

Title Impact of microRNA-145 to prevent vein graft disease in rabbit by regulation of smooth muscle cell phenotype

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Conflicts of interest None declared.

Abstract

Objective

Since microRNA-145 (miR-145) is a specific mediator in the regulation of the proliferation and differentiation of smooth muscle cells (SMCs), we investigated the impact of miR-145 on intimal hyperplasia in the rabbit model of vein graft disease using electroporation-mediated gene transfer.

Methods

The right jugular vein of male Japanese white rabbits was harvested and transduced with miR-145 encoding plasmids using an electroporator and then interposed in the carotid artery. Two or four weeks postoperatively, the venous graft was explanted, and the intimal thickness and intima / media area ratio were evaluated. Furthermore, three days after implantation, the myocardin and serum response factors were measured using the real-time polymerase chain reaction (PCR) method. Two weeks after implantation, immunohistochemical investigations using mature smooth muscle markers, myosin heavy chain smooth muscle-1 and -2 (SM-1 and SM-2), and proliferation marker Ki-67 were performed.

Results

MiR-145 transduction significantly reduced neointimal thickness at both two and four weeks

(two weeks: $52.1 \pm 15.7 \mu\text{m}$ vs. $113.2 \pm 26.9 \mu\text{m}$, $P < .05$, $n=6$; four weeks: $42.4 \pm 4.8 \mu\text{m}$ vs. $136.5 \pm 38.3 \mu\text{m}$, $P < .05$, $n=8$), and it also significantly reduced the intima / media area ratio at four weeks (0.22 ± 0.04 vs. 1.13 ± 0.23 , $P < .01$, $n=8$). Additionally, it upregulated the mRNA expression level of the myocardin compared to the grafts that did not receive gene transfer. SM-2 and Ki-67 revealed that miR-145 transduced grafts contained more SM-2-positive mature SMCs and less Ki-67-positive proliferating cells.

Conclusion

Non-viral transduction of miR-145 into the bypass graft may be a novel option for preventing intimal hyperplasia in vein graft disease.

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Ultramini abstract

MicroRNA-145 is a specific mediator in the regulation of smooth muscle cell phenotype. Since its transduction using the electroporation reduced neointimal thickness in the rabbit model of vein graft disease, non-viral transduction of microRNA-145 into the graft may be a novel option for preventing vein graft disease after surgical revascularization.

Introduction

The autologous vein graft has been widely used for revascularization in coronary artery bypass grafting (CABG) since its introduction in 1969.¹ Nevertheless, due to accelerated atherosclerosis, these vein grafts have a lower patency rate than those of other arterial grafts.² Thrombotic occlusion followed by intimal hyperplasia is the major cause of vein graft disease, and vascular smooth muscle cell (SMC) proliferation and migration into the intima are the key mechanisms in this process. Campbell et al. first described the phenotypic modulation of SMCs from the contractile state to the synthetic state.³ Phenotypic alternations of vascular SMCs have been identified as playing an important role in the response to atherosclerosis, arterial injury, and bypass vein graft.⁴⁻¹⁰ We previously demonstrated that C-type natriuretic peptide (CNP) suppressed neointimal lesion formation in the arterialized rabbit vein graft model using adenovirus-mediated gene transfer.¹¹ We also reported that overexpression of CNP in SMCs induced growth inhibition of proliferating SMCs with alteration of the phenotype from the synthetic state to the contractile state.¹²

MicroRNAs are a new class of small (~22 nucleotides) non-coding RNAs. In most cases, microRNAs negatively regulate expression of protein-coding genes by promoting degradation or suppressing translation of target mRNAs and modulating various biological functions.¹³ These days, microRNAs are being investigated as a new modality of gene therapy for ischemic heart disease and vascular diseases.¹⁴ Recent studies have reported that microRNA-145 (miR-145) is a

specific mediator in the regulation of proliferation, differentiation, and phenotype of SMCs and is necessary for maintaining a differentiated SMC phenotype.⁴⁻⁷

In recent years, electroporation-mediated gene transfer has emerged in *in vivo* study for future clinical applications designed at overcoming virus-related problems.¹⁵ Therefore, in this study, we investigated the impact of miR-145 on intimal hyperplasia in the rabbit model of vein graft disease using electroporation-mediated gene transfer.

Material and methods

Animals

Fifty male Japanese white rabbits weighing 2.5 to 3.5kg were used. The rabbits were maintained under a 12-hour light schedule and were fed a regular rabbit chow diet. Rabbits were randomly divided into two groups: a control group (n=25) without electroporation mediated-gene transfer and a microRNA-145 electroporation group (n=25). Three days after the operation, four animals were sacrificed for GFP immunostaining and five animals for RT-PCR study in each group. Eight animals from each group were sacrificed at two and four weeks respectively following the initial histological examination. This experiment was approved by the Kyoto University Animal Experiment Review Board (approval reference number: MedKyo 12185). All animals were used

in accordance with the guidelines for Animal Experiments of Kyoto University, which conforms to the law of Guide for the Care and Use of Laboratory Animals in Japan.

Plasmid preparation

Expression vector for human miR-145 (pMIW-cGFP-miR-145) was obtained from B-Bridge Co. Ltd. (Tokyo, Japan). In the construction of this vector, a green fluorescent protein (GFP) expression site was included to confirm successful gene transduction after electroporation (Appendix: Figure A).

Electric pulse delivery for gene electroporation

Electric pulses were delivered via an electric pulse generator (Super Electroporator NEPA21, NEPA GENE Co. Ltd. Chiba, Japan). Figure 1A shows a schematic representation of the electroporation device. Freshly isolated vein grafts were suspended in balanced salt saline (140 NaCl, 5.4 KCl, and 10 HCL [in mM], pH7.6) with 500 μ g/ml of vector and placed in an electrode chamber, as reported previously.^{16,17} First, to confirm the validity of the electroporation method, we performed a preliminary *in vitro* study using the DsRed expression vector in which several electric pulse parameters were tested (“poring pulse”: voltage 25, 50, and 75V; pulse duration 5 and 10ms; intervals 95 and 90ms; and number of pulses 2, 4, and 6; and “transfer pulse”: voltage 10V; pulse duration 5 and 10ms; intervals 95 and 90ms; and number of pulses 5 and 10; Appendix: Table 1). After the electroporation, grafts were suspended in α -MEM (Invitrogen,

Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycin (Invitrogen), and then cultured in standard plastic dishes for two days. The grafts were observed by fluorescence microscopy and also by immunostaining via monoclonal antibodies against DsRed. Secondly, in order to decide the optimal condition of the electroporation, several electric pulse parameters were tested using the miR-145 expression vector with a GFP expression site. These variables were based on the results of our experiment using the DsRed expression vector and earlier well-documented reports that utilized the electroporation method¹⁶⁻¹⁸ Finally, two electric poring pulses (25 V, 5-ms duration, 95-ms interval) followed by eight transfer pulses (10 V, 5-ms duration, 95-ms interval) were adopted based on the results of both the DsRed and GFP apparent expression shown in both Figure B in the Appendix and Figure 1C.

