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Altered expression of microRNA during fracture healing in diabetic rats

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Objectives

Diabetes mellitus (DM) is known to impair fracture healing. Increasing evidence suggests that some microRNA (miRNA) is involved in the pathophysiology of diabetes and its complications. We hypothesized that the functions of miRNA and changes to their patterns of expression may be implicated in the pathogenesis of impaired fracture healing in DM.

Methods

Closed transverse fractures were created in the femurs of 116 rats, with half assigned to the DM group and half assigned to the control group. Rats with DM were induced by a single intraperitoneal injection of streptozotocin. At post-fracture days five, seven, 11, 14, 21, and 28, miRNA was extracted from the newly generated tissue at the fracture site. Microarray analysis was performed with miRNA samples from each group on post-fracture days five and 11. For further analysis, real-time polymerase chain reaction (PCR) analysis was performed at each timepoint.

Results

Microarray analysis showed that there were 14 miRNAs at day five and 17 miRNAs at day 11, with a greater than twofold change in the DM group compared with the control group. Among these types of miRNA, five were selected based on a comparative and extended literature review. Real-time PCR analysis revealed that five types of miRNA (miR-140-3p, miR-140-5p, miR-181a-1-3p, miR-210-3p, and miR-222-3p) were differentially expressed with changing patterns of expression during fracture healing in diabetic rats compared with controls.

Conclusions

Our findings provide information to further understand the pathology of impaired fracture healing in a diabetic rat model. These results may allow the potential development of molecular therapy using miRNA for the treatment of impaired fracture healing in patients with DM.

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Keywords: Diabetes mellitus, microRNA, Fracture healing, Delayed union, Nonunion

Article focus

- microRNAs (miRNAs) play critical roles in many physiological and pathophysiological processes, and have recently emerged as key regulators in the complex process of fracture healing.
- This study examined and measured miRNA expression profiles in the fracture healing of diabetic rats and the changing patterns of expression of types of miRNA during fracture healing.

Key messages

- In this study, 31 miRNAs were dysregulated at fracture sites in diabetic animals compared with controls, as determined by microarray analysis.
- Microarray analysis and real-time polymerase chain reaction analysis revealed that five types of miRNA (miR-140-3p, miR-140-5p, miR-181a-1-3p, miR-210-3p, and miR-222-3p) were differentially expressed with changing patterns of expression during fracture healing in diabetic rats compared with controls.

Strengths and limitations

- Our findings provide further information to understand the pathophysiology of impaired fracture healing in DM and may

allow the potential development of molecular therapy using miRNA for the treatment of impaired fracture healing in patients with DM.

- It remains unclear whether the observed differences in expression of selected types of miRNA are due to a pathological process induced by DM or are caused by the impaired fracture healing itself.
- Further *in vivo* functional analyses, including gain-of-function tests in fracture healing in healthy rats and loss-of-function tests in DM rats, are required.

Introduction

Diabetes mellitus (DM) is one of the most prevalent chronic diseases. A total of 415 million people have DM worldwide and the number is predicted to rise to *642* million by 2040.[1] It is increasingly recognized that diabetes adversely affects bone health.[2] Clinical studies have demonstrated a significantly higher incidence of delayed union and nonunion with a doubling of the time to healing of fractures in diabetic, compared with nondiabetic, patients.[3-5] Although the association between DM and impaired fracture healing is well documented, there have been few investigations that looked at the molecular mechanisms by which DM affects the process of fracture healing.[5,6]

microRNA (miRNA) is a class of small non-coding RNA molecules that regulate gene expression. Recently, increasing evidence suggests that various types of miRNA are involved in the pathology of diabetes and its complications.[7] In the field of skeletal biology, several studies suggest that miRNA helps regulate chondrocyte, osteoblast, and osteoclast differentiation and function, indicating that miRNA are important regulators of bone formation, resorption, remodelling, and repair.[8] In addition, the involvement miRNA in fracture healing and nonunion has recently been demonstrated.[9-12] To date, however, in DM, the role of miRNA during fracture healing has not been directly studied.

We hypothesized that the function of various types of miRNA and changes to the patterns of expression may play an important role in the pathogenesis of impaired fracture healing in DM. The aim of this study was to examine miRNA expression profiles in fracture healing of the femur of rats with DM, using microarray analysis, and to elucidate the dynamic patterns of expression of the various types of miRNA during fracture healing.

