microRNA-200a regulates cell proliferation and differentiation in mandibular condylar cartilage during mouse development

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1. Summary

Mandibular condylar cartilage (MCC) is classified as a secondary cartilage and has a unique histological structure from that of the primary cartilage. MicroRNA (miRNA) is a small non-coding RNA that binds to the target gene and represses its translation, and plays an important role in cell differentiation, proliferation, and death. It was hypothesized that miRNA was involved in the regulation of cartilage formation during MCC growth. In a microarray analysis, miR-200a was characteristically expressed during embryonic development. The function of miR-200a was investigated by transfection of an inhibitor or mimic into MCC organ and cell cultures. A histological examination revealed a localized inhibitive effect of the mimic of miR-200a, and a widespread enhancive effect of the inhibitor on chondrocytic differentiation in the MCC organ culture system. An immunohistochemical examination and gene expression analysis demonstrated that the miR-200a inhibitor enhanced chondrogenesis, while the mimic had the opposite effect and enhanced cell proliferation. qRT-PCR analysis showed that miR-200a down-regulated the gene expression of chondrocyte markers in MCC organ culture and ATDC5 micromass.
culture. Moreover, transfection of the miR-200a mimic into ATDC5 cells indicated repressed cartilaginous matrix formation. These results suggest that miR-200a contributes to chondrogenesis in developing MCC through the control of proliferation and differentiation in MCC cells.
2. Introduction

In orthodontics, it has been long argued regarding the mechanisms of the
growth of mandible, and effectiveness of orthopedic treatment applied for the growth
regulation of maxillofacial complex. Orthognathic surgery has been applied for the
patients with severe jaw deformities, on the one hand, because of the difficulties of
growth modification of mandible in these patients. Indeed, clinical modalities to
control mandibular growth has been developed by many researchers, however, as of
yet, consensus regarding the effect of orthopedic appliances, such as functional
appliances and chin cap has not been obtained among the orthodontic researchers. In
this context, mandibular condylar cartilage (MCC) mainly contributes to the growth in
the length of mandibular ramous consequently with the whole size of the mandible as a
“growth cite” by endochondral ossification subsequently occurs after the
differentiation of chondrocyte in MCC. The amount and direction of the endochondral
bone growth in MCC is, therefore, one of the critical parameter to determine the
mandibular growth. Thus, it could be considered that it is very important to investigate
the mechanisms of the growth and development of MCC.
MCC is classified as a secondary cartilage and is distinct from primary cartilage, such as articular or growth plate cartilage (Silbermann et al., 1990; Mizoguchi et al., 1993). The development of MCC begins with the condensation of mesenchymal cells derived from the cranial neural crest (Chai et al., 2000) and cells in MCC synthesize type I, II, and X collagens and proteoglycans (Silbermann et al., 1987; Mizoguchi et al., 1990; Milam et al., 1991; Evanko et al., 1993; Ali et al., 1996). MCC functions as an articular cartilage and growth plate cartilage as the template of the longitudinal growth of the mandibular ramous by endochondral ossification as described above.

MicroRNA (miRNA) is one of the non-coding RNAs and plays an important role in the development, differentiation, and proliferation of various kinds of cells and organs (Bartel et al., 2004). It regulates gene expression post-transcriptionally by binding to complementary sequences in the 3’-untranslated region of the target messenger RNA (mRNA) (Wheeler et al., 2006). There have been a few studies in growth of long bones with regards to miRNA regulation in chondrogenesis in cartilage.
tissue and mesenchymal stem cells (Miyaki et al., 2009, Itoh et al., 2009). However, post-transcriptional regulation during the early stage of MCC development is still unclear.

In this study, mRNAs and miRNAs during the embryonic development of MCC were profiled in the mouse by microarray analysis and were noticed a rapid reduction in the expression of miR-200a. It was hypothesized that miR-200a would inhibit the chondrogenesis of MCC. I attempted to analyze the role of miR-200a by the transfection of an miR-200a mimic and inhibitor in developing MCC.
3. MATERIALS & METHODS

3.1 Experimental animals

Timed pregnant ICR mice were used on gestation day 14 (E14), 16 (E16), and 18 (E18) (within the day when the vaginal sperm plug was first observed, designated as day 0 of gestation). The MCC or whole heads of E14, E16, and E18 embryos were isolated and subsequently washed in cold phosphate-buffered saline (PBS) at pH 7.4 and were used for the following experiments. All protocols for the animal experiments were approved by the Animal Care and Use Committee of Kyushu University.

