Research Paper

The effect of OTX2 gene on the proliferation and differentiation of hFOB1.19 Osteoblasts by RNA interference

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ABSTRACT

The aim of this experiment is to down-regulate the expression of OTX2 gene and investigate the effect on the proliferation and differentiation of hFOB1.19 osteoblasts by RNA interference. According to OTX2 gene sequences in GenBank, we designed three target sequence siRNA and divided the experiment into 6 groups: synthesized OTX2-siRNA1 group, OTX2-siRNA2 group, OTX2-siRNA3 group, GAPDH-siRNA group, negative control group and blank control group. hFOB1.19 osteoblasts were maintained in incubator. Chemically synthesized OTX2-siRNA was transfected into the osteoblasts under the liposome mediation. The cells were collected after 24, 48 and 72 h respectively. 24 h after transfection, Trizol method was used to extract total RNA in cells, and ultraviolet spectrophotometer method used to quantify and detect the purity. The OTX2 mRNA level was detected by reverse transcription polymerase chain reaction (RT-PCR) after transfection. The products were separated by electrophoresis on agarose gel; total protein was extracted and quantified by Brandford assay, and the protein level detected by western blot. According to the results of RT-PCR and Western Blot experiment, OTX2-siRNA with the highest transfection efficiency was selected for subsequent tests. MTT assay was used to evaluate the proliferation inhibition of OTX2-siRNA on osteoblast; chemical colorimetry to detect the synthesis of alkaline phosphatase (ALP) 24 h after transfection. RT-PCR showed: OTX2 mRNA level lowered in synthesized OTX2-siRNA group and the OTX2-siRNA1 group lowered the most. Agarose gel electrophoresis showed that the band in experimental group was obviously weakened as compared with control groups (P<0.05). Western Blot showed: OTX2-siRNA down-regulated the transcription of OTX2 protein. The OTX2-siRNA group had the lowest protein expression level. There was significant difference between the experimental group and control group (P<0.01). Cell proliferation experiment results showed floating cells in OTX2-siRNA group were more than the control group obviously observed by inverted microscope; MTT experiment showed cell proliferation inhibition rate of OTX2-siRNA group was higher than the control group; there was significant difference between them. Alkaline phosphate detection results showed the alkaline phosphatase activity of OTX2-siRNA group was decreased compared with the control group (P<0.05). In conclusion, the chemically synthesized OTX2-siRNA effectively restrained mRNA and protein expression level of OTX2 gene in hFOB1.19 osteoblasts. Also, interference of OTX2 gene can inhibit the proliferation of hFOB1.19 osteoblasts. Finally, down-regulated expression of OTX2 gene can reduce the alkaline phosphate activity of hFOB1.19 osteoblast and inhibit differentiation.

Key words: hFOB1.19 Osteoblasts, OTX2, siRNA, RNAi, gene silencing.
INTRODUCTION

It is known that neural crest cells are specific pluripotent stem cells and can differentiate into different tissues and components including dental mesenchymal cells, osteoblasts, chondroblasts, so as to induce the occurrence of tissues and organs such as tooth and bone. Mammalian cranial neural crest cells migrate to the front nose and the first gill arch (mandibular arch) to form Meckel cartilage, the maxilla, mandible and other craniofacial structures. Therefore, the study of neural crest cells helps to clarify the molecular mechanisms of mandible development in genetic skeletal Class III crossbite.

OTX2 is one of the OTX family members, and is related to the development of the neural crest brain, mesencephalon and the diencephalon (Pantó et al., 2004) and promotes the development of the visual system such as the retina (Sakami et al., 2005; Kurokawa et al., 2004; Viczian et al., 2003). Furthermore, neural crest cells and the OTX2 gene expressions are involved in the formation of the mandibular distal region (Kimura et al., 1997; Simeone et al., 2001). OTX2 gene mutations can cause little or no jaw deformity (Herman et al., 2012; Shala et al., 2000), which reminds us that skeletal Class III due to excessive growth of the mandible is caused by high expression of OTX2 in osteoblasts.

RNA interference technique can specifically remove or turn off the expression of specific genes that cause gene silencing, which is an important way of regulation of gene expression as a self-protection mechanism on the level of gene regulation. Gene silencing was available in gene regulation. Gene silencing was an important way of regulation of gene expression. It can turn off the expression of specific genes that cause gene silencing, which is an important way of regulation of gene expression. Therefore, the study of neural crest cells helps to clarify the molecular mechanisms of mandible development in genetic skeletal Class III crossbite.