Materials and Methods

Animals. A total of 116 ten-week-old male Sprague–Dawley rats (Japan SLC Inc., Hamamatsu, Japan) were used in this study, with half assigned to the DM group and half to the control group. As an impaired fracture healing model, DM rats were created by a single intraperitoneal injection of 40 mg/kg streptozotocin (STZ) (Sigma-Aldrich, St. Louis, Missouri).[6] This experimental model reproducibly leads to type I DM. Rats with blood glucose levels over 16.7 mmol/l at one week after injection were used for experiments, and fractures were made two weeks after STZ injection. During experiments, blood glucose levels were measured once a week. Animals with blood glucose levels below 16.7 mmol/l were excluded from the study. No animal with induced DM was excluded based on our criteria. Control rats were injected with sodium chloride as a sham treatment. All animal procedures were performed under the approval and guidance of the Animal Care and Use Committee of Kobe University Graduate School of Medicine.

Surgical procedure. Closed transverse femoral shaft fractures were created in both groups using the method of Bonarens.[13] A transverse femoral shaft fracture was produced in all animals using a three-point bending apparatus with a drop weight. A Kirschner wire of 1.25 mm in diameter was introduced into the right femoral intramedullary canal by retrograde insertion, to stabilize the fracture.

Radiographic assessment of fracture repair. At days seven 14, 21, and 28 following fracture, eight randomly selected animals in each group were anaesthetized and radiographs of the fracture sites were acquired. On radiographic evaluation, four cortices (two on the anteroposterior and two on the lateral radiograph) on each callus were evaluated by two orthopedic surgeons blinded to the treatment group. Fracture healing was defined as a bony union when three of four cortices were bridged by callus and/or fracture lines disappeared completely.[14]

Histology of fracture sites. At days seven, 14, 21, and 28 after fracture, the fractured femur was harvested from three randomly selected animals in each group. The femur was fixed in 4% paraformaldehyde, decalcified, and embedded in paraffin wax. Sagittal 5 µm sections were cut and stained with safranin O/fast green for histological examination.

Strategy for miRNA expression analysis. Figure 1 shows the experimental design for miRNA expression analysis. Step 1: post-fracture days five and 11, the fracture callus from both groups were screened by microarray analysis for 727 types of miRNA in order to identify those expressed at high or low levels for both the DM group and the control group. The types of miRNA were extracted by filtering with a fold change of >2 or <0.5 as the threshold for significant change, low coefficient variation ($<50\%$), and high Hy3 signal (>10). Step 2: we selected types of miRNA from the array results that had been previously characterized from the published literature,[15-19] using a PubMed database search (https://www.ncbi.nlm.nih.gov/pubmed/), for their effects on inflammation, osteogenesis, chondrogenesis, endochondral ossification, and angiogenesis, all of which are important processes in normal fracture healing. Real-time polymerase chain reaction (PCR) analysis was performed to compare the

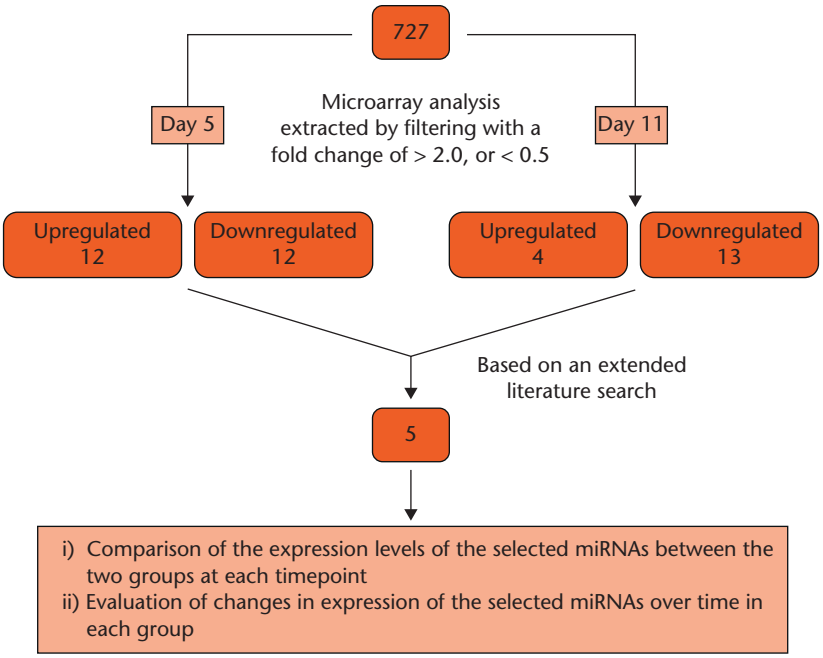

Fig. 1

Flowchart of the experimental design. The boxes indicate microRNA (miRNA) numbers. PCR, polymerase chain reaction.

expression levels of the selected types of miRNA between the two groups at post-fracture days five, seven, 11, 14, 21, and 28, and to investigate their changes in expression over time in each group.