Arterialized rabbit vein graft model

The operative procedure was performed under aseptic conditions. Anesthesia was achieved by administration of pentobarbital sodium (25mg/kg i.v.) and lidocaine hydrochloride (50mg/kg s.c.). After a longitudinal neck incision, a 15- to 20-mm segment of the right jugular vein was exposed, and all the branches of the vein were ligated. A 2Fr. Fogarty balloon catheter was inserted, and after the balloon was inflated with 0.2mL of air, the intima of the vein was denuded by three passages of the catheter. The vein was removed and kept moistened in heparinized saline (10 IU/mL) at room temperature. After the animals were given intravenous heparin (200

IU/kg), a 15- to 20-mm segment of the common carotid artery was meticulously isolated with vascular forceps and then removed. The prepared vein graft was interposed in the carotid artery in a reversed end-to-end fashion. Anastomoses were created with 10-0 nylon continuous suture under $\times 10$ magnification using an operative microscope. To facilitate the intimal hyperplasia, the internal carotid artery and one of the branches of the external carotid artery were ligated with 6-0 silk suture. The wound was closed layer to layer. Cefazolin sodium was given intravenously as a prophylactic measure.

Assessment of intimal hyperplasia

Two or four weeks after the operation, the vein grafts were harvested under general and local anesthesia, and the animals were sacrificed with an overdose of pentobarbital sodium. The harvested vein grafts were fixed with 10% phosphate buffered formalin at a pressure of 100mmHg for 20 minutes. The perfused vein graft was immersed in the same fixative overnight and subjected to histological examination. At least four sections were obtained from each vein graft and stained with both hematoxylin and eosin and elastica van Gieson. The neointima was defined as the area from the inner surface to the internal elastic lamina. The cross sectional intimal thickness was measured at six randomly selected views per section. The average of the six values was used to represent the intimal hyperplasia. The cross sectional intima / media area ratio was also calculated.

Immunohistochemical staining

Three days postoperatively, the vein grafts were explanted, and GFP immunostaining was performed in order to confirm the gene transduction of miR-145 via the electroporation method. Two weeks postoperatively, the vein grafts were explanted and analyzed by immunostaining via monoclonal antibodies against myosin heavy chain (MHC) smooth muscle-1 (SM-1) and smooth muscle-2 (SM-2) (Yamasa Corp., Tokyo, Japan). SM-1 is constitutively expressed in all types of adult SMCs, whereas SM-2 exists only in mature differentiated SMCs.⁸⁻¹⁰ Additionally, immunohistochemical staining with Ki-67 monoclonal antibody (DAKO Cytomation, Denmark) was performed to identify proliferative cells in the neointima. The quantitative analysis was performed as follows: The total and Ki-67 positive cells were counted in eight randomly selected high power fields (original magnification of $\times 400$) per section, and the number of Ki-67 positive cells / total number of cells counted was defined as the Ki-67 index.

Quantitative real-time polymerase chain reaction analysis

Three days after implantation, the vein grafts were explanted and preserved immediately in liquid nitrogen. Total RNA was then extracted from the vein using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) and reverse transcribed with the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, CA) following the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed on the StepOnePlus Real-Time PCR System

(Applied Biosystems, ON, Canada) using the SybrGreen quantitative PCR Mastermix (Qiagen, Venlo, The Netherlands). Custom-designed primers for rabbit serum response factor (SRF: 5'-CCACGGACCAGAGAATGAGT-3' and 5'-GGCTTCAGTGTGTCCTTGGT-3') and myocardin (Myocd: 5'-GCATCACGGATGGATTCTCT-3' and 5'-ACTGCAGCCCATTACTGTCC-3') were purchased from Life Technologies Japan Ltd. (Tokyo, Japan). Gene expression data were analyzed using the relative standard curve method. The expression level of mRNA was divided by the mRNA level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis

All values were expressed as the mean $\pm$ the standard error. The data were evaluated with an unpaired Student's t test using Statview Software version 5.0 (SAS Institute Inc., Cary, NC) and differences between groups were considered to be statistically significant when the *P* value was $<.05$.

Results

GFP immunostaining

Three days after implantation, several GFP-positive staining areas were detected in the media of

the miR-145-transduced graft, indicating the successful gene transduction of miR-145 into the bypass grafts via the electroporation method (Figure 1B and C).

Intimal hyperplasia

As shown in Figure 2A, in the arterialized vein grafts four weeks postoperatively, control grafts formed thick neointima. In contrast, miR-145-transduced grafts showed much thinner neointimal lesions. The statistical analysis of the cross section demonstrated that the intima / media area ratio of miR-145-transduced group was significantly lower than that of the control group (two weeks: 0.31 ± 0.06 vs. 1.25 ± 0.18 , $P < .01$, $n=6$; four weeks: 0.22 ± 0.04 vs. 1.13 ± 0.23 , $P < .01$, $n=8$) (Figure 2B).

Figure 2C shows the comparison of neointimal lesion thickness two and four weeks after implantation in each group. Intimal thickness was significantly lower in the miR-145-transduced group at both two and four weeks after implantation (two weeks: $52.1 \pm 15.7 \mu\text{m}$ vs. $113.2 \pm 26.9 \mu\text{m}$, $P < .05$, $n=6$; four weeks: $42.4 \pm 4.8 \mu\text{m}$ vs. $136.5 \pm 38.3 \mu\text{m}$, $P < .05$, $n=8$)

Immunohistochemical staining for SM-1 and SM-2

Two weeks after implantation, smooth muscle marker SM-1 immunostaining was equally detected in both control and miR-145-transduced vein grafts. Staining for SM-2, a mature SMC marker, revealed that the SM-2-positive area was barely detectable in the neointima of the

control grafts, whereas SM-2 was positive in the reduced neointimal lesion of the miR-145-transduced grafts (Figure 3).

Immunohistochemical staining for Ki-67

Figure 4A and B illustrates Ki-67 positive cells in the neointimal lesion of grafts two weeks after implantation. The frequency of Ki-67 positive cells was much higher in the control grafts. The Ki-67 index was significantly higher in the control group than in the miR-145-transduced group (11.1 ± 1.0 vs. 23.2 ± 1.3 , $n=2$).

Quantitative real-time PCR for myocardin and serum response factor

The mRNA expression level of myocardin was significantly upregulated in the miR-145 transduced grafts compared to the control grafts three days postoperatively (2.03 ± 0.99 vs. 0.90 ± 0.46 , $P<.001$, $n=5$). In contrast, the mRNA expression level of SRF did not reach a statistical difference between the miR-145 transduced grafts and the control grafts three days postoperatively (Figure 5A and B).

Discussion

The present study demonstrates that miR-145-transduced vein grafts inhibited the neointimal lesion formation compared to the control grafts in the arterialized rabbit vein graft model. To our

knowledge, this is the first report on the introduction of miR-145, which specifically regulates the SMC phenotype, to vein graft disease in rabbit via a non-viral gene transfer method.

Previously, Cordes et al. demonstrated the ability of miR-145 to efficiently direct the differentiation of vascular SMCs using transgenic mice experiments. They showed that miR-145 promotes the differentiation and represses the proliferation of SMCs.⁵ Our present study is based on their hypothesis that restoration of miR-145 possibly suppresses the smooth muscle hyperplasia observed in vascular injury and atherosclerosis.

