Abstract: The objectives of this study were to screen for the better Oligonucleotide (ODN) that promotes the proliferation of human bone-marrow mesenchymal stem cells (hBMSCs) and to investigate the mechanism of action of the ODN that has the greatest effect on the hBMSCs cell cycle. hBMSCs were isolated, cultured to the third passage and subjected to osteogenic, adipogenic and chondrogenic induction in order to examine their capacities for multi-differentiation. hBMSCs were seeded at different plating densities (3.0×10³/cm², 6.0×10³/cm², 1.2×10⁴/cm², 2.4×10⁴/cm²) and tested for seven consecutive days to determine the better plating density. A total of 12 experimental groups and 1 control group of hBMSCs (4 replicate wells in each group) were established and treated with ODN types MT01, FC003, or SAT05f at concentrations of 0.5mg/l, 1.0 mg/l, 2.0 mg/l or 4.0 mg/l; the control group was treated with an equal volume of PBS. Proliferation of hBMSCs was determined for 3 consecutive days after treatment via CCK-8 assay. The type and concentration of ODN that had a significant facilitatory effect on hBMSCs proliferation was selected and cell cycle analysis was done on days 1, 2 and 3 after ODN treatment; control groups were treated with an equal amount of PBS. The expressions of cyclin A, cyclin D1, cyclin dependent kinase (CDK) 2 and CDK 4 in hBMSCs were measured on day 2 after treatment using fluorescent quantitative real-time PCR. The isolated and cultured hBMSCs were found to have osteogenic, adipogenic and chondrogenic differentiation capacities. The better cell growth curve was found to occur at a plating density of 6.0×10³/cm². Optical density was significantly increased in hBMSCs treated with 0.5 mg/L FC003 on day 1 (P<0.01) compared to the control group. Optical density was significantly decreased on day 1 after treatment with 1.0 mg/L and 4.0 mg/l of SAT05f (P<0.01 and P<0.05, respectively). Optical density was significantly increased on days 1, 2 and 3 after treatment with a final concentration of 2.0 mg/L of MT01 (P<0.01, P<0.05, P<0.05, respectively). The percentage of cells in phase G0/G1 was significantly reduced, and the percentage of cells in phases S and G2/M was significantly increased (P<0.01) after treatment with 2.0 mg/l MT01 compared to the control group. Furthermore, the expressions of cyclin A, cyclin D1, CDK 2 and CDK 4 were significantly elevated (P<0.01) compared with the control group. In conclusion, a 2.0 mg/l concentration of MT01 significantly promotes hBMSCs proliferation as evidenced by the decrease in the percentage of cells in phase G0/G1 and the increase in the percentage of cells in phases S and G2/M. The underlying molecular mechanisms may include, but are not limited to, elevated expressions of cyclin A, cyclin D1, CDK 2 and CDK 4.

Keywords: Human Bone-marrow mesenchymal stem cells (hBMSCs), Proliferation, Oligonucleotide (ODN),

Introduction

Human bone-marrow mesenchymal stem cells (hBMSCs) are extensively investigated since they are easily cultured in vitro and have the capacities to proliferate and differentiate into multiple lineages. In addition hBMSCs are thought to be the ideal seed cells since they have few antigenic effects and a strong ability for tissue repair. In addition, because they are autologous, tissues obtained from differentiated hBMSCs will not cause immune rejection after transplantation. Therefore, hBMSCs are highly promising for use in clinical applications. Studies have demonstrated the value of hBMSCs for the diagnosis and treatment of cardiovascular, nervous, motor, digestive, autoimmune, circulatory and urinary system diseases, as well as for diseases of the eyes and bones. Finding an efficient way to increase proliferation of hBMSCs in vitro is imperative in order to reduce the amount of autologous bone marrow that must be collected.
Main consumable materials and reagents

