation rate difference between the male hUC-MSCs and the female hUC-MSCs.

Materials and methods

The experimental apparatus

CO_2 cell incubator (Thermo Inc., United States, Model: 3111); Inverted microscope (Leica Inc., Germany, Model: DMI 3000B); Flow cytometry instrument (Beckman Inc., United States, Model: FC-500); Microplate reader (Thermo Inc., United States, Model: Multiskan GO); Quantitative PCR instrument (Bio-rad Inc., United States, Model: IQ5).

Materials

1 × DMEM medium (Gibco Inc., United States); Fetal bovine serum (Gibco Inc., United States); Antibiotic mixture (Gibco Inc., United States); Trypsin-EDTA (Gibco Inc., United States); MTT reagent (Sigma Inc., United States); Dead cell dye 7-ADD (Beckman Inc., United States); Antibodies for the flow cytometry CD90-TITC, CD105-FITC, CD73-FITC, CD166-FITC, CD34-FITC, CD45-PC7, HLA-DR-FITC, CD14/79A/184-PE (Beckman Inc., United States); Kits for the adipogenoic, the chondrogenic, the osteogenic differentiation (Gibco Inc., United States); RNA extraction reagent Trizol (Life Tech Inc., United States); RNA purification kit (Beijing Tiangen Inc., China); cDNA synthesis kit (Bio-rad Inc., United States); qPCR Sybr green analysis kit (Bio-rad Inc., United States).

The preparation and identification of hUC-MSCs

The umbilical cords were collected from the cesarean healthy babies with term pregnancy in the Second Affiliated Hospital of Sichuan University. The collected umbilical cords were processed in 4-6 hours.

The hUC-MSCs were prepared with "Tissue cubes adherence method": the umbilical cords were taken out in a biological safety cabinet and washed with the physiological saline; the blood vessels were removed with the forceps and the surgical scissor; the umbilical cords were cut into the cubes with the size of 0.5-1 mm^3 with scissors; small tissue cubes were then evenly spread in the 60 cm culture dish and 2 ml cell culture medium was added in and the dish was put into the cell incubator; after 24 hours, another 1 ml the cell culture medium was added into the dish; 10 days later, the tissue cubes were removed with the physiological saline and the fresh medium was added into the dish and when cell confluence reached about 70%, the cell was passaged and prepared for the following experiments.

The prepared hUC-MSCs were identified from the cell morphologies, the surface antigens and the triple differentiation potentials (adipogenoic, chondrogenic and osteogenic).

The identification from the cell morphologies based on the microscopic images: the typical hUC-MSCs show the long spindle shapes.

The identification from the surface antigens based on the flow cytometry analysis: the typical hUC-MSCs have high expression levels for CD90, CD105, CD73 and CD166 and have very low expression levels for CD34, CD45, HLA-Dr and CD14/79A/184.

The identification from the triple differentiation potentials (adipogenoic, chondrogenic and osteogenic) based on the differentiation analysis: the typical hUC-MSCs show the differentiation into the adipocyte, the chondrocyte, and the

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osteocyte after the specific treatments.

For the cell morphologies, the prepared hUC-MSCs were observed under the inverted microscope and the images were taken and analyzed according to the above criterion.

For the cell surface antigens, the prepared hUC-MSCs were analyzed with the flow cytometry. The cell viability was monitored with 7-ADD (the dye for the dead cell), and the cell surface antigens CD90, CD105, CD73, CD166, CD34, CD45, HLA-DR and CD14/79A/184 were detected with the relative antibodies. The results were then analyzed according to the above criterion.

For the triple differentiation potentials (adipogenoic, chondrogenic and osteogenic), the prepared hUC-MSCs were treated with the specific kits. The results were then analyzed according to the above criterion.

The male and the female hUC-MSCs were consecutively prepared at six different time points, and the time interval was about 21 days.

The measurement of the cellular proliferation rate

200 μl/well and 2500 cells/well of the hUC-MSCs were inoculated into the 96 well plate and triple wells were prepared for the every condition. After 24h, MTT assay was started and this result was recognized as the cell amount for day 0 and after that, the MTT assay was continued at day 1, 2, 3, 4, 5, 7, 8, 10, 11. At each time point, the operations were the following: 22 μl MTT original solution (5 mg/ml) was added to the well and the plate was incubated in 37℃ cell incubator for 4 h; the medium in each well was removed and the blue particles (formazen granules) at the bottom of the well were kept; 150 μl DMSO was added into the well to dissolve the particles; the plate was read at 570 nm on the microplate reader (OD 570nm).

