

Therapeutic angiogenesis by local sustained release of microRNA-126 using poly lactic-co-glycolic acid nanoparticles in murine hindlimb ischemia

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ABSTRACT

Objective: Recent studies demonstrate that microRNAs show promising potential, including angiogenesis, in therapeutic intervention. MicroRNA-126 (miR-126) is reported to regulate angiogenesis by blocking Sprouty-related EVH1 domain-containing protein 1 (SPRED1), an endogenous inhibitor of vascular endothelial cell growth factor. In this study, we investigated the angiogenic effects of the sustained release of miR-126 loaded with poly lactic-co-glycolic acid (PLGA) nanoparticles (NPs) in a murine hindlimb ischemia model.

Methods: We induced mice hindlimb ischemia through femoral artery excision. We randomly assigned the mice to two groups and performed an intramuscular injection of miR-126-loaded PLGA NPs (miR-126) or scrambled miR-loaded PLGA NPs (control) shortly after induction of ischemia.

Results: The miR-126 expression levels in the ischemic limb at 3 days after treatment were significantly higher in mice treated with miR-126-loaded PLGA NPs than in those with scrambled miR, indicating the fair efficiency of local miR transduction (control vs miR-126: 0.33 ± 0.12 vs 0.74 ± 0.42 ; $P < .05$; $n = 6$). Laser Doppler perfusion imaging revealed that limb blood flow in mice treated with miR-126-loaded PLGA NPs was significantly higher at 14 days after treatment (sham vs control vs miR-126: 0.62 ± 0.09 vs 0.58 ± 0.05 vs 0.72 ± 0.07 ; $P < .001$; $n = 12$). Immunohistochemical analysis indicated that CD31-positive cell density and α -smooth muscle actin-positive vessel density were significantly higher in miR-126-treated mice (control vs miR-126: 0.33 ± 0.12 vs 0.74 ± 0.42 ; $P < .05$; $n = 6$). SPRED1 messenger RNA expression levels were significantly lower in miR-126-treated mice (control vs miR-126: 1.00 ± 0.05 vs 0.81 ± 0.07 ; $P < .05$; $n = 6$). Western blotting indicated that protein levels of pERK/ERK mediated by SPRED1 were significantly higher in miR-126-treated mice (control vs miR-126: 0.29 ± 0.10 vs 0.54 ± 0.21 ; $P < .05$; $n = 6$).

Conclusions: This study suggests that sustained release of miR-126-loaded PLGA NPs might be an effective method in therapeutic angiogenesis for hindlimb ischemia. (J Vasc Surg 2017;■:1-7.)

Clinical Relevance: Severe clinical limb ischemia resistant to conventional treatments, including medication, percutaneous angioplasty, and bypass surgery, remains a serious health care problem. Therapeutic angiogenesis that induces angiogenesis at the ischemic site could be a novel and effective approach for this severe condition. Here, we developed a therapeutic approach combining microRNA-126 and a drug delivery system using poly lactic-co-glycolic acid nanoparticles, which showed novel therapeutic potential on a mouse hindlimb ischemia model. This therapeutic approach could be a promising new strategy after verification of the safety and long-term therapeutic effects in clinical studies in the future.

Recently, endogenous small noncoding RNAs, called microRNAs (miRNAs), have been identified as important post-transcriptional inhibitors of gene expression in many biologic processes.¹⁻³ Moreover, miRNAs have been shown to play important roles in regulating the pathophysiologic process of various diseases, including vascular diseases.⁴⁻⁶ The biologic roles of miRNAs in vascular diseases have led to an increased interest in

miRNA regulation as a therapeutic and diagnostic approach. Recent studies demonstrated that miRNAs show promising potential in therapeutic interventions directed toward ischemic diseases.^{7,8}

Several specific miRNAs have been identified as angiogenic regulators. Among them, miRNA-126 (miR-126) is reported to regulate angiogenesis by blocking Sprouty-related EVH1 domain-containing protein 1 (SPRED1), an

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endogenous inhibitor of vascular endothelial cell growth factor (VEGF) through the intracellular VEGF receptor pathway.⁹⁻¹² Accordingly, deletion of the miR-126 affects vascular integrity and angiogenesis.^{9,10} miR-126 is known to be located in intron 7 of the *egfl7* gene and is highly expressed in endothelial cells.^{13,14} These findings imply that miR-126 could be an attractive target for therapeutic intervention for ischemic diseases.

To maximize the therapeutic effect of miRNA-based therapies, a drug delivery system (DDS) for the target tissue optimized for miRNA delivery is indispensable. Poly lactic-co-glycolic acid (PLGA) nanoparticles (NPs), a family of Food and Drug Administration-approved biodegradable polymers, are reported to have excellent biocompatibility and biodegradability and are popular among the various available biodegradable polymers because of their long clinical experience, favorable degradation characteristics, and possibility of sustained drug delivery.^{15,16} It is also reported that the PLGA NPs are effectively and rapidly incorporated in endothelial cells.¹⁷ Here, we hypothesized that the DDS using PLGA NPs and miR-126 would serve as a novel strategy for the treatment of ischemic diseases, including clinical limb ischemia. In this study, we investigated the angiogenic effects of the local sustained release of miR-126 loaded with PLGA NPs in a murine hindlimb ischemia model.