3.2 Mandibular condylar cartilage (MCC) explant culture

MCC derived from E14 mouse embryos was isolated as described above. They were cultured in a modified Trowell organ culture system in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma, St. Louis, MO) supplemented with 100U of penicillin and streptomycin (Invitrogen, Carlsbad, CA) and 100 µg/ml L-ascorbic acid (Sigma) in 5% CO₂ at 37 °C for 0, 1, 2, and 3 days. In preliminary experiments,
MCC explants were cultured under the several different conditions of medium (0% fetal bovine serum (FBS)/DMEM, 2% FBS/DMEM, 0% FBS/BGJb and 2% FBS/BGJb) for 1, 2, 3, 5 and 7 days (Fig. 3-1A). Histological analysis at day 7 demonstrated that samples at 0% and 2% FBS/DMEM kept cartilaginous features, while those at 0% and 2% FBS/BGJb were reduced cartilaginous structure and increased osteoid tissue (Fig. 3-1B). Eventually, a serum-free DMEM was selected for MCC organ culture as appropriate condition.
Figure 3-1 MCC organs under different culture conditions. (A) Mandibular organ culture samples E14 mouse embryos. MCC explants were cultured under the different conditions of medium (0% FBS/DMEM, 2% FBS/DMEM, 0% FBS/BGJb and 2% FBS/BGJb) for 1, 2, 3, 5 and 7 days. Scale bar = 500 μm. (B) Photographs of the histological section stained with H&E after 7 day culture. Scale bar = 500 μm.
3.3 Transfection of miR-200a mimic or inhibitor into MCC organ culture

MCC explants were preincubated for 4 hrs prior to transfection. 150nM Anti-mmu-miR-200a miScript miRNA Inhibitor (Qiagen), 150 nM miR-200a miScript miRNA Mimic (Qiagen) and AllStars negative control siRNA Labeling with Alexa Fluor 488 solutions (Qiagen) were labeled with 0.05% Fast Green solution (Sigma) and injected into explants using a micromanipulator and a microinjector (Narishige, Tokyo, Japan) with a glass capillary needle prior to electroporation. Details of electroporation condition were described in previous study (Terao et al., 2007). After transfection, the MCC explants were cultured for 3 days.

3.4 MCC cell culture

Isolated MCCs were treated in 0.25% trypsin EDTA and 0.25 mg/ml collagenase type 2 (Invitrogen) in 0.1 M PBS for 10 min at 37 °C and were dissociated into single cell suspensions. Cells were resuspended in DMEM supplemented with 10% FBS, 2.4 mg/ml N- (2-hydroxyethyl) piperazine-N’- (ethanesulfonic acid) (HEPES) (Nacalai Tesque, Kyoto, Japan), 0.2% sodium bicarbonate (Sigma), and
100U each of penicillin and streptomycin.

3.5 ATDC5 cell culture

Chondrogenic cell line, ATDC5 cells were obtained from the RIKEN cell bank (Tsukuba, Ibaraki, Japan). Cells were maintained in a 1:1 mixture of DMEM and Ham’s F12 medium (DMEM/F12, Gibco) supplemented with 5% FBS containing 100U each of penicillin and streptomycin (Invitrogen) in 5% CO₂ at 37 °C.

3.6 ATDC5 micromass culture

ATDC5 cells were plated in 12-well plates at 2.6 × 10⁵ cells per well and transfected with miR-200a mimic, miR-200a inhibitor and negative control siRNA. After incubation for 2 days, transfected ATDC5 cells in monolayer were trypsinized and resuspended at a density of 2.0 × 10⁶ cells/ml, and three 10 µl drop was plated around the center of well on 6-well plate and maintained to settle down for 1 hr. And then, medium supplemented with insulin-transferrin-selenite (Gibco) was added gently.
Medium was changed every other day, and cells were harvested for 9 days in 5% CO₂ at 37 °C.

3. 7 Microarray and data analysis

3. 7. 1 Extraction of total RNA

Total RNA was prepared from TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

3. 7. 2 Microarray analysis for mRNAs and miRNAs

Total RNA was labeled and hybridized according to the manufacturer’s instructions. Cyanine-3-labeled miRNA samples were hybridized to Mouse miRNA microarray 8x15K Version 2 or Whole Mouse Genome Microarray Kit version 2 (Agilent Technologies, Palo Alto, CA) according to the manufacturer’s instructions.