After all MTT data were collected, the plot of scattered dots was drawn in Excel with the time as the X-axis and with the OD 570nm as the Y-axis. The trend line and the formula were added for each plot. The slope of the formula was used to estimate the cellular proliferation rate (Fig. 4).

The detection of the transcriptional expression levels of the cellular proliferation-related genes

The RNAs of each batch of the hUC-MSCs were prepared and purified. 12 cellular proliferation-related genes were selected (*E2F1*, *MYBL2*, *BUB1*, *PLK1*, *CCNE1*, *CCND1*, *CCNB1*, *STK1*, *FOXM1*, *MKI67*, *MCM*, *PCNA*), whose up-regulation promotes the cellular proliferation [7], and *GAPDH* was used as the reference gene. The primer information was listed in Table 1.

Table 1. The primer information of the proliferation-related genes

Gene	Forward primer	Backward primer	The amplicon length （b.p.）
E2F1	CAGACCCTGCAGAGCAGATG	CATCGATCGGGCCTTGTTT	120

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MYBL2	AGGCTGGCATCGAACTCATC	CCACAATGTCAAGAGCCAGAGA	120
BUB1	CCACAGGAGCCAGGACCTT	TGCAGCGAATACCCCATACA	120
PLK1	GAGCAAGAAAGGGCACAGTTTC	ATCCGGAGGTAGGTCTCTTTTAGG	120
CCNE1	TGGTTCCATTTGCCATGGTT	CCAGCAAATCCAAGCTGTCTCT	120
CCND1	GCTCCTGGTGAACAAGCTCAA	GAAGGTCTGCGCGTGTTTG	120
CCNB1	CAAATGAAATTCAGGTTGTTGCA	CATGGCAGTGACACCAACCA	120
STK1	TCAGCGGGTCTTGTGTCCTT	ACACTGGTTGCCTGCAATTG	120
FOXM1	AGGAAGCGCATGACTTTGAAA	GTCGTGCAGGGAAAGGTTGT	120
MKI67	AGAAGGCGTGTGTCCTTTGG	CAGGTGGAGTGTGCATTACCA	120
MCM	TTCCCAAAGTGGCTGATCCT	TCAGCTACAGCTTTGGCCATT	120
PCNA	GGTCCAGGGCTCCATCCT	GAGACGTGGGACGAGTCCAT	120
GAPDH	GGAGTCCACTGGCGTCTTCA	TTCACACCCATGACGAACATG	120

The statistical analysis

The proliferation rates of six batches of the prepared hUC-MSCs were compared with the paired t test method and the same batch of the male and the female hUC-MSCs were paired. The qPCR results of the sixth batch of the hUC-MSCs were compared with the independent sample t test method. $P < 0.05$ indicated that there was a significant difference and $P < 0.01$ indicated that there was a very significant difference.

Results

The preparation and identification of the hUC-MSCs

The hUC-MSCs from the male babies and the female babies were prepared with the "Tissue cubes adherence method". The representative cell morphologies were shown in Fig.1: the hUC-MSCs of both genders were long spindle shaped, which conformed to the publicly accepted cell morphology of the hUC-MSCs.