RNA extraction and miRNA microarray analysis. On post-fracture days five and 11, total RNA, including miRNA, was extracted from the newly generated callus (n = 5 in each group) using miRNeasy Mini Kit (Qiagen, Venlo, The Netherlands). Extracted total RNA was labelled with a 3D Gene miRNA labelling kit (Toray, Kamakura, Japan). Labelled RNAs were hybridized onto 3D Gene Rat miRNA Oligo chips (Toray). The annotation and oligonucleotide sequences of the probes conformed to the miRBase miRNA database. After stringent washes, fluorescent signals were scanned with the 3D Gene Scanner and analyzed using 3D Gene Extraction software (Toray).

Real-time PCR analysis. Based on the array results, five types of miRNA were selected for further real-time PCR analysis. Real-time PCR was performed on RNA from the newly generated callus collected on post-fracture days five, seven, 11, 14, 21, and 28 (n = 6 in each group at each timepoint). Tissue specimens were homogenized and total RNA was extracted. RNA was reverse-transcribed into single-strand complementary DNA using the miRCURY locked nucleic acid (LNA) Universal RT microRNA PCR kit (Exiqon A/S, Vedbaek, Denmark). Real-time PCR analysis was performed in duplicate with a StepOne Sequence Detector (Applied Biosystems Inc., Foster City, California), using SYBR Green master mix and microRNA LNA PCR primer sets (both from Exiqon A/S, Vedbaek, Denmark). U6, a small nuclear RNA, was used as an internal control to normalize differences in miRNA levels in each sample.[9,12] The relative abundance of each miRNA was calculated using the comparative ΔΔCT method[9,12] and is presented as the fold change relative to levels in the post-fracture day five control sample.

Statistical analysis. All quantitative data are presented as means and standard errors of the mean. The chi-squared test was used to compare the radiological results between the groups at each timepoint. The values of the DM group and the control group were compared at each timepoint using the Mann–Whitney U test. The Kruskal–Wallis test and Mann–Whitney U test with Bonferroni correction were used to compare timepoints in each group. A p-value of < 0.05 was defined as statistically significant.

Results

Radiological assessment of fracture repair. At day 21, enlargement of the callus was observed and three of the eight animals (37.5%) had achieved fracture union in the control group. In contrast, the callus size was smaller and no animal achieved union in the DM group. At day 28 in the control group, seven of the eight animals (87.5%) had achieved union compared with no animals in the DM group, and remodelling processes were observed (Fig. 2). The union rates of the two groups on day 28 were significantly different (p = 0.0004, chi-squared test).

Histology of fracture sites. Figure 3 shows the histology of fracture healing in control and DM rats. On day 14, animals in the control group had formed a thick callus consisting of chondrocytes and newly formed woven bone, while, in the DM group, smaller cartilage developed. In the control group on day 21, the callus on each side of the fracture were nearly united, with newly formed woven

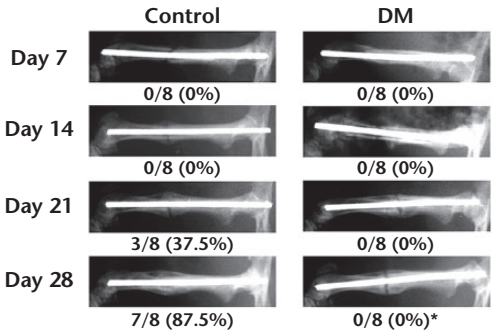

Fig. 2

Radiographs of femurs during fracture healing in the control and diabetes mellitus (DM) groups. Representative radiographs are shown and the proportion of rats with fracture union is indicated at the bottom of each image. *p < 0.05 compared with the control group (chi-squared test).