All hybridized microarray slides were scanned by an Agilent scanner. Relative hybridization intensities and background hybridization values were calculated using the Agilent Feature Extraction Software program (9. 5. 3. 1).
3. 7. 3 \hspace{1cm} \textbf{Data analysis and filter criteria}

Raw signal intensities and flags for each probe were calculated from the hybridization intensities (gProcessedSignal), and spot information (including gIsSaturated), according to the procedures recommended by Agilent. Microarray data analysis was supported by Cell Innovator Inc. (Fukuoka, Japan).
3.8 Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) analysis

3.8.1 mRNA quantification by qRT-PCR

250 ng of total RNA was reverse transcribed to cDNA by using the SuperScript III reverse transcription kit (Invitrogen). The quantification of mRNA expression was performed by intercalator methodology with a Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies). The thermal cycling program of mRNA for quantitative PCR consisted of 15 sec of predenaturation at 95 °C and 40 cycles for denaturation, 45 sec of annealing at the optimized temperature indicated in Table 3-1, and 2 min of extension at 72 °C, followed by a final extension cycle of 10 min. Primers for qRT-PCR (Table 3-1) were obtained from PrimerBank.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>GenBank Accession No.</th>
<th>Nucleic acid sequences of primers</th>
<th>PCR annealing temperature (°C)</th>
<th>Product size (bp)</th>
<th>PrimerBank ID</th>
</tr>
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<tr>
<td>Sox9</td>
<td>NM_011448</td>
<td>AGTACCCGCATCTGCACAAC</td>
<td>60</td>
<td>88</td>
<td>165932320b1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ACGAAGGGTCTCTCTCTGCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Col2a1</td>
<td>NM_001113515</td>
<td>CAGGATGCCCCAAAATAGGG</td>
<td>62</td>
<td>132</td>
<td>30353888a1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ACCACGATCACCTCTGGGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-cadherin</td>
<td>NM_007664</td>
<td>AGGCCAGTCTTACCGAAGG</td>
<td>60</td>
<td>101</td>
<td>6680902a1</td>
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<tr>
<td></td>
<td></td>
<td>TCGCTGCTTTCATACGACTTT</td>
<td></td>
<td></td>
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<tr>
<td>Dlx5</td>
<td>NM_010056</td>
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<td>60</td>
<td>79</td>
<td>7106293a1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCGATTTCTGAGACGGGATT</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>β-actin</td>
<td>NM_007393</td>
<td>GTCAGGCTGACATCCGTTAAGA</td>
<td>60</td>
<td>245</td>
<td>145966868c1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCCGGACTCATCGTACTCC</td>
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(http://pga.mgh.harvard.edu/primerbank). mRNA level was quantitatively analyzed by
a real-time PCR method using an Applied Biosystems ABI Prism 7500 (Foster City, CA) according to the supplier’s recommendations. The expression levels of mRNA were normalized against β-actin.

3.8.2 MicroRNA quantification by qRT-PCR

Reverse transcription of miRNA was carried out by using miScript Reverse Transcription Kit (Qiagen). PCR was performed for miRNA by using miScript Primer Assay kit (Qiagen). The thermal cycling program of miRNA for quantitative PCR consisted of 15 min of predenaturation at 95 °C and 40 cycles for denaturation, 30 sec of 3 annealing at 55 °C, and 34 sec of extension at 70 °C, followed by a final extension cycle of 10 min. PCR was then performed with and miRNA-specific forward primers and a universal reverse primer. Hsa-let-7f was chosen as a housekeeping gene (because of mro-let-7f identical to Has-let-7f) to normalize the expression levels of miR-200a.
3. 9 Transfection of miR-200a mimic or inhibitor into MCC organ culture

MCC explants were preincubated for 4 hrs prior to transfection. An anti-mmu-miR-200a miScript miRNA Inhibitor (Qiagen), miR-200a miScript miRNA Mimic (Qiagen) and AllStars negative control siRNA Labeling with Alexa Fluor 488 solutions at 150 nM (Qiagen) were labeled with 0.05% Fast Green (Sigma) solution and injected into explants using a micromanipulator and a microinjector (Narishige, Tokyo, Japan) with a glass capillary needle prior to electroporation. Details of electroporation condition were described in previous study (Terao et al., 2007). After transfection, the MCC explants were cultured for 3 days.