METHODS

Animals. Male 10-week-old C57BL/6J wild-type mice were purchased from CLEA Japan (Osaka, Japan). All animal experimental protocols were approved by the Animal Experimentation Committee of Kyoto University. All animal experiments were performed according to the Guidelines for Animal Experiments of Kyoto University, which conform to Japanese law and the *Guide for the Care and Use of Laboratory Animals*.

Preparation of miR-126-loaded PLGA NPs. We prepared cholesterol-conjugated 27-nt double-stranded RNAs (dsRNAs) to facilitate cellular uptake and designed to stabilize mature miR-126 as previously reported.¹⁸ The RNA oligonucleotides with a partial phosphorothioate backbone (*) and a cholesterol group (Chol) were synthesized as follows: miR-126 dsRNA sense: 5'-Chol-CGGCGCAUUAUUACUCACGGUACGAGU-3', miR-126 dsRNA antisense: 5'-ACU(*)C(*)G(*)U(*)A(*)C(*)C(*)G(*)U(*)G(*)A(*)G(*)U(*)A(*)A(*)U(*)A(*)A(*)U(*)G(*)C(*)G(*)CCG-3'. The following control dsRNA included a random sequence of nucleotides and was not complementary to previously known miRNA: control dsRNA sense: 5'-Chol-CGGGUCUCCACGCGCAGUACAUUUCGU-3', control dsRNA antisense: 5'-ACG(*)A(*)A(*)A(*)U(*)G(*)U(*)A(*)C(*)U(*)G(*)C(*)G(*)C(*)G(*)U(*)G(*)G(*)A(*)G(*)A(*)C(*)CCG-3'. The dsRNAs were purchased from Hokkaido System Science (Sapporo, Japan).

ARTICLE HIGHLIGHTS

- **Type of Research:** Case-control study to assess angiogenesis in a murine model of hindlimb ischemia
- **Take Home Message:** Intramuscular injection of nanoparticle-associated microRNA-126 increased limb perfusion 14 days after induction of ischemia, and it was associated with increased angiogenesis.
- **Recommendation:** In a murine model, intramuscular injection of sustained-release microRNA-126 increased ischemic limb blood flow and contributed to angiogenesis.

Anionic PLGA NPs encapsulated with miR-126 or miR-control were prepared by a previously reported emulsion solvent diffusion method in purified water.^{15,16,19} The diameter and surface charge of the miR-126-loaded PLGA NPs were 322 nm and +50 mV, respectively. The diameter and surface charge of the miR-control-loaded PLGA NPs were 329 nm and +57 mV, respectively. The miR-126-loaded PLGA NPs contained 4.59% (wt/vol) miR-126, and the miR-control-loaded PLGA NPs contained 4.86% (wt/vol) miR-control.

Animal preparation and experimental protocol. The mice were randomly divided into five groups: a sham group, two control groups (scrambled miR-loaded PLGA NPs; 5 µg/50 µg), and two miR-126 groups (miR-126-loaded PLGA NPs; 5 µg/50 µg; Fig 1). We assigned 24 mice per group, and 12 of 24 were used for laser Doppler perfusion imaging, 6 mice for day-3 analyses of real-time polymerase chain reaction (PCR), and 6 mice for day-7 analyses of real-time PCR or Western blotting. The animals were anesthetized with pentobarbital, 65 mg/kg intraperitoneally, for the surgical procedure as well as for laser Doppler measurements. A skin incision was made in the left hindlimb. The proximal femoral artery and the proximal portion of the saphenous artery were ligated. After all side branches were dissected free, the entire femoral artery was excised in all groups including the sham group.²⁰ We injected miR-126-loaded PLGA NPs or miR-control-loaded PLGA NPs into the ischemic thigh muscles just after induction of ischemia. We prepared 25 µL of solution including control-miR or miR-126 PLGA NPs for each animal. We divided the volume into 5 × 5 µL and injected it into five different sites of the musculature. We did not optically observe acute local inflammatory responses after miR administration, which potentially relates to the initial burst of local delivery.²¹

Hindlimb blood perfusion. Hindlimb blood perfusion was scanned using a laser Doppler perfusion image analyzer (OZ-1; OMEGAZONE, OMEGAWAVE Inc, Tokyo,

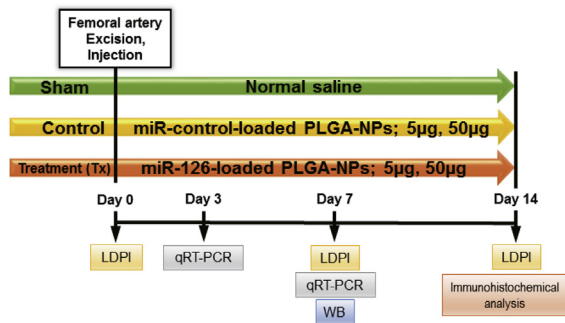


Fig 1. Experimental protocol. LDPI, laser Doppler perfusion imaging index; miR, MicroRNA; PLGA-NPs, poly lactic-co-glycolic acid nanoparticles; qRT-PCR, quantitative real-time polymerase chain reaction; WB, Western blotting.