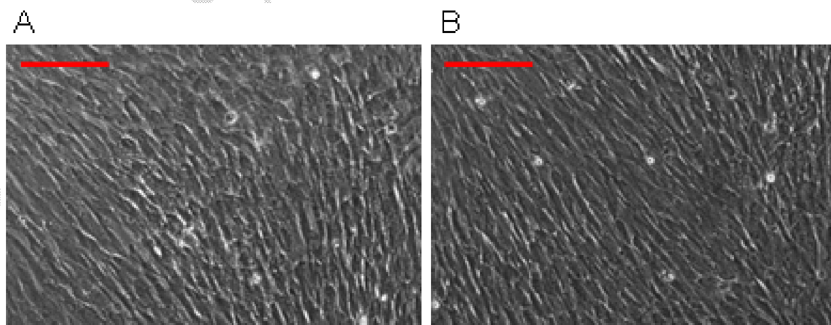
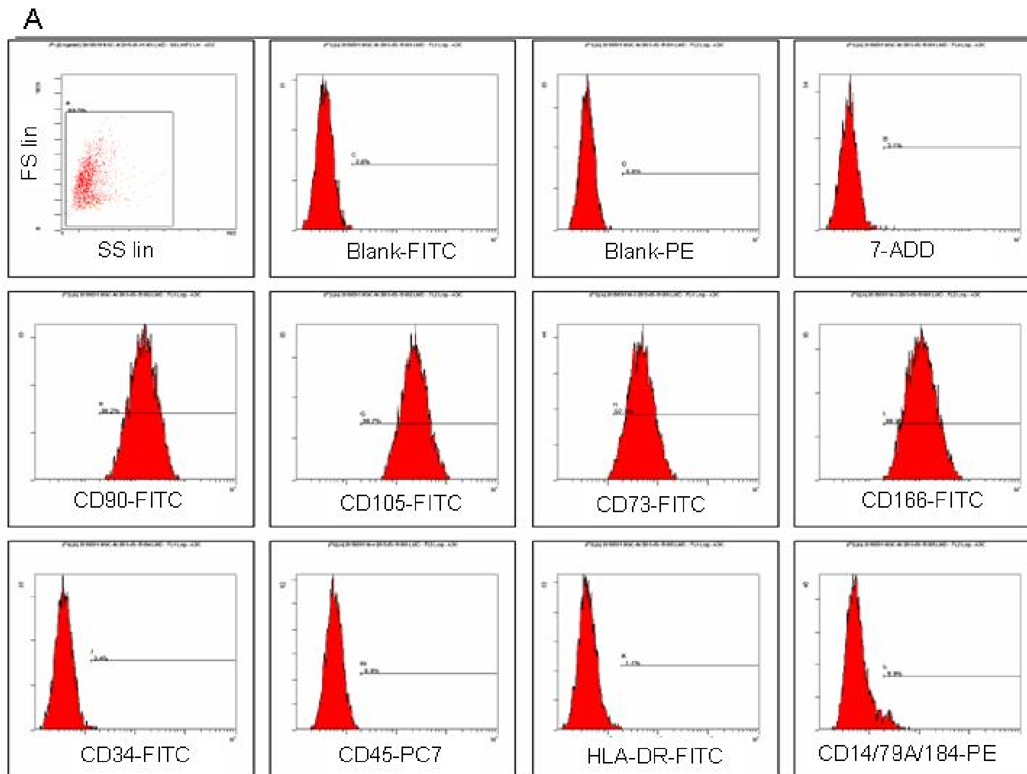


Figure 1. The representative microscopic images of the male and female hUC-MSCs. A: male; B: female. The scales inside the images represent 100 μm

Then, the cellular surface antigens were analyzed with the flow cytometry. The representative results were shown in Fig.2: the hUC-MSCs of both genders had high expression levels for CD90, CD105, CD73 and CD166, meanwhile they had very low

expression levels for CD34, CD45, HLA-Dr and CD14/79A/184, which conformed to the publicly accepted cellular surface antigen characteristics of the hUC-MSCs.



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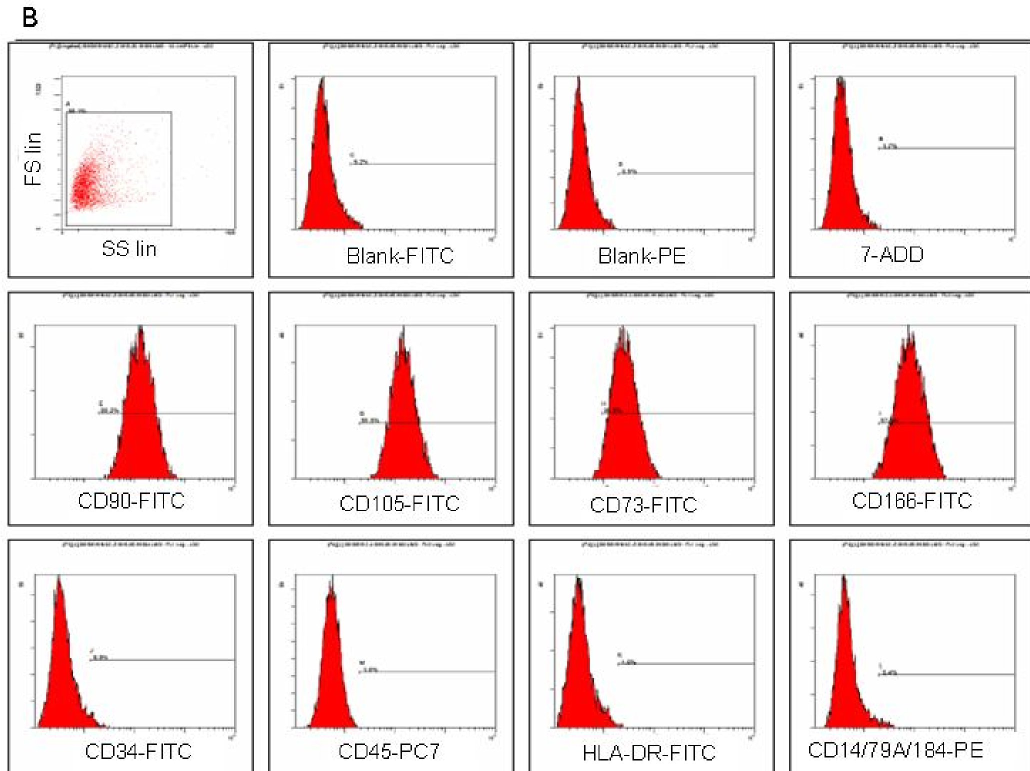

