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Original article

Acceleration of healing of the medial collateral ligament of the knee by local administration of synthetic microRNA-210 in a rat model

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Abstract

Background: Injury to the medial collateral ligament (MCL) of the knee joint is the most common ligament injury of the knee. Ligament healing generally takes a long time. Micro-ribonucleic acid (miRNA) is one of the noncoding RNAs and plays a crucial role in physiological function; miRNA (miR)-210 is known as a potent factor of angiogenesis, which is an important initiator of ligament healing. The purpose of this study is to examine the effect of local injection of double-stranded (ds) miR-210 on the healing of the MCL of rat knee joint.
Methods: MCLs of Sprague-Dawley rats were cut transversely. After the fascia and skin were sutured, dsmiR-210 or control dsRNA was injected into the injured site of MCL. At 2 weeks and 4 weeks, histological analysis and immunofluorescence staining of vascular endothelial growth factor, isolectin B4, collagen type 1, and Ki67 as well as a mechanical test were performed. Analysis of complementary deoxyribonucleic acid (cDNA) microarray data was performed at 1 week.
Results: Histological analysis showed that parallel fibres in the injured site were organised at 2 weeks and became thicker at 4 weeks in the miR-210-treated group, whereas the injured site in controls was filled with loose fibrous tissues and was thinner than that in the miR-210-treated group. The number of blood vessels in the miR-210-treated group was significantly higher than that in controls ($p < 0.05$), and vascular endothelial growth factor, Ki67, and collagen type 1 in the miR-210-treated group were intensely expressed in the repaired site as compared to the control group. The mechanical test indicated that the ultimate failure load in the miR-210-treated group was significantly higher than that in the control group at 2 weeks. The cDNA microarray analysis showed significant upregulation of genes related to cell proliferation and cell differentiation, and genes involved in negative regulation of apoptosis.
Conclusion: This study showed that local injection of dsmiR-210 could accelerate MCL healing in rat, which is likely due to stimulation of angiogenesis at the healing site.

Keywords: angiogenesis; knee; medial collateral ligament; microRNA; microRNA-210

Introduction

Injury to the medial collateral ligament (MCL) of the knee is the most common knee ligament injury that occurs mainly in young individuals during sports activity.[1,2] MCL injury, even acute Grade 3 injury, can heal by nonoperative treatment.[3,4] It generally takes 5–7 weeks for athletes to heal and return to full competition under a well-controlled rehabilitation programme.[5] However, some cases do not heal and lead to continuous instability, subsequently causing dysfunction of the knee joint. Novel strategies for more effective and early healing of MCL injury by nonoperative treatment should be developed.

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Recently, the importance of micro-ribonucleic acid (miRNA) in the pathogenesis of human diseases such as cancers, heart diseases, and neuron disorders has been recognised. These miRNAs are a set of approximately 22-nucleotide, small noncoding RNAs that negatively regulate gene expressions through binding to the 3′ untranslated region of mRNA of their target genes.[6,7] The miRNAs play a crucial role in various cellular processes such as cell proliferation, differentiation, and apoptosis, which result in tissue specialisation and homeostasis. Therefore, an abnormal expression of miRNAs has been linked to several diseases.[8−11] Therapeutic trials to regulate endogenous expression of miRNA *in vivo* by administration of synthetic miRNA or antisense miRNA with some modifications have been reported.[12−14] Lanford et al[15] demonstrated the successful treatment of chimpanzees with hepatitis C virus infection by the administration of a locked nucleic acid-modified oligonucleotide complementary to miR-122. Thus, the therapeutic strategy targeting miRNAs is a novel and promising avenue to treat human diseases.

Angiogenesis has been recognised as an important initiator of healing and remodelling of tissues including ligaments.[16−18] Several reports demonstrated induction of angiogenesis/vasculogenesis by administration of drugs, cytokines, and cells to the injured ligaments.[19−22] A well-known potent factor of angiogenesis is miRNA (miR)-210. It is upregulated in endothelial cells in response to hypoxia, which affects cell survival, migration, and differentiation; even in normoxic condition, overexpression of miR-210 could induce the formation of capillary-like structures with vascular endothelial growth factor (VEGF)-driven cell migration.[23−25] It has been reported that local administration of miR-210 can promote tissue healing *in vivo*.[26,27] Therefore, we hypothesised that local injection of synthetic miR-210 into the injured MCL could accelerate ligament healing. The purpose of this study is to examine the effect of local injection of synthetic miR-210 on the acceleration of ligament healing in a rat MCL injury model.

Materials and methods

All procedures were performed according to the guidelines for animal experimentation of Hiroshima University, and with the approval of the Committee of Research Facilities for Laboratory Animal Sciences, Graduate School of Biomedical Sciences, Hiroshima University.

Preparation of double-stranded RNA

Double-stranded (ds) miR-210 (sequences 66-CUG-UGC-GUG-UGA-CAG-CGG-CUG-A-87 and 87-AGC-CCC-UGC-CCA-CCG-CAC-ACU-G-66; B-Bridge International, Mountain View, CA, USA) and ds control RNA [sequences 51-ATC-CGC-GCG-ATA-GTA-CGT-A-31 and 31-overhang dTdT/dTdT (sense/antisense) siRNA negative control; B-Bridge International] were prepared for the local injection according to a previous report.[27] The dsRNA was mixed with atelocollagen, which is a highly purified collagen type 1 isolated from calf dermis by pepsin treatment (Koken, Tokyo, Japan). Equal volumes of atelocollagen (10 μL) as a carrier of dsRNA and dsRNA solution (20 μg/10 μL) were combined and mixed by rotation at 4°C for 20 minutes, and a total of 20 μL dsRNA−atelocollagen complex was injected into the injured site of MCL.