Japan). Ischemic and nonischemic limb perfusion was measured 0 (post), 7, and 14 days after surgery. The ratio of ischemic to nonischemic hindlimb perfusion was calculated. Before analysis, we fully warmed the animals using a bed heater (5 minutes) to obtain consistent results free from temperature artifacts.²²

Immunohistochemical analysis. The mice were euthanized and perfusion fixed with 4% paraformaldehyde 14 days after surgery. The histologic evaluation was performed in 5-µm paraffin-embedded sections of the gastrocnemius muscle 14 days after hindlimb ischemia. Capillary density and arteriolar density in the ischemic muscle were determined by immunohistochemical staining with anti-mouse platelet endothelial cell adhesion molecule 1 antibody (CD31; Abcam, Cambridge, UK) and α -smooth muscle actin (α -SMA; Dako, Glostrup, Denmark), respectively. Digital images of five microscopic fields from four different sections from each animal were stored. Capillary density was expressed as the number of CD31-positive cells per square millimeter, and arteriolar density was expressed as the number of circumvented brown signals of α -SMA per square millimeter as previously described.²²

Quantitative real-time PCR analysis. The gastrocnemius muscle was explanted and preserved immediately in liquid nitrogen at 3 or 7 days after surgery. Total RNA was then extracted from the gastrocnemius muscle using TRIzol (Invitrogen, Carlsbad, Calif). miRNA was measured using TaqMan MicroRNA Assays (Applied Biosystems, Oakville, Ontario, Canada). Real-time PCR was performed on the StepOnePlus Real-Time PCR System (Applied Biosystems). miRNA expression levels were normalized by U6 small nuclear RNA expression. For the quantification of messenger RNA (mRNA), complementary DNA was synthesized using Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, Mass), and analysis of mRNA expression levels was performed by a StepOnePlus Real-Time PCR System (Applied Biosystems) and THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka,

Table. Gene-specific oligonucleotide primer sequences

Gene	Primer sequence 5'-3'
SPRED1	F-CCGCCGACTTCTGACAACGAT R-TTGAGTCATCTCTGGTCATCAC
PIK3R2	F-CCGGGACATACGTGGAGTTC R-TGGGACTATGTGGCTGACT
β -Actin	F-GATCTGGCACCACACCTTCT R-GGGGTGTTGAAGGTCTCAAA

PI3KR2, Phosphoinositide 3-kinase regulatory subunit 2; *SPRED1*, Sprouty-related EVH1 domain-containing protein 1.

Japan). Expression was normalized by the housekeeping gene β -actin (the expression was stable). The primer sequences are listed in the Table. Custom-designed primers were purchased from Hokkaido System Science. The control 50-µg group was set as 1 in all cases. The analyses for miR-126 were performed on days 3 and 7, and those for SPRED1 and phosphoinositide 3-kinase regulatory subunit 2 (PI3KR2) were performed on day 7.

Western blotting. Muscle tissues were lysed using lysis buffer (100 mM Tris-HCl [pH 7.4], 75 mM NaCl, and 1% Triton X-100 [Nacalai Tesque, Kyoto, Japan]) with Complete Mini protease inhibitor (Roche, Basel, Switzerland), aprotinin (Sigma-Aldrich, St. Louis, Mo), 50 mM NaF, and 1 mM Na_2VO_4 as previously reported.¹⁸ Protein concentrations were quantified using a bicinchoninic acid protein assay kit (Bio-Rad, Hercules, Calif). For Western blotting, 20 µg of protein was electrophoresed with NuPAGE 4-12% Bis-Tris (Invitrogen) gels and transferred to a Protran nitrocellulose transfer membrane (Whatman, Maidstone, UK). The membrane was incubated with the primary antibody at 4°C overnight, followed by incubation with the secondary antibody conjugated with horseradish peroxidase (1:2000). The signals were detected and visualized using ECL-Plus chemiluminescent detection reagent (Amersham Biosciences, Little Chalfont, UK). The primary antibodies were used as follows: anti-phosphorylated-ERK, anti-ERK (1:1000; Cell Signaling Technology, Danvers, Mass); anti-phosphorylated-AKT, anti-AKT (1:1000; Cell Signaling Technology); anti-SPRED1 (1:1000; Abcam); anti-glyceraldehyde 3-phosphate dehydrogenase (1:1000; Cell Signaling Technology). Anti-rabbit immunoglobulin G (GE Healthcare, Chicago, Ill) and anti-mouse immunoglobulin G (GE Healthcare) were used as secondary antibodies, each at a 1:2000 dilution. Luminescence was detected using an ImageQuant LAS 4000mini (GE Healthcare). Quantification of the band intensity was performed with ImageJ software (U.S. National Institutes of Health, Bethesda, Md). The analyses were performed on day-7 samples.