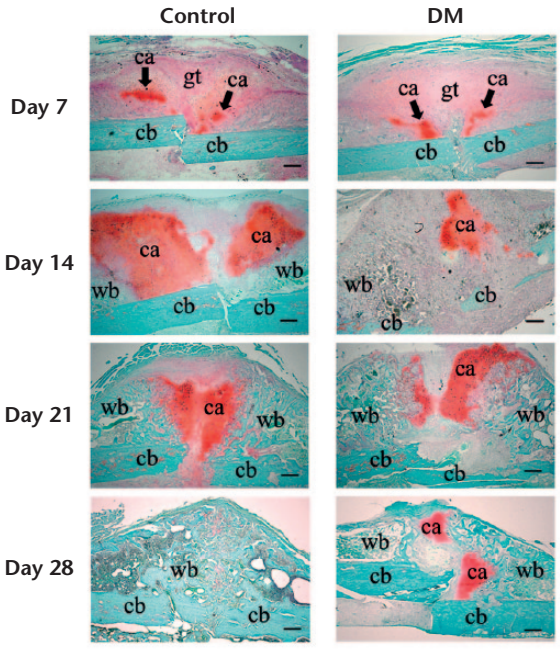

Fig. 3

Histology of fracture sites in control and diabetes mellitus (DM) groups on post-fracture days seven, 14, 21, and 28. Sections were stained with safranin-O/fast green. gt, granulation tissue; cb, cortical bone; ca, cartilage; wb, woven bone. Bar = 500 µm.

bone predominated in the callus. In contrast, animals in the DM group exhibited poor bridging callus formation and fibrous tissue interposition existed at the fracture site. The area of cartilage was much smaller in the DM group (Supplementary material). Finally, on day 28 the callus in the control group had united and active remodelling was underway. In comparison, in the DM group cartilage remained between the woven bones, suggesting delayed union.

miRNA microarray analysis. Using a miRNA-based array screening, we tested the expression of 727 types of rat miRNA. There were 14 differentially expressed types of miRNA (12 increased and two decreased), with a greater than twofold change in the DM group, compared with the control group at days five, and 17 differentially expressed types of miRNA (four increased and 13 decreased) at day 11 (Table I). These data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus with the accession number GSE76365.

Table I. Highly up- or downregulated types of microRNA (miRNA) in the diabetes mellitus group compared with expression in control group on post-fracture days five and 11

Day 5		Day 11	
miRNA	**Fold change**	**miRNA**	**Fold change**
miR-451-5p	3.44	miR-379-3p	2.68
miR-532-5p	3.23	miR-376a-3p	2.09
miR-551b-3p	3.04	miR-221-3p	2.03
miR-339-3p	2.50	miR-379-5p	2.02
miR-181a-1-3p	2.44	miR-6216	0.46
miR-3065-3p	2.44	miR-210-3p	0.46
miR-222-3p	2.37	miR-6324	0.43
miR-133a-5p	2.24	miR-675-3p	0.39
miR-181b-5p	2.15	miR-330-3p	0.38
miR-3593-3p	2.14	miR-455-3p	0.37
miR-183-3p	2.13	miR-6317	0.33
miR-125b-1-3p	2.05	miR-3557-3p	0.33
miR-6324	0.49	miR-455-5p	0.32
miR-151-3p	0.48	miR-207	0.32
		miR-3075	0.31
		miR-140-5p	0.30
		miR-140-3p	0.29

Selection of five types of miRNA for real-time PCR analysis. Among 31 types of miRNA described in Table I, we selected five for further real-time PCR analysis based on our results and an extended literature search of the PubMed database (Fig. 4). The types of miRNA included miR-140-3p, miR-140-5p, miR-181a-1-3p, miR-210-3p, and miR-222-3p. Representative validated target genes of the five selected types of miRNA are shown in Table II. We compared the expression levels of those selected between the two groups at each timepoint and investigated their changes in expression over time.

miR-140-3p and miR-181a-1-3p. miR-140-3p and miR-181a-1-3p are associated with inflammation.[15,16] The expression level of miR-140-3p at day 14 was significantly lower in the DM group than in the control group ($p = 0.002$; Mann–Whitney U test with Bonferroni correction; Fig. 5a). In the control group, miR-140-3p expression peaked on day 14 and significantly decreased on day 21 ($p = 0.006$). In contrast, the expression in the DM group peaked on day 11 and then declined over time. The expression in the DM group peaked earlier than in the control group. The miR-181a-1-3p expressions at days seven and 21 were significantly higher in the DM group than in the control group ($p = 0.026$ and $p = 0.004$, respectively; Mann–Whitney U test with Bonferroni correction; Fig. 5c), while the expression level at day 14 was significantly lower in the DM group than in the control group ($p = 0.015$). In the control group, miR-181a-1-3p