Cheng et al. revealed in their *in vitro* and *in vivo* studies that miR-145 was a vascular SMC phenotypic modulator which controlled the formation of vascular neointimal lesions. They demonstrated that, using balloon-injured rat arteries, the differentiation of vascular SMCs was accelerated by an adenovirus vector expressing miR-145.⁴

We recently revealed the essential function of miR-145 in the differentiation process of SMCs from undifferentiated human ES cells. Overexpression of miR-145 in the ES-derived precursor of SMCs (ES-pre-SMCs) accelerated SMC differentiation, as confirmed by the analysis of smooth muscle marker expression, morphological change, and contractile function.⁷ Based on these findings, we believe that miR-145 works as an essential regulator for SMC differentiation.

Although various protein-based signaling cascades have been implicated in controlling the SMC phenotype,^{11,12,22} applying microRNA to vascular SMCs shows great potential due to the manner

in which it functions. The key characteristic of microRNA is its ability to regulate the expression of multiple genes because it can bind to a number of mRNA targets.¹³ Cordes et al. revealed that miR-145 targeted a network of smooth muscle-related transcription factors, including Kruppel-like factor 4 (KLF4), myocardin, and Elk-1 (a member of the ETS oncogene family), which can promote the differentiation and repress the proliferation of SMCs.⁵

Among these factors, myocardin is a cardiac and smooth muscle-specific factor for SMC differentiation which was discovered by Wang et al.¹⁹ They subsequently recognized that myocardin was a master regulator of smooth muscle cell differentiation.²⁰ It is a potent co-activator for SRF and is also a component of a molecular switch for the SMCs' fate, which is sufficient for it to affect both the structural and physiological attributes of this cell type. SRF weakly activated the miR-143/145 enhancer upstream of a luciferase reporter, but co-transfection of myocardin synergistically and robustly activated luciferase activity.⁵

Based on these findings, although numerous factors and cofactors are concerned with SMC differentiation, we believe miR-145 has additive and synergic effects on its downstream factors for differentiation.

To date, many investigators have presented the results of procedures which inhibit the vein graft disease in animal models.^{11,12,21-24} Nevertheless, none of these methods can be applied to clinical research or usage because they make use of virus-mediated gene transduction. For example, the

use of adenoviral vectors is one of the most popular methods in virus-mediated gene transfer. Its procedures have been already established, and its efficacy of gene transduction is considered satisfactory.^{11,12,22-24} But ethical issues and cytotoxicity still remain due to difficulties in virus handling and regulation. We recently documented the three goals which need to be achieved for clinical usage in gene therapy: suitable vectors, suitable genes, and an appropriate delivery system.²⁵ In this study, we chose the electroporation method in order to overcome virus-related problems and establish an appropriate delivery system for clinical application. Eefting et al. also chose electroporation-mediated gene transfer in their mouse arterial bypass model, resulting in the effective *in vivo* suppression of vein graft disease.¹⁵ In fact, the efficacy of gene transduction using the electroporation method is controversial. In addition, electroporation is not without its disadvantages: heat injury being the most common problem. Previously, Matsumoto et al. established the optimal settings of *in vivo* gene transfer to the rabbit carotid artery via electric pulse. They found that transgene expression was increased in nearly a voltage-dependent manner up to 20V; however, administration at over 30V resulted in a marked decrease of cells in the arterial wall due to apoptosis induced by electroporation.¹⁸ Similarly, we observed vacuolar degeneration and the disappearance of nuclei in the vasculature after high energy delivery to the vein grafts (Appendix: Table 2 and Figure C). Thereafter, Yamaoka et al. established the optimal parameters for gene transfer into the rabbit vein graft model (pulse-on time: 5ms; interval time: 95ms; number of pulses: 10; and voltage: 10 [from the inner lumen side] and 30V [from the

adventitia side]).¹⁶ On the basis of their method, we modified these parameters in our experiments. Our electroporation device has mainly two characteristics: “poring pulse” and “transfer pulse.” The former is for creating temporary pores (small holes) in cell membranes with minimal damage, and the latter is for delivering the target molecules into cells with minimal damage. Based on the established optimal settings of electroporation¹⁶⁻¹⁸ and taking both our histological findings and the distinguishing characteristics of the electric device into account, we adapted the former documented duration (5ms) and interval time (95ms) by using a decrease in the number of poring pulses and an increase in the number of transfer pulses. Consequently, we were able to achieve the successful gene transduction of miR-145, as shown in Figure 1C.

SMC proliferation and migration into the intima are the key mechanisms in the pathogenesis of atherosclerosis and vein graft disease after surgical revascularization. Nagai et al. have demonstrated that smooth muscle MHC isoforms are useful markers for vascular SMC differentiation.⁸ In the present study, there were many more SM-2-positive mature SMCs in the miR-145-transduced grafts than in the control grafts, which indicated that the dedifferentiation of SMCs was inhibited. Since Ki-67 positive cells were observed more frequently in the control grafts than in the miR-145-transduced grafts, it was determined that the neointima of the control grafts contained a significant number of proliferative cells. From these observations, we speculate that these cells originated from a synthetic phenotype of SMCs due to the fact that the control grafts did not contain mature SM-2-positive cells. We therefore believe that proliferation

of the synthetic phenotype was inhibited in the miR-145 transduced grafts, compared to in the control grafts. This is consistent with the theory of Elia et al.⁶ who asserted that miR-145 gene expression functioned as a brake to dedifferentiation rather than as a stimulus for differentiation.

The precise mechanisms of the function of miR-145 should be clarified, and further investigation is needed. We believe that downstream factors such as KLF4 and Elk-1 would be good candidates for future study.

Conclusion

Our present study demonstrates that non-viral transduction of miR-145, which specifically regulates the SMC phenotype, into the bypass graft inhibits neointimal lesion formation and may be a novel option to prevent vein graft disease after surgical revascularization.

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Table 1. Four examples of more than 20 settings of four electric parameters in a preliminary *in vitro* study using a DsRed expression vector

	“Poring pulse”					“Transfer pulse”					Total energy (J)
	Vol (V)	Dur (ms)	Int (ms)	Freq (n)	Delivered energy (J)	Vol (V)	Dur (ms)	Int (ms)	Freq (n)	Delivered energy (J)	
Control	-	-	-	-	-	-	-	-	-	-	-
Sample 1	25	5	50	2	0.306	10	5	95	10	0.034	0.340
Sample 2				4	0.425		5	95	10	0.036	0.461
Sample 3				4	0.478		10	90	10	0.066	0.544
Sample 4				6	0.626		10	90	5	0.070	0.695

Abbreviations: Vol, voltage; Dur, pulse duration; Int, interval time; Freq, number of pulses

Table 2. Electric parameters in a preliminary *in vivo* study using a miR-145 expression vector based on the *in vitro* study using a DsRed expression vector

	“Poring pulse”					“Transfer pulse”					Total energy (J)
	Vol	Dur	Int	Freq	Delivered	Vol	Dur	Int	Freq	Delivered	
	(V)	(ms)	(ms)	(n)	energy (J)	(V)	(ms)	(ms)	(n)	energy (J)	
miR-145	25	5	50	6	0.832	10	10	90	5	0.090	0.922

Abbreviations: Vol, voltage; Dur, pulse duration; Int, interval time; Freq, number of pulses

Figure legends

Figure 1. Schematic illustration of the electroporation device (A) and representative photographs of GFP immunostaining three days after implantation. (B) Control. (C) miR-145 (Scale bar 100µm). GFP positive cells were detected in the media of the miR-145-transduced grafts.

