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Research Article

Potential of miR-106b in Regulating Osteogenesis of Canine Wharton's jelly Derived Mesenchymal Stem Cells in an *in vitro* Model

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Abstract

Wharton's jelly derived-MSCs isolated from canine umbilical cord matrix have been a promising source of MSCs to be used in bone regeneration. MicroRNA (miRNA) is a post-transcriptional regulator of gene expression during osteogenesis. In this study, cMSCs were induced to osteogenesis and miR-106b transcription was measured at 0, 7, 14 and 21 days following induction. Moreover, cMSCs were transfected with a miR-106b mimic and an inhibitor and induced to osteogenesis. Osteocalcin, osteopontin and RUNX2 genes expression and levels of miR-106b was performed using qPCR. Alkaline Phosphatase (ALP) activity was quantified and detected at the same period of observation. Morphologically, cMSCs transfected with an inhibitor of miR-106b appeared as osteocyte-like cells when compared to the same cells transfected with the mimic of miR-106b. cMSCs showed miR-106b transcription after 7 days of osteoinduction was at a low level compared to the positive control, whereas transfected cells with the miR-106b mimic showed miR-106b to be upregulated. After inhibition of miR-106b expression in osteoinduced cMSCs, ALP activity was increased. Osteocalcin, osteopontin and RUNX2 mRNA transcription were upregulated at 21 days after osteoinduction following miR-106b inhibition. This study demonstrated that miR-106b influences *in vitro* osteogenesis of cMSCs and could be a promising for veterinary medicine.

Keywords: Canine Mesenchymal Stromal Cells; Gene Expression; microRNA; Osteodifferentiation

Introduction

Mesenchymal Stem Cells (MSCs) are multi-potent progenitor cells and can be cultured from adult and foetaltissues. In humans, as well as veterinary medicine, MSCs have been successfully harvested from a wide range of tissues [1,2]. As an alternative source of MSCs, foetalor neonatal MSCs appear to be more primitive and have greater multi-potentially than their adult counterparts [3]. Their intermediate state between adult and embryonic stem cells also makes them an ideal candidate for reprogramming pluripotent status [4]. In humans, Wharton´s jelly, an umbilical cord tissue, has been used as a source of MSCs, and studies in many different animal species have also shown their capacity to be classified as a promising source of stem cells [3,5].

Canine Wharton's jelly derived MSCs have been previously isolated and characterized; however, few studies have utilized these MSCs in *in vivo* treatments [6,7]. Previously, canine Wharton's jelly derived MSCs have been isolated and differentiated towards osteogenic lineages [8,9]. There are many protocols of culture and differentiation of MSCs into osteogenic lineages in the literature [10-12]. However, Platelet-Rich Plasma (PRP) and dexamethasone exposure were the inducers of osteogenesis most frequently applied to canine Wharton's jelly derived MSCs to date [6,8,9]. Additionally, dogs are considered to be a good model system for the investigation into human orthopaedic diseasebecause these two species share similar bone pathologies.

MicroRNAs (miRNAs) are small endogenous single-stranded noncoding RNAs that are present in many organisms and repress gene expression by binding to mRNA. Moreover, miRNAs seem to have important regulatory roles in osteocyte proliferation, differentiation and function [13]. There is recent evidence that miRNAs have a crucial role in regulating the complex process of osteoblastic bone formation and osteoclastogenesis [13]. miR-106b is a member of the miR-106b-25 cluster and has been implicated in the immune response and tumourigenesis[14,15]. A recent study has demonstrated that silencing miR-106b accelerates osteogen-

esis of human MSCs derived from human normal term placentas [16]. Despite several studies that describe the role of miRNAs in many biological processes, there is a lack of information in veterinary science related to miRNAs and stem cells.

In this study, we investigated whether miR-106b is expressed in canine MSCs derived from Wharton's jelly before and after osteoinduction, during 0, 7, 14 and 21 days of *in vitro* observation. In addition, we also determined whether transfecting MSCs with a mimic and/or inhibitor of miR-106b interferes with mineralization and osteoblastic gene expression.

Materials and Methods

Cell Culture of Canine MSCs

Chemicals were obtained from Sigma-Aldrich® (St. Louis, MO, USA), Invitrogen (Invitrogen®, Life Technologies™, Carlsbad, CA, USA) and Applied Biosystems™ (Applied Biosystems™, CA, USA). All tissue culture plastic was purchased from BD Falcon™ (BD Falcon, Bedford, USA) unless otherwise specified. Canine MSC cultures were obtained from canine Wharton´s jelly, collected from umbilical cords, as described previously [9], and were stored at -86°C until used. The culture was established in Stemline® Mesenchymal stem cell expansion medium with 2 mM L-glutamine and incubated at 37°C in 5% CO_2 in a humidified incubator. The medium was refreshed every 24 h, and images were acquired to observe the morphology at five-day intervals. The cell lines were then expanded until they reached subconfluence of 80-90%, at which time they were harvested by detachment after a 5-mins incubation period with 0.25% trypsin. Cells were then replated into culture flasks at a 1:5 split ratio. For passaging, 1 x 106 cells were replated in 25 cm2 flasks in the same conditions as in the culture, and the cells were analysed for their capacity for differentiation.