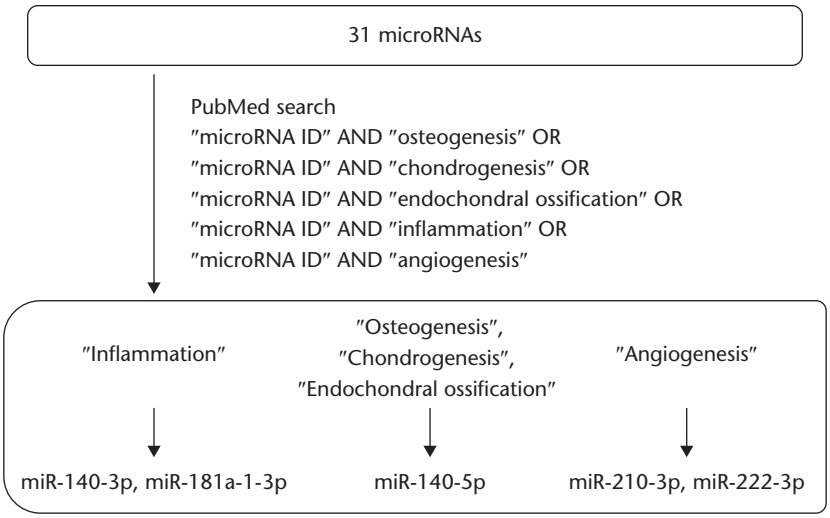

Fig. 4

Flowchart of types of microRNA (miRNA) selected from the array results for further investigation with real-time polymerase chain reaction by the extended literature search performed using the PubMed search engine.

Table II. Validated target genes of the five selected types of microRNA (miRNA)

miRNA	Target genes	References
miR-140-3p	NCOA1, NRIP1	Takata et al[15]
miR-140-5p	BMP-2, Dnpep	Hwang et al,[17] Nakamura et al[20]
miR-181a-1-3p	IL-1α	Xie et al[16]
miR-210-3p	Ephrin-A3	Claes et al[23]
miR-222-3p	c-kit	Poliseno et al[31]

NCOA1, nuclear receptor co-activator 1; NRIP1, nuclear receptor-interacting protein 1; BMP-2, bone morphogenetic protein-2; IL-1α, interleukin-1 alpha

expression significantly increased on day 14 (p=0.003) and peaked on day 21. The expression in the DM group increased until day 21 and then declined.

miR-140-5p. miR-140-5p is associated with osteogenesis,[17] chondrogenesis,[18] and endochondral ossification.[20] The miR-140-5p expression level at day 14 was significantly lower in the DM group than in the control group (p=0.015; Mann–Whitney U test with Bonferroni correction; Fig. 5b), while the expression at day seven was significantly higher in the DM group than in the control group (p=0.004). In the control group, the expression peaked on day 14 and significantly decreased on day 21 (p=0.014). In contrast, the expression in the DM group peaked on day 11 and then declined over time. The expression in the DM group peaked earlier than that in the control group.

miR-210-3p and miR-222-3p. miR-210-3p and miR-222-3p are associated with angiogenesis.[19] miR-210-3p and miR-222-3p expression at day 7 was significantly higher in the DM group than in the control group (p=0.004 and p=0.041, respectively; Mann–Whitney U test with Bonferroni correction; Figs 5d and 5e), while the expression level at day 14 was significantly lower in the DM group than in the control group (p=0.015 and p=0.004, respectively; Mann–Whitney U test with Bonferroni correction). The miR-222-3p expression at day 28 was significantly higher in the DM group than in the control group (p=0.026). In the DM group, miR-210-3p expression peaked on day seven and then declined over time. miR-222-3p expression in the DM group peaked on day five and then declined over time. The expression of both types of miRNA in the DM group peaked earlier than that in the control group.