Animal model

Twelve-week-old male Sprague-Dawley rats were used in this study. A total of 41 rats were anaesthetised with an intraperitoneal injection of 1 mL/kg pentobarbital sodium. Medial skin incision was applied at the right knee joint of the rats, and the MCL was cut transversely using a scalpel. The fascia and skin were sutured, and briefly, the dsRNA−atelocollagen complex was injected into the injured site of MCL. Twenty-one rats (control animals) were given ds control RNA injection, and another 21 rats were assigned to the miR-210-treated group. Five rats, on which sham surgery was performed, were used for mechanical testing.

Histological analysis

At 2 weeks and 4 weeks after injection, 10 rats in each group (2 weeks, 5 rats; 4 weeks, 5 rats) were sacrificed by an overdose of anaesthesia. The MCL was harvested and embedded in optical cutting temperature compound (Sakura Finetek USA, Inc., Torrance, CA, USA). Frozen serial sections of 6 μm thickness were prepared and Masson trichrome staining was performed. All specimens were given a histological score from 0 to 6 according to the following parameters: degree of retraction, tissue coverage, predominant cell type, and collagen fibre organisation as described previously.[28] All specimens were evaluated by two authors (T.N. and M.A.U.) without any knowledge of treatment, in a blinded fashion.

Mechanical testing

The ultimate failure loads of the MCL in both groups ($n = 5$) at 2 weeks and 4 weeks, and the sham group ($n = 5$) were measured using a conventional tensile tester (1840NT/500; AIKOH Engineering, Osaka, Japan). All specimens were dissected free of all skin, subcutaneous muscle, ligament, and menisci, leaving the MCL. The dissected specimens were set into a cylinder of polymethylmethacrylate cement so that the load was directed along the longitudinal axis of the MCL. The ultimate failure load was measured at a cross-head speed of 200 mm/minute until the femur−MCL−tibia complex failed. Normal ligaments of the rats that received sham operation used as the basic standard.

Immunohistochemistry

For immunofluorescence staining of VEGF, isolectin B4, collagen type 1, and Ki67, frozen sections at 2 weeks were fixed with 4.0% paraformaldehyde at 4°C for 5 minutes, and

stained with rabbit polyclonal anti-VEGF (Abcam, Cambridge, MA, USA), fluorescein-labelled GSL I-isolectin B4 (Vector Laboratories, Burlingame, CA, USA), rabbit anti-Ki67 (Neomarkers, Fremont, CA, USA), and goat polyclonal anticollagen type 1 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). The secondary antibodies were Alexa Fluor 568-conjugated goat antirabbit immunoglobulin G for VEGF and Ki67, and Alexa Flour 488-conjugated rabbit antigoat immunoglobulin G for collagen type 1 (all from Molecular Probes/Invitrogen, Carlsbad, CA, USA). DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride) solution was applied for 5 minutes to detect nuclear staining.

For the evaluation of newly formed vessels in the MCL, five microscopic areas (250 μm × 250 μm) from each specimen at 200× magnification were randomly chosen ($n = 5$ in each group). Capillaries were recognised as isolectin B4-positive tubular structures and counted by the two authors (T.N. and M.A.U.).

Microarray analysis

Total RNA for DNA microarray was extracted from the repaired site in each group using TRIzol (Life Technologies, Carlsbad, California, USA), according to the manufacturer's instructions. One microgram of the total RNA was used for the DNA microarray analysis. After amplification, the RNA sample in each group ($n = 1$ in each group) was labelled with Cy3 or Cy5, and hybridised with 3D-Gene Rat Oligo chip 24k (Toray Industries Inc., Tokyo, Japan). After hybridisation, hybridisation signals were scanned using 3D Gene Scanner 3000 (Toray Industries Inc.), and all the data from scanned images were scaled by global normalisation. From microarray data, the differentially expressed genes during MCL healing were analysed by gene ontology and pathway enrichment analysis. Gene ontology analysis was performed using GeneCodis2.0 software to integrate differentially expressed genes and to evaluate groups of genes with similar biological function.[29–31] GenMAPP (gene map annotator and pathway profiler), which allows us to view and analyse microarray data on biological pathways, was also used[32] to identify which pathways are affected by miR-210 during MCL healing.

Statistical analysis

The Mann–Whitney *U* test was used for the detection of differences in the histological scores and vessel numbers between the two groups, and multiple comparisons in the mechanical testing were performed using the Tukey–Kramer's *post hoc* test. A p value of < 0.05 was considered statistically significant.

