Role of microRNA-29b in interleukin-3 stimulated migration and proliferation of vascular smooth muscle cell

Jiyun Lee

Department of Medical Science

The Graduate School, Yonsei University

Role of microRNA-29b in interleukin-3 stimulated migration and proliferation of vascular smooth muscle cell

Directed by Professor Ki-Chul Hwang

The Master's Thesis
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Jiyun Lee

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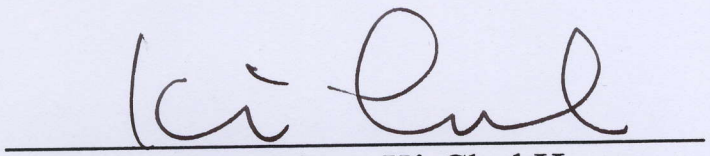
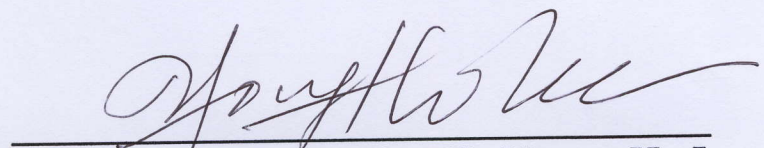
This certifies that the Master's Thesis
of Jiyun Lee is approved.



Thesis Supervisor: Ki-Chul Hwang

Thesis Committee Member#1: Ho Geun Yoon



Thesis Committee Member#2: Young Ho Lee

The Graduate School

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ABSTRACT

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The Graduate School, Yonsei University

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Interleukin-3 (IL-3) level is especially known to increase significantly during vessel damage in the cardiovascular system and is involved in the proliferation and migration of smooth muscle cells (SMCs) through the activation of the ERK1/ERK2 MAPkinase and PI-3K/Akt pathways. MicroRNA-29b is known to regulate cell growth and target Mcl-1 and MMP2, which are highly important molecules in the proliferation and migration of cancer cells. However, the roles of microRNA-29b in other cell types still remain unknown. Therefore, we

hypothesized that microRNA-29b may control SMCs that have gone through the proliferation and migration processes induced by IL-3 stimulation by inhibiting its own specific targets. In this study, IL-3 stimulated the proliferation and migration of SMCs, while microRNA-29b inhibited these effects. These findings were confirmed by a proliferation and migration assay. The expression levels or activation of Mcl-1 and MMP2, which are considered important proteins related to the IL-3-simulated signaling pathway, were also increased with IL-3 stimulation. MicroRNA-29b treatment resulted in significant reductions of Mcl-1 and MMP2 in RT-PCR and the immunoblot assay. In addition to Mcl-1 and MMP2, the upstream signal proteins stimulated by IL-3 were also investigated. IL-3 activated ERK and AKT, but microRNA-29b did not suppress the activation of these proteins. Finally, the luciferase assay showed that microRNA-29b was able to selectively downregulate Mcl-1 and MMP2 expression. This study demonstrated that microRNA-29b successfully suppressed the proliferation and migration of SMCs induced by IL-3 treatment through the inhibition of the signaling pathway related to Mcl-1 and MMP2.

Key words: smooth muscle cell, proliferation, migration, microRNA-29b

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I. INTRODUCTION

Cardiovascular diseases including atherosclerosis remain a leading cause of death, despite surgical improvements such as stent placement and percutaneous transluminal coronary angioplasty (PTCA). Although these surgical methods are a safe and effective way to treat coronary artery disease, restenosis can occur in 30-40% of patients within 3-6 months [1]. Even effective drugs that have been coated with stents cannot completely prevent restenosis, even

though the process inhibits the migration and proliferation of vascular smooth muscle cells (vSMCs). Therefore, new therapeutic methods need to be developed in order to overcome these limitations.

Restenosis is a recurrence of stenosis – a re-blockage of a treated vessel initiated from an inflammatory reaction due to endothelial cell damage [2] and produced by numerous proinflammatory cytokines and growth factors from macrophages.

One type of cytokine, Interleukin-3 (IL-3), is an important cytokine produced by activated T-cells and plays a critical role in inflammation and immune responses such as stimulating basophil and mast cell responses [3]. IL-3 is also known to regulate the survival and proliferation of hematopoietic stem cells in the embryonic stage of development [4]. IL-3 level has been shown to be significantly higher in patients with coronary artery disease and much higher in patients with restenosis versus patients without restenosis [5]. In human atheromas, the IL-3 level was also observed to be increased to stimulate the migration and proliferation of SMCs [6].

IL-3 can cause phenotypic changes in SMCs, which then transform from a contractile phenotype to a synthetic phenotype in order to migrate and

proliferate, ultimately forming a neointima. SMCs are still highly emphasized in vascular function, especially in terms of contractility depending on the phenotype. Contractile SMCs exhibit an elongated spindle shape and form a large proportion of the contractile filaments, whereas synthetic SMCs show a less elongated morphology and are involved in protein synthesis. Beside IL-3, it is also known that SMCs can be transformed by fibroblast growth factor (FGF)-2, platelet-derived growth factor (PDGF)-B, or transforming growth factor (TGF)-β [7].