Discussion

miRNA plays a critical role in many physiological and pathophysiological processes and have recently emerged as key regulators in the complex process of fracture healing.[9-11] In the present study, 31 miRNA was dysregulated at fracture sites in DM animals compared with controls, as determined by the microarray analysis. The rationale for choosing two timepoints (post-fracture days five and 11) for microarray analysis was that these represent the moments of key cellular events of the fracture healing process in healthy rats. The process of fracture healing can be divided into three overlapping phases: inflammation; repair; and remodelling.[21] Post-fracture day five corresponds to the transitional period from the inflammatory phase, which is recognized as the first step of fracture healing to the repair phase.[22] Post-fracture day 11 corresponds to the repair phase, which is recognized as a timepoint that represents the process of intramembranous

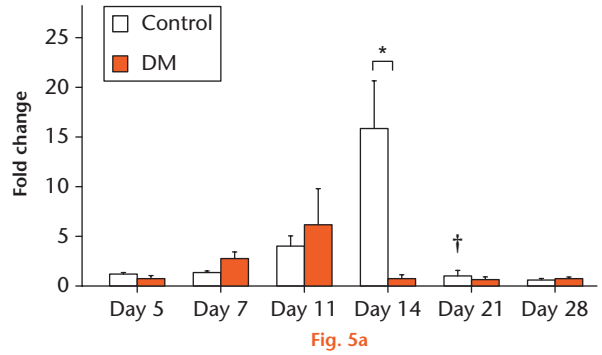

Fig. 5a

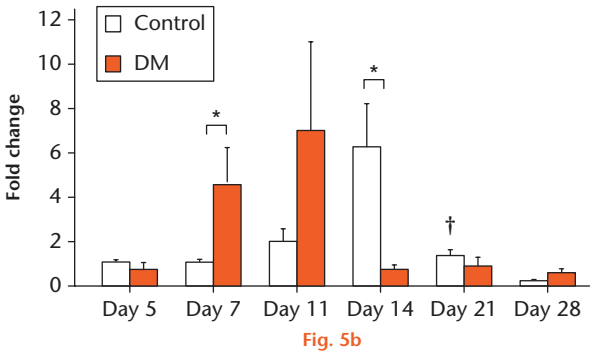

Fig. 5b

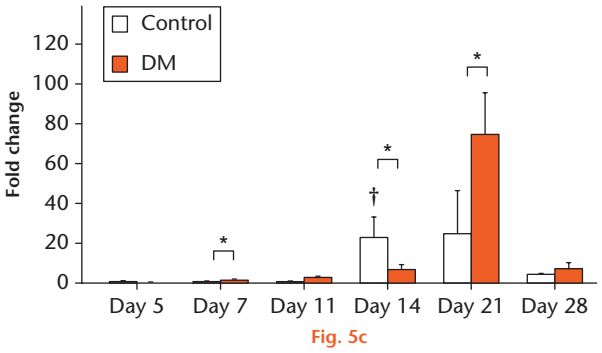

Fig. 5c

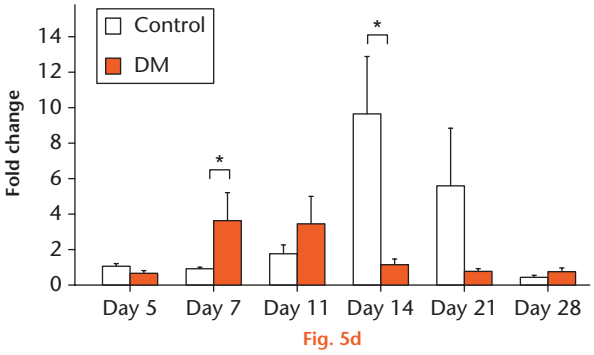

Fig. 5d

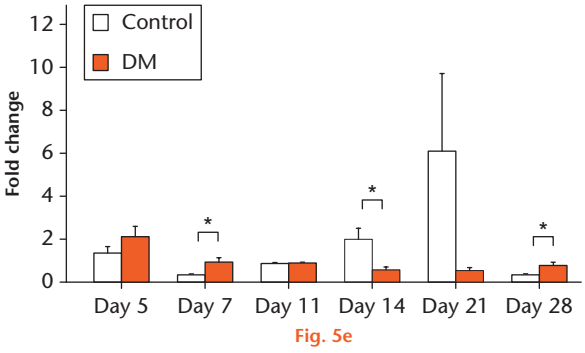

Fig. 5e

Graphs showing expression of a) miR-140-3p, b) miR-140-5p, c) miR-181a-1-3p, d) miR-210-3p, and e) miR-222-3p in the control group (white bars) and diabetes mellitus (DM) group (orange bars) on post-fracture days five, seven, 11, 14, 21, and 28, as analyzed by real-time polymerase chain reaction (PCR). All graphs show the fold change in expression when the expression in the control group on day five was normalized as 1. Values are the mean and standard error of the mean (n = 6 in each group at each timepoint). *$p < 0.05$ for indicated groups at the same timepoint. †$p < 0.05$ *versus* values on the former timepoint in the control group. p-values calculated using Mann–Whitney U test with Bonferroni correction.