Cell Transfection

Prior to osteogenic differentiation, canine MSCs were transfected with miR-106b mimic and miR-106b inhibitor, and untransfected cells were considered to be the negative control. The cells were plated in 6-well plates, and when they reached 25-30% of confluency, the medium was removed and 3 washes with phosphate buffered solution were performed. Then, 3 ml of Opti-MEM® reduced serum medium was added, together with 100 pmol RNA oligomer (miR-106b mimic and inhibitor) mixed with 5 µl of Lipofectamine™ 2000 (Invitrogen). The plates were incubated for 8 h, and when cell confluency reached 45-55%, osteoinduction was performed. All analyses were performed at 0, 7, 14 and 21 days after osteogenic differentiation as described below.

Osteogenic Differentiation, ALP Activity and Staining

For the osteogenic differentiation, cells at passage 5 were plated at 1 x 106 cells / ml in 6-well culture plates under the same culture conditions described previously [9]. Undifferentiated cells were analyzed as a negative control. After differentiation, the cells were stained with Von Kossa to confirm calcium deposition [7]. It is well established that ALP cleaves the phosphate group from p-nitrophenyl phosphate to form p-nitrophenol, from 10 to 21 days after osteoinduction of canine MSCs derived from Wharton's jelly [9]. ALP activity was measured using the Alkaline Phosphatase detection kit (AKP/ALP™, Sigma-Aldrich®). For ALP staining, all procedures followed steps described previously [9].

RNA Reverse Transcription and quantitative Real-Time PCR (qRT-PCR)

At 0, 7, 14 and 21 days after osteogenic differentiation, cells were collected and total RNA was extracted using the TRizol™ total RNA isolation method [9]. cDNA synthesis was conducted using an Enhanced Avian™ RT First Strand Synthesis Kit (Sigma-Aldrich®) and primers and probes for qRT-PCR specific to dog (*Canisfamiliaris*) osteopontin, osteocalcin, and Runx2 genes were commercially purchased (Applied Biosystems™). Assays to quantify mature miRNAs were carried out using TaqMan™ microRNA probes, with both primers and probes mapping within a single exon sequence (Applied Biosystems™). Quantification of gene expression was performed by the 2 –ΔΔCt method using the dog GAPDH gene as a reference [9]. The results are expressed as relative gene expression, which indicates how many times (fold change) the gene expression is higher (upregulated) or lower (down-regulated) in all studied groups.

Statistical Analysis

Statistical analysis was performed using Graph PadInstat 6.00 for Windows (Graph Pad Software, LaJolla, CA, USA). Four replicates of each experiment were performed, and the results are reported as the mean±s.d. One-way ANOVA was used for multiple comparisons. A p-value of < 0.05 was considered to be significant.

Results

Canine MSC Culture and Osteoblast Differentiation is Associated with miR-106b Expression

Canine MSCs isolated from Wharton's jelly were characterized for their osteogenic differentiation and exhibited changes in cell morphology 21 days after induction (Figure 1A-D).

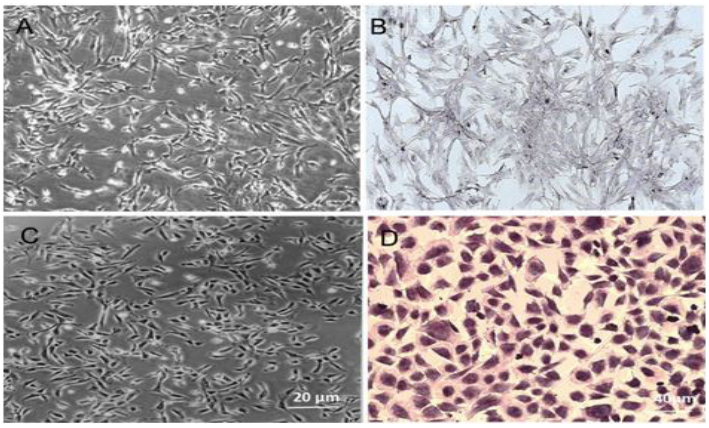

Figure 1A-D: Morphology of canine MSCs (cMSCs) isolated from Wharton's jelly source closely resembled MSCs-like cells (A and C). Micrographs were taken under phase contrast microscopy. After osteogenic differentiation cMSCs transfected with mimic miR-106b (B) and by inhibitor miR-106b (D) showed by Von Kossa's staining. Scale bars 20µm.