Figure 2. (A) Representative photographs of vein grafts four weeks after implantation. (Elastica van Gieson staining. Arrowheads indicate the internal elastic lamina. Scale bar 100µm.) (B) Cross sectional intima / media area ratio four weeks after implantation. (C) Intimal thickness two and four weeks after implantation. * $P<.05$. Neointimal lesion formation was significantly suppressed in the miR-145-transduced group two and four weeks after implantation.

Figure 3. SM-1 and SM-2 immunostaining of the vein grafts two weeks after implantation. (Arrowheads indicate internal elastic lamina. Scale bar 100µm.) Staining for SM-2, a mature SMC marker, revealed that the SM-2-positive area was barely detectable in the neointima of the control grafts, whereas SM-2 was positive in the reduced neointimal lesion of the miR-145-transduced grafts.

Figure 4. Ki-67 immunostaining of the vein grafts two weeks after implantation. (A) Control. (B) miR-145. (White arrowheads indicate Ki-67-positive cells. Dashed line indicates internal elastic lamina. Scale bar 50µm.) The frequency of Ki-67 positive cells was much higher in the control grafts, which suggests that there were more proliferative cells in the control group.

Figure 5. Serum response factor (A) and myocardin (B) mRNA expression in explanted grafts were evaluated by quantitative RT-PCR three days postoperatively. The mRNA expression level of myocardin was significantly upregulated in the miR-145 transduced grafts (2.03 ± 0.99 vs. 0.90 ± 0.46 , $P < .001$, $n=5$).

Appendix: Figure A. Schema of the construct of the miR-145 expression vector, which includes miR-145 and a Green Fluorescent Protein expression site.

Appendix: Figure B. DsRed fluorescent image (left) and DsRed immunostaining (right) two days after electroporation (original magnification $\times 200$, Lu: lumen side, Ad: adventitia side). An apparent DsRed expression in the lumen side was observed, indicating successful gene transduction after adopting the electric parameters of Sample 4 in Table 1.

Appendix: Figure C. Representative photographs of degenerative vasculature in the preliminary *in vivo* study using a miR-145 expression vector. Electric parameters are described in Table 2.

Vein grafts were explanted two weeks after implantation. (Hematoxylin and eosin staining, Lu: lumen side, Ad: adventitia side) (A) Black arrowheads indicate the vacuolar degeneration in the vasculature (original magnification $\times 100$). (B) Between white arrowheads the disappearance of nuclei was observed in almost all layers of the vasculature (original magnification $\times 200$).

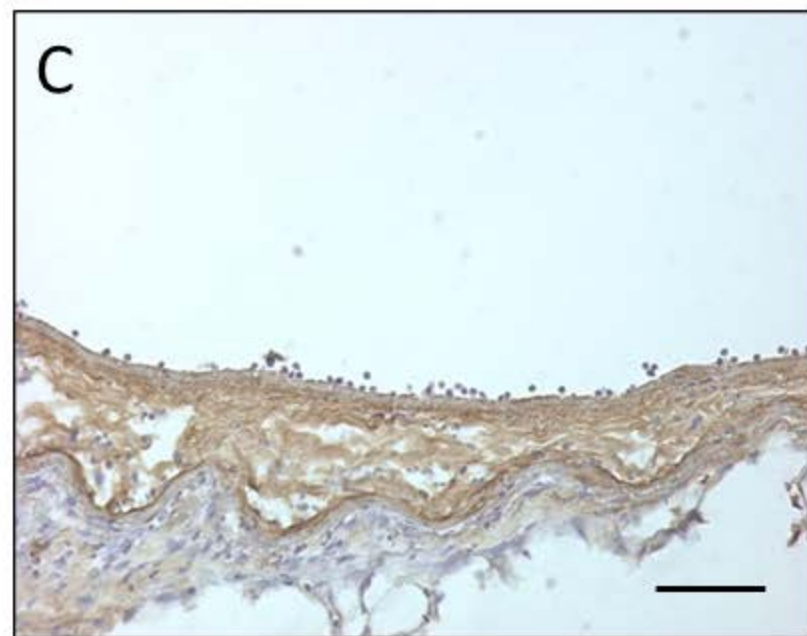
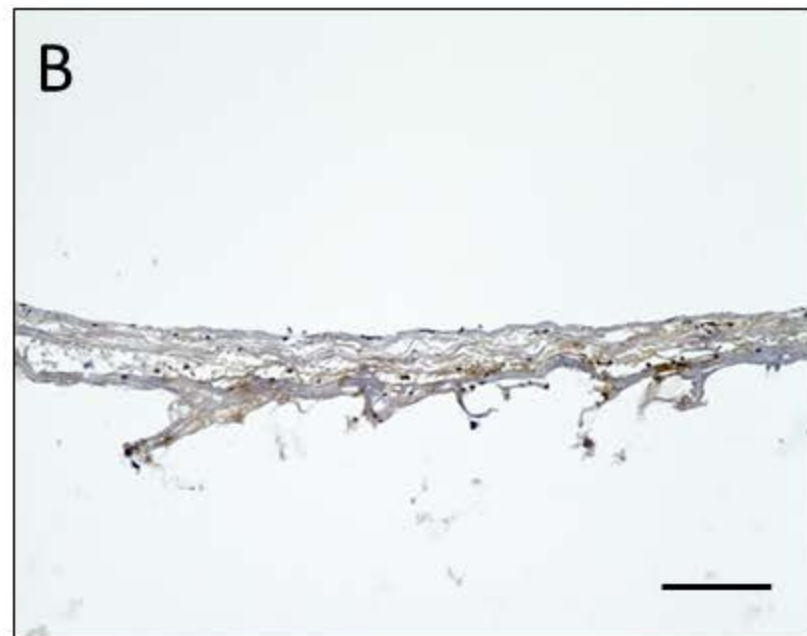
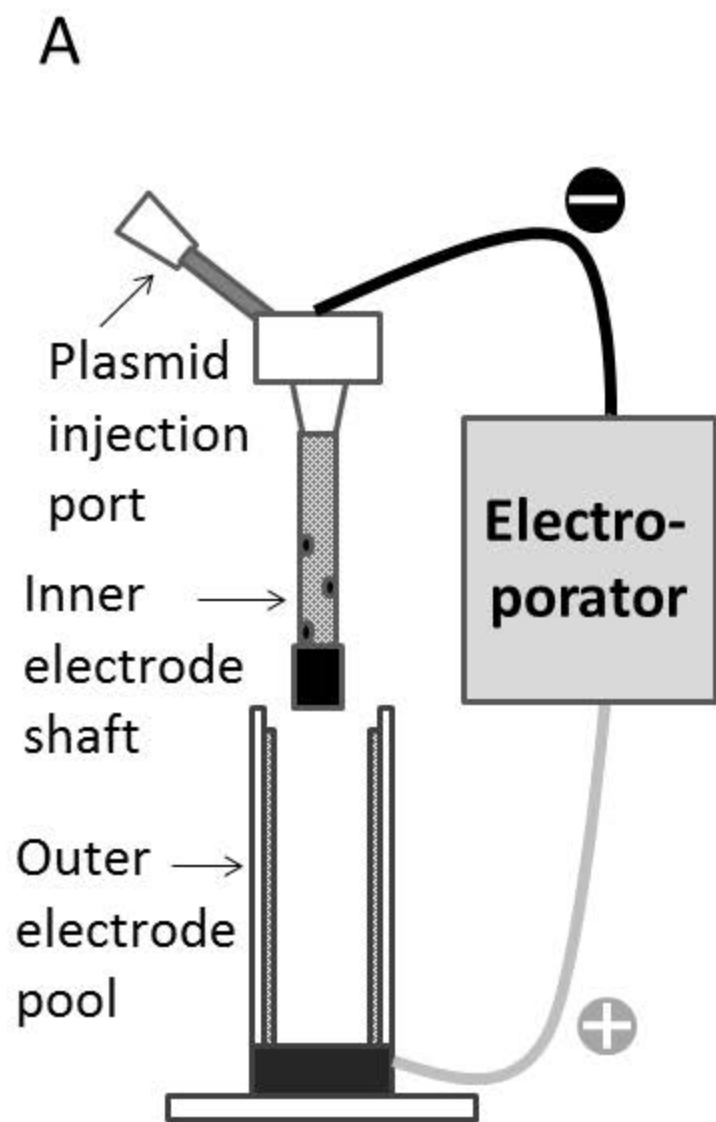


Figure 1.