The hBMSCs were a gift from the Research Center of Plastic Surgery Hospital, Chinese Academy of Medical Sciences. Human bone marrow aspirates were harvested from the iliac crest of patients who underwent alveolar cleft correction surgery (n = 4). This study was approved by the ethic committee of plastic surgery at CAMS, PUMC, and informed consent from all of the patients was obtained. The ODNs (MT01, FC003, SA T05f) were a gift from Professor Liying Wang, Jilin University School of Basic Medicine, and were synthesized by TaKaRa Biotechnology (Dalian) Co., Ltd.; CCK-8 (Dôjindo Laboratories, Japan); fluorescein isothiocyanate (FITC) anti-human CD73, CD90, CD105, CD34, CD45, CD19, CD116, human leukocyte antigen (HLA)-DR stain (eBioscience, USA); Osteogenic induction medium (DMEM complete medium containing 10^{-8} \text{mol/l} \text{dexamethasone}, 10^{-2} \text{mol/l} \text{-glycerol phosphate} \text{and} \ 50 \text{mg/l} \text{vitamin} \text{C}), adipogenic induction medium (containing 0.5×10^{-3} \text{mol/l} \text{3-Isobutyl-1-methylxanthine}, 10^{-5} \text{mol/l} \text{insulin}, 2.0×10^{-4} \text{mol/l} \text{indomethacin}, 10^{-3} \text{mol/l} \text{dexamethasone}), chondrogenic induction medium (LG-DMEM containing 10 \% \text{FBS}, 1.0 \% \text{penicillin-streptomycin}, 10^{-7} \text{mol/l} \text{dexamethasone}, 10^{-2} \text{mg/l} \text{TGF-\beta}1, \text{vitamin} \text{C} 50 \text{mg/l}, 40 \text{mg/l} \text{proline}, 1×\text{ITS}); Muse Cell Cycle Kit (Merck Millipore, USA), primers for cyclin A, Cyclin D1, CDK 2, CDK 4 (TaKaRa Biotechnology (Dalian) Co., Ltd., China).

Methods

Isolation, culture and identification of hBMSCs

hBMSCs were collected from healthy adults via density gradient centrifugation. Cell morphology was observed at different phases. Third-passage hBMSCs were seeded at 1.0×10^4/cm^2 in 6-well plate, and osteogenic, adipogenic or chondrogenic induction medium was added. The medium was changed every 72 h. After 2-3 weeks, the cells were subjected to Alizarin red, Oil Red O and toluidine blue staining. Stem cell surface molecules were identified using flow cytometry.

Establishment of hBMSC better plating density

Third-passage hBMSCs were seeded at concentrations of 3.0×10^3/cm^2, 6.0×10^3/cm^2, 1.2×10^4/cm^2, or 2.4×10^4/cm^2 in a 96-well plate. A volume of 200 μl of complete medium was then added to each well and the medium was changed on day 4. Optical density was determined for 7 consecutive days using a microplate reader (test wavelength 450 nm, reference wavelength 630 nm) and the cell growth curves were analyzed to determine the better plating density.

Comparison of the effects of three ODNs at four concentrations on hBMSCs proliferation (Table1)

Third-passage hBMSCs were seeded at the better plating density in a 96-well plate and 200 μl of complete medium was added to each well. A total of 12 experimental groups were established based on ODN type (MT01, FC003, SAT05f) and final concentration (0.5 mg/l, 1.0 mg/l, 2.0 mg/l, 4.0 mg/l). There were four replicate wells in each group. The control group was treated with an equal amount of PBS. The CCK-8 assay was performed as above for three consecutive days in order to determine which ODN had the greatest effect on the proliferation of hBMSCs.

The effect of ODN MT01 on the hBMSCs cell cycle

Third-passage hBMSCs were seeded at the better plating density in a 6cm petri dish. After the cells adhered to the wall, 2.0 mg/l MT01 was added to the dish; control cells were treated with an equal volume of PBS. Cell cycle analysis was done on days 1, 2 and 3. Sample handling and detection were conducted according to the Muse™ Cell Analyzer kit instructions.

Molecular mechanism(s) underlying the effect of MT01 on hBMSCs proliferation (Table2)

hBMSCs were treated as in 1.3.4. Sample handling and detection were conducted according to the LightCycler@480 fluorescent quantitative real-time PCR analyzer kit instructions. Cyclin A, cyclin D1, CDK 2 and CDK 4 expressions were measured on the second day after the addition of MT01.