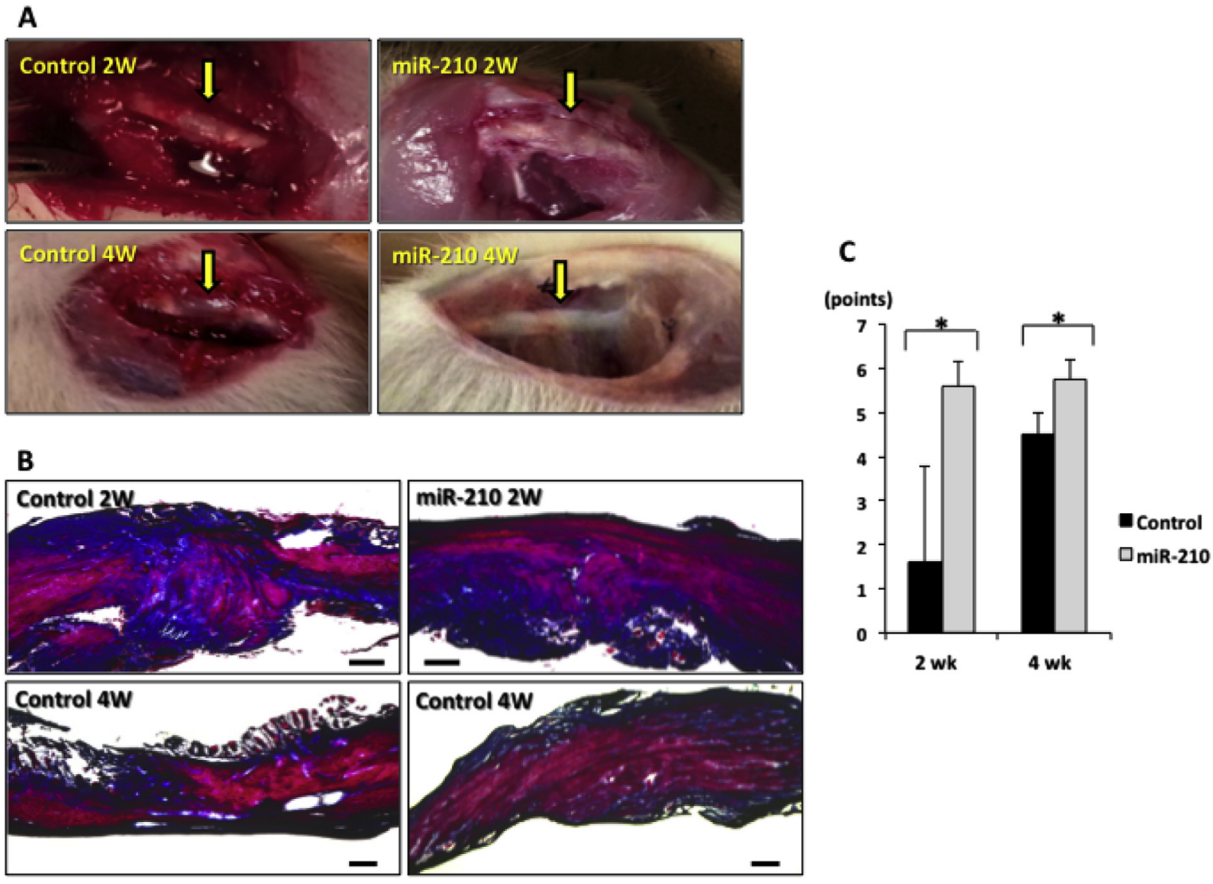

Figure 1. (A) Gross appearance of medial collateral ligament (MCL). Arrow indicates the injured site of MCL. (B) Masson trichrome staining of MCL in both groups. Bar indicates 200 μm. (C) Histological score of both groups. *$p < 0.05$. miR-210 = microRNA-210.

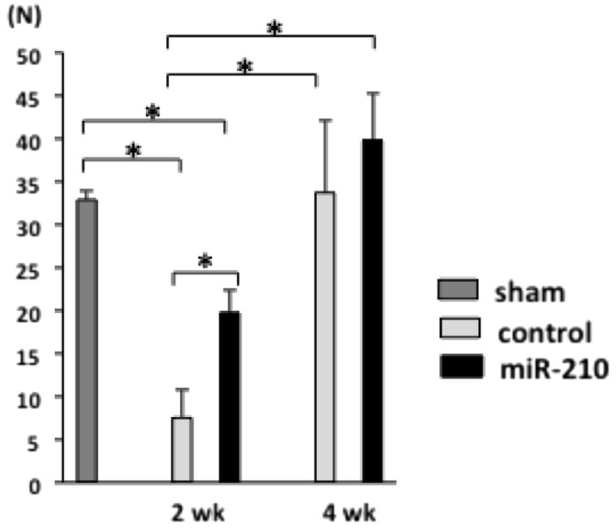

Figure 2. Ultimate failure load of medial collateral ligament (MCL). *$p < 0.05$. miR-210 = microRNA-210.

Results

Macroscopic observation

At 2 weeks after local injection, the fibres in the miR-210-treated group were macroscopically observed to be continuous and bulkier as compared to the control group. In the control group, the resected area of MCL was filled with scar tissues. At 4 weeks, the repaired site of MCL in the control group was thinner than that in the miR-210-treated group (Figure 1A).

Histological analysis

Histological analysis using Masson trichrome staining revealed that parallel fibres in the injured site were organised at 2 weeks and became thicker at 4 weeks in the miR-210-treated group, as compared to the control group. On the other hand, the injured sites in the control group were filled with loose fibrous tissues and were thinner than those in miR-