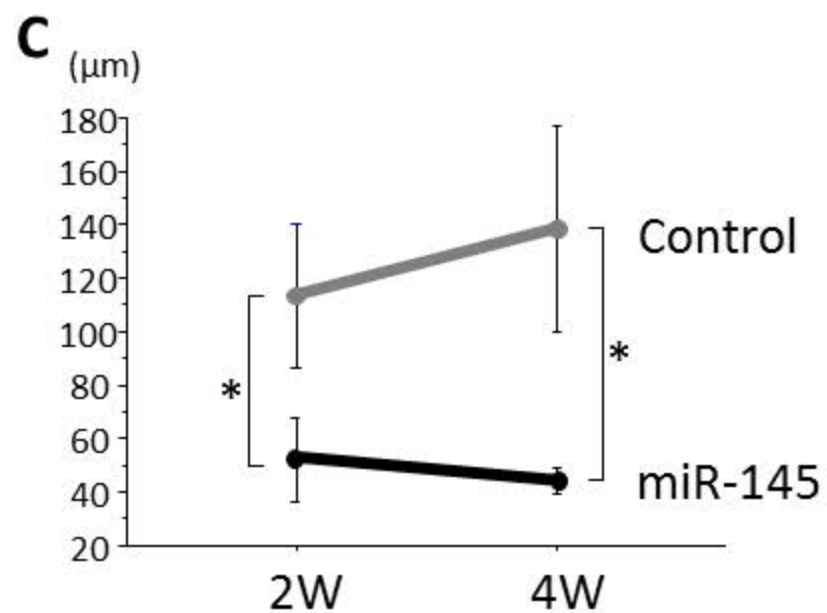
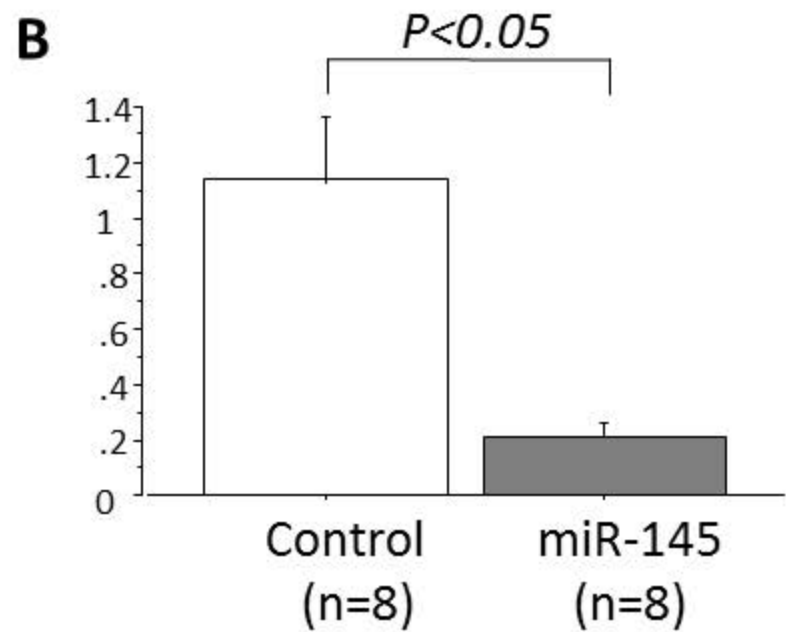
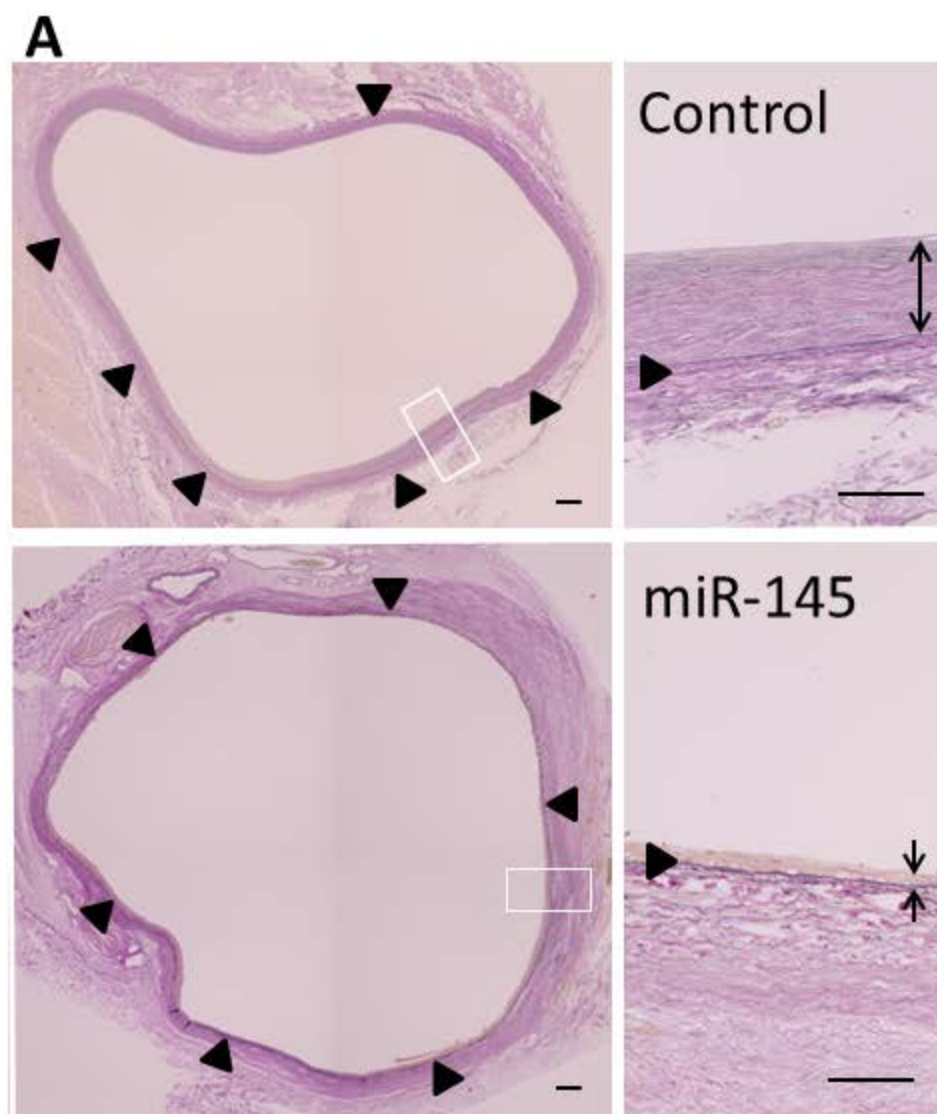


Figure 2.