Statistical analysis

Statistical differences were compared by one-way ANOVA analysis and defined as p < 0.01 or p < 0.05.

Results

hBMSCs isolation and culture

The hBMSCs that had been isolated, purified and cultured using density gradient centrifugation grew well. The third-passage hBMSCs were of spindle shape and were arranged like vortices after a significant amount of proliferation (Fig. 1-A).

Multiple induction of hBMSCs and detection of stem cell surface molecules

A large amount of range-mineralized nodules were present in the petri dish when the fixed cells were stained with Alizarin red, Oil Red O and toluidine blue staining. Stem cell surface molecules were identified using flow cytometry.

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A large amount of range-mineralized nodules were present in the petri dish when the fixed cells were stained with Alizarin red after undergoing 21 days of osteogenic induction (Fig. 1-B). A large number of dark red lipid droplets formed when cells were stained with Red O Oil after undergoing 15 days of adipogenic induction (Fig. 1-C). Light blue metachromatic granules were present in the cytoplasm when the fixed cells were stained with
Figure 1 A. Third-passage hBMSCs. B. Alizarin red staining after 21 days of osteogenic induction. C. Oil Red staining after 15 days of adipogenic induction. D. Toluidine blue staining after 21 days of chondrogenic induction.

Better plating density of hBMSCs on a 96-well plate
The growth curve for hBMSCs was better at a plating density of 6×10^3/cm^2 (Fig. 3-A).

The effects of four different concentrations of three types of ODNs on hBMSCs proliferation
Optical density was significantly higher on day 1 after treatment with 0.5 mg/L FC003 (Fig. 3-B, P<0.01) compared to the control group. Conversely, optical density was significantly lower on day 1 after treatment with 1.0 and 4.0 mg/L SAT05f (Fig. 3-C, P<0.01 and P<0.05, respectively). Optical density was significantly higher on all 3 days after treatment with 2.0 mg/l MT01 (Fig. 3-D; day 1, P<0.01; day 2 and 3, P<0.05).

The effect of MT01 on the hBMSCs cell cycle (Table 3)
The percentage of cells in phase G0/G1 was significantly lower and the percentage of cells in phases S and G2/M was significantly higher (P<0.01) on day 1, 2 and 3 after treatment with 2.0 mg/l MT01, compared with the control group.

The molecular mechanisms underlying the effect of MT01 on hBMSCs proliferation
The expressions of cyclin A, cyclin D1, CDK 2 and CDK 4 were significantly elevated in the group treated with MT01 compared with the control group (P<0.01)(P<0.01)(Fig. 4-A,B,C,D).

Toluidine blue after undergoing 21 days of chondrogenic induction. The extracellular matrix was also stained light blue (Fig. 1-D).

The positive expression rates of CD90, CD73 and CD105 were 99.6 %, 99.9 % and 96.5 %, respectively (Fig. 2-A,B,C). The overall positive expression rate of CD34/45/116 was 0.51 % (Fig. 2-D).

Figure 2. A/B/C/D. Results of detection of stem cell surface molecules

Figure 3. A/B/C/D. Results of detection of stem cell surface molecules
Figure 3. A. Growth curves of hBMSCs at different plating densities; B. The effect of four different concentrations of ODN FC003 on hBMSCs proliferation (**: $P<0.01$, *: $P<0.05$); C. The effect of four different concentrations of ODN SAT05f on hBMSCs proliferation (**: $P<0.01$, *: $P<0.05$); D. The effect of four different concentrations of ODN MT01 on hBMSCs proliferation (**: $P<0.01$, *: $P<0.05$).

Table 1. ODNs (3 different sequences) were co-cultured with hBMSCs to screen for those that increased the proliferation of cells.