Once SMCs recognize an IL-3 cytokine through the IL-3 receptor, several signaling pathways related to proliferation and migration are activated. As the most representative signaling pathway related to proliferation and migration, the activation of the ERK1/ERK2 MAP kinase and PI3K-AKT pathways are well known, and it is reported that these signals can cause an increase of myeloid cell leukemia sequence 1 (Mcl-1) expression and the activation of matrix metalloproteinases (MMPs) [6,8]. Mcl-1 is known as an anti-apoptotic Bcl-2 family member and plays an important role in cell survival. Unfortunately, its mechanism for cell survival is not known. Recently, one research group revealed that an isoform of Mcl-1 was imported into the mitochondrial matrix and contributed to basic cell functions such as normal

mitochondrial fusion, ATP production, and membrane potential. This result suggests the possibility that Mcl-1 may control the survival of normal cells as well as cancer cells [9]. MMPs are matrix-degrading enzymes, such as zinc endopeptidases, that degrade ECM and are necessary for cell migration. MMP2s and MMP9s are secreted from SMCs [10]. Fitzgerald M et al. reported that human atherosclerotic plaques display increased expression levels of MMP2 and MMP9 in SMCs [11]. MMPs are associated with the proliferation and migration of SMCs and are expressed and activated in atherosclerotic lesions. Since MMPs were first found in vertebrates, almost 30 isoforms have been discovered [12]. MMP2s and MMP9s are especially thought to be important and have been actively studied in cancer metastasis [13-14]. MMPs are also involved in physiological and pathological processes in cardiovascular health including roles in obesity, diabetes, and cardiovascular diseases [15]. Various stimuli can cause the activation of MMPs such as reactive oxygen species (ROS), tumor necrosis factor (TNF-a), and interleukins (IL). In addition, different protein kinases can control the expression and activation of MMPs [16-17]. However, the relationship between IL-3 and MMPs remains unknown.

Since the first microRNA was discovered through the study of Caenorhabditis elegans [18], many microRNAs have been revealed to play critical roles in the

induction of pathological conditions. In the past several years, these microRNAs have been extensively studied as a powerful therapeutic tool for many diseases such as cancers [19], neurodegenerative diseases [20], cardiovascular diseases [21-22] and autoimmune diseases [23], and even as a regulator for differentiation and regeneration [24-25]. MicroRNAs are noncoding RNAs consisting of 18-22 nucleotides and are a novel class of gene regulators, which degrade transcripts or repress transcription by binding to the 3'-untranslated regions (3'-UTRs) of specific mRNAs [26-27]. Studies related to the microRNA-29 family have demonstrated that microRNA-29, especially microRNA-29b, is important for tumor suppression by regulating proliferation and migration through the suppression of SPARC and COL4A2 [28-29]. Not much is known regarding the mechanisms or targets of microRNA-29b in SMCs. Therefore, we hypothesized that microRNA-29b may regulate the IL-3-induced proliferation and migration of SMCs through its putative gene targets, which can affect vascular smooth muscle remodeling and possibly be used as a tool for novel molecular therapy.

II. MATERIALS AND METHODS

1. Materials

Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Gibco BRL (Grand island, NY, USA). Recombinant rat Interleukin-3 (IL-3) was obtained from R&D Systems (R&D Systems Inc., MN, USA). The antibody of Mcl-1 was purchased from Abcam (AbcamPlc, CB, UK). Antibodies of MMP2, ERK, and phospho-ERK were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antibodies of AKT and phospho-AKT were purchased from Cell Signaling (Cell Signaling Technology, Danvers, MA, USA). Horseradish peroxidase-conjugated secondary antibodies of mice or rabbits were obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA, USA). A Western blotting detection system was obtained from Amersham Biosciences (Amersham Pharmacia Biotech, Tokyo, Japan).

2. Isolation and culture of rat aortic smooth muscle cells

Thoracic aortas from 6- to 8-week-old Sprague–Dawley rats were removed and transferred on ice in serum-free DMEM containing 100 units/ml of penicillin and 100 μg/ml of streptomycin. Each aorta was freed from the connective tissue,

transferred into a Petri dish containing 5 ml of an enzyme dissociation mixture containing DMEM with 1 mg/ml of collagenase type I (Sigma-Aldrich Inc., St. Louis, MO, USA) and 0.5 mg/ml elastase (USB, OH, USA), and incubated at 37°C for 30 min. The aorta was then transferred into DMEM, and the adventitia was stripped off with forceps under a binocular microscope. Afterward, the aortic specimen was transferred into a plastic tube containing 5 ml of the enzyme dissociation mixture and incubated for 2 hr at 37°C. The suspension was centrifuged (1,500 rpm for 10 min), and the pellet was resuspended in DMEM with 10% FBS. The cells were cultured over several passages (up to 10). SMCs were cultured in DMEM supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin in 75-cm^2 flasks at 37°C in a humidified atmosphere of 95% air and 5% CO_2 (Forma Scientific Inc., Marjetta, OH, USA).

3. Cell proliferation assay

SMCs were plated in triplicate wells of 96-well plates at 2×10^3 cells/well. The cells were pretreated with 0.1% FBS DMEM for 48 hr and then treated with IL-3(80 ng/ml) for 48 hr. After treatment, MTT[3-(4,5-dimethylthiazol-2-yl)-2,5 -diphenyl-2H-tetrazoliumbromide] solution (Dojindo, Japan) in a 0.5 mg/ml

final concentration was added to each well and incubated at 37 ºC for 2 hr to allow for MTT reduction. The absorbance of the samples was measured at 450 nm using a microplate reader. The experiments were performed in triplicate.