MATERIALS AND METHODS

Cell culture and transfection

The experiments were divided into six groups: synthesized OTX2-siRNA1 group, OTX2-siRNA2 group, OTX2-siRNA3 group, GAPDH-siRNA group, negative control group and blank control group. siRNA gene sequences were taken from GenBank; three target sequences designed by the Shanghai GenePharma Technology Co., Ltd, the synthetic sequences are:

OTX2-siRNA1 5′-GGGGCGAGGAUGGUAUUAATT-UUAAACCAUACCUGACCCCTT-3′
OTX2-siRNA2 5′-CAUGGACUGGGAUCAUATT-AUAUGUCCACAGUCAUGTT-3′
OTX2-siRNA3

24 h before transfection, osteoblasts were seeded in 6-well plates at 2 × 10^5/ well and 1.5 ml DMEM/F12 serum-free medium containing antibiotics added and attached overnight until the cells reached 80 to 90% confluence. Dilute siRNA (final concentration 33nM) with 250 ul Opti-MEM medium, gently pipetting 3 to 5 times. The transfection reagent was gently mixed and 5.0 μl dilute Lipofectamine TM 2000 with 250 μl Opti-MEM medium and pipetted 3 to 5 times gently and kept at room temperature for 5 min. The transfection reagent and SiRNA dilution was mixed, pipetted 3 to 5 times gently and allowed to stand at room temperature for 20 min. The transfection complex was then added to a 6-well cell plate in 37°C, 5% CO₂ incubator.

Reverse Transcription (RT)-PCR Analysis

Total RNA was extracted by TRIZOL reagent. First-strand cDNA synthesis was performed with reverse transcriptase kit. The PCR primers 5′-GATGTCGAGAGCAACTGGTTG-3′ (upstream) and 5′ GACCTCATCTGCTGTGTTG -3′ (downstream) were used. PCR reaction conditions: 94°C 1 min, 94°C 30 s, 55°C 30 s, 72°C 1 min, 30 cycles, 72°C 5 min. 5 μl PCR products were mixed with a 1 μl sample buffer for 2% agarose gel electrophoresis and ultraviolet photography scan analysis taken. To the average ratio of OTX2 and GAPDH absorbance values to semi-quantitatively reflected OTX2 mRNA expression of strength (Table 1).

Western Blot detection of protein expression levels

The total protein was extracted 24 h after transfection by Bradford and mixed with sample buffer solution. 15% min, 5 min, 30 g, 5 Marker, 80V, 100V, min, 50 PBS, 1 * Bio-Rad were used. Stay overnight at 4C; closed liquid drained, joined Western washing solutions for 5 to 10 min, adding an anti hybrid shaking at room temperature for 2 h in appropriate proportions by Western anti dilution in buffer solution in closed, were incubated for 60 min; membrane washing liquid washing 3 times, every 10 min; using ECL reagents developing, fixing to detect protein expression. Stern and Blot RT-PCR were selected to carry out the following experiments. The best transfection efficiency of siRNA was detected.

MTT observed proliferation of osteoblasts

Osteoblasts were trypsinized after transfection, the cell suspension prepared in 2 × 104 ml were plated in 96- well plates, each well 200 ul with three parallel holes.
Table 1. The PCR reaction system.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Values (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>4.1</td>
</tr>
<tr>
<td>5×PCR Buffer</td>
<td>2.0</td>
</tr>
<tr>
<td>TaKaRa Ex Taq HS</td>
<td>0.1</td>
</tr>
<tr>
<td>OTX2 Upstream specific primers</td>
<td>0.5</td>
</tr>
<tr>
<td>OTX2 Downstream specific primers</td>
<td>0.5</td>
</tr>
<tr>
<td>cDNA</td>
<td>2.0</td>
</tr>
<tr>
<td>dNTP</td>
<td>0.8</td>
</tr>
<tr>
<td>Total volume</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Transfected 24, 48 and 72 h was added to each well MTT (5 mg / ml) 20 μl, the incubator set and cultured at 4 h and the supernatant discarded carefully. Dimethyl sulfoxide 150 μl, oscillation 5 min, the crystal was fully dissolved and the absorbance read (OD) at 490 nm using a plate reader. Results were recorded with time as the abscissa absorbance value for the vertical cell growth curve. Cell proliferation inhibition rate = (1 - experimental group, the average OD value / control group mean OD value) × 100%.

Statistical analysis

The experimental data were expressed as mean ± standard deviation (x ± s) with the use of statistical software SPSS 17.0 and multi-parameter groups using ANOVA.

