Repair of Canine Medial Orbital Bone Defects With miR-31–Modified Bone Marrow Mesenchymal Stem Cells

Yuan Deng, Huifang Zhou, Ping Gu, and Xianqun Fan

Department of Ophthalmology, Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

PURPOSE. To investigate the role of miR-31 genetically modified bone marrow mesenchymal stem cells (BMSCs) composited with porous β-tricalcium phosphate (β-TCP) scaffolds in repairing canine medial orbital wall defects.

METHODS. A circular bone defect (10 mm in diameter) was created on the canine medial orbital wall. After canine BMSCs were isolated and transfected with lentiviral vectors encoding miR-31, anti-miR-31 (anti-miR), and negative control (miR-Neg) in vitro, they were seeded onto porous β-TCP scaffolds and implanted to repair the orbital defects. Spiral computed tomography (CT) scans were conducted at 4 and 16 weeks after surgery. Micro-CT and histological analysis were performed at 16 weeks after surgery. The results were analyzed to evaluate the extent of bone repair.

RESULTS. Examination with CT revealed good recovery in the anti-miR group at 16 weeks after surgery. In addition, the micro-CT analysis showed that the bone mineral density and new bone volume increased in the anti-miR group and decreased in the miR-31 group compared with that in the miR-Neg group. Histologic analysis confirmed that the formation of new bone and extent of β-TCP degradation were enhanced in the anti-miR and attenuated in the miR-31 group. In situ hybridization and immunohistochemical analysis further confirmed the micro-CT findings.

CONCLUSIONS. The use of BMSCs with suppression of miR-31 expression combined with β-TCP scaffolds can efficiently repair medial orbital wall defects in dogs.

Keywords: miR-31, bone marrow mesenchymal stem cells, orbital bone defects, bone repair, β-tricalcium phosphate

Orbital bone defects caused by trauma, tumor operation, infection, or congenital malformation often require repair and reconstruction. Autogenous and allogeneic bone transplantation are quite effective tools for repairing orbital defects, but are also associated with challenges such as limited bone resources, time-consuming procedures, and risk of infection. Several types of artificial materials, including polymeric porous polyethylene, hydroxyapatite, and titanium, can be used for orbital bone repair. However, these materials are usually undegradable or difficult to degrade, which increases the risk of rejection, infection, cyst formation, and implant metastasis.

In our previous studies, autogenous bone marrow mesenchymal stem cells (BMSCs) combined with β-tricalcium phosphate (β-TCP) scaffolds were used to repair orbital rim or orbital wall defects in a canine model, and the results showed that the repairing effect of osteoinductive BMSCs was superior to that of noninduced BMSCs. These findings were further confirmed by using bone morphogenic protein-2 (BMP-2) gene-modified BMSCs combined with biocoral scaffolds to heal critical-sized orbital wall defects in canines. In addition, repair of orbital rim segmental defects was enhanced by BMP2 and VEGF gene-modified BMSCs in rabbits.

MicroRNAs (miRNAs) are small (approximately 22 nucleotides in length), noncoding RNAs that regulate the expression of proteins by binding to the 3′ untranslated region of a target mRNA, thereby inhibiting mRNA translation. Recently, the functions of several miRNAs in stem cell differentiation have been elucidated, which enabled us to determine that miRNA-31 (miR-31) plays an important role in the osteogenic differentiation of BMSCs by targeting the key osteogenic transcriptional factor special AT-rich sequence-binding protein 2 (Satb2). Our previous data showed that miR-31 negatively regulates the osteoinductive differentiation of BMSCs, and that suppression of miR-31 expression significantly enhances the effects of BMSCs on caviar bone repair.

In order to investigate the role of miR-31-modified BMSCs in repairing orbital bone defects, autologous BMSCs transfected with lentiviral vectors encoding miR-31, miR-Neg (negative control), or anti-miR (anti-miR-31), and composited with β-TCP scaffolds, were implanted into orbital wall bone defects in a canine model. Furthermore, long-term follow-up was carried out, and the effectiveness of repairing orbital wall defects using miR-31-modified BMSCs was evaluated.