4. Cell migration assay

The effect of IL-3 on the migration of SMCs was examined by performing a modified Boyden chamber assay in Trans well cell culture chambers using a collagen-treated polycarbonate membrane with 8-μm pores in a 24-well plate (Nunc, Rochester, NY, USA). Preconfluent SMCs were suspended in 0.1% FBS DMEM to a concentration of 8×10^3 cells/ml, and 10% FBS DMEM was added to the lower chamber containing IL-3 (80 ng/ml) and incubated for 9 hr at 37 ºC. The filter was then removed, and the cells on the upper side of the filter were scraped off with a cotton tip. Cells that migrated to the lower side of the filter were fixed in methanol and stained with hematoxylin. Three randomly chosen fields were counted at 200x magnification with an inverted microscope. The experiments were performed in triplicate and were repeated at least three times.

5. Wound healing assay

For the wound healing assay, a rectangular lesion was created using a cell

scraper. The cells were then rinsed twice with serum-free medium and incubated with IL-3 (80 ng/ml) for 48 hr. After the designated times, three randomly selected fields at the lesion border were acquired using a CCD camera (Olympus, Japan) attached to an inverted microscope. In each field, the distances from the margin of the lesion to the ten most migrated cells were measured, and the mean values of the distances were taken as the mobility value of the cells in each culture dish.

6. Immunoblot analysis

The cells were washed once in PBS and lysed in a lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_2-EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined using the Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Proteins were separated in a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). After blocking the membrane with Tris-buffered saline-Tween 20 (TBS-T, 0.1% Tween 20) containing 5% nonfat

dried milk for 1 hr at room temperature, the membrane was washed twice with TBS-T and incubated with primary antibody for 1 hr at room temperature or overnight at 4°C. The membrane was washed three times with TBS-T for 10 min and then incubated for 1 hr at room temperature with horseradish peroxidase-conjugated secondary antibodies. After extensive washing, the bands were detected by an enhanced chemiluminescence reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The band intensities were quantified using NIH Image J version 1.34e software.

7. RT-PCR analysis

Total RNA was extracted with a 500 μl/ 60 mm plate Tri-reagent (Sigma-Aldrich Inc., St. Louis, MO, USA). After extraction, 100 μl chloroform was poured above the Tri-reagent, and the sample was vortexed for approximately 10 seconds. The sample was then centrifuged at 12,000 g at 4 ºC for 15 min, resulting in three layers in the tube. The transparent upper layer was collected in new tubes. To these tubes, 250 μl of 2-propanol was added and then vortexed for approximately 30 seconds. Afterward, the sample was centrifuged at about 12,000 g at 4 ºC for 10 min. The pellet was retained and washed with 75% ethanol (Sigma-Aldrich Inc., St. Louis, MO, USA)-mixed

diethylpyrocarbonate (DEPC; Sigma-Aldrich Inc., St. Louis, MO, USA) water. The washed pellet was centrifuged at approximately 7500 g at 4℃ for 5 min. The supernatant was once again discarded and the pellet was dried at room temperature for around 7 min. Finally, 30 μl of nuclease free water (NFW) was added to the pellet. The quality and quantity of the RNA were determined by OD260/OD280 with a DU 640 spectrophotometer (Effendorf, Hambug, Germany).

Complementary DNA (cDNA) was synthesized with RT-&GO^{TM}; 1 μg of total RNA was added to 1 μl of anchored primer (dT), 2 μl of dithiothreitol (DTT), and NFW to yield a total volume of 9 μl. To prevent the formation of secondary structures, the mixture was incubated at 70 ºC for 5 min, and 8 μl of RT-&GO^{TM} mastermix was added. The sample was incubated at 42 ºC for 1 hr. Finally, the sample was inactivated using reverse transcriptase at 70 ºC for 15 min. Similar to the isolation of total RNA, the sample was detected by OD260/OD280 using a DU 640 spectrophotometer; 1 μg of cDNA, 10 pmol of primer (forward and backward), 0.1 mM of dNTP mixture, 1.25 U of Taq polymerase, and 10 X reaction buffer were mixed with NFW to a final total volume of 25 μl. The primers used to amplify the sample were GAPDH: 5'-ACC ACA GTA CAT GCC ATC AC-3' and 5'-TCC ACC ACCCTG TTG CTG TA-3'(450bp), MMP2:

5’-AGG ACA AGT GGT CCG AGT AAA G-3’ and 5’-CCA CTT CCG GTC ATC ATC GTA GT-3’(510bp), and MMP9: 5’-AAG GAT GGT CTA CTG GCA C-3’ and 5’-AGA GAT TCT CAC TGG GGC-3’(280bp). The PCR condition was fixed as follows: A cycle of denaturation at 94 ºC for 3 min followed by 35 cycles of denaturation at 94 ºC for 30 seconds, annealing at 48 ºC to 60 ºC for 30 seconds, and elongation at 72 ºC for 30 seconds. The sample was then kept at a temperature of 72 ºC for 10 min. When the PCR assay was completed, the PCR product was separated by electrophoresis in a 1.2% agarose gel (Bio-Rad, Hercules, CA, USA) and was Gel-Doc (Bio-Rad, Hercules, CA, USA) visualized after staining with ethidium bromide (EtBr; Sigma-Aldrich Inc., St. Louis, MO, USA).