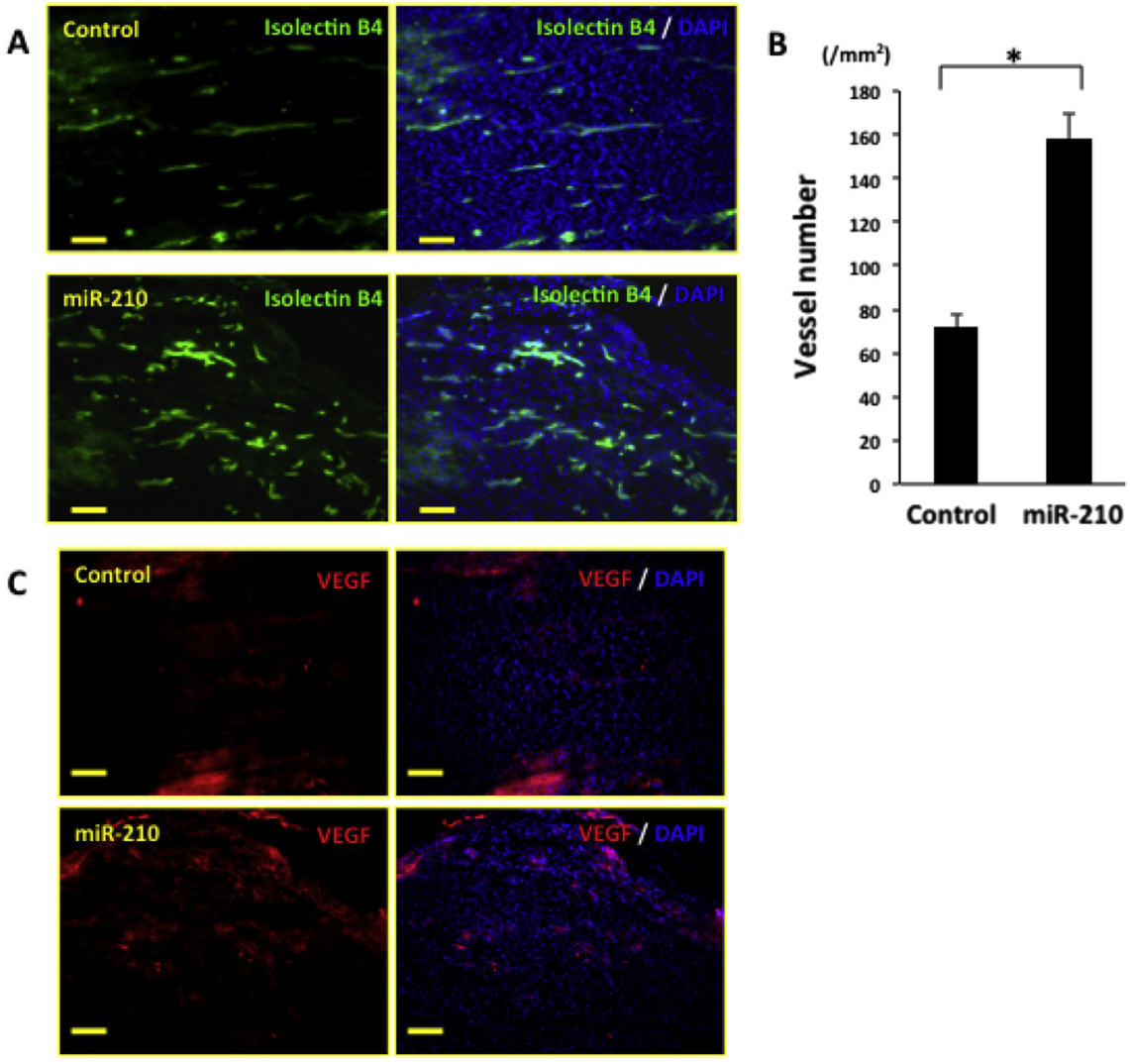

Figure 3. (A) Immunohistochemistry of isolectin B4 in both groups. Bar indicates 100 μm. (B) Vessel number in both groups. (C) Immunohistochemistry of vascular endothelial growth factor (VEGF) in both groups. Bar indicates 100 μm. *$p < 0.05$. miR-210 = microRNA-210; VEGF = vascular endothelial growth factor; DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride) is a fluorescent stain that binds strongly to A-Trich regions in DNA.

210-treated group (Figure 1B). The histological score of tissue samples from the miR-210-treated group was significantly higher than that from the control group at 2 weeks (1.6 ± 2.2 vs. 5.6 ± 0.54) and 4 weeks (4.5 ± 0.5 vs. 5.8 ± 0.4; Figure 1C). A mechanical test showed that the ultimate failure load in the miR-210-treated group (19.8 ± 2.7 N) was significantly higher than that in the control group (7.5 ± 3.4 N) at 2 weeks, but it was significantly lower than that in the sham group (32.9 ± 1.2 N; $p < 0.05$). At 4 weeks, there was no significant difference of the ultimate failure load between the miR-210-treated group (39.8 ± 5.5 N) and the control group (33.7 ± 8.3 N), although the ultimate failure load in the miR-210-treated group was likely to be higher than that in the control group ($p = 0.14$; Figure 2).

Immunofluorescent staining

To examine the angiogenesis during MCL healing, isolectin B4 staining was performed to detect the blood vessels. At 2 weeks, abundant blood vessels in the repaired tissues were observed in the miR-210-treated group as compared to the control group (Figure 3A). The number of blood vessels in the repaired tissues of the miR-210-treated group (158.1 ± 11.5/mm^2) was significantly higher than that in the control group (72 ± 6.1/mm^2; $p < 0.05$; Figure 3B). Immunohistochemistry staining of VEGF (an essential protein for angiogenesis and vasculogenesis) in the repaired tissues revealed that VEGF in the miR-210-treated group was intensely expressed in the repaired sites as compared to that in the control group (Figure 3C).