MATERIALS AND METHODS

Isolation and Culture of BMSCs

Under general anesthesia with ketamine (10 mg/kg) and sodium pentobarbital (2.5 mg/kg), bone marrow was harvested from the ilium of beagle dogs, as previously described. Bone marrow (1 mL) was mixed with 10 mL of α-minimum essential medium (Gibco, Grand Island, NY, USA) supplemented with...
10% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 U/mL streptomycin, and then placed in a 10-cm dish. All cell cultures were incubated in a humidified atmosphere with 5% CO2 at 37°C, subcultured to passage 3, and then used for gene transduction.

**Gene Transduction**

The green fluorescent protein (GFP)-labeled lentiviral vectors encoding miR-31 precursor (miR-31), miR-31 self-complementary oligonucleotides (anti-miR), and an irrelevant sequence (negative control, miR-Neg) were constructed as previously described. For transduction, lentiviral vectors with a multiplicity of infection of 50 and an 8 l

**Quantitative Real-Time Polymerase Chain Reaction (qPCR)**

The total RNA was extracted from the transfected BMSCs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) at days 4, 7, and 14, and cDNA was synthesized using a reagent kit (PrimeScript RT; Takara, Dalian, China). The qPCR reaction was performed using a qPCR detection kit (miRCute miRNA; Tiangen Biotech, Beijing, China) and a PCR detection system (7500 Real-Time PCR Detection System; Applied Biosystems, Foster City, CA, USA), as described previously. The relative expression levels of mature miR-31 were normalized to the expression of U6 and are presented as the fold-change relative to expression of miR-Neg-modified cells.

**Construction of Cell-Material Composites in Vitro**

The porous β-TCP scaffolds (Shanghai Bio-Lu Biomaterials Co., Ltd, Shanghai, China) were molded into 3-mm-high symmetric cylinders with diameters of 10 mm, as described previously.5 The scaffolds had a volume porosity of 75%, with a pore diameter of 450 ± 50 μm, and a fully interconnected geometry with an interconnection pore size of 150 ± 50 μm. Next, the transduced BMSCs were concentrated and evenly seeded onto scaffolds using negative-pressure suctioning. The cell-seeded scaffolds were subjected to quiescence in an incubator at 37°C, 5% CO2, 5% O2, and 100% saturation for 24 hours. The transduction efficiency of the lentiviral vectors was determined based on the proportion of GFP-positive BMSCs.

**Micro-CT Analysis**

At 16 weeks after surgery, the animals were euthanized under general anesthesia, and the specimens were harvested using a saw and fixed in 4% paraformaldehyde. The morphology of the reconstructed orbital wall was determined using micro-CT (eXplore Locus; GE Healthcare Biosciences, London, UK) with the following parameters: x-ray tube potential of 80 kV, tube current of 0.45 mA, and 15-mm voxel resolution, as described previously.15 After the micro-CT scan, the bone was visualized using commercial software (MicroView; GE Healthcare) by 3D isosurface renderings, and then the ratio of new bone volume to tissue volume (BV/TV) and the bone mineral densities (BMDs) of the specimens were measured by the software.

**Van Gieson’s Picrofuchsin Stain**

Half of the harvested specimens (n = 4) were dehydrated in increasing concentrations of ethanol and embedded in methylmethacrylate. The sagittal sections of the central segment were cut using a microtome (Leica; Hamburg, Germany), which were then manually ground and polished to a final thickness of 40 μm. Three sections per sample were subjected to Van Gieson’s picrofuchsin staining to compare the ratio of the newly formed bone area to the residual β-TCP materials area. An image analysis system (Image-Pro Plus; Media Cybernetics, Inc., Rockville, MD, USA) was used to determine the percentage of new bone or β-TCP residual area relative to the total area, as described previously.16

**Immunohistochemistry**

The remaining samples (n = 4) were decalcified in 10% ethylene diaminetetraacetic acid for 2 weeks, embedded in paraffin, and cut into coronal cross sections. Immunohistochemistry for osteocalcin (Ocn; 1:100 dilution; Abcam, Cambridge, MA, USA) and green fluorescent protein (GFP; 1:100 dilution; Abcam) was then performed on the sections from each group following previously described methods.17 In brief, the sections were dehydrated, treated with an antigen-retrieval agent, and incubated with the primary antibody at 4°C overnight. Subsequently, the biotinylated secondary antibody (Dako, Carpinteria, CA, USA) and a streptavidin-biotin complex were applied, and a 3’,3-diaminobenzidine substrate was used to stain the sections. The results were analyzed with a light microscope.