To evaluate the biological function of miR-106b in osteogenesis, a miR-106b mimic and inhibitor were transfected into canine MSCs. Cells transfected with the mimic changed their morphology from spindle-shaped to fusiform in a late stage of differentiation (Figure 1A and B). Moreover, when canine MSCs were transfected with the miR-106b inhibitor, their morphology changed to that of polygonal cells (Figure 1C and D). We then measured the level of expression of miR-106b in canine MSCs during osteogenesis without transfection and found that its expression significantly decreased during MSC differentiation (Figure 2A). However, when miR-106b transcription was assessed in transfected MSC cells with mimic miR-106b, we observed higher levels of transcript than when the inhibitor was transfected (Figure 2B; $p<0.05$).

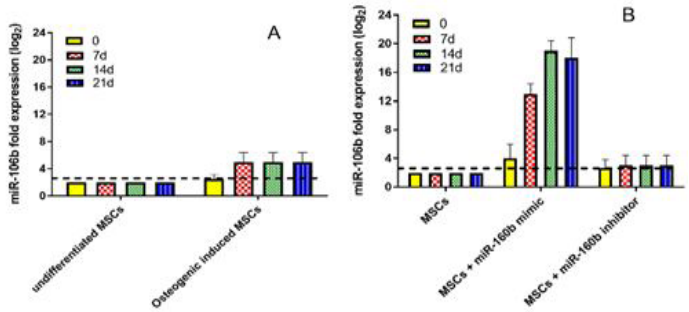

Figure 2A-B: Data from fold change presented as mean±SD (n=3 for all experiments). miR-106b expression in undifferentiated and osteoinduced cMSCs (A) at 0, 7, 14 and 21 days of observation. miR-106b expression in undifferentiated, transfected with mimic miR-106b and with inhibitor miR-106b, respectively (B).

Quantification of Mineralization

Alizarin Red S (ARS) staining was used to detect the calcium mineralization of three experimental conditions of canine MSCs: MSCs without miR-106b transfection, miR-106b mimic, and inhibitor transfection (Figure 3A and B). ALP activity was higher after 21 days following osteoinduction when compared with canine MSCs transfected with a miR-106b mimic (Figure 3A; $p< 0.05$). In contrast, when canine MSCs were transfected with a miR-106b inhibitor, ALP activity was higher after the same period of observation, compared to untransfected and miR-106b mimic-transfected canine MSCs (Figure 3A and b; $p<0.05$). After staining with ARS, calcium-rich deposits were visible as red in colourand appeared more intense in canine MSCs without transfections when compared to the mimic and inhibitor miR-106b transfected cells, respectively (Figure 4A, B and C). However, the morphology was distinct among groups; miR-106b inhibition produced a monolayer of uniform polygonal cells (Figure 4C), in contrast to the fusiform shape, seen following either no transfection or miR-106b mimic transfection (Figure 4A and B).

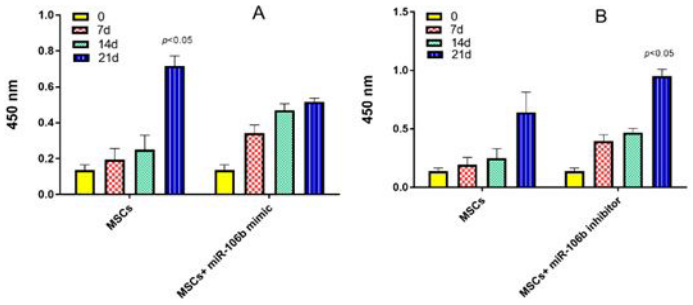

Figure 3A-B: Relative Alkaline Phosphatase (ALP) activity. cMSCs untransfected and miR-106b mimic transfected cells showing statistically difference after 21 days after osteoinduction ($p< 0.05$)**(A)**. Same results were obtained when cMSCs where transfected with miR-106b inhibitor **(B).** Data were obtained from three consecutive experiments.

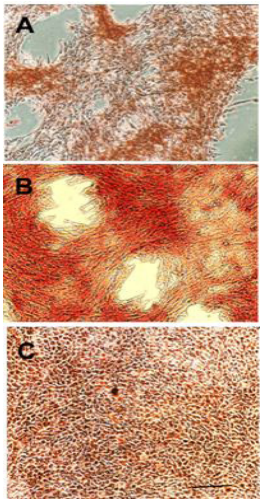

Figure 4A-C:Alizarin Red staining showing extracellular matrix deposition and mineralization of cMSCs submitted to osteo-differentiation without miR-106b transfection (A), cMSCs transfected with mimic miR-106b (B) and respective inhibitor (C). Bar 40µm.