DNA microarray analysis

The cDNA microarray analysis of tissues from the repaired sites of both groups was performed at 1 week after the procedure using 3D-Gene Rat Oligo chip 24k (Toray Industries Inc.). Gene ontology analysis revealed that the expression of genes related to cell proliferation, differentiation, and negative regulation of apoptosis was remarkably upregulated and gene expression related to negative regulation of cell proliferation, negative regulation of cell growth, and positive regulation of apoptosis was downregulated (Table 1). These results indicated that miR-210 could stimulate cell differentiation and proliferation as well as negatively regulate cell apoptosis in the repaired site of MCL. To confirm the effect of miR-210 in cell proliferation as well as to identify target cells of miR210 treatment in the repaired site, immunohistochemistry was performed. A lot of Ki67 (a marker of cell proliferation)-positive cells were observed in the repaired site of MCL in the miR-210-treated group as compared to that of the control group, and these cells were merged with isolectin B4- and collagen type 1-positive cells (Figure 4). Thus, miR-210 might increase endothelial cells and collagen type 1-producing cells during MCL healing. Pathway enrichment analysis showed that retinol metabolism, prostaglandin synthesis and regulation, adipogenesis, triacylglyceride synthesis, type 2 interferon signalling, and cholesterol metabolism were upregulated (>2-fold change; Table 2).

Table 1
The result of gene ontology analysis.

Up regulation (>2 fold change)	**Gene count**
GO:0045944: positive regulation of transcription from RNA polymerase II promoter	281
GO:0042493: response to drug	181
GO:0043066: negative regulation of apoptosis	155
GO:0008284: positive regulation of cell proliferation	139
GO:0045893: positive regulation of transcription, DNA-dependent	137
GO:0014070: response to organic cyclic compound	126
GO:0006355: regulation of transcription, DNA-dependent	111
GO:0000122: negative regulation of transcription from RNA polymerase II promoter	105
GO:0006351: transcription, DNA-dependent	100
GO:0030154: cell differentiation	91
GO:0045892: negative regulation of transcription, DNA-dependent	90
GO:0051384: response to glucocorticoid stimulus	78
GO:0007275: multicellular organismal development	74
GO:0007507: heart development	65
GO:0006357: regulation of transcription from RNA polymerase II promoter	54
Down regulation (<0.5 fold change)	**Gene count**
GO:0008285: negative regulation of cell proliferation	129
GO:0006355: regulation of transcription, DNA-dependent	128
GO:0043065: positive regulation of apoptosis	113
GO:0007275: multicellular organismal development	100
GO:0030308: negative regulation of cell growth	100
GO:0045893: positive regulation of transcription, DNA-dependent	100
GO:0007519: skeletal muscle tissue development	92
GO:0006811: ion transport	81
GO:0043066: negative regulation of apoptosis	81
GO:0030154: cell differentiation	64
GO:0042493: response to drug	61
GO:0006096: glycolysis	56
GO:0005975: carbohydrate metabolic process	54
GO:0006810: transport	51

Discussion

Previous reports showed that several therapeutic trials using cells, cytokines, and platelet-rich plasma for accelerating MCL healing were conducted.[21,22,33] Among the cytokines for ligament healing, VEGF plays a crucial role in angiogenesis, a crucial event in the healing process of injured MCL.[22] In the natural healing process of ligaments, the VEGF expression crested between 5 days and 9 days postinjury.[17,34] Additional expression of VEGF such as local administration does not affect the mechanical properties, which indicated that proper levels of VEGF and other factors may be required for functional ligament healing.[35,36] Another report demonstrated that local administration of peripheral blood-derived CD34+ cells could promote MCL healing via vasculogenesis.[21] In this study, we have shown that local injection of dsmiR-210 could accelerate MCL healing. The ultimate failure load in the miR-210-treated group was significantly higher than that of the control group at 2 weeks. At 4 weeks, ultimate failure load in the miR-210-treated group was likely to be high compared to that in the control group, but there was no statistically significant difference. The ultimate failure load in both groups