Figure 2. The representative images of the identification of the male and female hUC-MSCs by flow cytometry. A: male; B: female

Finally, the differentiation potentials of the prepared hUC-MSCs toward the adipocyte, the chondrocyte, and the osteocyte were analyzed with special kits. The representative results were shown in Fig.3: the hUC-MSCs of both genders both demonstrated the differentiation potentials toward the adipocyte (red parts), the chondrocyte (blue parts), and the osteocyte (red parts), which conformed to the publicly accepted differentiation potentials of the hUC-MSCs.

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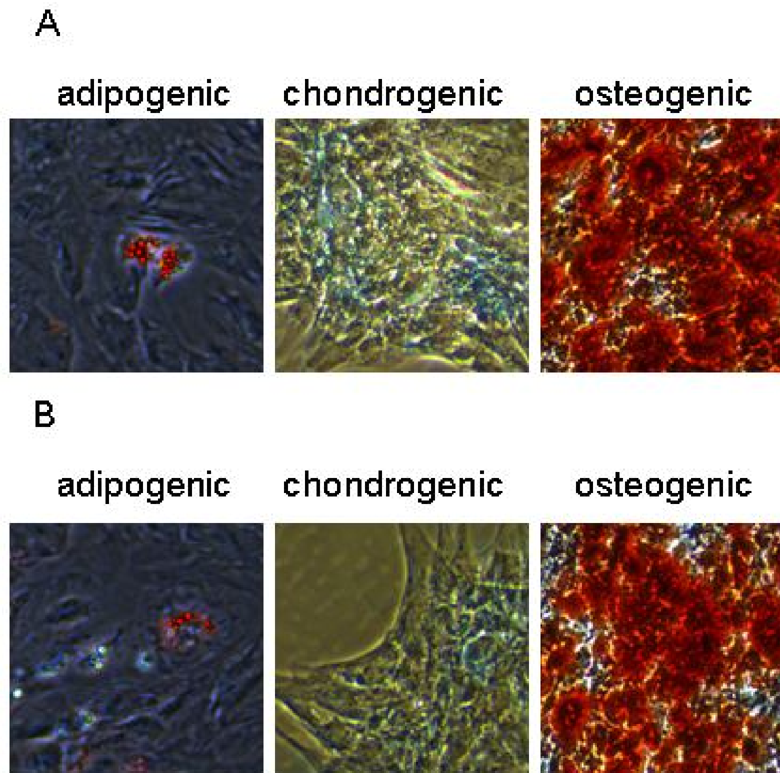

Figure 3. The representative images of the identification of the differentiation potentials for the male and female hUC-MSCs. A: male; B: female

In summary, according to the results of the cell morphology, the cellular surface antigens and triple differentiation potentials, the prepared cells were real hUC-MSCs.

The proliferation rate comparison between the male and the female hUC-MSCs

The proliferation rates of the hUC-MSCs of both genders were estimated with the MTT assay for six times consecutively. The estimating method for the proliferation rates was shown in Fig.4: according to the method previously described, the plot of scattered dots was drawn in the software Excel; then the trend line and the formula were added; the slope in the formula was used as the estimate of the proliferation rate. For example, in Fig.4, the proliferation rate estimate of the male hUC-MSCs was 0.2106 and the proliferation rate estimate of the female hUC-MSCs was 0.1353. The comparative result for the six consecutive times was shown in Table 2: the data were analyzed with the paired t test and the P value is 0.0363 (< 0.05), so it could be said that under the same preparative conditions, the proliferation rate of the male hUC-MSCs was higher than that of the female hUC-MSCs. In order to eliminate the influence of maternal age, the ages of the mothers were investigated, and the age was not the factor affecting the proliferation rate.