**In Situ Hybridization (ISH)**

Paraffin-embedded sections that were 6-μm thick were mounted on glass slides, and ISH was performed according to the manufacturer’s protocol (Exiqon, Vedbaek, Denmark), as previously described.18,19 In brief, sections were deparaffinized with xylene and rehydrated with ethanol, and then treated with 15 μg/mL protease K at 37°C for 10 minutes. After sections were prehybridized in hybridization buffer (Exiqon) at 62°C for 15 minutes, a 40-nM digoxigenin (DIG)-labeled oligonucleotide probe of miR-31 was added to the hybridizatio-
tion solution and hybridized at 50°C overnight. Subsequently, the sections were treated with DIG blocking reagent (Roche, Mannheim, Germany) in maleic acid buffer containing 2% sheep serum, and an alkaline phosphate-conjugated anti-DIG antibody (Roche) was applied. After the sections were washed in staining solution, they were incubated in NBT/BCIP developing solution (Roche) at 37°C for 30 minutes and counterstained with Nuclear Fast Red at 25°C for 1 minute.

Statistical Analysis
The data are expressed as the mean values ± SD. The differences between the experimental and control groups were statistically evaluated by analysis of variance followed by the Student-Neuman-Keuls post hoc test or with the nonparametric Kruskal-Wallis procedure, based on the results of normal distribution and equal variance assumption tests, using the a statistical software package (SPSS, version 17.0; SPSS, Inc., Chicago, IL, USA). Differences between groups were considered to be statistically significant when P < 0.05.

RESULTS
Gene Transduction and Transplantation of BMSCs
Three days after BMSCs were transfected with lentivirus encoding miR-Neg, miR-31, or anti-miR, emission of green fluorescence was determined by fluorescence microscopy observation (Fig. 1A). The expression levels of miR-31 in the gene-modified cells were determined using qPCR analysis at 4, 7, and 14 days. The qPCR results showed that, on days 4, 7, and 14, miR-31 was significantly overexpressed in the miR-31 group and was lower in the anti-miR group than in the miR-Neg group (P < 0.05; Fig. 1B). Twenty-four hours after BMSCs were seeded onto β-TCP scaffolds, the composites were submitted to SEM, which showed that the BMSCs were well attached to the surfaces of the scaffolds (black arrow, Figs. 1C, 1D). Next, the composites (miR-Neg/BMSCs/β-TCP, miR-31/BMSCs/β-TCP, or anti-miR/BMSCs/β-TCP) were transplanted into the bone defects (10 mm in diameter) made in the canine medial orbital walls.

CT Examination
Four weeks after implantation, the miR-Neg, miR-31, and anti-miR groups all showed adequate repair of the medial wall defects (Figs. 2A1–3, black arrows). The implants were smooth and maintained their initial shape, but were substantially thicker than the surrounding orbital wall. No notable inflammatory response was observed. Sixteen weeks after implantation, the 3D images showed that the implants of the anti-miR group had integrated successfully within the surrounding orbital bone tissue and that the joint lines were blurred and similar in thickness to the normal orbital wall (Fig. 2B3). The implant surfaces of the miR-Neg and miR-31 groups became uneven and displayed different degrees of degradation at 16 weeks after surgery (Figs. 2B1–2), and the joint lines were still clearly visible in the boundaries of the implants. The coronal views showed that the implants of the anti-miR group appeared even thinner and were again similar in thickness to the adjacent orbital walls (Fig. 2C3). The implant surfaces of the miR-Neg and miR-31 groups appeared thicker than the surrounding orbital wall (Figs. 2C1–2, white arrows).
Micro-CT Imaging

The microstructure of the newly formed bone was also evaluated in each group using micro-CT imaging. The majority of the orbital bone defects were filled with a substantial amount of newly formed bone tissue in the anti-miR group (Fig. 3A). In the sagittal view, a small amount of nondegraded and residual \( \beta \)-TCP material was distributed among the new bone. A small amount of new bone and a large amount of \( \beta \)-TCP material residue and honeycomb scaffold structure could also be found in the peripheral area of the repaired defect (Fig. 3A).