RESULTS

Expression of mRNA OTX2 in osteoblasts after transfection with RT-PCR

The expression levels of mRNA OTX2 were down regulated in the groups of OTX2-siRNA2 and OTX2-siRNA3, respectively (0.196±0.017), (0.331±0.024), (0.235±0.021) and GAPDH-siRNA group (0.523±0.029)(0.496±0.026)(0.539±0.033). Statistical analysis showed that the difference was statistically significant (P<0.05), as shown in Figures 1 and 2; the expression of OTX2 gene was inhibited by targeting OTX2-siRNA gene.

Expression of OTX2 protein in osteoblasts after transfection with Blot Western

The expression of OTX2 protein was down regulated, the positive rate of protein expression in specific OTX2-siRNA group 1 was (22.9 ±1.3)%, OTX2-siRNA group 2 was (30.4 ±2.8)%, OTX2-siRNA group 2 was (24.2 ±1.6)%, blank control group was (93.1 ±1.7)%, negative control group was (91.6 ±2.1)% , GAPDH-siRNA group was (90.5 ±2.8)%. Compared with the control group, the difference was significant (P < 0.01) (Figures 3 and 4). After RT-PCR and Blot Western experiment, the OTX2-siRNA1 was selected for the following experiment.

Proliferation inhibition of osteoblasts by MTT assay

Observation under inverted microscope and 24 h after transfected with specific OTX2-siRNA into some cells of bone cells became round 72 h after transfection, cell
Figure 1. OTX2 - siRNA transfection of osteoblast OTX2 mRNA level. 1. OTX2-siRNA1 group; 2. OTX2-siRNA2 group; 3. OTX2-siRNA3 group; 4. GAPDH-siRNA group; 5. negative control group; 6. blank control group.

Figure 2. Agarose gel electrophoresis. 1. GAPDH-siRNA group; 2. negative control group; 3. OTX2-siRNA1 group; 4. OTX2-siRNA2 group; 5. OTX2-siRNA3 group; 6. blank control group.
shrinkage and floating cells increased; and the blank control group and non-specific siRNA group of most normal cell morphology was adherent good. MTT method was used to detect the inhibitory growth rate (Table 2) and the cell growth curve drawn (Figure 5). The results showed that at each time point in the experimental group,
Table 2. The effect of OTX2-siRNA on cell proliferation.

<table>
<thead>
<tr>
<th>Group</th>
<th>24 h OD inhibition ratio</th>
<th>Percentag (%)</th>
<th>48 h OD inhibition ratio</th>
<th>Percentage (%)</th>
<th>72 h OD inhibition ratio</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank group</td>
<td>0.36±0.05</td>
<td>-</td>
<td>0.69±0.09</td>
<td>-</td>
<td>1.19±0.11</td>
<td>-</td>
</tr>
<tr>
<td>Blank control</td>
<td>0.33±0.03</td>
<td>8.3</td>
<td>0.65±0.09</td>
<td>5.8</td>
<td>1.14±0.04</td>
<td>4.2</td>
</tr>
<tr>
<td>negative control</td>
<td>32±0.03</td>
<td>11.1</td>
<td>0.63±0.07</td>
<td>8.7</td>
<td>1.11±0.06</td>
<td>6.7</td>
</tr>
<tr>
<td>OTX2-siRNA</td>
<td>0.25±0.04*</td>
<td>30.6</td>
<td>0.41±0.03*</td>
<td>40.6</td>
<td>0.76±0.04*</td>
<td>36.1</td>
</tr>
</tbody>
</table>

* compared with control group P<0.05.

Figure 5. hFOB1.19 growth curve.

the bone cells significantly inhibited the proliferation (P < 0.05) as compared with the control group; the control group had no obvious inhibitory effect (P > 0.05).

Expression of alkaline phosphatase (ALP) after transfection of specific OTX2-siRNA cells

Alkaline phosphatase assay (Table 3), the ALP activity in the gene transfection group and the supernatant of cells was significantly lower than that of the control group (P < 0.05), indicating that the expression of OTX2 gene inhibited the activity of osteoblasts.

DISCUSSION

Class III malocclusion is one of the common clinical cranio maxillofacial malocclusion deformity caused by the abnormal size of the upper and lower jaw. Research proves that both skeletal and non-skeletal Class III malocclusion deformities are affected by both genetic and environmental factors. A combination of the two factors can make excessive growth of the mandibular, resulting in mandibular prognathism and crossbite deformity. The study found that Gsc, Gli2, Gli3, DLx5, TGFβ2 genes can affect the morphological development through the different ways in different developmental stage of rat mandibular (Nakamura et al., 2005; Savontaus et al., 2004). Foxo1 and Runx2 synergy affected the development of the rat's lower jaw (Teixeira et al., 2010). The P561T gene can inhibit the development of mandible in the early stage (Sasaki et al., 2009).