Statistical analysis. The data were analyzed using JMP Pro 11 (SAS Institute, Cary, NC). All data are presented as mean $\pm$ standard deviation. The laser Doppler data

were tested with one-way analysis of variance. Differences between groups were determined with the Student *t*-test. Data were considered significant when $P < .05$.

RESULTS

Increased blood perfusion by the miR-126-loaded PLGA NP treatment. No deaths or leg necrosis was observed during the observation period. Laser Doppler perfusion imaging revealed that limb blood flow in the high-dose miR-126 group was higher than that in the other groups at 14 days after treatment (Fig 2, A). The laser Doppler perfusion imaging index in the high-dose miR-126 group was significantly higher than in the other groups, including the low-dose miR-126 group, at 14 days after treatment (sham vs low-dose control vs high-dose control vs low-dose miR-126 vs high-dose miR-126: 0.62 ± 0.09 vs 0.63 ± 0.03 vs 0.58 ± 0.05 vs 0.65 ± 0.07 vs 0.72 ± 0.07 ; $P < .001$; $n = 12$; Fig 2, B). No significant difference in laser Doppler perfusion imaging index was observed among the sham, low-dose control, high-dose control, and low-dose miR-126 groups.

Increased capillary and arteriolar density after miR-126-loaded PLGA NP treatment. CD31-positive lumen density, which indicates capillary angiogenesis, in the high-dose miR-126 group was significantly higher than in the other groups (sham vs control vs miR-126: 241 ± 108 vs 211 ± 45 vs 437 ± 89 ; $P < .01$; $n = 6$; Fig 3, A and B). Moreover, α -SMA-positive vessel density, which indicates arteriogenesis, in the high-dose miR-126 group was significantly higher than in the other groups (sham vs control vs miR-126: 13.2 ± 4.7 vs 13.2 ± 4.3 vs 35.2 ± 12.7 ; $P < .001$; $n = 6$; Fig 3, C and D). The densities of capillary and arteriole in the high-dose miR-126 group were almost comparable with those in normal nonischemic mice hindlimb (Fig 3, A and C).

miR-126 loaded PLGA NP treatment partially controlled VEGF receptor pathway activity. The miR-126 expression levels in the ischemic limb at 3 and 7 days after treatment were significantly higher in mice treated with miR-126-loaded PLGA NPs compared with those with scrambled miR, indicating the fair efficiency of local miR transduction and upregulation (control vs miR-126: day 3, 1.00 ± 0.15 vs 2.27 ± 0.53 [$P < .05$; $n = 6$]; day 7, 1.00 ± 0.11 vs 1.32 ± 0.29 [$P < .05$; $n = 6$]; Fig 4, A).

Next, we investigated the gene expression level of downstream molecules of the VEGF receptor pathway. SPRED1 mRNA expression levels on day 7 were significantly lower in miR-126-treated mice, which is consistent with previous reports that miR-126 downregulates SPRED1 (control vs miR-126: 1.00 ± 0.05 vs 0.81 ± 0.07 ; $P < .05$, $n = 6$; Fig 4, B). The protein level examined by Western blotting on day 7 also revealed the downregulation of SPRED1 (control vs miR-126: 0.36 ± 0.09 vs 0.13 ± 0.02 ; $P < .05$; $n = 6$; Fig 4, D). We also found that protein levels of pERK/ERK, which is negatively regulated

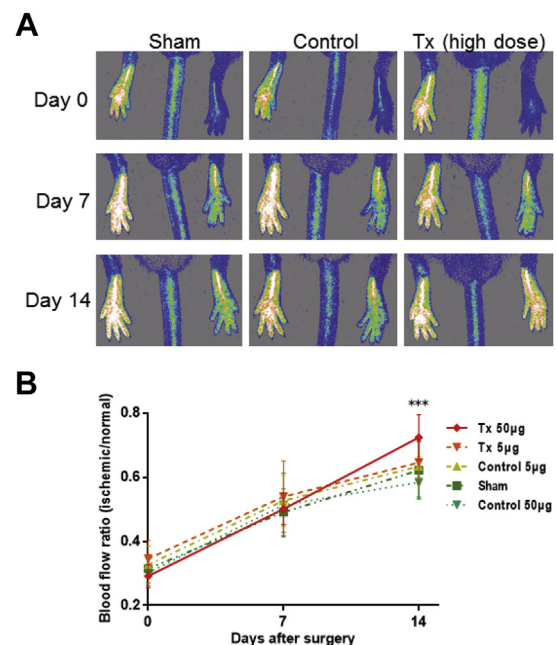


Fig 2. Laser Doppler perfusion after administration of microRNA-126 (miR-126)-loaded poly lactic-co-glycolic acid nanoparticles (PLGA NPs). **A**, Representative laser Doppler perfusion imaging. $n = 12$ in each group. Tx, Treatment group. **B**, Time course of ischemic/nonischemic blood perfusion ratio. *** $P < .001$ compared with other groups; $n = 12$ in each group.

by SPRED1, on day 7 were significantly higher in miR-126-treated mice (control vs miR-126: 0.29 ± 0.10 vs 0.54 ± 0.21 ; $P < .05$; $n = 6$; Fig 4, E).