8. MicroRNA transfection

Transfections of microRNA were performed using a silentfact reagent. Briefly, cells were seeded at the density of 2×10^5 cells per 35 mm of culture plate. The silentfact reagent was diluted with Opti-MEM and combined with its indicated microRNA mimic for each plate. The microRNA and silentfact reagent were added to each plate containing fresh medium and cells. After 6 hr of incubation in a CO_2 incubator at 37°C, the medium was changed to 10% FBS DMEM.

9. Real-time polymerase chain reaction (PCR)

Total RNA was isolated with the Tripure isolation reagent (Roche, Basel, Switzerland). In brief, 10 ng of purified total RNA was used for reverse transcriptase (Taqman® MicroRNA Reverse Transcriptase Kit, Applied Biosystems) in combination with the Taqman® MicroRNA Assays for the quantification of specific microRNAs and U6 control transcripts, according to the manufacturer's conditions. The amplification and detection of specific products were performed in a Light Cycler 480 II (Roche, Basel, Switzerland) at 95°C for 10 min, followed by 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds. The threshold cycle (Ct) of each target gene was automatically defined, located in the linear amplification phase of the PCR, and normalized to that of the control U6 (ΔCt value). The relative difference in expression level of each microRNAs in the cells (ΔΔCt) was calculated and presented as fold induction (2-ΔΔCt)

10. Luciferase assay

The predicted target gene of microRNA-29b was searched using a public database (TargetScan, www.targetscan.org). We synthesized the 3'-UTRs of Mcl-1 and MMP2 that contained the binding site of microRNA-29b. The

control sequences containing several mutated bases within the binding sites were also synthesized. The corresponding gene was then cloned into the pmirGLO vector. HeLa was plated at a 2.5x 10^4 cells/ml concentration in a 24-well plate. After 48 hr, the pmirGLO vector containing the Mcl-1 and MMP2 binding sites for microRNA-29b was co-transfected with microRNA-29b or the negative control using Lipofectamine 2000 (Life Technologies,Grand Island, NY, USA). The Renilla luciferase was used to normalize the cell number and the transfection efficiency. Luciferase activity was measured with the Dual Luciferase assay (Promega, Madison, WI, USA) according to the manufacturer's instructions after 48 hr on the luminometer (Promega, Madison, WI, USA). Each assay was repeated three times.

11. Statistical analysis

Data are expressed as mean ±SE. The statistical analysis of two groups was estimated by Student`s t-test. Comparison of more than two groups was completed by one-way ANOVA using the Bonferroni test. $P<0.05$ was considered significant.

III. RESULTS

1. microRNA-29b inhibits IL-3-induced SMC proliferation.

To determine an optimal IL-3 concentration for the proliferation of SMCs, 20~100 ng/ml of IL-3 was applied for 48 hr. IL-3 exhibits the maximum proliferative effect at a concentration of 80 ng/ml, while 100 ng/ml of IL-3 showed reduced proliferation in comparison (Figure 1). In SMCs treated with 80 ng/ml of IL-3, microRNA-29b was added to determine whether it could inhibit cell growth induced by IL-3 stimulation. MicroRNA-29b significantly attenuated the proliferation of SMCs under IL-3 stimulation compared to the proliferation of IL-3 alone and that of the control group. Additionally, microRNA-29b was treated to determine its own effect on SMC proliferation without any stimulus. MicroRNA-29b did not exhibit a significant reduction on the proliferation of SMCs (Figure 2).

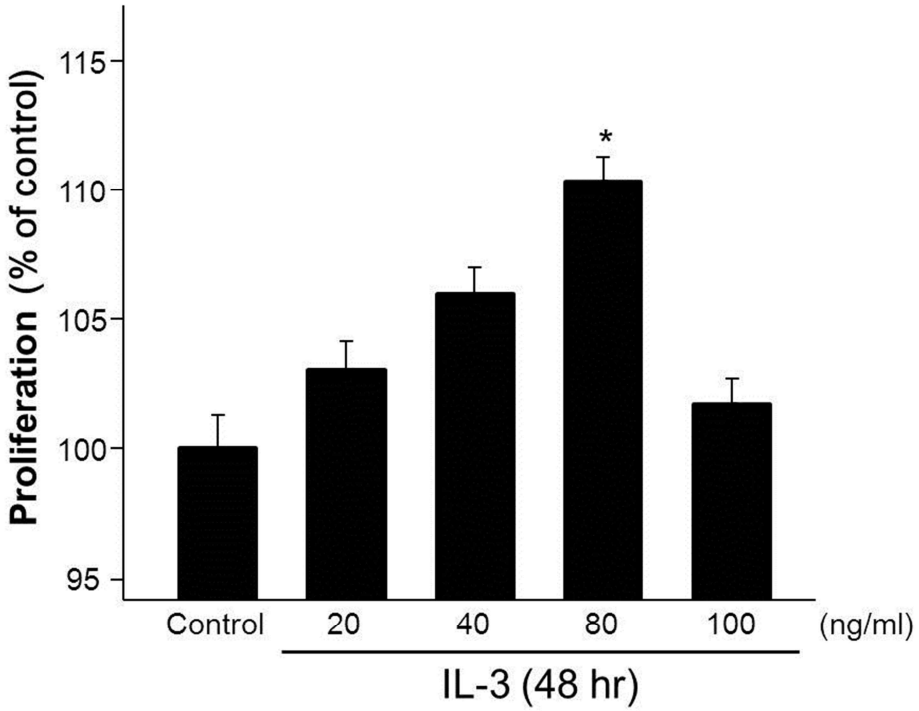

Figure 1. The effect of IL-3 on the proliferation of SMCs The effect of IL-3 on SMC proliferation was measured using an MTT assay. The SMCs were treated with 20~100 ng/ml of IL-3 for 48 hr under growth conditions at 37 ºC and 5% CO_2. IL-3: Interleukin-3, SMC: smooth muscle cell *P< 0.05 vs. control.