The quantity of newly formed bone within the defects was determined using commercial software (GE Healthcare). The percentage of new bone volume relative to tissue volume (BV/TV) in the miR-Neg group (36.32 ± 5.60%) was significantly lower (\( P < 0.05 \)) than that in the anti-miR group (41.82 ± 6.54%), but was markedly higher (\( P < 0.05 \)) than that in the miR-31 group (20.50 ± 3.47%; Fig. 3B). The BMDs were 0.460 ± 0.053 g/cm\(^3\) in the miR-Neg group, 0.278 ± 0.046 g/cm\(^3\) in the miR-31 group, and 0.607 ± 0.042 g/cm\(^3\) in the anti-miR group (Fig. 3C), showing the same pattern as the BV/TV ratios. These findings suggest that miR-31 impairs the potential of BMSCs in promoting orbital bone repair.

Histological Examination

To confirm the findings described above, histological assessment of nondecalcified specimens was conducted using Van Gieson’s picrofuchsin staining at 16 weeks after surgery. Calcified bone stains bright red, whereas \( \beta \)-TCP appears black under light microscopy. In the anti-miR group, the implant and the broken ends of the orbital wall were fixed by synostosis (Fig. 4A). In the miR-Neg and miR-31 groups, the defects were largely held by the \( \beta \)-TCP scaffold, with only a few red bone structures forming at the broken margin (Fig. 4A). Quantitative analysis using an image analysis software (Media Cybernetics, Inc.) was used to calculate the percentages of new bone area relative to total area, which were 19.45% ± 2.89% in the miR-Neg group, 10.34% ± 3.45% in the miR-31 group, and 38.4% ± 4.24% in the anti-miR group (Fig. 4B). The percentages of the residual \( \beta \)-TCP material area relative to total area were 28.43% ± 4.23% in the miR-Neg group, 38.7% ± 5.49% in the miR-31 group, and 19.54% ± 3.24% in the anti-miR group (Fig. 4C). Therefore, histological analysis further supported the micro-CT findings.

Immunohistochemical analysis of Ocn was used to determine the presence of the transplanted BMSCs in the orbital bone defect in the decalcified specimens. Osteocalcin was apparent in the new bone matrix or fibrous tissue in the miR-Neg and anti-miR groups at 16 weeks after surgery (Figs. 5A, 5C), while negative staining was found in the miR-31 group (Fig. 5B). Positive staining of GFP in the new bone matrix or fibrous matrix demonstrated the viability of the transplanted BMSCs in all groups (Figs. 5D–F, black arrow).

To further investigate the expression levels of miR-31 in transplanted BMSCs, the paraffin-embedded decalcified specimens were evaluated using ISH. High-level expression of miR-31 was observed in the miR-31 groups (Fig. 5I), whereas the anti-miR group (Fig. 5J) showed low-level miR-31 expression relatively to the miR-Neg group (Fig. 5H). The correlation analysis between Ocn and miR-31 levels showed that low-level miR-31 expression was associated with osteogenic differentiation and bone regeneration in vivo (Fig. 5).

DISCUSSION

With improvements in surgical procedures, including reconstruction of orbital structures and repositioning of the eyeball by transplantation of bone or artificial material, it is crucial to...
FIGURE 3. Micro-computed tomography imaging. (A) The top and bottom panels represent the 3D images and sagittal view of harvested specimens in each group at week 16 postoperation. (B) The percentage of new BV/TV. (C) The bone mineral density. *P < 0.05.

FIGURE 4. Histological analysis of new bone formation and β-TCP degradation using van Gieson’s picrofuchsin stain at week 16 postoperation. (A) From top to bottom: miR-Neg/BMSCs/β-TCP, miR-31/BMSCs/β-TCP, and anti-miR/BMSCs/β-TCP implants (original magnification ×4 and ×40). New bone stains red, whereas β-TCP appears black. (B, C) There were significant differences between the miR-Neg, miR-31, and anti-miR groups with respect to new bone area and amounts of β-TCP residue.
consider the nature and appropriate selection of implants. Transplantation of autogenous bone such as the ilium spongy bone is considered to be the gold standard for clinical bone repair, but the drawbacks include the limited supply, risk of bleeding, infection, and chronic pain. Furthermore, allogeneous bone grafts present a potential risk of virus infection and immunological rejection. Several types of artificial materials, including polymeric porous polyethylene, hydroxyapatite, and titanium, have been widely used in clinical orbital repair; however, these materials are all either nondegradable or difficult to degrade. Hence, they remain as a foreign body, potentially leading to rejection, infection, cyst formation, or implant displacement.