3. 10 Tissue preparation for histological and immunohistochemical examination

3. 10. 1 Fixation, embedding and sectioning

The MCC explants or whole heads of experimental animals were fixed with 4% paraformaldehyde in 0.1 M PBS for 1hr at 4 °C. Specimens were dehydrated in a graded series of ethanol and embedded in paraffin. Then 5 µm-thick sections were cut
for histological examinations.

3.10.2 Immunohistochemistry

The protocol for immunohistochemistry used in the present study was described in the previous studies (Takahashi et al., 1995; Yamada et al., 2002). Briefly, the sections were deparaffinized, and treated with 2.5% hyaluronidase from bovine testis (Sigma) in PBS for 1 hr at 37 °C. After washed in PBS, incubate with Blocking Reagent (Roche Diagnostics, Indianapolis, IN) in PBS for 2 hrs at room temperature. The sections were then incubated in the moisture chamber overnight at 4 °C with rabbit anti-type I, II and X collagen antibodies (LSL Co., Tokyo, Japan) and mouse anti-Proliferating Cell Nuclear Antigen (PCNA) antibodies (Cell Signaling Technology, Beverly, MA) diluted 1:1000 with 0.05% normal bovine serum in PBS. After a through rinse with 0.01M PBS, the sections were incubated with IgG Alexa Fluor 488-conjugated donkey anti-rabbit (Jackson ImmunoResearch, West Grove, PA), diluted 1:500 with 0.01 M PBS, for 2 hrs at room temperature. After several rinses with 0.01 M PBS, the sections were mounted in Vectashield mounting medium with
4’-6’-diamidino-2-phenylindole (DAPI) (Vector Labs, Burlingame, CA) or PermaFluor aqueous mounting medium (Thermo Scientific, Waltham, MA). Images were captured with an Olympus IX71 inverted fluorescence microscope (Olympus, Tokyo, Japan) and recorded on a CCD camera (DP72, Olympus). For regular histological observations, sections were stained with hematoxylin and eosin (H&E).

3.11 Cell proliferation assay

Cultures of MCC cells or ATDC5 cells were plated in 96-well plates at 2.0 × 10⁴ cells per well and transfected with 5 nM miR-200a mimic, 5 nM miR-200a inhibitor and 5 nM negative control siRNA by Hi-PerFect reagent (Qiagen). The cell proliferation occurring in the MCC monolayer culture was evaluated using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc, Kumamoto, Japan). After the addition of the cell counting reagent, the cells were incubated at 37 °C for 1 hr. Absorbance was measured at a wavelength of 450 nm using an iMark microplate reader (BioRad, Hercules, CA).
3.12 Alcian Blue staining

ATDC5 cells micromass cultures were fixed with 2% acetic acid in 95% ethanol for 15 min at room temperature (RT) on day 6, rehydrated and stained overnight at RT with 0.5% alcian blue 8GX (Sigma) in 0.1N HCl. The cultures of all groups were microphotographed using a digital camera (Coolpix 8400, Nikon), and then alcian blue stained matrix was solubilized by 8 M guanidine HCl (Wako Pure Chemicals, Tokyo, Japan) overnight to measure the absorbance at a wavelength of 595 nm using the microplate reader.

3.13 Statistical analysis

All data were subjected to statistical analysis using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test. Statistical significance was considered to be $p<0.01$ or $p<0.05$. 
4. Results

4.1 Histological analysis during normal MCC development.

Firstly, histological features of MCC and adjacent tissues during normal embryonic development were confirmed. Eyes, tongue, brain and tooth germ were observed, but condensation of mesenchymal cells was seldom detected at E13 (Fig. 4-1 A1, A2). At E14, Meckel’s cartilage was clearly detected and mesenchymal cell condensation was located above it in the future region of MCC (Fig. 4-1 B). At E16, temporomandibular joint (TMJ) cavity and condylar anlagen, which contained fibrous layer, proliferative cell layer, transitional cell layer, maturative cell layer and hypertrophic cell layer were observed (Fig. 4-1C). The layers were clearly established by E18 (Fig. 4-1B).
Figure 4-1 Normal MCC development in E13 to E18 mouse embryo. (A) Photomicrographs of temporomandibular joint in frontal sections staining with H&E at E13. Scale bar = 200 µm. (B) The condylar cartilage at E14, E16 and E18. m: Meckel’s cartilage, t: Tongue, e: Eye, tg: Tooth germ, b: Brain. Scale bar = 200 µm. (C) Phase-contrast photomicrograph of the MCC of E16. The condylar cartilage is divided into following five cell layers: F; fibrous layer, P; proliferative cell layer, T; transitional cell layer, M; maturative cell layer, and H; hypertrophic cell layer. Scale bar = 50 µm.
4.2 The expression profiles of mRNA and miRNA in MCC during normal embryonic development.