On the other hand, mRNA expression levels of PI3KR2, which is reported to be downregulated by miR-126,^{9,23,24} were not affected by the miR-126 treatment at 7 days after treatment (control vs miR-126: 1.00 ± 0.05 vs 0.84 ± 0.13 ; $P = .2943$; $n = 6$; Fig 4, C). Protein levels of pAKT/AKT negatively regulated by SPRED1 were also not affected at 7 days after treatment (control vs miR-126: 0.80 ± 0.13 vs 0.95 ± 0.14 ; $P = .4709$; $n = 6$; Fig 4, F).

DISCUSSION

In this study, we showed that the sustained release of miR-126 with PLGA NPs significantly improved the hindlimb ischemia of mice. The improved recovery of blood flow was likely mediated by regulation of the VEGF receptor pathway by the in vivo overexpression of miR-126.

Recent studies have indicated that miRNAs play important regulatory roles in almost every physiologic process. The expression profiles of miRNAs in various cardiovascular diseases have been examined,²⁵ and several miRNAs have been reported to regulate vascular development and angiogenesis in endothelial cells.^{26,27} These findings encourage researchers to explore miRNA-based angiogenic therapies, which can control intracellular signaling pathways targeting the regulations of multiple mRNAs controlled by the miRNAs.

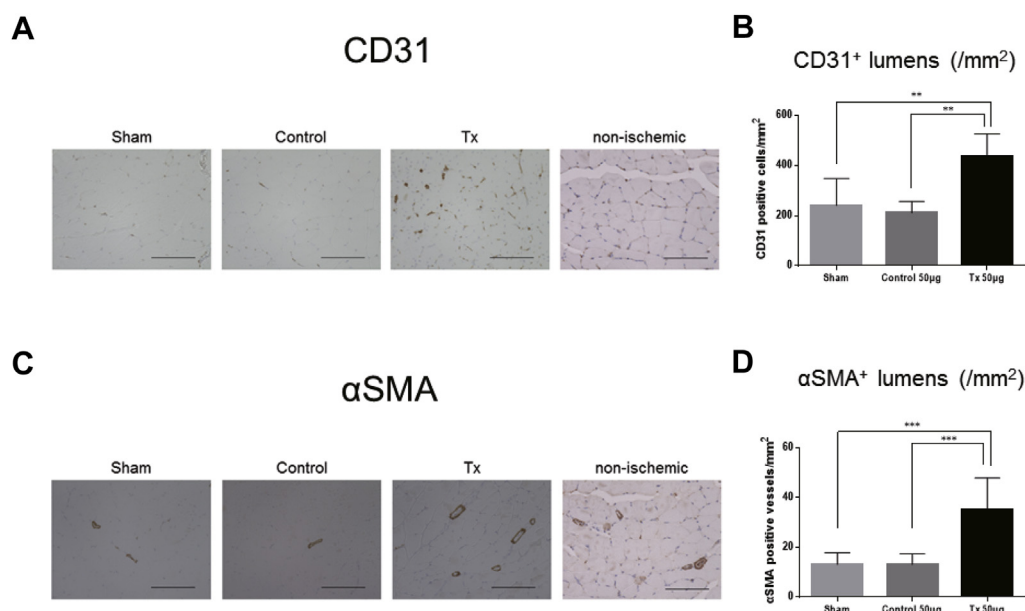


Fig 3. Neovascularization after administration of microRNA-126 (miR-126)-loaded poly lactic-co-glycolic acid nanoparticles (PLGA NPs). **A** and **B**, CD31-positive cell density. The right panel in **(A)** shows the data from normal nonischemic hindlimb. $n = 6$ in each group. $^{**}P < .01$. The scale bars indicate 100 μm . **C** and **D**, α -Smooth muscle actin (α -SMA)-positive vessel density. The right panel in **(C)** shows the data from normal nonischemic hindlimb. $n = 6$ in each group. $^{***}P < .001$. The scale bars indicate 100 μm . Tx, Treatment group.

The relationship between miR-126 and various cardiovascular diseases has been recently reported.^{10,24,28} miR-126 is one of the miRNAs that are highly enriched in endothelial cells and is recognized to be important for regulating vascular integrity and angiogenesis by targeting SPRED1 and PI3KR2 in the VEGF receptor signaling pathway.^{9,10} miR-126 would be an attractive therapeutic target for ischemic diseases.