<table>
<thead>
<tr>
<th>Number</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MT01</td>
<td>5'-ACCCCTCTACCCCTCTACCCCTCT-3'</td>
</tr>
<tr>
<td>2</td>
<td>FC003</td>
<td>5'-TCTCTCTCTCTCTCTCTCTCTCT-3'</td>
</tr>
<tr>
<td>3</td>
<td>SAT05f</td>
<td>5'-CCTCCTCCCTCTCCCTCTCCCTCT-3'</td>
</tr>
</tbody>
</table>

Table 2. The primers used for qRT-PCR of various genes.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Oligonucleotide UP (5'-3')</th>
<th>Oligonucleotide DW (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>ATG GGG AAG AGT AAG GTC</td>
<td>TAA AAG CAG CCC TGG TGA CC</td>
</tr>
<tr>
<td>CYCLIN-A</td>
<td>AAG TTT GAT AGA TGC TGA CCC</td>
<td>TGG AGG TAG GTC TGG TGA AG</td>
</tr>
<tr>
<td>CYCLIN-D1</td>
<td>GAC TCT CAT TCG GGA TGA TTG GA</td>
<td>TTT GGT TCG GCA GCT TGC TA</td>
</tr>
<tr>
<td>CDK-2</td>
<td>TAT GGC TGA TTA CAA GCC CAG</td>
<td>GAA ATC CGC TGA TTA GGG TC</td>
</tr>
<tr>
<td>CDK-4</td>
<td>TTC TGC AGT CCA CCA CAT ATG CAA CA</td>
<td>GGT CGG TCT CAG AGT TTC CAC</td>
</tr>
</tbody>
</table>

Table 3. The effect of ODN MT01 (final concentration 2 mg/L) on the hBMSCs cell cycle

<table>
<thead>
<tr>
<th></th>
<th>1 d (%)</th>
<th>2 d (%)</th>
<th>3 d (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>54.2±0.23</td>
<td>51.7±0.28''</td>
<td>53.6±0.16''</td>
</tr>
<tr>
<td>G0/G1</td>
<td>15.2±0.08</td>
<td>16.5±0.17''</td>
<td>15.9±0.61''</td>
</tr>
<tr>
<td>S</td>
<td>30.6±0.18</td>
<td>31.8±0.11''</td>
<td>30.5±0.38''</td>
</tr>
<tr>
<td>G2/M</td>
<td>30.5±0.38''</td>
<td>26.5±0.62</td>
<td>12.7±0.27</td>
</tr>
</tbody>
</table>

**: compared to the control group, $P<0.01$

**Discussion**

hBMSCs are an important type of seed cells that are used in tissue engineering. They have multi-differentiation potential and are of critical significance for the development and regeneration of tissue. The concentration of BMSCs in bone marrow is only about $10^3$-$10^4$ that of nucleated cells, hence, the isolation, purification and in vitro amplification of BMSCs are especially important. BMSCs of relatively high purity can be obtained using...
density gradient centrifugation for isolation\(^1\), therefore, density gradient centrifugation was used to isolate and purify hBMSCs in the present study. Identification of hBMSCs was done per the standards proposed by Dominici\(^1\) and published in Cytotherapy in 2006. These are thought to represent the semi-official views of the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy. These standards are as follows: (1) Under standard culture conditions, the MSCs can be affixed to a plastic wall and grow; (2) the MSCs can express CD105, CD73 and CD90, but do not express CD45, CD34, CD14, CD11b, CD79 alpha, CD19 or HLA-DR surface molecules; and (3) the MSCs can differentiate into osteoblasts, fat cells and chondrocytes. The hBMSCs used in the current study met the above standards. The stem cell characteristics were validated morphologically and they could be induced to multi-differentiate. A previous study that examined the influence of \(\beta\)-hydroxybutyrate on the activity of bovine abomasum smooth muscle cells found that, compared to the MTT assay, the CCK-8 assay had a lower rate of data deviation and was more accurate and sensitive. Therefore in the present study, the optical density was determined via CCK-8 assay, using a microplate reader at a test wavelength of 450 nm and a reference wavelength of 630 nm.