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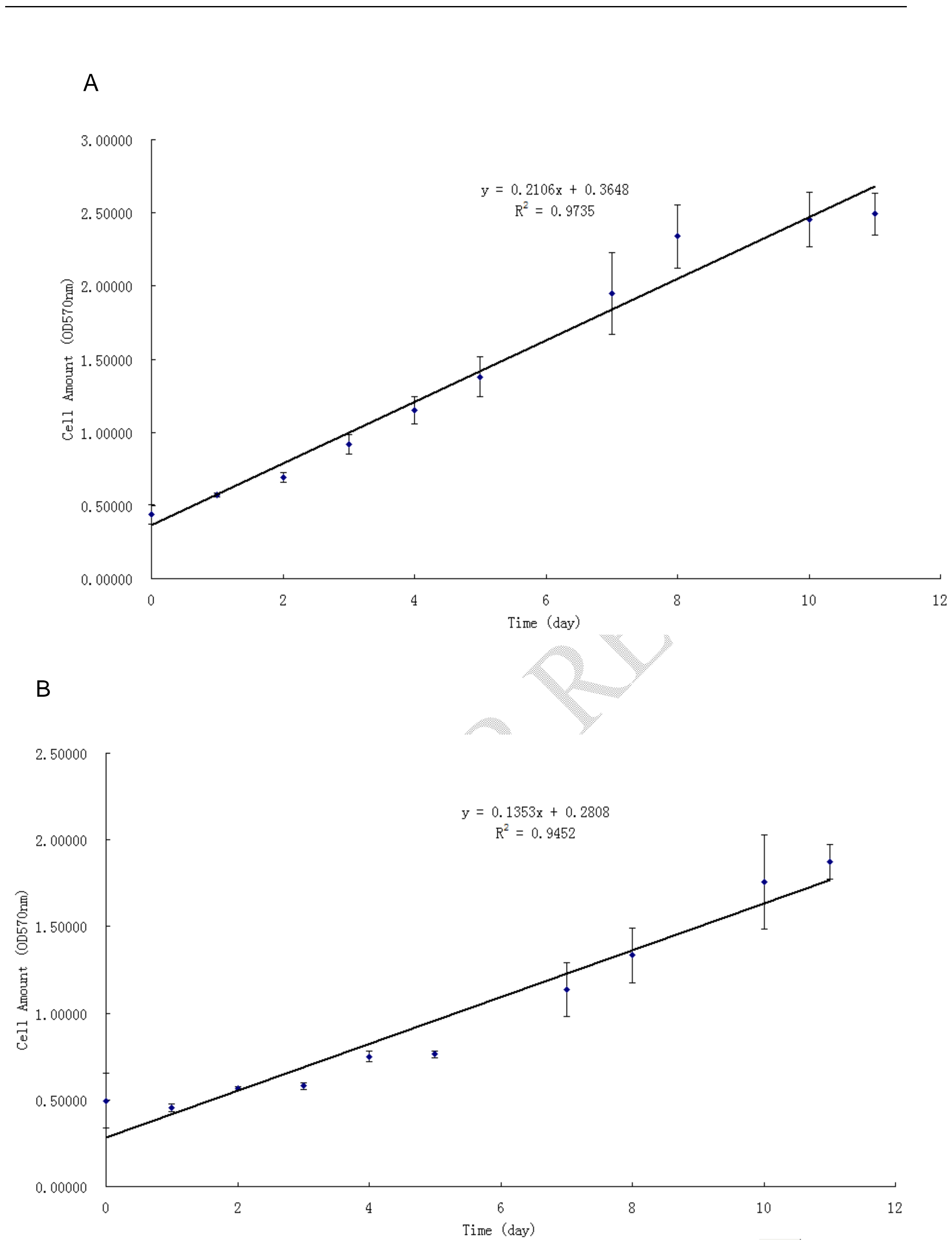

Figure 4. The representative images of the analyses of the growth rates for the male and female hUC-MSCs. A: male; B: female