The DDS of miRNA is crucial for miRNA-based therapy. Although several techniques of targeted miRNA delivery exist,^{19,29,30} the efficacy of the delivery techniques is still unclear, and the optimal delivery technique remains to be determined. The efficiency of miRNA-based therapy for proangiogenesis depends on sufficient and specific delivery to a target organ, such as the vascular endothelium. Furthermore, a viral vector-free method of miRNA delivery would be advantageous for the miRNA-based therapy. A PLGA delivery system is reported to be a viral vector-free method of gene delivery,^{15,16,18} and the nucleic acids loaded in PLGA can gradually be released during the degradation after local administration.

miR-126-loaded PLGA NP-mediated delivery increased neovascularization and improved tissue perfusion in a murine hindlimb ischemia model. Analysis of the neovascularization revealed increased capillary and arteriolar formation and improved limb perfusion. Investigations for the mechanisms underlying these effects revealed that miR-126 administration downregulated a reported direct target, SPRED1. It is reported that the downregulation of SPRED1 led to the activation of the MAPK/ERK pathway,¹⁰ which contributed to the activation of

angiogenesis-related genes.³¹ Although it is not clearly shown in this study, it might be possible that the downregulation of SPRED1 after miR-126 administration into the murine hindlimb ischemia activated the MAPK/ERK-mediated angiogenesis. In contrast, another reported direct target of miR-126, PI3KR2, and the downstream pathway, including the activation of AKT, were not affected in the present experiments. The divergence in working pathways might be caused by unknown pathways mediated by miR-126 and should be further investigated.

There are several limitations in this study. First, we did not include the treatment group of miR-126 in saline (without PLGA). To clarify the precise effect of DDS mediated by PLGA, we should compare the therapeutic effect of miR-126 with or without PLGA and check the expression level of miR-126 in a later stage (after day 7), which will be performed in future studies. Second, the experiments represent an experimental acute-phase hindlimb ischemia without atherosclerosis, which is a common cause of clinical limb ischemia. We may need to further validate the therapeutic effect of miR-126 on disease model animals and clinical studies and include other functional evaluations, such as muscle volumetry or walking distance, in the future. Third, we employed a single administration of miR-126 PLGA NPs with only two doses (5 μg /50 μg). Optimal treatment dose and timing should be further investigated before clinical studies. Fourth, as arteriogenesis is clinically reported to be more relevant in blood flow recovery in limb ischemia,³² it would be better to employ a more sensitive method for