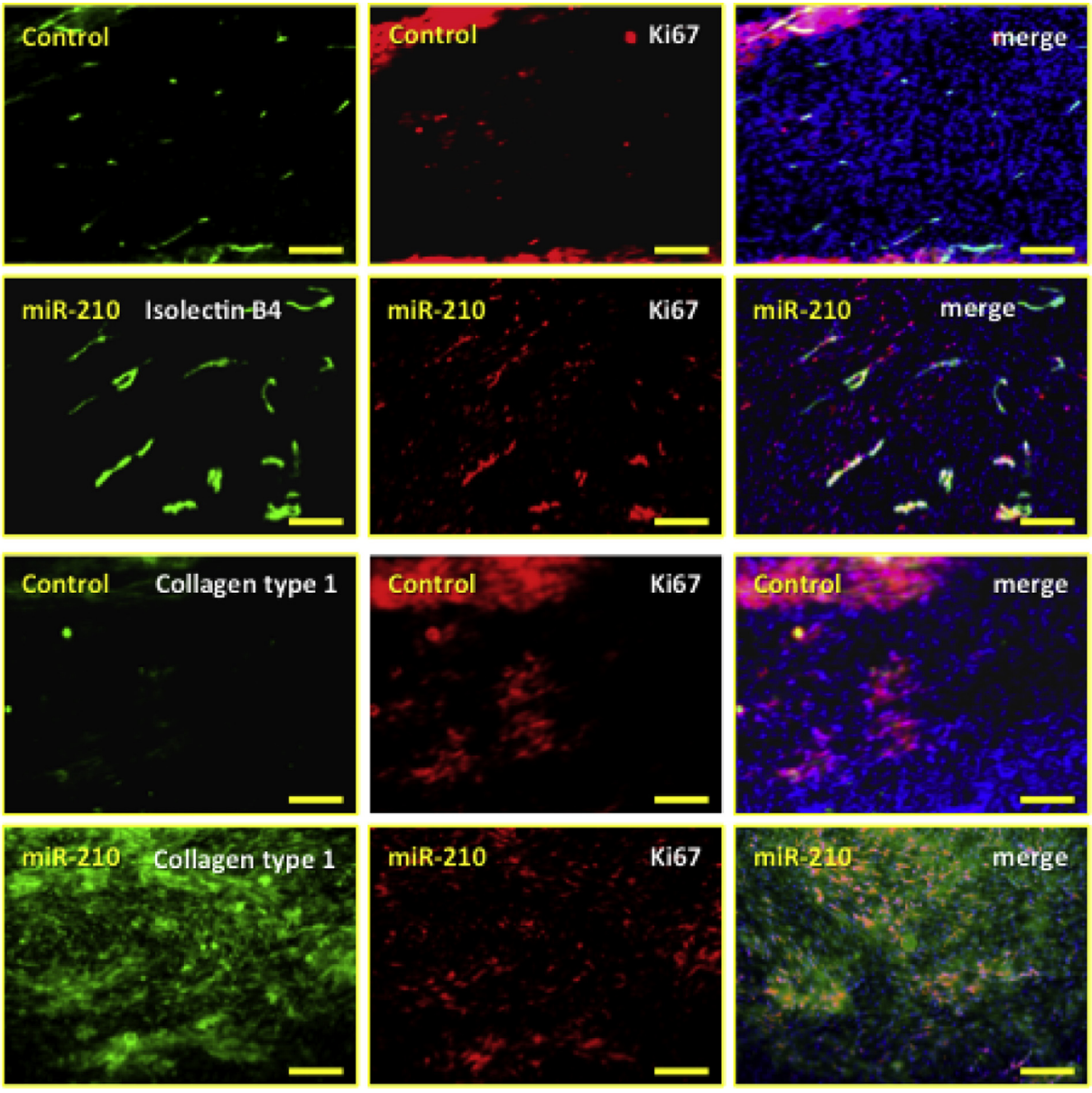

Figure 4. Immunohistochemistry of isolectin B4, Ki67, and collagen type 1 in both groups. Bar indicates 100 μm. miR-210 = microRNA-210.

was almost the same as that in the sham group; therefore, the effect of the administration of dsmiR-210 on the mechanical properties might not be detected in the rat model. At 2 weeks, collagen type 1 expression and angiogenesis were upregulated in the miR-210-treated group. Most athletes with ligament injury generally can return to full competition 5–7 weeks after injury.[5] Early intervention for the injured MCL would be important for upregulation of angiogenesis and synthesis of collagen type 1, subsequently yielding better mechanical properties. In this study, a single-dose injection was administered. However, even with a single-dose injection, the expression of many genes in the injected site was altered at 1 week. Previous reports showed that a single-dose injection of miRNA could enhance the therapeutic effects; therefore, only a single-dose injection to the injured MCL will be feasible.[13,27,37,38] However, our study showed only one time point of injection. The effect of repeated injections of miR-210 on MCL healing, including the adverse effects, is unclear. As a single injection of miR-210 could enhance collagen type 1 expression, there is a possibility that repeated injections of miR-210 can show better results than a single injection. The results of this study suggested that local injection of miR-210 can be a potential novel treatment strategy to accelerate the healing of an injured ligament, thus enabling injured athletes to return to full competition earlier.

An advantage of miRNA therapy is that it does not require tissue harvesting, including isolation and purification of cells or platelet-rich plasma. Thus, miRNA therapy is simple in its construction and potentially used as a novel drug. Furthermore, multifactorial effects of miRNA for obtaining functional ligament properties will be expected as its targeting many genes based on computational analysis. In addition to its effect

Table 2
The results of pathway enrichment analysis.

		MAPP name	Gene number	Z Score	PermuteP
>2 fold change	1	Retinol metabolism	8	5.424	0
	2	Prostaglandin synthesis and regulation	7	4.93	0
	3	Adipogenesis	10	2	0.06
	4	Triacylglyceride synthesis	3	2.13	0.065
	5	Type II interferon signaling	4	2.079	0.066
	6	Cholesterol metabolism	3	2.035	0.094
<0.5 fold change	1	Electron transport chain	44	12.524	0
	2	Striated muscle contraction	22	11.405	0
	3	TCA cycle	16	9.26	0
	4	Oxidative phosphorylation	25	9.224	0
	5	Glycolysis and gluconeogenesis	16	7.285	0
	6	Glycogen metabolism	8	2.967	0.007
	7	Beta oxidation meta pathway	8	2.967	0.007
	8	Fatty acid beta oxidation	8	2.766	0.014
	9	Diurnally regulated genes with circadian orthologs	9	2.614	0.019
	10	Mitochondrial LC-fatty acid beta-oxidation	5	2.775	0.02
	11	Beta oxidation of unsaturated fatty acids	3	3.358	0.022
	12	Fatty acid beta oxidation 1	6	2.231	0.041