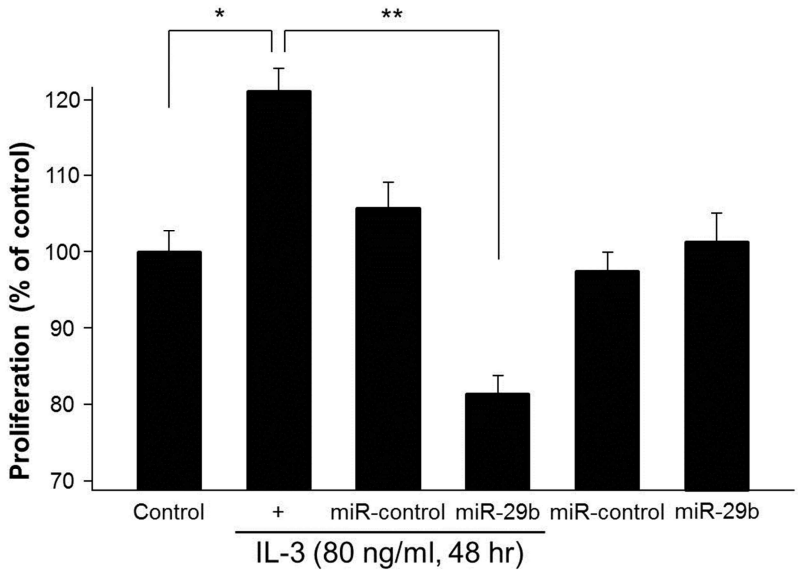

Figure 2. The effect of microRNA-29b on the proliferation of IL-3 stimulated SMCs SMCs were transfected with 100 nM of microRNA-29b for 4 hr and then treated with 80 ng/ml of IL-3 for 48 hr. *P< 0.05 vs. control. **P< 0.05 vs. IL-3.

2. microRNA-29b attenuates IL-3 stimulated Mcl-1 expression but not the MAPK and PI3K signaling pathways.

Since the Mcl-1 gene is known to be induced by IL-3 as well as other growth factors or cytokines, we tested whether microRNA-29b inhibition increased the expression of Mcl-1 induced by IL-3 in SMCs. IL-3 was treated for 6 hr in SMCs because Mcl-1 is known to have its half-life for protein expression around 4-6 hr [30]. IL-3 increased Mcl-1 expression up to 4.5-fold, and microRNA-29b attenuated Mcl-1 expression to approximately 30% under IL-3 stimulation (Figure 3). We examined whether microRNA-29b treatment can affect the activations of these kinases under IL-3 stimulation. IL-3 significantly increased the phosphorylation of both ERK1/2 and AKT as expected, but microRNA-29b did not alter the phosphorylation level of those kinases. This data demonstrates that microRNA can target Mcl-1 but not the upstream regulators of MAPK and AKT (Figure 4).