In order to find specific miRNAs involved in MCC development, microarray analysis was performed to profile mRNAs and miRNAs among the developing MCCs isolated from E14, E16 and E18 ICR mice. Signal intensity of mRNA and miRNA showed in a heat-map (Fig. 4-2A and B). Gene expression of chondrogenic markers increased during MCC development (Fig. 4-2C). Signal values of several characteristic miRNAs in MCC were shown in Fig. 4-2D. The expression of miR-429, -200c, -205, -200a and -135a decreased as the MCC development proceeded based on the result of microarray analysis. It has been known that miR-429, -200a and -200c compose of miR-200 family based on their sequence homology. Thus, miR-200a, as a representative of the family was chosen for further experiments. It was expected that the miRNAs that decrease the expression could target the genes related with chondrogenic differentiation in MCC.
Figure 4-2 Expression profiles of mRNA and miRNA in MCC during normal embryonic development. (A) Heatmap of mRNA signal value. (B) Expression intensity (heat map) for chondrogenic marker genes during the normal development of MCCs. (C) Heatmap of mRNA signal value at E14, E16 and E18. (D) Signal values of several characteristic miRNAs in MCC at E14.
4.3  Expression of miR-200a during normal MCC development

The expression of miR-200a was significantly decreased on E16 and E18 (Fig. 4-3A) (N=4, p<0.01).

4.4  Expression of cartilage maker genes in normal MCC development.

To evaluate the impairment of chondrogenesis, qRT-PCR analysis was performed to assess the expression changes of chondrogenic markers, type II collagen and Sox9. The expression of type II collagen was significantly increased during MCC development (Fig. 4-3B) (N=4, p<0.05). The expression of Sox9 was significantly up-regulated on E16 (Fig. 4-3C) (N=4, p<0.05).
Figure 4-3 Changes of the expression of (A) miR-200a, (B) type II collagen and (C) Sox9 in MCC during embryonic development. Statistically significant differences are indicated by *: p<0.05, N = 4.
4. 5 Transfection of Alexa-488 conjugated negative control siRNA

The bright field image was acquired by stereomicroscopy, and fluorescent image was captured by confocal laser-scanning microscope at 6 hrs after transfection (Fig. 4-4). The Alexa 488 labeled negative control siRNA in the living explants was observed in the condylar head of the explant under the fluorescent confocal microscope.

![Image](image_url)

**Figure. 4-4** The effect of the transfection of Alexa 488-conjugated siRNA as a control. BF: bright field, DF: dark field. Scale bar = 500 µm.

4. 6 Histological observation of MCC organ culture without transfection.

The staining with type I collagen antibody was observed mainly in fibrous layer (Fig. 4-5 B), and the staining with type II collagen was shown in the proliferative, transitional and hypertrophic cell layer (Fig. 4-5 C). The staining for type X collagen was strong in the area of hypertrophic cell layer (Fig. 4-5 D). Furthermore, there were
large number of PCNA-positive cells in fibrous layer and underlying proliferative cell layer (Fig. 4-5 E, F and G).

4.7 Histological analysis after transfection of miR-200a mimic or inhibitor into MCC organ culture.

To investigate the role of miR-200a in MCC development, miR-200a mimic (mimic group) or inhibitor (inhibitor group) was transfected into MCC organ culture. In inhibitor group, thinning of fibrous undifferentiated layer on the upper aspect of MCC was found, and the condyles were occupied by matured chondrocytes when compared with the other groups (Fig. 4-6 A2 and B2). In the mimic group, the hypertrophic cell layer in the center region of MCC disappeared and undifferentiated fibrous tissue was observed (Fig. 4-6 A3 and B3).