The use of tissue-engineered bone is a new therapeutic strategy for repairing bone tissue defects. Our previous studies showed that the tissue-engineered bones from osteogenically induced BMSCs and a biodegradable β-TCP scaffold could efficiently repair the orbital rim or wall defects in canines, indicating that osteogenic differentiation could promote the orbital bone repair or regeneration induced by BMSCs. These findings were further confirmed by using BMP-2 and VEGF gene-modified BMSCs in repairing orbital rim segmental defects of rabbits. An increasing number of miRNAs have been implicated as regulators of different aspects of bone development, osteoblast differentiation, and osteoporosis pathophysiology. In particular, miR-31 has been identified as an important miRNA in this respect and plays a key role in regulating the osteogenic differentiation of BMSCs; indeed, systematic evaluations have shown that suppression of miR-31 significantly improves the expression of osteogenic-specific marker genes and enhances the osteogenesis of BMSCs or adipose tissue-derived stem cells.

In this study, the role of miR-31-modified BMSCs in orbital bone regeneration was evaluated by repairing a medial orbital wall defect in a canine model. Micro-CT examination showed that anti-miR treatment could significantly improve reconstruction and ossification in canine orbital bone through the application of BMSCs. In contrast, only a small amount of new bone formation was observed in the miR-31 and miR-Neg groups. In addition, the BMD and BV/TV ratio of newly formed bone in the miR-Neg group were significantly higher than those in the miR-31 group and were lower than those in the anti-miR group. Histological examination by van Gieson's picrofuchsin staining further supported the CT and micro-CT findings. The immunohistochemical results showed that Ocn, an osteoblast marker, was expressed at a high level in the anti-miR group but at a low level in the anti-miR group relatively to the miR-Neg group. Our results suggested that miR-31 expression is significantly associated with the osteogenic differentiation of BMSCs in vivo, in which downregulation of miR-31 plays an important role in osteogenesis and orbital bone regeneration.
The orbital wall is a distinctive area that has poor blood supply. However, the degradation rate of β-TCP-based materials in vivo is closely related to blood supply. The CT images showed that the composite of the anti-miR group degraded gradually and approached the thickness of the surrounding normal wall. The degradation rates of the miR-31 and miR-Neg groups were relatively slow compared with that of the anti-miR group. Therefore, these results show that the degradation rate of the material increased when applied in combination with bone-regenerated BMSCs, and this combination was conducive to providing space for new bone ingrowth. These findings are consistent with those of a previous study showing that the microenvironment of bone formation and the extracellular matrix facilitate osteoclast adhesion, mineral resorption, and scaffold degradation.

Orbital floor fractures as devastating forms of trauma accounting for approximately 60%–70% of all human orbital fractures are most commonly caused by assault and traffic accidents. The combined weight of the human orbital contents on the floor would reach to approximately 0.13 MPa, which can be supported by our β-TCP scaffold (mechanical strength > 0.2 MPa). However, the potential application of this strategy in repair of orbital floor fracture should be further investigated in future.

CONCLUSIONS

In summary, the expression level of miR-31 was significantly associated with the osteogenic differentiation of BMSCs in vivo, and the downregulation of miR-31 played an important role in osteogenesis and orbital bone regeneration. In addition, the composite of miR-31 suppressed the ability of BMSCs and porous β-TCP scaffolds to repair a full-thickness orbital wall defect successfully. Our results provide preclinical data supporting the potential application of miRNA-modified BMSCs and degraded materials to repair orbital bone defects.

Acknowledgments

Supported by the National Natural Science Foundation of China (81170876, 31271029, and 8132710801), the Research Fund for the Doctoral Program of Higher Education of China (20130073110015), the Shanghai Municipality Commission for Science and Technology (14JC1493103, 13JC1403800).

Disclosure: Y. Deng, None; H. Zhou, None; P. Gu, None; X. Fan, None.

References