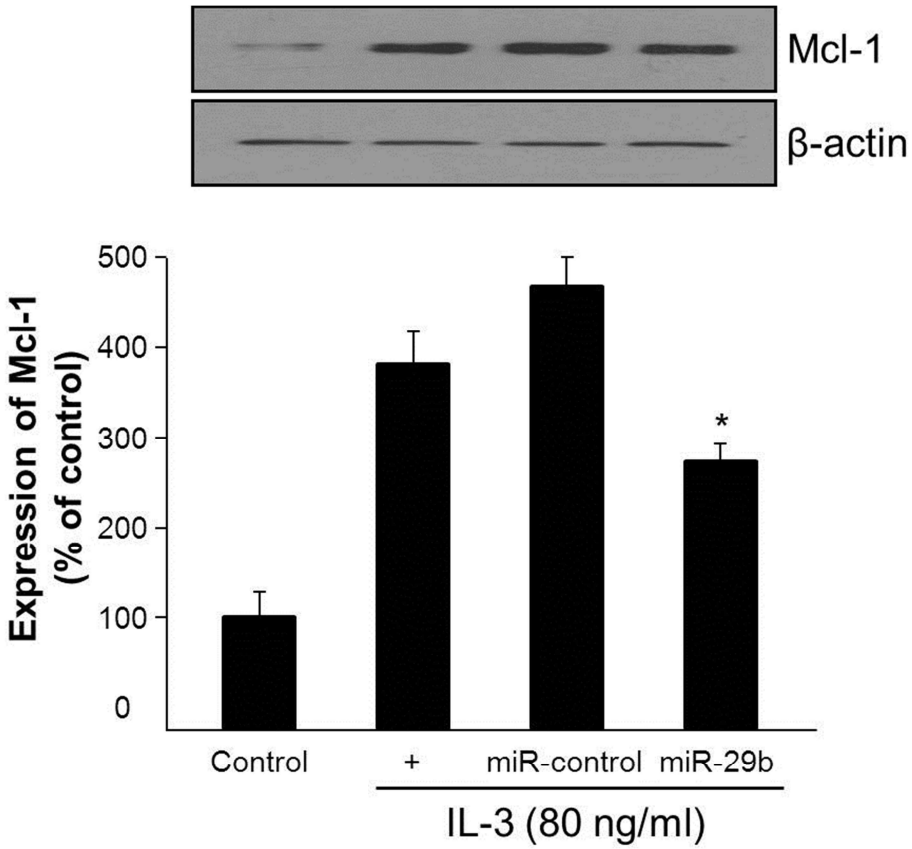

Figure 3. The change of Mcl-1 by microRNA-29b on IL-3-stimulated SMCs

SMCs were starved with 0.1% FBS DMEM and then transfected with 100 nM of microRNA-29b after 80 ng/ml of IL-3 treatment for 6 hr. Mcl-1 expression was measured by immunoblot analysis. Mcl-1: myeloid cell leukemia sequence 1. *P< 0.05 vs. IL-3.

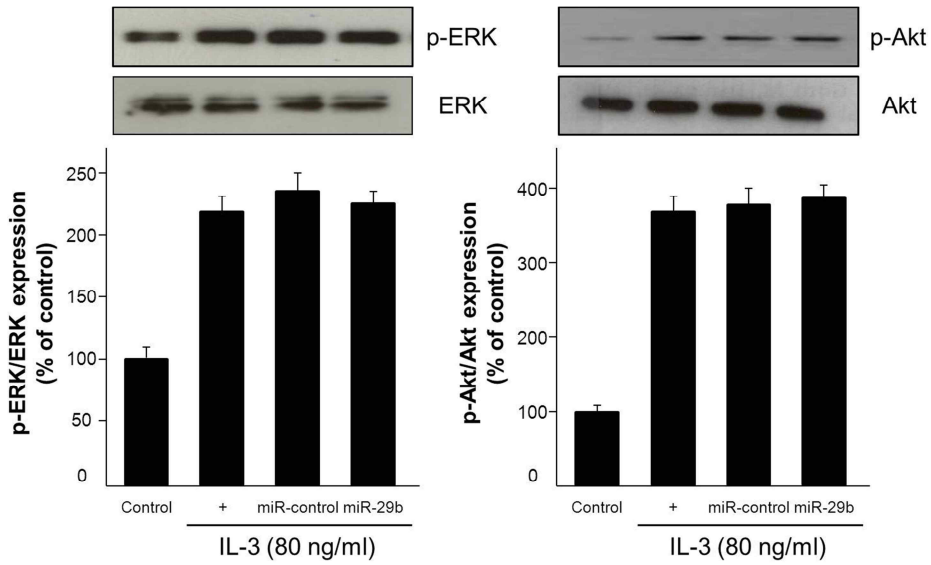

Figure 4. The effect of microRNA-29b on the expression of proliferation signals at IL-3-stimulated SMCs Altered phosphorylation levels of proliferation signals were assessed in IL-3-stimulated SMCs with or without microRNA-29b using immunoblot analysis.

3. microRNA-29b attenuates IL-3-induced SMC migration through MMP activation.

To test the inhibitory effect of microRNA-29b on IL-3-induced SMC migration, a migration assay using a Boyden chamber and wound healing assay was performed. MicroRNA-29b significantly reduced the migration of SMC in both experiments (Figures 5 and 6). First, the expression levels of two MMPs were checked. IL-3 induced both MMP2 and MMP9 expression levels in a time-dependent manner for up to 48 hr (Figure 7). To determine whether microRNA-29b can regulate MMP2 or MMP9 under IL-3 stimulation, an immunoblot was performed. MicroRNA-29b selectively inhibited the expression level of MMP2 but not that of MMP9 (Figure 8).

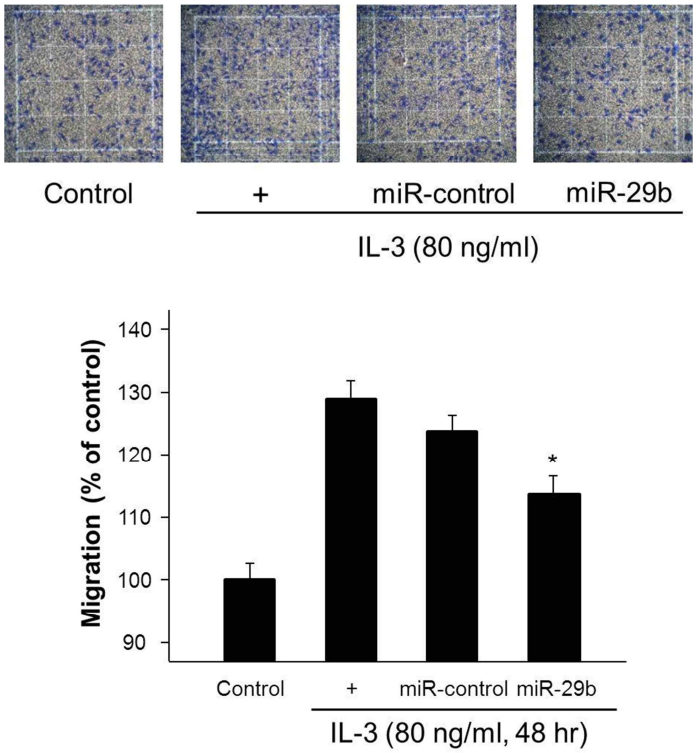

Figure 5. The effect of microRNA-29b on the migration of SMCs treated with IL-3 The effect of microRNA-29b on SMCs treated with IL-3 was measured using Boyden chambers. The SMCs were transfected with microRNA-29b before starvation with 0.1% FBS DMEM and then treated with 80 ng/ml or IL-3 for 12 hr and migrated for 9 hr under conditions of 37 ℃ and 5 % CO_2. *P< 0.05 vs. IL-3.