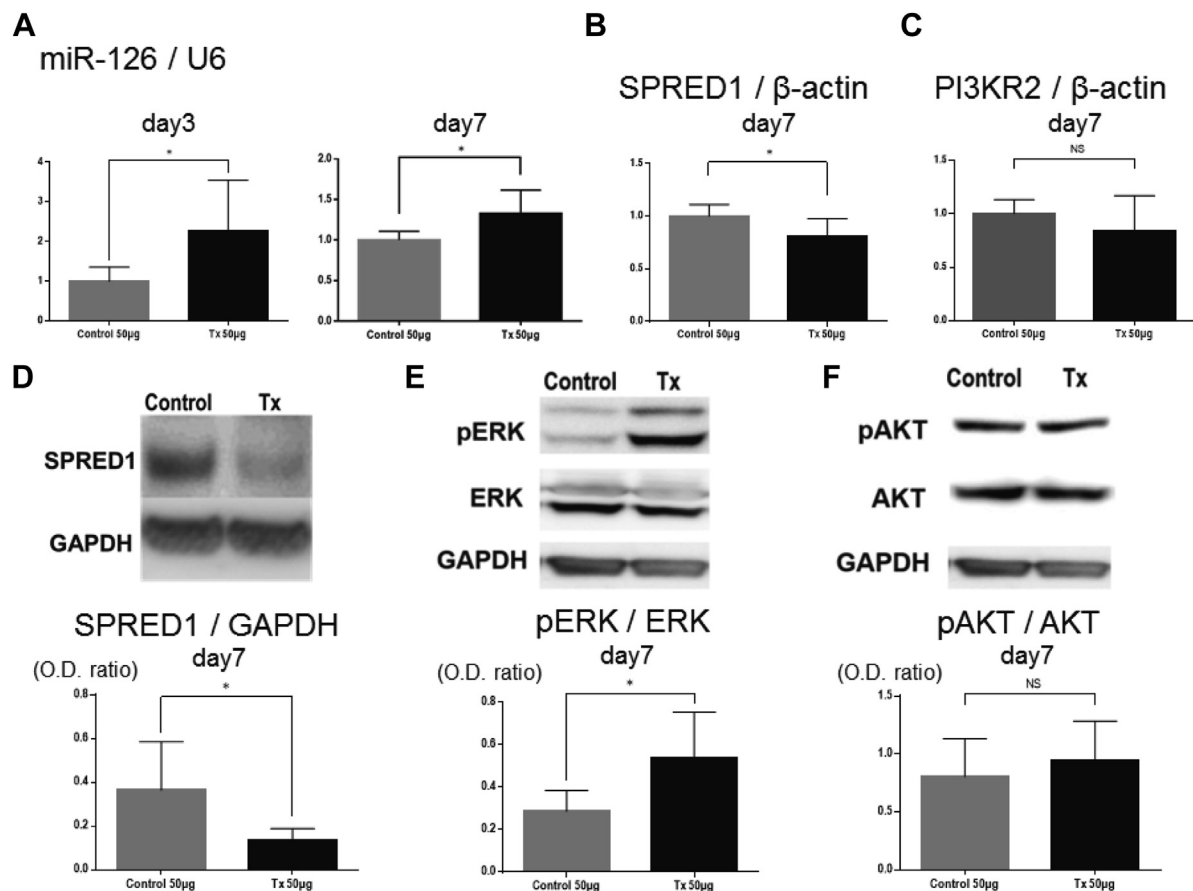


Fig 4. Regulation of vascular endothelial cell growth factor (VEGF) receptor pathways after administration of microRNA-126 (*miR-126*)-loaded poly lactic-co-glycolic acid nanoparticles (PLGA NPs). **A-C**, Quantitative real-time polymerase chain reaction (PCR). **A**, *miR-126* expression levels. $n = 6$ in each group; day 3 (*left panel*) and day 7 (*right panel*). **B**, Sprouty-related EVH1 domain-containing protein 1 (*SPRED1*) messenger RNA (mRNA) expression levels on day 7. $n = 6$ in each group. **C**, Phosphoinositide 3-kinase regulatory subunit 2 (*PI3KR2*) mRNA expression levels on day 7. $n = 6$ in each group. **D-F**, Western blotting. **D**, Protein levels of *SPRED1* on day 7. $n = 6$ in each group. **E**, Protein levels of phosphorylated ERK on day 7. $n = 6$ in each group. **F**, Protein levels of phosphorylated AKT on day 7. $n = 6$ in each group. $^*P < .05$. *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase; *NS*, not significant. *O.D.*, optical density; *Tx*, treatment group.

the quantification of arterioles, such as micro-computed tomography or angiography, than α -SMA staining. Last, there is the possibility that intramuscular administration may affect tissues other than the target of the present study. Moreover, the effect of *miR-126* toward the unexpectedly transduced cells remains unknown. Although the possibility would be lower than with systemic administration, the unexpected effect of *miR-126* and the distribution of *miR-126* after administration should be clarified in future experiments.

CONCLUSIONS

The sustained release of *miR-126* using PLGA NPs showed an excellent therapeutic potential in hindlimb ischemia in mice. This finding serves as a fundamental step for the development of future *miRNA*-based therapeutic angiogenesis for clinical limb ischemia.

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AUTHOR CONTRIBUTIONS

Conception and design: ST, HM, KMinakata, KY, TI, KO, RS, KMinatoya

Analysis and interpretation: ST, MI

Data collection: ST

Writing the article: ST, HM

Critical revision of the article: ST, HM, KMinakata, MI, KY, TI, KO, RS, KMinatoya