In inhibitor group, fibrous layer reacting type I collagen antibody was found to be thinner than siRNA control group (Fig. 4-6 A4, A5, B4 and B5). An area reacting to type I collagen antibody was found in the center of condylar explants, where miR-200a mimic was transfected (Fig. 4-6 A6 and B6). Staining for type II collagen
was observed in cartilaginous tissue in control and inhibitor group (Fig. 4-6 A7, A8, B7 and B8), but the staining was divided into two regions in mimic group (Fig. 4-6 A9 and B9). Intense staining for type X collagen was expanded in hypertrophic area in inhibitor group when compared with control (Fig. 4-6 A10, A11, B10 and B11), while the staining in mimic group is separated by the transfected area (Fig. 4-6 A12 and B12).
Figure 4-5 Immunohistochemistry for type I, II and X collagens and Proliferating Cell Nuclear Antigen (PCNA) of MCC without transfection. (A) Histological specimens stained with H&E of MCC at day 3; Photomicrographs of immunoreactivity. (B) type I collagen. (C) type II collagen. (D) type X collagen. (E) PCNA. (F) DAPI staining and (G) merged image of both PCNA and DAPI. Scale bar = 500 μm.
Figure 4-6 The effect of the transfection of miR-200a mimic or inhibitor. (A) Representative photographs of the histological section stained with H&E at day 3 (A1-A3) and fluorescent immunohistochemistry of type I (A4-A6), type II (A7-A9) and type X (A10-A12) collagen in cultured MCC after transfection. Scale bar = 500 μm. (B) Representative photographs of the histological section stained with H&E at day 3 (B1-B3) and fluorescent immunohistochemistry of type I (B4-B6), type II (B7-B9) and type X (B10-B12) collagen in cultured MCC after transfection of higher magnification. Note that open arrowhead indicates hypertrophic layers, open arrow indicates fibrous layer, closed arrow indicates undifferentiated tissue in MCC. Scale bar = 100 μm.
4.8 qRT-PCR analysis after transfection of miR-200a mimic or inhibitor into MCC organ culture.

qRT-PCR analysis of chondrogenic markers and miR-200a expression after transfection of siRNA control, miR-200a inhibitor and mimic. MiR-200a expression was 0.7-fold in the inhibitor group and 38-fold in the mimic group than that in the control group on day 1 after transfection, and 0.3-fold in the inhibitor group on day 3 (Fig. 4-7A). The miR-200a inhibitor significantly promoted the expressions of Col2a1, Sox9 and N-cadherin on day 3 (Fig. 4-7 B, C and D) (p<0.05). The expression of Dlx5 was significantly reduced in mimic group compared to inhibitor group on day 3 (Fig. 4-7 E) (p<0.05).
Figure 4-7 Changes of the expression of (A) miR-200a, (B) type II collagen, (C) Sox9, (D) N-cadherin, (E) Dlx5 in MCC organ culture, after transfection of siRNA control, miR-200a inhibitor or mimic. Statistically significant differences are indicated by **: $p < 0.01$, *: $p < 0.05$, (N = 4).
4.9 The Effect of miR-200a on proliferative activity of MCC

Analysis of PCNA positive cells in MCC organ culture was carried out in siRNA control, miR-200a mimic and inhibitor group. PCNA-positive cells were observed in the proliferative cell layers of all groups (Fig. 4-8). The number of PCNA positive cells was significantly higher in the mimic group (Table 4-1) \((p<0.01)\). The cell proliferation rate in the mimic group was also higher than that in the control and inhibitor groups \((p<0.05, p<0.01, \text{respectively})\). However, there was no significant difference between the number of PCNA positive cells in the control group and that in the inhibitor group.
Figure 4-8 MCC treated with miR-200a mimic showed increased cell proliferation. (A) H&E staining (A1-A3); PCNA staining (A4-A9); low-magnification images (A4-A6), and high-magnification images (A7-A9); DAPI staining (A10-A12) and merged images (A13-A15). Scale bar: 500 μm in A1-A6, and 200 μm in A7-A9. Note that closed arrow indicates fibrous layer, closed arrowhead indicates undifferentiated tissue.