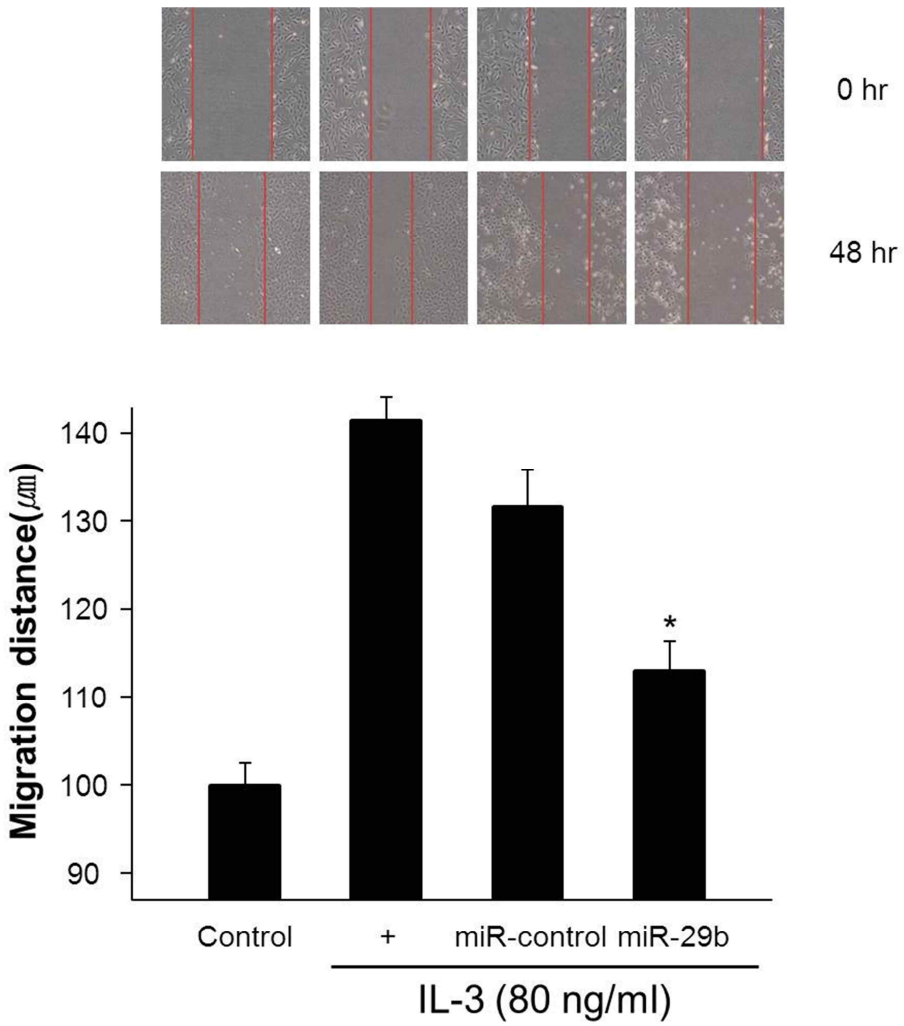

Figure 6. The effect of microRNA-29b on the migration of IL-3 stimulated SMCs microRNA-29b was transfected and then treated with 80 ng/ml of IL-3 for 48 hr. The migration rate was investigated using a wound healing assay. *P< 0.05 vs. IL-3.