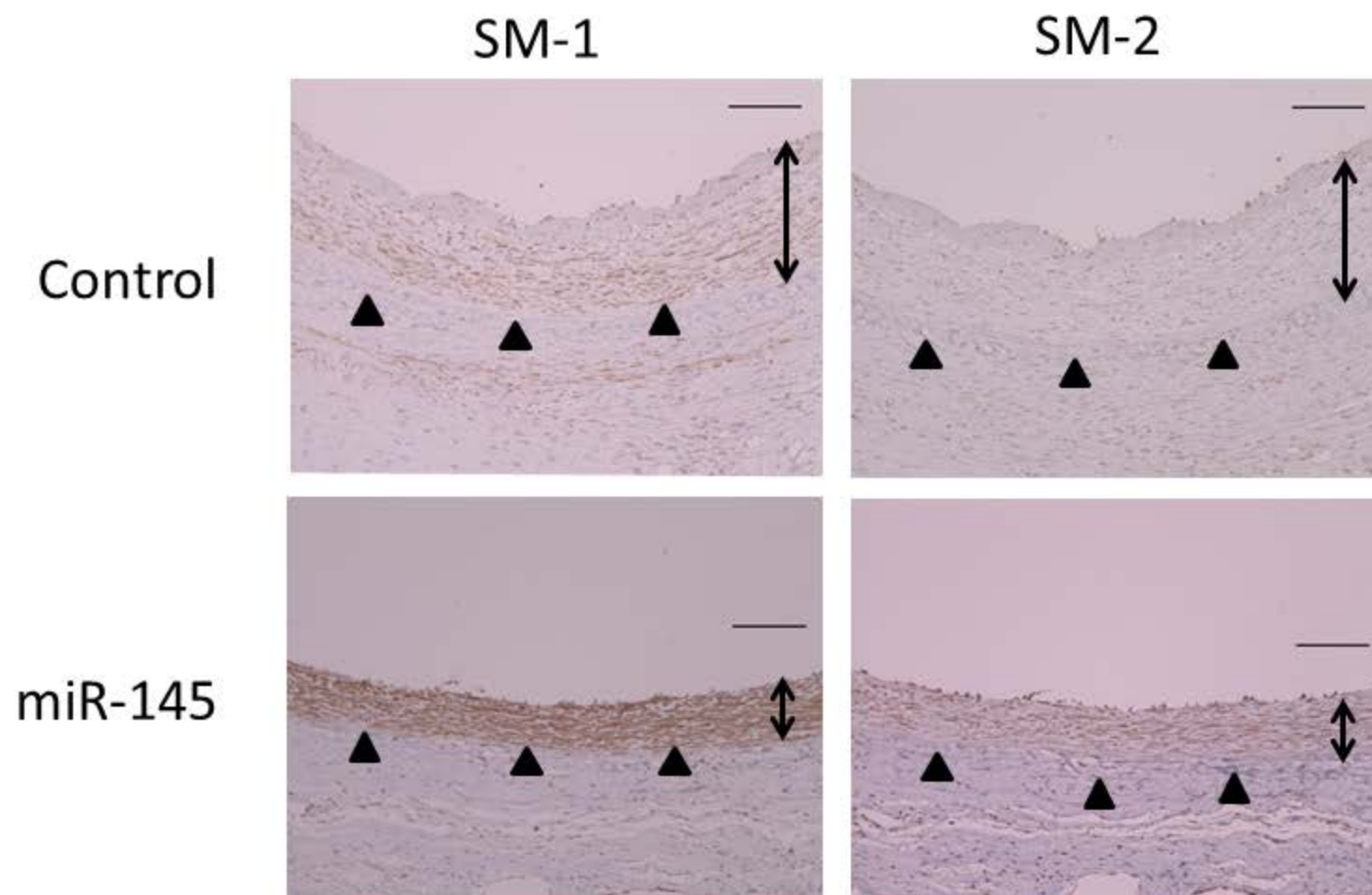


Figure 3.

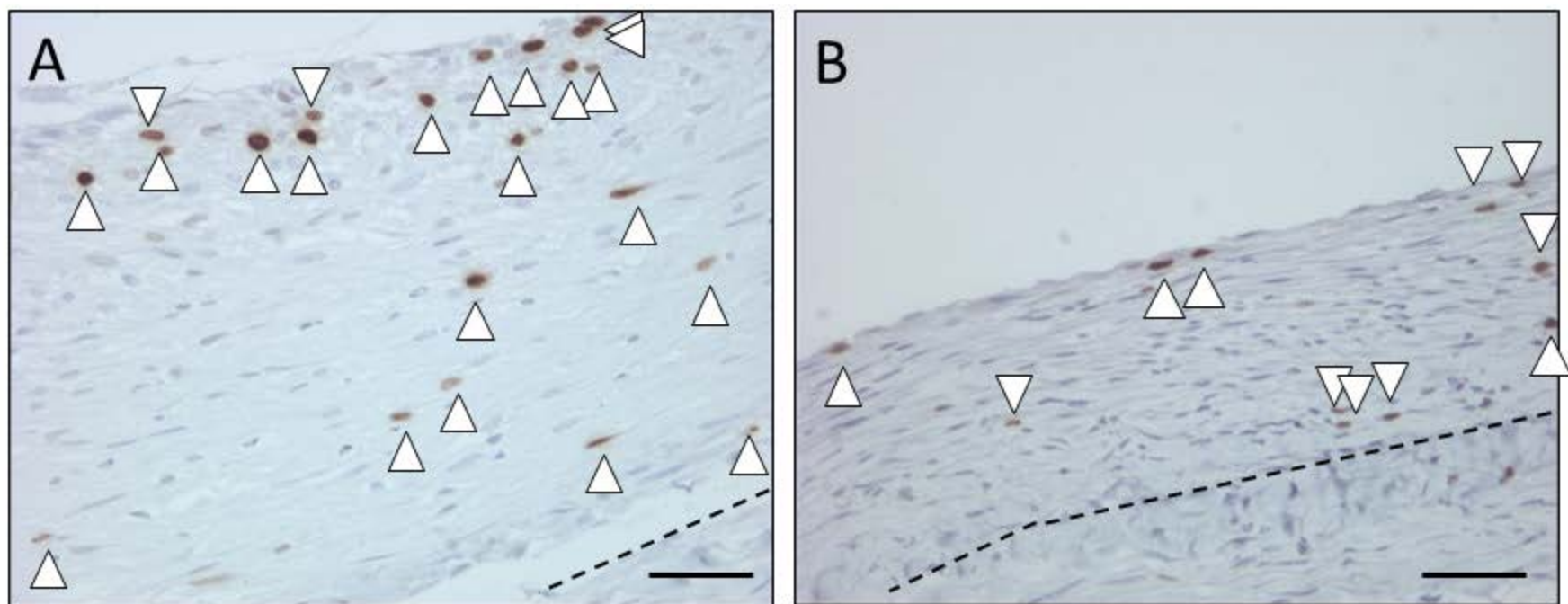
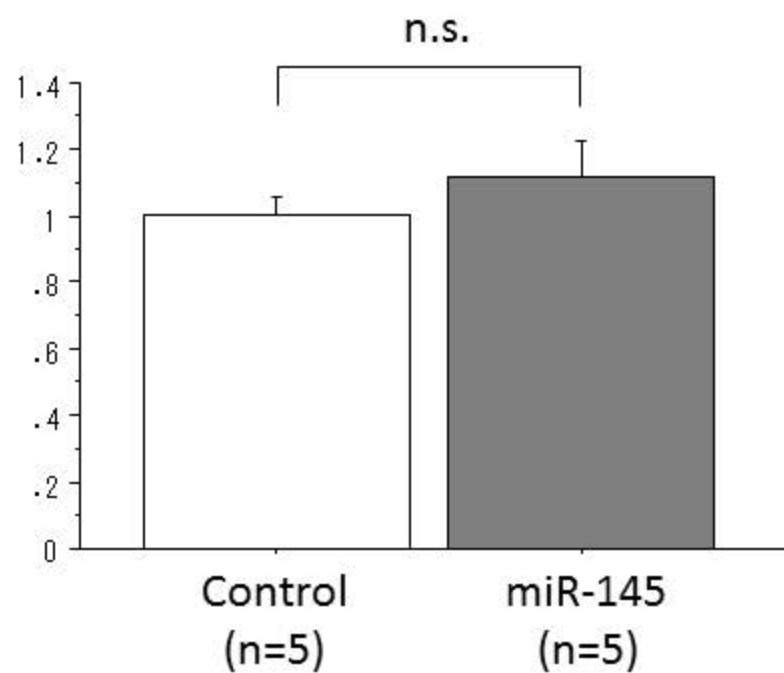