on angiogenesis, miR-210 has also been reported to play roles in other biological processes, including mitochondrial metabolism, DNA repair, regulation of apoptosis, and cell proliferation.[25] Gene ontology enrichment analysis of our study indicated significant upregulation of genes that are positively involved in transcription, cell proliferation, and cell differentiation, as well as genes that negatively regulate apoptosis in the samples derived from miR-210-treated genes, as compared to those from the control group. These results suggested that gene expression related to tissue repair was upregulated by miR-210 treatment in the injured sites of MCL, an effect that seem to specifically stimulate angiogenesis and collagen type 1 production. Pathway enrichment analysis showed that retinol metabolism was activated by miR-210 administration during MCL healing. The retinol metabolism pathway plays an important role in normal development and disease.[39] Retinoic acid, which is the main derivative of retinol (vitamin A), is essential for regulating several biological processes including differentiation, proliferation, and apoptosis. Moreover, retinoids regulate stem cell differentiation via transcription activation.[40] The potential of retinoic acid in regenerative medicine has been examined.[41] Thus, the results of this study are consistent with those reported in the literature. Further studies are needed to explore the role of retinoic acid in the healing process of MCL and its relationship with miR-210. In this study, microarray analysis was performed at 1 week postinjection because there was a significant difference in the histological findings at 2 weeks. The early change of the gene expression is important to analyse the mechanism of healing. Significant events such as angiogenesis in the tendon healing occur at around 1 week; therefore, we performed microarray analysis at 1 week.

For clinical application, the biological safety of miRNA treatment should be validated. Expressions of miRNA have a role in several human diseases such as cancer. In this study, no adverse effects were observed, but we could not confirm the distribution of administered miR-210 in the rats. It has been reported that miR-210 negatively regulates cancer cell proliferation.[42] However, further studies are needed to examine its safety, including investigation of its adverse effects, prior to its application in clinical settings. Clinical trials of miRNA replacement therapy or antisense oligonucleotides of miRNA are currently ongoing; hence, miRNA targeting therapy is gradually being recognised.[43,44]

In conclusion, our study showed that local injection of miR-210 could accelerate MCL healing. Further studies are required to gain more detailed insights into its mechanism of action. Local injection of miR-210 into the injured MCL is one of the potential therapeutic options in the future since accumulating evidence suggested that the effectiveness and safety of miRNA therapy are promising.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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References

1. Peterson L, Junge A, Chomiak J, Graf-Baumann T, Dvorak J. Incidence of football injuries and complaints in different age groups and skill-level groups. *Am J Sports Med*. 2000;28:S51–S57.
2. Najibi S, Albright JP. The use of knee braces, part 1: prophylactic knee braces in contact sports. *Am J Sports Med*. 2005;333:602–611.
3. Reider B, Sathy MR, Talkington J, Blyznak N, Kollias S. Treatment of isolated medial collateral ligament injuries in athletes with early functional rehabilitation. A five-year follow-up study. *Am J Sports Med*. 1994;22:470–477.
4. Lind M, Jakobsen BW, Lund B, Hansen MS, Abdallah O, Christiansen SE. Anatomical reconstruction of the medial collateral ligament and posteromedial corner of the knee in patients with chronic medial collateral ligament instability. *Am J Sports Med*. 2009;37:1116–1122.
5. Wijdicks CA, Griffith CJ, Johansen S, Engebretsen L, Laprade RF. Injuries to the medial collateral ligament and associated medial structures of the knee. *J Bone Joint Surg Am*. 2010;92:1266–1280.
6. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116:281–297.
7. Ambros V. The functions of animal microRNAs. *Nature*. 2004;431:350–355.
8. De Santis G, Ferracin M, Biondani A, et al. Altered miRNA expression in T regulatory cells in course of multiple sclerosis. *J Neuroimmunol*. 2010;226:165–171.

