Potential Role of MicroRNA in Fracture Healing in Rats

Takahiro Waki, M.D., Sang Yang Lee, M.D., Ph.D., Takahiro Niikura, M.D., Ph.D., Takashi Iwakura, M.D., Ph.D., Yoshihiro Dogaki, M.D., Etsuko Okumachi, M.D., Shunsuke Takahara, M.D., Masahiro Kurosaka, M.D., Ph.D.
Kobe University Graduate School of Medicine, Kobe, Japan.


Introduction: It is estimated that approximately 7.9 million fractures are sustained in the United States each year, and 5-10% of fractures fail to heal and result in delayed union or persistent nonunion [1]. To enhance fracture healing, a variety of treatment techniques have been explored. The recent discovery of microRNA (miRNA) introduces a novel type of regulatory control over gene expression. miRNA is a class of noncoding RNA that regulates gene expression by binding to the 3'-untranslated region (3'-UTR) of their target messenger RNA (mRNA), leading to translational repression or mRNA degradation [2]. miRNA has been shown to play key roles in biological processes such as tissue repair, development, disease, and cancer [3].

The role of miRNA during the process of fracture healing remains unknown. The purpose of this study was to examine miRNA expression patterns specific for fracture healing in rats and to clarify the important miRNAs during fracture healing.

Methods: Animal Model: Seventy male Sprague-Dawley rats were used in this study. Animals were randomized to receive either a surgical treatment that has been shown to produce a nonunion or to a standard stabilized closed femoral shaft fracture that is known to successfully heal. The details of these procedures have been previously described [4, 5]. Briefly, to produce standard healing models, a 1.25-mm diameter K-wire was inserted retrograde into the right femoral intramedullary canal and a closed transverse femoral shaft fracture was produced using a three-point bending apparatus with a drop weight [4]. To produce the nonunion, the fractured site was additionally exposed and the periosteum was cauterized circumferentially for a distance of 2 mm on each side of the fracture [5].

Tissue Harvest: Five animals from each group were euthanized at post-fracture day 14 for microarray analysis and five animals from each group were euthanized at post-fracture day 3, 7, 10, 14, 21, and 28 for real-time PCR. At the abovementioned time points, the newly generated specific tissues, that is, the fracture callus for the standard healing and the fibrous tissue surrounding the fracture site for the nonunion, were harvested.

RNA Extraction: Total cellular RNA including miRNA was extracted from the harvested tissues using miRCURY RNA Isolation Kit (Exiqon).

miRNA Microarray Analysis: RNA samples from 5 different animals in each group at post-fracture day 14 were pooled. The microarray experiments were performed using miRCURY LNA microRNA Hi-Power Labeling Kit and miRCURY LNA Array (Exiqon). Each customized microarray chip contained a probe set based on the miRNA database version 16.0.

Real-time PCR Analysis: Based on the results of the miRNA microarray analysis, we selected five miRNAs, of which expression was remarkably increased in the standard healing group compared to the nonunion group. Real-time PCR was performed using SYBR Green master mix and microRNA LNA PCR primer sets (Exiqon), and StepOne Sequence Detector (Applied Biosystems). Expression levels of miRNAs were normalized to U6 levels and expressed relative to the levels in the standard healing group at post-fracture day 14 (ΔΔCT methods).

Results: miRNA Microarray Analysis: Using a miRNA-based array screening, we tested the expression of 2791 miRNAs. We identified 17 differentially expressed miRNAs (8 increased and 9 decreased) with a greater than two-fold change in standard healing group compared to nonunion group. Table 1 shows five selected miRNA, miR-140-3p, miR-140-5p, miR-181a-5p, miR-181d, and miR-451a with increased expression in standard healing group at 2 weeks after fracture.

Real-time PCR Analysis: Expression levels of all five miRNAs at day 14 were significantly higher in standard healing group than nonunion group, and they all showed a peak at day 14 (Figure 1A–E). In addition to this, the expression levels of miR-140-3p, miR-140-5p, miR-181a-5p, miR-181d, and miR-451a were significantly higher in standard healing group than nonunion group at days 7 and 10, at days 7 and 10, at days 7, 10, and 21, at days 3 and 10, and at day 28, respectively. As for time course changes in the expression levels of all five miRNAs in standard healing group at post-fracture day 14 (ΔΔCT methods).

Discussion: This is the first study to show miRNA expression patterns specific for fracture healing in rats. miRNA microarray revealed that five miRNAs (miR-140-3p, miR-140-5p, miR-181a-5p, miR-181d, and miR-451a) increased remarkably in the standard healing group compared to the nonunion group (Table 1). Real-time PCR analysis showed that all five miRNAs in standard healing group showed a peak at day 14 and then dramatically declined.

miR-140-3p and miR-140-5p have been reported to regulate endochondral bone development [6]. Loss of miR-140 in mice caused growth defects of endochondral bones, resulting in dwarfism and craniofacial deformities. miR-451a was reported to regulate cyclooxygenase-2 (COX-2) [7]. Zhang et al. demonstrated that COX-2 plays an essential role in both endochondral and intramembranous bone formation during skeletal repair [8]. In the previous study which used the same rat fracture model as we used, COX-2 mRNA levels showed peak expression during the first 14 days of healing [9], which was consistent with our results.
miR-140-3p, miR-181a, and miR-181d were reported to negatively regulate inflammation via NF-κB pathway [10]. Inflammation is an important factor during fracture healing, with molecular factors and immune cells appearing locally at the fracture site in a distinct spatial and temporal manner. Disturbances to this finely tuned sequence of events leads to impaired fracture healing, as demonstrated in certain gene knockout animal model (such as IL-6 and TNF deficient mice) [11]. Taken together, all five miRNAs which we here clarified using microarray analysis and real-time PCR analysis may play an important role in fracture healing. Recently, several in vivo studies provided evidence suggesting the potential therapeutic usefulness of silencing or enhancing miRNA. Therefore, further functional analyses to define the precise role of miRNAs during fracture healing may provide novel attractive therapeutic tools for fracture healing and/or offer new strategies to enhance fracture healing.

**Significance:** To characterize miRNA profiles in fracture is important for understanding the molecular mechanism for fracture healing. This study may provide new evidence for the clinical application of miRNA for enhancement of fracture healing.

**Acknowledgments:**

# Table 1

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Fold change</th>
<th>Target genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-140-3p</td>
<td>2.15</td>
<td>NEFA, UBE2C, ZNF295</td>
</tr>
<tr>
<td>miR-140-5p</td>
<td>2.43</td>
<td>HDAC4, FGF2, VEGFA</td>
</tr>
<tr>
<td>miR-181a-5p</td>
<td>3.02</td>
<td>METAP1, PLCL2, ZNF594</td>
</tr>
<tr>
<td>miR-181d</td>
<td>3.53</td>
<td>ZNF799, CREB1F, ZNF788</td>
</tr>
<tr>
<td>miR-451a</td>
<td>2.65</td>
<td>MIF, CAB39, ABCB1</td>
</tr>
</tbody>
</table>

2012.

# Figure 1

A, B, C, D, E: Bar charts showing the fold change and target genes for different miRNAs over time.