Table 4-1 Proliferating Activity of Cartilage Cells (mean ± SD)

<table>
<thead>
<tr>
<th>siRNA/control</th>
<th>Inhibitor</th>
<th>Mimic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (S)</td>
<td>Group (I)</td>
<td>Group (M)</td>
</tr>
<tr>
<td>(N = 9)</td>
<td>(N = 9)</td>
<td>(N = 9)</td>
</tr>
<tr>
<td>PCNA</td>
<td>1121.4 ± 364.9 (** vs. M)</td>
<td>876.3 ± 272.2 (** vs. M)</td>
</tr>
<tr>
<td>Total cell</td>
<td>3258.1 ± 814.5 NS</td>
<td>2915 ± 723.2 (** vs. M)</td>
</tr>
<tr>
<td>PCNA/Total cell (%)</td>
<td>34.6 ± 9.7 (** vs. M)</td>
<td>29.8 ± 4.5 (** vs. M)</td>
</tr>
</tbody>
</table>

Groups which showed significantly different values are indicated by capitalized initials of group names in parentheses. N, number of samples. NS, not significant. Statistically significant differences are indicated by*: p < 0.05, **: p < 0.01.
4.10 Transfection of a mimic for miR-200a monolayer MCC cell culture

Cell proliferation derived from MCC with transfection of the miR-200a mimic was higher than that in the control and inhibitor groups on day 5 ($p<0.01$, $p<0.05$, respectively) (Fig. 4-9A).

4.11 Transfection of a mimic for miR-200a monolayer ATDC5 cell culture

Cell proliferative activity of ATDC5 in the monolayer culture with transfection of the miR-200a mimic was significantly higher than that in the control and inhibitor groups on day 3 ($p<0.05$) (Fig. 4-9B).
Figure 4-9 Effect of miR-200a mimic and inhibitor on the cell proliferation. (A) MCC cell monolayer culture, (B) ATDC5 monolayer culture. **: p<0.01, *: p < 0.05, (N = 3).
4. 12 Effects of miR-200a mimic and inhibitor on chondrogenesis in ATDC5 micromass culture

The effect of miR-200a mimic and inhibitor on the expression of chondrogenic marker genes and accumulation of cartilage matrix production were examined in ATDC5 micromass culture. The area of the chondrogenic nodule in the inhibitor group significantly increased on day 6 (Fig. 4-10A and B). The absorbance at 595 nm of extracted alcian blue showed that the proteoglycan content in the miR-200a mimic group was lower than that in the control and inhibitor groups \(p<0.05, p<0.01\), respectively) (Fig. 4-10C).

The gene expressions of Col2a1, Sox9, N-cadherin and Dlx5 in the inhibitor group were significantly higher than those in the mimic group on day 3 and day 6 (Fig. 4-10A, B, C and D) \(p<0.05, p<0.01\), respectively).
Figure 4-10 The effect of miR-200a mimic and inhibitor on cartilage matrix formation. 
(A) The effect of miR-200a mimic and inhibitor on cartilage matrix formation by alcian blue staining in ATDC5 micromass culture. Scale bar = 1 mm. (B) Representative graph of alcian blue positive nodule area of ATDC5 micromass culture. (C) Representative graph of alcian blue positive nodule absorbance of ATDC5 micromass culture. Statistically significant differences are indicated by **: p < 0.01, *: p < 0.05, (B and C: N = 3).
Figure 4-11 Graphs indicate the results of qRT-PCR in ATDC5 micro mass culture of qRT-PCR. The expression of Col2a1 (A), Sox9 (B), N-cadherin (C), and Dlx5 (D) after transfection of siRNA control (open bars), miR-200a inhibitor (shaded bars) and mimic (closed bars). Statistically significant differences are indicated by **: p < 0.01, *: p < 0.05, N = 3.
5. Discussion

Formation of MCC begins with mesenchymal cell condensation in the future region of the TMJ. As MCC development proceeded, the expression of cartilage specific markers significantly increased, while the expression of miR-200a decreased based on the microarray analysis. The present study is the first report of microRNA analysis in MCC as secondary cartilage. Some chondrocyte-specific microRNAs have been found in the mouse limb bud (Yang et al., 2011). Several chondrogenesis-related miRNAs, such as miR-140, -145, -221, and -337 were found to be involved in the differentiation from mesenchymal cells to chondrocytes (Dunn et al., 2009; Kim et al., 2010; Zhong et al 2012; Dong et al., 2012). miR-140 was specifically expressed in cartilage tissue regulated proliferation and hypertrophy by repressing histone deacetylase 4 (Miyaki et al., 2010; Tuddenham et al., 2006). miR-145 has been shown to regulate the chondrogenesis of mesenchymal stem cells by targeting Sox9 (Yang et al., 2011). Focusing on the results of the present study, miR-200a inhibited
chondrogenic differentiation and enhanced proliferation. These results implied the involvement of miR-200a in the early formation of MCC during the fetal stage.