9. Nakasa T, Miyaki S, Okubo A, et al. Expression of microRNA-146 in rheumatoid arthritis synovial tissue. *Arthritis Rheum*. 2008;58:1284−1292.
10. Song Q, Xu Y, Yang C, et al. miR-483-5p promotes invasion and metastasis of lung adenocarcinoma by targeting RhoGDI1 and ALCAM. *Cancer Res*. 2014;74.
11. Gupta P, Cairns MJ, Saksena NK. Regulation of gene expression by microRNA in HCV infection and HCV-mediated hepatocellular carcinoma. *Virol J*. 2014;11:64.
12. Tazawa H, Tsuchiya N, Izumiya M, Nakagama H. Tumor-suppressive miR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells. *Proc Natl Acad Sci U S A*. 2007;104:15472−15477.
13. Nakasa T, Ishikawa M, Shi M, Shibuya H, Adachi N, Ochi M. Acceleration of muscle regeneration by local injection of muscle-specific microRNAs in rat skeletal muscle injury model. *J Cell Mol Med*. 2010;14:2495−2505.
14. Nakasa T, Shibuya H, Nagata Y, Niimoto T, Ochi M. The inhibitory effect of microRNA-146a expression on bone destruction in collagen-induced arthritis. *Arthritis Rheum*. 2011;63:1582−1590.
15. Lanford RE, Hildebrandt-Eriksen ES, Petri A, et al. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science*. 2010;327:198−201.
16. Woo SL, Hildebrand K, Watanabe N, Fenwick JA, Papageorgiou CD, Wang JH. Tissue engineering of ligament and tendon healing. *Clin Orthop Relat Res*. 1999;367:S312−S323.
17. Molloy T, Wang Y, Murrell G. The roles of growth factors in tendon and ligament healing. *Sports Med*. 2003;33:381−394.
18. Bray RC, Leonard CA, Salo PT. Correlation of healing capacity with vascular response in anterior cruciate and medial collateral ligaments of the rabbit. *J Orthop Res*. 2003;21:1118−1123.
19. Mammoto T, Seerattan RA, Paulson KD, Leonard CA, Bray RC, Salo PT. Nerve growth factor improves ligament healing. *J Orthop Res*. 2008;26:957−964.
20. Yuan T, Zhang CQ, Wang JH. Augmenting tendon and ligament repair with platelet-rich plasma (PRP). *Muscle Ligaments Tendons J*. 2013;3:139−149.
21. Tei K, Matsumoto T, Mifune Y, et al. Administrations of peripheral blood CD34-positive cells contribute to medial collateral ligament healing via vasculogenesis. *Stem Cells*. 2008;26:819−830.
22. Nishimori M, Matsumoto T, Ota S, et al. Role of angiogenesis after muscle derived stem cell transplantation in injured medial collateral ligament. *J Orthop Res*. 2012;30:627−633.
23. Fasanaro P, D'Alessandra Y, Di Stefano V, et al. MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3. *J Biol Chem*. 2008;283:15878−15883.
24. Fasanaro P, Greco S, Lorenzi M, et al. An integrated approach for experimental target identification of hypoxia-induced miR-210. *J Biol Chem*. 2009;284:35134−35143.
25. Devlin C, Greco S, Martelli F, Ivan M. miR-210: more than a silent player in hypoxia. *IUBMB Life*. 2011;63:94−100.
26. Hu S, Huang M, Li Z, et al. microRNA-210 as a novel therapy for treatment of ischemic heart disease. *Circulation*. 2010;122:S124−S131.
27. Shoji T, Nakasa T, Yamasaki K, et al. The effect of intra-articular injection of microRNA-210 on ligament healing in a rat model. *Am J Sports Med*. 2012;40:2470−2478.
28. Kanaya A, Deie M, Adachi N, Nishimori M, Yanada S, Ochi M. Intra-articular injection of mesenchymal stromal cells in partially torn anterior cruciate ligaments in a rat model. *Arthroscopy*. 2007;23:610−617.
29. Carmona-Saez P, Chagoyen M, Tirado F, Carazo JM, Pascual-montano A. GENECODIS: a web-based tool for finding significant concurrent annotations in gene lists. *Genome Biol*. 2007;8:R3.
30. Nogales-Cadenas R, Carmona-Saez P, Vazquez M, et al. GeneCodis: interpreting gene lists through enrichment analysis and integration of diverse biological information. *Nucleic Acids Res*. 2009;37:W317−W322.
31. Tabas-Madrid D, Nogales-Cadenas R, Pascual-Montano A. GeneCodis3: a non-redundant and modular enrichment analysis tool for functional genomics. *Nucleic Acids Res*. 2012;40:W478−W483.
32. Dahlquist KD, Salomonis N, Vranizan K, Lawlor SC, Conklin BR. GenMAPP, a new tool for viewing and analyzing microarray data on biological pathways. *Nat Genet*. 2002;31:19−20.
33. Yoshioka T, Kanamori A, Washio T, et al. The effects of plasma rich in growth factors (PRGF-endoret) on healing of medial collateral ligament of the knee. *Knee Surg Sports Traumatol Arthrosc*. 2013;21:1763−1769.
34. Gharaibeh B, Lavasani M, Cummins JH, Huard J. Terminal differentiation is not a major determinant for the success of stem cell therapy—cross-talk between muscle-derived stem cells and host cells. *Stem Cell Res Ther*. 2011;2:31.
35. Yoshikawa T, Tohyama H, Katsura T, et al. Effects of local administration of vascular endothelial growth factor on mechanical characteristics of the semitendinosus tendon graft after anterior cruciate ligament reconstruction in sheep. *Am J Sports Med*. 2006;34:1918−1925.
36. Ju YJ, Tohyama H, Kondo E, et al. The angiogenic peptide vascular endothelial growth factor on properties of the *in situ* frozen−thawed anterior cruciate ligament in rabbits. *Am J Sports Med*. 2006;34:84−91.
37. Kawanishi Y, Nakasa T, Shoji T, et al. Intra-articular injection of synthetic microRNA-210 accelerates avascular meniscal healing in rat medial meniscal injured model. *Arthritis Res Ther*. 2014;16:488.
38. Usman MA, Nakasa T, Shoji T, et al. The effect of administration of double stranded MicroRNA-210 on acceleration of Achilles tendon healing in a rat model. *J Orthop Sci*. 2015;20:538−546.
39. Das BC, Thapa P, Karki R, et al. Retinoic acid signaling pathways in development and diseases. *Bioorg Med Chem*. 2014;22:674−683.
40. Gudas LJ, Wagner JA. Retinoids regulate stem cell differentiation. *J Cell Physiol*. 2011;226:322−330.
41. Christie VB, Barnard H, Batsanov AS, et al. Synthesis and evaluation of synthetic retinoid derivatives as inducers of stem cell differentiation. *Org Biomol Chem*. 2008;6:3497−3507.
42. Tsuchiya S, Fujiwara T, Sato F, et al. MicroRNA-210 regulates cancer cell proliferation through targeting fibroblast growth factor receptor-like 1 (FGFRL1). *J Biol Chem*. 2011;286:420−428.
43. Bader AG. miR-34—a microRNA replacement therapy is headed to the clinic. *Front Genet*. 2012;3:120−140.
44. Ling H, Fabbri M, Calin GA. MicroRNAs and other noncoding RNAs as targets for anticancer drug development. *Nat Rev Drug Discov*. 2013;12:847−865.