and endochondral ossification, including osteogenesis, chondrogenesis, and vascular invasion.[22] Imbalance in inflammatory responses, reduced proliferation, differentiation of osteoblast and chondrocyte function, and alteration in vascularization have been implicated as possible pathological mechanisms underlying the impaired fracture healing in DM.[2] We focused on the types of miRNA associated with inflammation, osteogenesis, chondrogenesis, endochondral ossification, and angiogenesis, and selected five types of miRNA (miR-140-3p, miR-140-5p, miR-181a-1-3p, miR-210-3p, and miR-222-3p) for further analysis. We have shown for the first time using real-time PCR analysis that miRNA is expressed differentially with changing patterns of expression during fracture healing in diabetic rats when compared with control rats. These findings provide insights into the involvement of types of miRNA in impaired fracture healing in DM.

Inflammation is a critical factor during fracture healing, with inflammatory cells and molecular factors (e.g. TNF, IL-a, IL-6, and lactate) appearing locally at the fracture site in a distinct spatial and temporal manner.[22] Highly regulated inflammatory signalling during fracture healing is essential for initiating bone regeneration. Disturbance to the finely tuned inflammatory responses at the site of fracture impairs vascularization, reduces bone formation, disturbs osteoclast function, and consequently may lead to delayed or nonunion.[23] miR-140-3p and miR-181a-1-3p are involved in the regulation of inflammatory responses.[15,16] miR-140-3p negatively regulates nuclear factor-κB (NF-κB) inflammatory signalling by regulating the expression of nuclear receptor

co-activator 1 (NCOA1) and nuclear receptor-interacting protein 1 (NRIP1), both of which are NF-κB co-activators.[15] One of the primary molecular responses to tumor necrosis factor-α (TNF-α) signalling is NF-κB activation.[24] In contrast, miR-181a-1-3p regulates inflammatory responses by directly targeting and downregulating interleukin-1 alpha (IL-1α) in monocytes and macrophages.[16] In rats with DM in the current study, altered patterns of expression of these inflammation-regulating types of miRNA – miR-140-3p and miR-181a-1-3p – might lead to dysregulation of normal inflammatory response in fracture healing, thus contributing to impaired fracture healing.

Osteogenesis and chondrogenesis are essential components of the endochondral ossification in fracture healing.[21] miR-140-5p is associated with osteogenesis[17] and chondrogenesis.[18] Bone morphogenetic protein-2 (BMP-2) is a direct target of miR-140-5p.[17] Hwang et al[17] demonstrated that miR-140-5p is commonly enriched, or upregulated, in undifferentiated mesenchymal stem cells and is a negative regulator of osteogenic differentiation. The process of fracture healing closely resembles that of normal skeletal development which occurs by intramembranous and endochondral ossification.[25] miR-140-5p plays a major role in regulating skeletal development. Nakamura et al[20] demonstrated that miR-140-5p plays an essential role by regulating the processes of endochondral ossification. They identified Dnpep as a miR-140-5p target gene, the upregulation of which plays a causal role in the skeletal defects of miR-140-null mice by reducing BMP signalling. In this study, the miR-140-5p expression level at day 14 was significantly lower in the DM group, and its expression peaked earlier. The altered expression of miR-140-5p at the fracture site in rats with DM might cause the impaired fracture healing during the repair phase when regulating osteogenesis, chondrogenesis, and endochondral ossification.