Table 2. The comparison of the proliferation rates of the male and the female hUC-MSCs in the consecutive 6 times

Repeat	The proliferation rate (OD 570nm/day)	
	The Male	The female

	hUC-MSCs	hUC-MSCs
1st time	0. 0931	0. 0680
2nd time	0. 1606	0. 0853
3rd time	0. 0538	0. 0472
4th time	0. 2106	0. 1353
5th time	0. 0996	0. 0778
6th time	0. 1138	0. 1016
7th time	0. 2122	0. 113
8th time	0. 1398	0. 1045
9th time	0. 1404	0. 1032
10th time	0. 1356	0. 0995
11th time	0. 1239	0. 0902
12th time	0. 1397	0. 1056
13th time	0. 1794	0. 1204
14th time	0. 1644	0. 1129
$P = 0.0363$ ($P < 0.05$)		

The transcriptional express level comparison of the proliferation-related genes between the male and the female hUC-MSCs

The transcriptional expression levels of the selected 12 proliferation-related genes were representatively compared between the male and the female hUC-MSCs of the 6th preparation. The up-regulation of those 12 genes was beneficial to the cellular proliferation. The primer sequences were listed in Table 1 and the measurement results were shown in Table 3: for most of the genes, the male hUC-MSCs were significantly higher than the female counterpart, which preliminarily demonstrated the possible cause for the difference in the proliferation rates between the male and the female hUC-MSCs.

Table 3. The comparison of the transcriptional expression levels of the proliferation-related genes from the male and the female hUC-MSCs

Gene	Male *vs.* female	*P* value
E2F1	↓ 80%	0.2627
MYBL2	↑ 189%	0.0025
BUB1	↑ 64%	0.0939
PLK1	↑ 140%	0.0001
CCNE1	↓ 49%	0.0043
CCND1	↓ 28%	0.0003

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CCNB1	↑ 467%	0.0002
STK1	↑ 41%	0.0051
FOXM1	↑ 69%	0.0312
MKI67	↑ 145%	0.0000
MCM	↑ 75%	0.0003
PCNA	↑ 120%	0.0001

Discussion

This research, for the first time, investigated the difference of the proliferation rate between the male and the female hUC-MSCs and the cause on the gene level was preliminarily clarified.

In the summary of the data on the proliferation rates of the male and the female hUC-MSCs, the paired t test method was used because: (1) the preparative conditions were similar for the cells in the same batch, but they might be different for the cells in different batches, so the independent sample t test method could not be used; (2) the conditions were similar for the cells in the same batch and they could be paired.

Currently, there are no reports about the difference between the male and the female hUC-MSCs and there are only some reports about the gender difference for other types of mesenchymal stem cells: Shamsul et al. compared the age and gender factors on the proliferation of the human bone marrow mesenchymal stem cell, and found that under the age of 40, the proliferation of the human derived marrow mesenchymal stem cell had no gender difference in [8]; Zanotti et al. compared the bone formation in the mice of different gender, and found that the bone formation in mice had gender difference [9]; Hong et al. investigated the effects of the steroid hormones on the proliferation and the bone differentiation of the bone marrow mesenchymal stem cell of rats of different gender, and found the relative responsiveness to steroid hormones had gender difference [10]. From the above reports, it was shown that the researchers already began to notice the gender difference of mesenchymal stem cells. But there were not many such reports and the investigational depth of the few existent reports was not deep enough. This research was the first report for the gender difference of the hUC-MSCs.

The selected 12 proliferation-related genes were publicly recognized [7]. From the obtained expression pattern, it was shown that the male hUC-MSCs had higher expression levels than the female hUC-MSCs for most of those 12 genes, which preliminarily clarified the possible molecular mechanism of the difference in the proliferation rates between the male and the female hUC-MSCs.

The results of this study might have an important reference value for the stem cell therapy and might prompt the operator to treat the male and female hUC-MSCs differently. Based on the findings in this research, during the stem cell therapy process using hUC-MSCs, in one hand, the male hUC-MSCs can be selected in purpose to obtain the relative faster amplification of hUC-MSCs, in the other hand, some special substances may be added to the cell culture to accelerate the amplification of the

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female hUC-MSCs in the situation that the female hUC-MSCs must be used. Certainly, the above potential applications must base on the premise that the functions and the therapy effects of the hUC-MSCs were not altered. It will be the next research direction to explore which special materials will be used to increase the proliferation rate of the female hUC-MSCs but not to change its functions.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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