Figure 4.

A. SRF/GAPDH Ratio



B. Myocardin/GAPDH Ratio

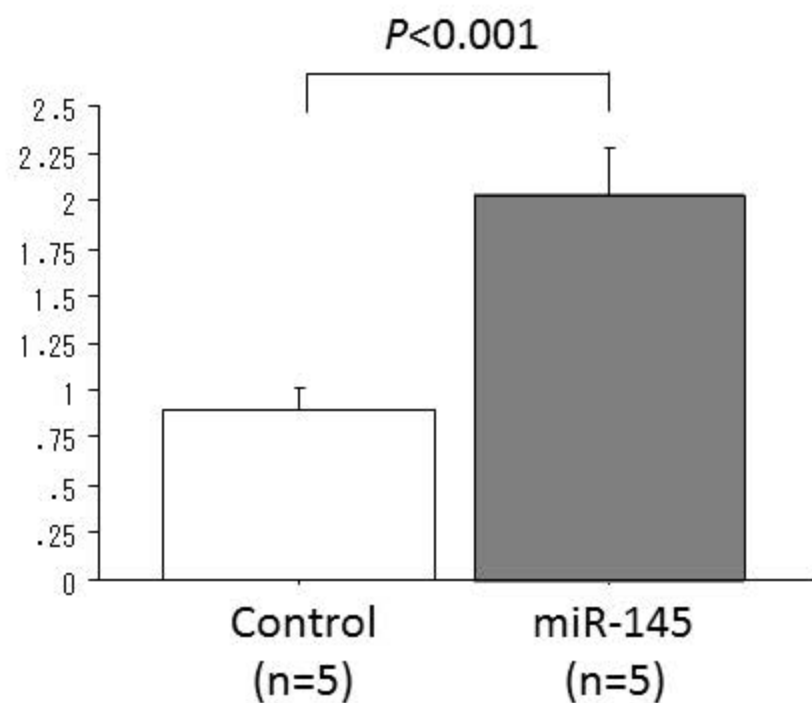


Figure 5.

pMIW-cGFP-miR145

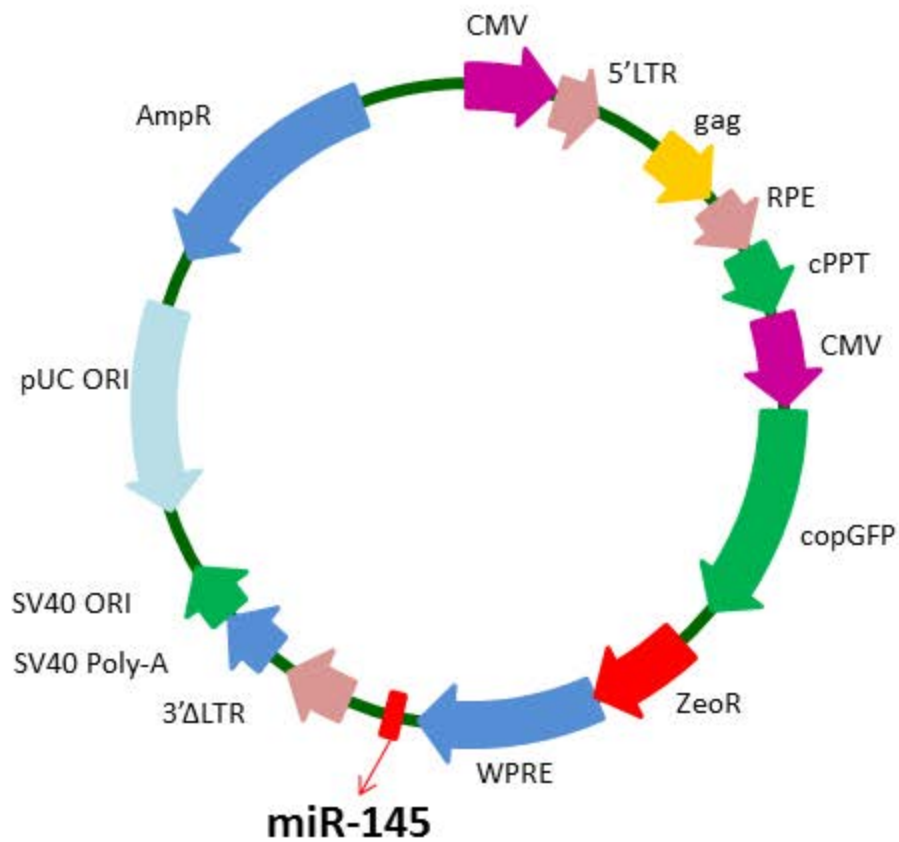


Figure A.