Osteoblastic Genes and miR-106b Transcription

After osteogenic induction, expression levels of osteocalcin, osteopontin and RUNX2, which are related to osteogenesis, were measured at 0, 7, 14 and 21 days of observation in cells transfected with a mimic or an inhibitor of miR-106b (Figure 5A-C). The expression of osteocalcin was significantly upregulated after 21 days of osteoinduction when miR-106b inhibitor was transfected (Figure 5A). This phenomenon was also observed for osteopontin and RUNX2gene expression (Figure 5B and C). Interestingly, miR-106b was upregulated in transfected canine MSCs during osteogenic induction (Figure 2B), yet this may not influence the expression of osteoblastic genes, which were considered to be at a lower level (Figure 5A, B and C).

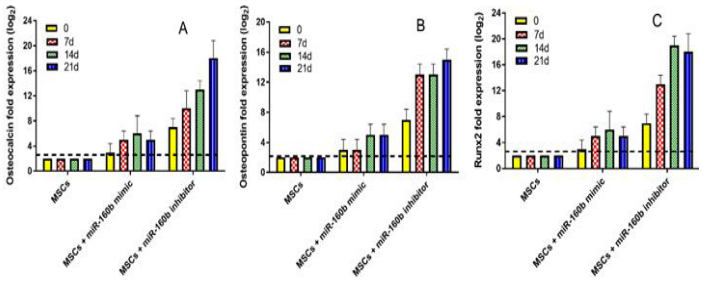

Figure 5A-C: Relative mRNA expression of osteoblast related genes osteocalcin (A), osteopontin(B) and RUNX2 (C) at 0, 7, 14 and 21 days assayed by qRT-PCR. The gene expressions were referenced by GADPH expression for cMSCs (not transfected), transfected cMSCs with mimic miR-106b and with respective inhibitor. Data of fold change are presented as mean±SD ($p<0.005$) in three consecutive experiments.

Discussion

Stem cell studies in veterinary medicine continue to produce rapid experimental and clinical results. In particular, canine-derived MSCs have been isolated from adipose tissues [17], umbilical cord [8], bone marrow [10,18-20] and Wharton's jelly [8,9,21]. Moreover, Wharton's jelly derivedcanine MSCs were first isolated and characterized five years ago [7]. In addition, many reports describe different procedures of maintenance and culture of Wharton´s jelly derived canine MSCs and their advantages over other MSC sources [1-4,21].

This study used Wharton's jelly derived canine MSC cells, which have been well characterized previously [5,7,9]. However, the application of Platelet-Rich Plasma (PRP) as as a supplement [9] was not included in this study due to controversial results found in the literature [20]. In fact, the osteogenic potential of canine MSCs obtained from Wharton's jelly in repairing bone defects has been previously evaluated [8,9] and was shown to be appropriate for in vitro bone engineering techniques. In our previous study, when canine MSCs were isolated and characterized from canine Wharton's jelly, expression levels of osteocalcin, osteopontin and RUNX2,considered to be directly associated with osteogenesis, were upregulated after 21 days of osteoinduction under different culture conditions [9]. However, our findings here are similar tothe results found when same culture conditions were applied in the first characterization of canine MSCs derived from Wharton's jelly [7].

Here, it has been demonstrated that miR-106b is a negative regulator of osteogenesis of canine MSCs derived from Wharton's jelly. The in vitro evidence indicated that miR-106b expression could inhibit the mRNA transcription of Runx2, osteocalcin, and osteopontin, followed by a decrease in ALP activity. However, inhibition of miR-106b expression reversed the phenotype. Recently, emerging evidence has shown that miRNAs are closely involved in regulating key steps of osteogenesis in MSCs [13]. MicroRNAs (miRNAs) are small noncoding RNA molecules of only 22 nucleotides in length that regulate protein expression by binding the 3´untranslated region of a target miRNA [13]. Despite many reports of miRNA inhibition and/or activation in human MSCs, there is a lack of information on this topic in veterinary science.

Osteogenesis is a process regulated by several signals. One of these is Runx2 signalling, the expression of which interferes with osteogenesis via a complex network of miRNAs [13]. Recently, repair of canine medial orbital bone defects with miR-31 -modified bone marrow mesenchymal stem cells was described [22]. A previous study showed that miR-31 negatively regulates osteoinduction of bone marrow-derived canine MSCs [22]. Similar work has demonstrated that suppression of miR-106b in human MSCs isolated from bone marrow had a positive effect on differentiation into osteo-like cells [13]. Despite some studies in human MSCs, little if any information is available about the participation of miRNAs in animal MSCs. For example, miR-125b was the first miRNA reported to influence osteogenesis by regulating MSC proliferation [22]. Since then, several miRNAs have been shown to induce or inhibit MSC differentiation [13]. Although evidence suggests that miRNAs take part in MSCs proliferation and differentiation, more studies are required to elucidate the exact pathway of a complex network of several miRNAs that act in concert to regulate expression of a single gene.

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