Final approval of the article: ST, HM, KMinakata, MI, KY, TI, KO, RS, KMinatoya

Statistical analysis: ST

Obtained funding: KMinatoya

Overall responsibility: ST

REFERENCES

- Ambros V. The functions of animal microRNAs. *Nature* 2004;431:350-5.
- Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281-97.
- Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* 2008;9:102-14.
- Suárez Y, Sessa WC. MicroRNAs as novel regulators of angiogenesis. *Circ Res* 2009;104:442-54.
- Dang LT, Lawson ND, Fish JE. MicroRNA control of vascular endothelial growth factor signaling output during vascular development. *Arterioscler Thromb Vasc Biol* 2013;33:193-200.
- Caporali A, Emanuelli C. MicroRNA regulation in angiogenesis. *Vascul Pharmacol* 2011;55:79-86.
- Zhang Q, Kandic I, Kutryk MJ. Dysregulation of angiogenesis-related microRNAs in endothelial progenitor cells from patients with coronary artery disease. *Biochem Biophys Res Commun* 2011;405:42-6.
- Minami Y, Satoh M, Maesawa C, Takahashi Y, Tabuchi T, Itoh T, et al. Effect of atorvastatin on microRNA 221/222 expression in endothelial progenitor cells obtained from patients with coronary artery disease. *Eur J Clin Invest* 2009;39:359-67.
- Fish JE, Santoro MM, Morton SU, Yu S, Yeh RF, Wythe JD, et al. miR-126 regulates angiogenic signaling and vascular integrity. *Dev Cell* 2008;15:272-84.
- Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, Hill JA, et al. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. *Dev Cell* 2008;15:261-71.
- Kolch W. Coordinating ERK/MAPK signalling through scaffolds and inhibitors. *Nat Rev Mol Cell Biol* 2005;6:827-37.
- Nicoli S, Standley C, Walker P, Hurlstone A, Fogarty KE, Lawson ND. MicroRNA-mediated integration of haemodynamics and vegf signalling during angiogenesis. *Nature* 2010;464:1196-200.
- Rodríguez A, Griffiths-Jones S, Ashurst JL, Bradley A. Identification of mammalian microRNA host genes and transcription units. *Genome Res* 2004;14:1902-10.
- Campagnolo L, Leahy A, Chitnis S, Koschnick S, Fitch MJ, Fallon JT, et al. EGFL7 is a chemoattractant for endothelial cells and is up-regulated in angiogenesis and arterial injury. *Am J Pathol* 2005;167:275-84.
- Tahara K, Sakai T, Yamamoto H, Takeuchi H, Kawashima Y. Establishing chitosan coated PLGA nanosphere platform loaded with wide variety of nucleic acid by complexation with cationic compound for gene delivery. *Int J Pharm* 2008;354:210-6.
- Tahara K, Samura S, Tsuji K, Yamamoto H, Tsukada Y, Bando Y, et al. Oral nuclear factor- κ B decoy oligonucleotides delivery system with chitosan modified poly(D,L-lactide-co-glycolide) nanospheres for inflammatory bowel disease. *Biomaterials* 2011;32:870-8.
- Kubo M, Egashira K, Inoue T, Koga J, Oda S, Chen L, et al. Therapeutic neovascularization by nanotechnology-mediated cell-selective delivery of pitavastatin into the vascular endothelium. *Arterioscler Thromb Vasc Biol* 2009;29:796-801.
- Izuhara M, Kuwabara Y, Saito N, Yamamoto E, Hakuno D, Nakashima Y, et al. Prevention of neointimal formation using miRNA-126-containing nanoparticle-conjugated stents in a rabbit model. *PLoS One* 2017;12:e0172798.
- Morrissey DV, Lockridge JA, Shaw L, Blanchard K, Jensen K, Breen W, et al. Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. *Nat Biotechnol* 2005;23:1002-7.
- Limboung A, Korff T, Napp LC, Schaper W, Drexler H, Limbourg FP. Evaluation of postnatal arteriogenesis and angiogenesis in a mouse model of hind-limb ischemia. *Nat Protoc* 2009;4:1737-46.
- Cha C, Jeong JH, Kong H. Poly(ethylene glycol)-poly(lactic-co-glycolic acid) core-shell microspheres with enhanced controllability of drug encapsulation and release rate. *J Biomater Sci Polym Ed* 2015;26:828-40.
- Niiyama H, Huang NF, Rollins MD, Cooke JP. Murine model of hindlimb ischemia [published online ahead of print January 21, 2009]. *J Vis Exp* doi: 10.3791/1035.
- Gao J, Zhou XL, Kong RN, Ji LM, He LL, Zhao DB. microRNA-126 targeting PI3KR2 promotes rheumatoid arthritis synovial fibro-blasts proliferation and resistance to apoptosis by regulating PI3K/AKT pathway. *Exp Mol Pathol* 2016;100:192-8.
- van Solingen C, Seghers L, Bijkerk R, Duijs JM, Roeten MK, van Oeveren-Rietdijk AM, et al. Antagomir-mediated silencing of endothelial cell specific microRNA-126 impairs ischemia-induced angiogenesis. *J Cell Mol Med* 2009;13:1577-85.
- Welten SMJ, Goossens EA, Quax PHA, Nossent AY. The multifactorial nature of microRNAs in vascular remodeling. *Cardiovasc Res* 2016;110:6-22.
- Wei Y, Schober A, Weber C. Pathogenic arterial remodeling: the good and bad of microRNAs. *Am J Physiol Heart Circ Physiol* 2013;304:H1050-9.
- Kuehnbacher A, Urbich C, Zeiher AM, Dimmeler S. Role of Dicer and drosha for endothelial microRNA expression and angiogenesis. *Circ Res* 2007;101:59-68.
- Katare R, Rawal S, Munasinghe PE, Tsuchimochi H, Inagaki T, Fujii Y, et al. Ghrelin promotes functional angiogenesis in a mouse model of critical limb ischemia through activation of proangiogenic microRNAs. *Endocrinology* 2016;157:432-45.
- Zimmermann TS, Lee AC, Akinc A, Bramlage B, Bumcrot D, Fedoruk MN, et al. RNAi-mediated gene silencing in non-human primates. *Nature* 2006;441:111-4.
- Endo-Takahashi Y, Negishi Y, Nakamura A, Ukai S, Ooaku K, Oda Y, et al. Systemic delivery of miR-126 by miRNA-loaded Bubble liposomes for the treatment of hindlimb ischemia. *Sci Rep* 2014;4:3883.
- Wakioka T, Sasaki A, Kato R, Shouda T, Matsumoto A, Miyoshi K, et al. Sprd is a Sprouty-related suppressor of Ras signaling. *Nature* 2001;412:647-51.
- van Weel V, van Tongeren RB, van Hinsbergh VW, van Bockel JH, Quax PH. Vascular growth in ischemic limbs: a review of mechanisms and possible therapeutic stimulation. *Ann Vasc Surg* 2008;22:582-97.

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