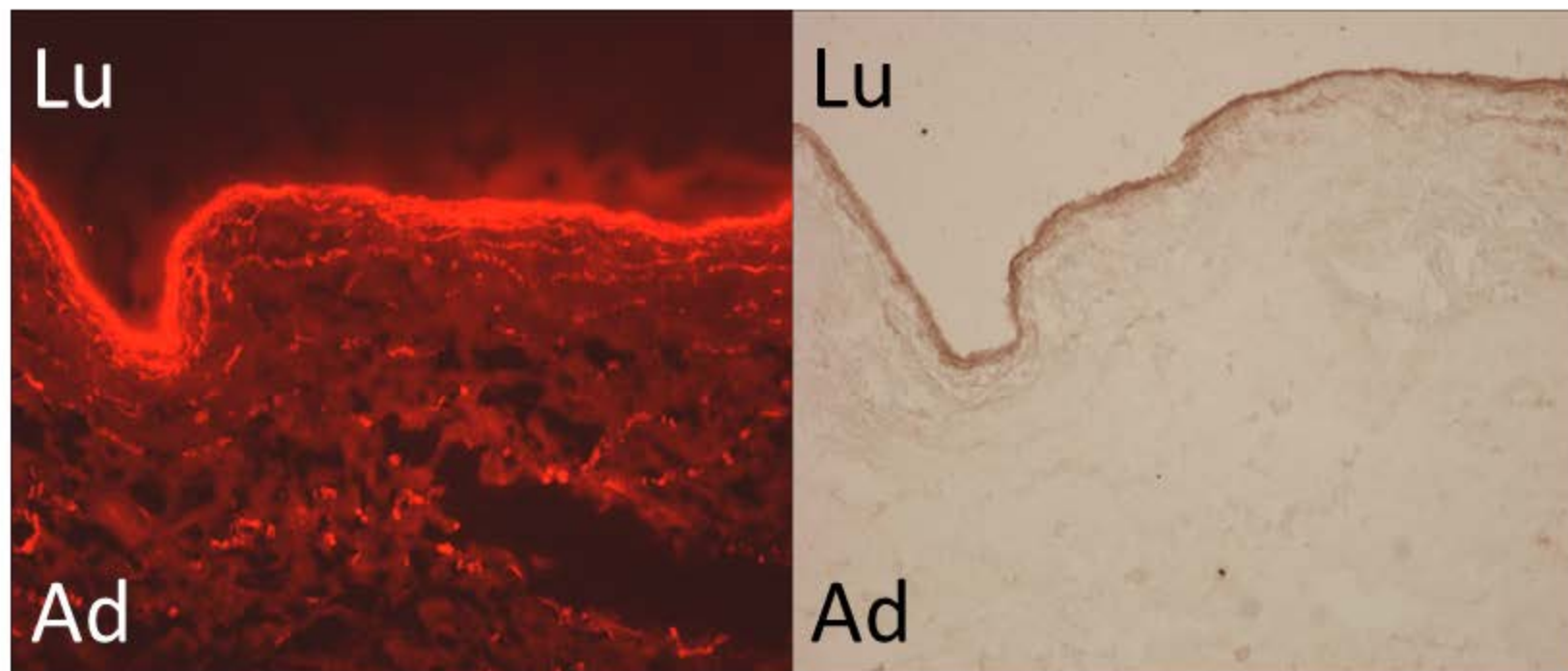


Figure B.

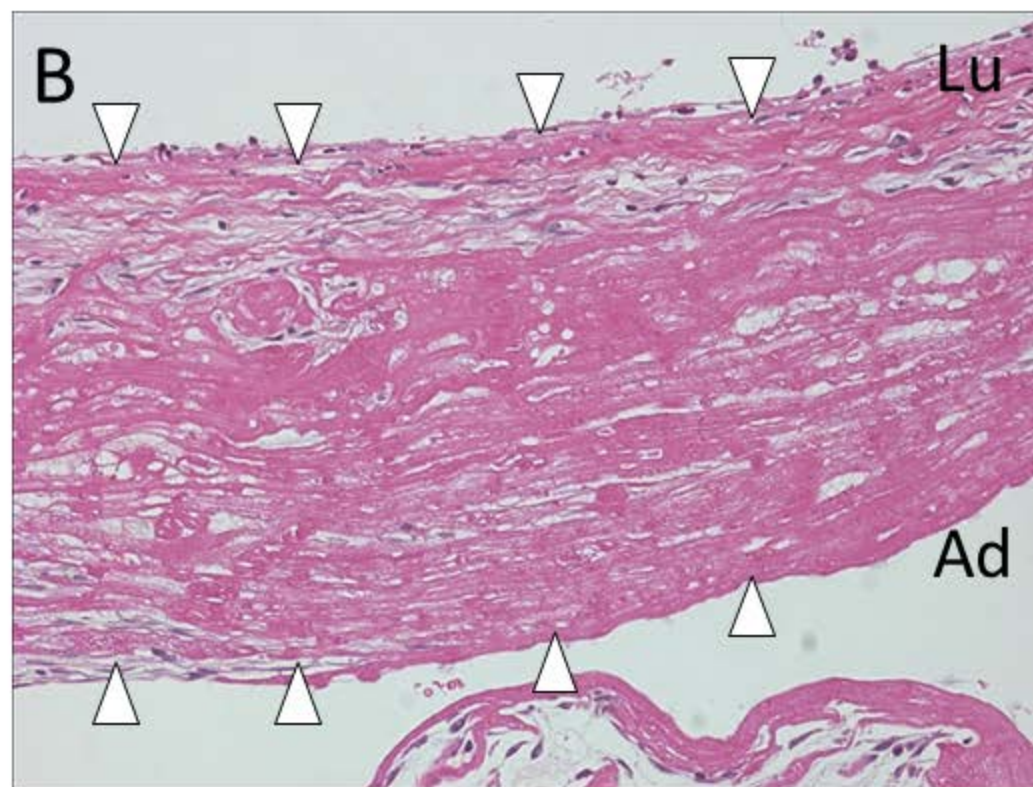
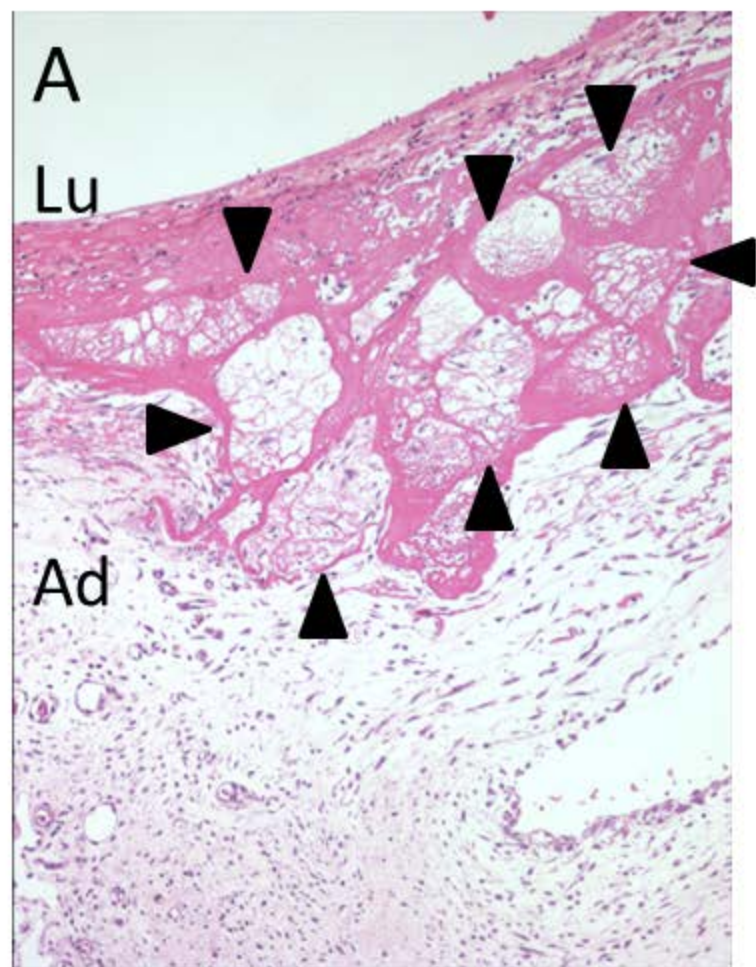


Figure C.