OTX2 gene is one of the members of Otx family located in 14q22.3, containing five exons and encoding 297 amino acid. OTX2 is an important functional gene in embryonic development widely expressed in various tissues, such as the brain, eyes and jaw, etc. Gene expression patterns and function research of Otx2 gene in the brain and eye development has been relatively clear, but the role of the
mandible development still needs further study. According to the literature, OTX2 and other genes may affect the formation of the first gill arch, which can affect the development of the mandible (Tian et al., 2011). OTX1 gene belongs to the OTX family, which is widely distributed in bones, nervous system, eyes, and other tissues (Shala et al., 2000). Preliminary experiment shows that OTX1 gene may be a susceptibility gene (Tian et al., 2011). These suggest that the OTX2 gene may be involved in the formation of skeletal crossbite by regulating the development of the mandible.

In this study, RNA interference technology was used to transfected into osteoblasts and the expression of mRNA and protein down regulated. The principle is: siRNA was unwound into positive-sense strand and antisense strand under the action of intracellular RNA helicase. In this experiment, the OTX2-siRNA gene was transfected into osteoblasts and the expression of OTX2 gene down regulated. RT-PCR results showed that the expression of mRNA was significantly decreased by transfection of OTX2-siRNA. Blot Western showed that the expression of OTX2 protein was down regulated by OTX2-siRNA. The expression of mRNA OTX2 and protein was significantly down regulated by OTX2-siRNA. The RNA interference target sequence in this experiment selects has good effect; the results of RNA interference was highly effective and stable, which can be used as an effective tool and means for the study of cell proliferation and osteogenic activity.

MTT is a method for detecting cell survival and growth. The detection principle of succinate dehydrogenase of mitochondria in living cells to exogenous MTT reduction for water insoluble blue purple crystal formazan deposition in cells was carried out but the dead cells were without this feature. Dimethyl sulfoxide to formazan cell lysis and enzyme standard instrument at the wavelength of 490 nm determination of the light absorption value was carried out and the absorbance value was observed to be greater and the cell activity stronger. The results of this experiment showed that the proliferation of osteoblasts was significantly inhibited and the difference was significant in the control group. The expression of OTX2 gene was significantly inhibited by OTX2-siRNA gene expression.

Studies indicated that multiple signal transduction pathways and cytokines were involved in the regulation of osteoblast differentiation such as Wnt, BMP and PI3K/Akt. According to the literature, BMP-2 can promote the expression of the gene of the osteoblast cell alkaline phosphatase, increase its activity and promote the differentiation of osteoblasts (Wang et al., 2011; Liu et al., 2009). Alkaline phosphatase, an enzyme protein secreted by osteoblasts, is a specific marker of osteoblast differentiation and its content can be directly reflected by the function of osteoblasts (Zhang et al., 2011; Canturk et al., 2001; Kojima et al., 2004). Therefore, the effect of OTX2-siRNA interference on the differentiation of osteoblasts was evaluated by detecting the content of alkaline phosphatase in culture fluid and cells. The results showed that the content of ALP was significantly lower than that of the control group, which indicated that the activity of the alkaline phosphatase decreased and the differentiation of osteoblasts was inhibited by RNA interference. OTX2-siRNA interference in the specific regulation mechanism of osteoblast differentiation and the signal transduction pathway involved in the study also need to be studied further.

This study shows that OTX2-siRNA can inhibit the proliferation and the differentiation of hFOB1.19 osteoblast and decrease the activity of alkaline phosphatase by down regulating the expression of OTX2 mRNA and protein suggesting that OTX2 may be related to the expression, proliferation and differentiation of osteoblasts. We will further study the relationship between OTX2 gene and mandible development by using immuno-histochemistry.

**Conclusion**

The chemically synthesized OTX2-siRNA can effectively restrain mRNA and protein expression level of OTX2 gene in hFOB1.19 osteoblasts. Interference of OTX2 gene can inhibit the proliferation of hFOB1.19 osteoblasts. Down-regulated expression of OTX2 gene can also reduce the alkaline phosphatase activity of hFOB1.19 osteoblast and inhibit differentiation.

**REFERENCES**


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**Table 3.** The activity of alkaline phosphatase in the supernatant and cells of each group.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Blank control group</th>
<th>Negative control group</th>
<th>OTX2-siRNA group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant ALP</td>
<td>0.776±0.038</td>
<td>0.769±0.034</td>
<td>0.357±0.022*</td>
</tr>
<tr>
<td>Intracellular ALP</td>
<td>3.314±0.045</td>
<td>3.312±0.042</td>
<td>2.993±0.028*</td>
</tr>
</tbody>
</table>

*Compared with the control group, P<0.05.


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