Angiogenesis and bone formation are coupled during fracture healing.[26] The lack of oxygen (hypoxia) and the subsequent generation of angiogenic factors are critical in achieving successful fracture healing.[27] miR-210-3p and miR-222-3p are associated with angiogenesis.[19] miR-210-3p is a key player in angiogenesis in response to hypoxia.[28] Overexpression of miR-210-3p enhances the formation of capillary-like structures and the vascular endothelial growth factor (VEGF)-driven migration of normoxic endothelial cells, whereas blocking inhibits the formation of the capillary-like structures and decreases the migration in response to VEGF. The relevant target for miR-210-3p in hypoxia is Ephrin-A3, a molecule that has an essential function in angiogenesis. Ephrin-A3, as an ephrin ligand family member, can join the Eph/ephrin system controlled by the VEGF pathway, regulating angiogenesis.[29] Shoji et al[30] demonstrated that the intra-articular injection of double-stranded miR-210-3p can accelerate the healing of partially torn anterior cruciate ligaments through enhancement of angiogenesis in a rat model. In contrast, miR-222-3p is an anti-angiogenic miRNA. miR-222-3p regulates angiogenesis in response to stem cell factor, one of the main growth factors involved in cell fate and angiogenesis, by directly repressing the levels of c-Kit and attenuating cell survival, migration, and vessel formation.[31] The altered expression patterns of miR-210-3p and miR-222-3p in the DM group may cause dysregulation of normal angiogenesis during fracture healing, leading to impaired fracture healing.

Our findings have clinical implications. Due to the increasing number of diabetic patients, impaired fracture healing associated with DM may be expected to become more prevalent. Currently the alternative treatment options for impaired fracture healing in diabetic patients are limited. The recent discovery of different types of miRNA, and their ability to regulate global gene expression patterns in a variety of tissues and processes, suggests that strategy targeting miRNAs has therapeutic potential. Several therapeutic trials in different clinical situations, targeting types of miRNA have been conducted.[32] Notably, data from the first clinical phase 2 study showed that the anti-miR-122 drug, miravirsen (Santaris Pharma A/S, Copenhagen, Denmark), was safe and well-tolerated, and provided prolonged antiviral activity in patients with chronic Hepatitis C.[33] This implies that miRNA-based therapeutics are likely to have a role in future clinical medicine. In orthopaedic research, the involvement of types of miRNA in fracture healing has recently been demonstrated.[9-12,34-36] Wang et al[34] showed that synovial fluid levels of miR-9 and miR-181a-1-3p were significantly downregulated five days after tibial plateau fracture on mice. Waki et al[9] examined miRNA expression profiles during standard fracture healing of the rat femur and identified highly expressed types of miRNA at the fracture site, miR-140-3p, miR-140-5p, miR-181a-5p, miR-181d-5p, and miR-451a, which are associated with the process of fracture healing. More recently, Hadjiargyrou et al[11] detected that 20 miRNA displayed combined up- and downregulated expression within the time course in the mouse femoral fracture model. Although not all types of miRNA have yet been shown to influence fracture healing *in vivo*, these findings could potentially use types of miRNA as therapeutic targets to affect or influence fracture healing. The types of miRNA identified in our study could represent key tools for the development of molecular therapy for the treatment or prevention of impaired fracture healing in patients.

One limitation of this study is that we did not demonstrate any regulation exerted by these five types of miRNA and hence we have only shown an association with, but not the causation of, impaired fracture healing. It remains unclear whether the observed differences in expression of these types of miRNA are due to a pathological process

induced by DM or are caused by the impaired fracture healing itself. Further *in vivo* functional analyses, including 'gain-of-function' tests in fracture healing in healthy rats and 'loss-of-function' tests in DM rats, will be required to define the precise role of each miRNA during fracture healing. Another limitation is that our study did not validate or identify target genes of the five selected types of miRNA. Some types of miRNA may target any of the previously reported target genes in one cell type but not in others, given the diversity of cell types found within the fracture callus.[11] The contribution of the differently regulated miRNA patterns identified in this study to the downregulation of specific genes, in the context of the fracture healing process, needs further investigation. The third limitation of the study is that microarray analysis was not performed at later timepoints, i.e. the remodelling phase. This study focused on the first two phases of fracture healing based on the hypothesis that dysregulation of certain microRNAs would affect the progression of endochondral ossification in the DM group during inflammatory and repair phases. It would be of interest to investigate further the expression of miRNA during the remodelling phase of bone healing, and this will be the subject of future experiments.

In conclusion, the present study shows that five types of miRNA – miR-140-3p, miR-140-5p, miR-181a-1-3p, miR-210-3p, and miR-222-3p – identified using microarray analysis and real-time PCR analysis may contribute to impaired fracture healing in DM. Our findings provide useful information to further understand the pathology of impaired fracture healing in DM, and may allow the development of molecular therapy using miRNA for the treatment of impaired fracture healing in patients.

Supplementary material

Further data relating to this paper are available alongside the online version of this article at www.bjr.boneandjoint.org.uk

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Conflict of Interest Statement
- None declared