Oligodeoxynucleotides (ODNs) containing unmethylated nucleotide motifs are immunostimulatory in vertebrates, and some ODNs containing CpG motifs are used for treating cancer, virus-associated diseases, and infections\(^14-17\). Recently, specific ODNs were found to have an effect on modulating osteoclast- and osteoblast-lineage cells\(^11\). In previous studies\(^6-8\), the specific ODN that could promote the proliferation of cells was examined using a single ODN concentration (1.0 mg/l), however these studies did not explore the ODN mechanism of action. Based on these previous reports, it has been hypothesized that certain ODNs may affect the proliferation of hBMSCs. Three types of ODNs (MT01, FC003, SA T05f) were tested at different concentrations (0.5 mg/l, 1.0 mg/l, 2.0 mg/l, 4.0 mg/l) and it was determined that 2 mg/l of MT01 most significantly promoted the proliferation of hBMSCs. To test this hypothesis, density gradient centrifugation was applied to isolate and culture hBMSCs. These hBMSCs then underwent morphological examination and multi-differentiation induction to verify stem cell characteristics. In addition, hBMSCs were seeded in 96-well plate at four different plating densities to determine the better plating density by comparing the growth curve, in order to lay the foundation for subsequent experiments. Then tests on three types of ODN (MT01, FC003, SA T05f) at four final concentration (0.5 mg/l, 1.0 mg/l, 2.0 mg/l, 4.0 mg/l) and one control group (equal amount of PBS added) showed that compared to the control group, the experimental group with ODN MT01 at a final concentration of 2.0 mg/l added exhibited significantly higher optical density on all three days after adding ODN.

Cell division is the basis for the formation of biological organisms and tissue growth. It plays a key role in maintaining normal tissue, repairing tissue damage and in tissue regeneration. Through evolution, eukaryotic cells developed a complex network, called the cell cycle regulation system, which consists of a variety of proteins and controls cell cycle progression. Cyclin and CDK complexes are central to the cell cycle regulation system. Their cyclic formation and degradation trigger specific events in cell cycle progression and contribute to the irreversible conversion from phase G\(_1\) to phase S, from phase G\(_2\) to phase M and from the middle phase to the late phase\(^19\). A previous report showed that after hBMSCs were incubated with erythropoietin (EPO), the cell proliferation index was significantly increased in a dose and time dependent manner. The effects on the proliferation of hBMSCs were the highest in the group treated with 5 U/ml. Compared with the control group, EPO significantly decreased the percentage of G\(_2\)/G\(_1\) cells and increased the percentage of S and G\(_2\)/M cells. EPO promotes more hBMSCs to enter the DNA synthesis stage leading to the production of many proliferative cells in a dose and time dependent manner\(^19\). The results of the present study showed that the percentage of G\(_2\)/G\(_1\) cells in the experimental group was lower, whereas the percentage of S and G\(_2\)/M cells was higher, compared to the control group. These results are in agreement with a previous report. Thus, it appears that MT01 promotes hBMSCs proliferation by influencing regulatory factors related to the cell cycle.

CDKs are serine/threonine kinase family members and play important roles at the beginning and throughout different stages of the cell cycle. During the cell cycle, cyclin/CDK complexes are activated to control the progression of the entire cell cycle. Cyclin D/CDK4 or CDK 6 controls the progression of phase G\(_1\); cyclin E/CDK 2 controls the transition from phase G\(_1\) to phase S;
and cyclin A/CDK 2 plays an important role in the S phase and in the DNA replication process. In the current study, the expressions of cyclin A, cyclin D1, CDK 2 and CDK 4 were significantly higher in the MT01 group, compared with the control group. Therefore, the potential mechanisms underlying the facilitatory effect of MT01 on hBMSCs proliferation may include, but are not limited to, an elevated amount of cyclin D1/CDK 4 complex that shortens phase G, or an elevated amount of cyclin A/CDK 2 complex that shortens phase S. This is consistent with the cell cycle analysis results. However, the possibility that MT01 may also regulate other relevant factors cannot be ruled out.

In summary, MT01 appears to promote hBMSCs proliferation by regulating factors related to the cell cycle. These results shed new light on, and provide a preliminary theoretical basis for, in vitro amplification of seed cells for tissue engineering. However, many more studies still need to be performed before practical application of MT01.

Acknowledgements

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References