The results of present study demonstrated that the expression of N-cadherin and Dlx5 were significantly increased in miR-200a inhibitor group compared to mimic group. The online database miRBase showed other candidates of target genes, N-cadherin, Dlx5, transforming growth factor (TGF) β2, TGFβ receptor type 2 and matrix metalloproteinase 13. A recent report has demonstrated that miR-200a is involved in osteoblast differentiation by regulating the expression of Dlx5 (Itoh et al., 2009). As Dlx5 or TGFβ signaling has been shown to be one of the critical factors for MCC formation (Itoh et al., 2003; Oka et al., 2007; Oka et al., 2008), Dlx5 and/or TGFβs may be the targets of miR-200a in MCC development. Histological and immunohistochemical results demonstrated that the miR-200a inhibitor enhanced the widespread expansion of the hypertrophic cell layer, while the mimic strongly inhibited the differentiation of chondrocytes into which they were transferred. It is suggested that miR-200a could repress the expression of soluble factors because the inhibitor caused an effect in the long range, while the mimic immediately affected the
cells expressing it. If the cell surface or intracellular proteins are affected, the affected area must be limited to the site where they were transferred.

Transfection of miR-200a mimic significantly increased proliferation in MCC organ culture. Several studies have reported that miR-200 family (miR-141, miR-200a, miR-200b, miR200a and miR-429) is involved in cancer development (Elson-Schwab et al, 2010; Soubani et al., 2012). In Drosophila and MCF-7 cells, miR-200a induced the cell proliferation through USH/FOG2 by regulating PI3K (Hyun et al., 2009). Thus, it could be considered that the miR-200a promoted the proliferation through similar signaling pathway.

Cartilage dose not contain blood vessel (Gerber et al., 1999) and exist always in the under-nutrition and hypoxia. The nutrition necessary for chondrocyte is diffused from synovial fluid. In the present study, it was observed that the histological appearance of cultured MCC in serum-free DMEM medium showed the layered structure similar to normal MCC. Bone growth was specifically occurred in BGJb medium in the cultured MCC, since BGJb was known to be a specifically arranged medium for osteoblast differentiation. It was observed that organ culture in DMEM with
high-glucose showed specific layered structure close to MCC. In addition, while MCC from neonatal or E17 were used in previous studies (Silberman et al., 1987, Maour et al., 1993, Serrano et al., 2011), however this is the first report that enabled E14 mouse MMC organ culture. Thus, it made possible to survey the earlier stage of MCC formation by using this organ culture system.

The electroporation, which was previously applied to rat embryonic mandibular organ culture (Terao et al., 2007), was exploit to MCC organ culture system in order to transfec miRNA inhibitor or mimic. Previous study reported that transfection efficiency was strongly related to vector size by using various sizes of plasmid vector coding sequence the enhanced green fluorescent protein (EGFP). The smaller the vector size was, the better transfection efficiency observed. In this study, it was suggested that using smaller-molecules, such as siRNA, miRNA mimic or inhibitor, enabled to achieve efficient intracellular delivery. The transfection system for miRNA analysis was established by combining MCC organ culture with electroporation. This system will provide better understanding of the mechanism of MCC development during early embryonic stage.
6. Conclusions

1) Transfection of miRNA inhibitor or mimic into MCC organ culture system was established. This method will be useful to illuminate the comprehensive mechanism of regulation by miRNAs on MCC development.

2) miR-200a could down-regulate the gene expression of N-cadherin, Sox9 and type II collagen and subsequently inhibited chondrogenic differentiation of MCC.

3) miR-200 could enhance the proliferation of the cells in proliferative cell layer of MCC in the early development of mandibular condyle.

Molecular mechanism during craniofacial development, in part, was elucidated by analysis of the microRNA functions during MCC development in mouse. The present research provides the mandibular basis of MCC development, which could contribute to future development of clinical orthodontics showing the possibility of therapeutic utilization of miRNA.
7. Acknowledgements

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8. References