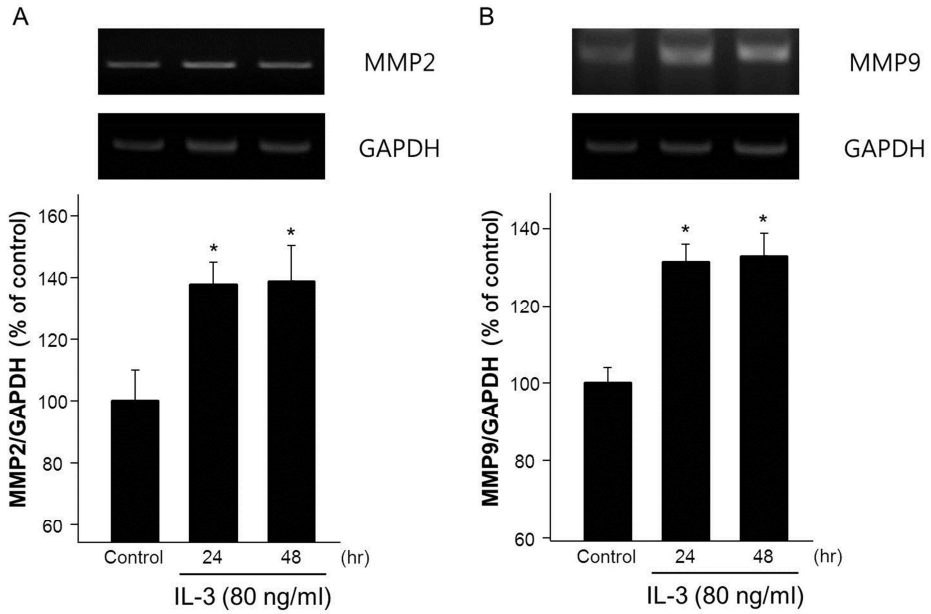

Figure 7. The expression levels of MMP2 and MMP9 SMCs were treated with 80 ng/ml of IL-3 for 24 or 48 hr. (A) RT-PCR analysis for MMP2 mRNA in SMCs. (B) RT-PCR analysis for MMP9 mRNA in SMCs. MMP2: Matrix metalloproteinase 2, MMP9: Matrix metalloproteinase 9. *P< 0.05 vs. control.

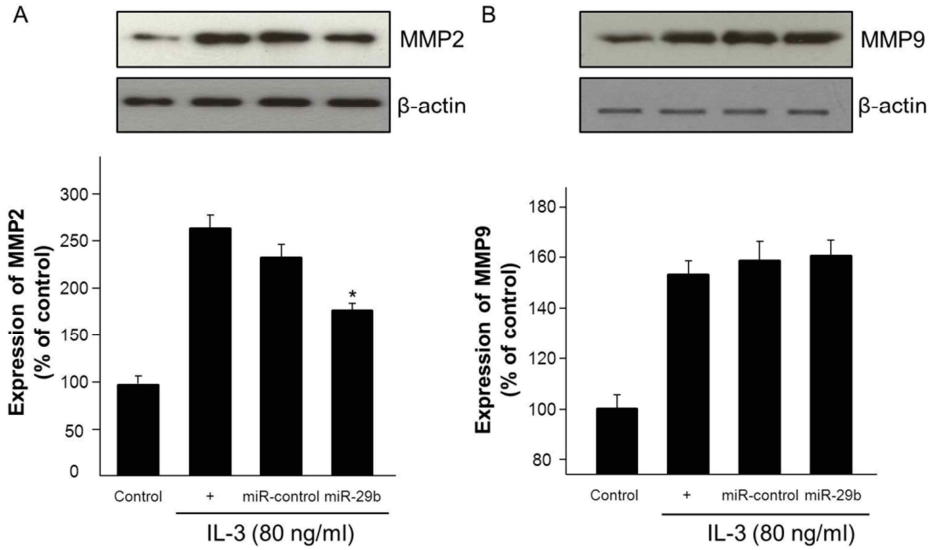

Figure 8. The effect of microRNA-29b on the expression of MMP2 and MMP9 microRNA-29b was transfected and then treated with 80 ng/ml of IL-3. (A) Immunoblot analysis for MMP2 in SMCs. (B) Immunoblot analysis for MMP9 in SMCs. *P< 0.05 vs. IL-3.

4. microRNA-29b directly targets Mcl-1 and MMP2 in SMC.

We used a Targetscan program to assess the possibility of whether microRNA-29b can target these genes in the 3' UTR, and the putative target sites were noted (Figure 9). According to the predictions based on the program, microRNA-29b significantly decreased the luciferase activities of Mcl-1 and MMP2 up to approximately 50% and 40%, respectively (Figure 10). Our results confirmed that microRNA-29b treatment inhibited proliferation and migration by blocking Mcl-1 and MMP2.

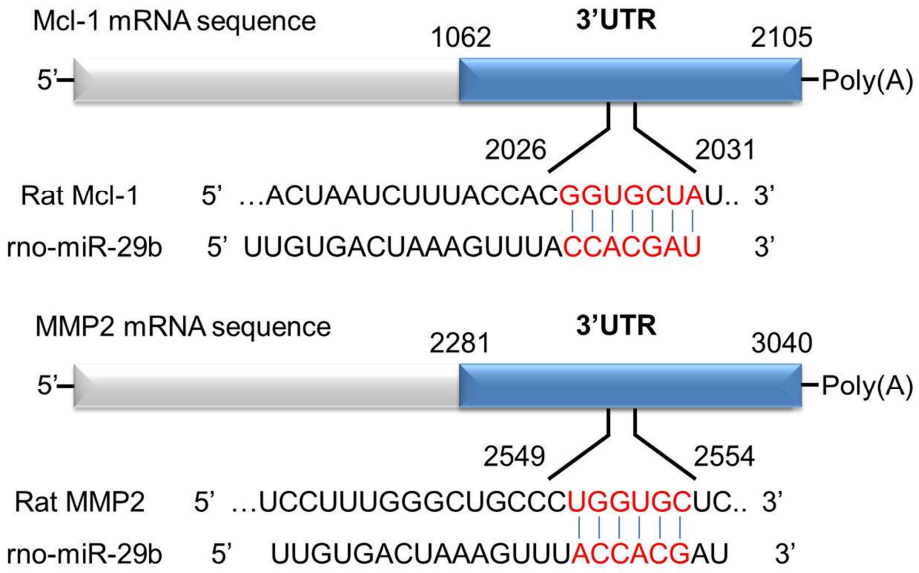

Figure9. The target sequences of microRNA-29b: Mcl-1 and MMP2 The sequences of the predicted target of microRNA-29b were checked using the Targetscan program.

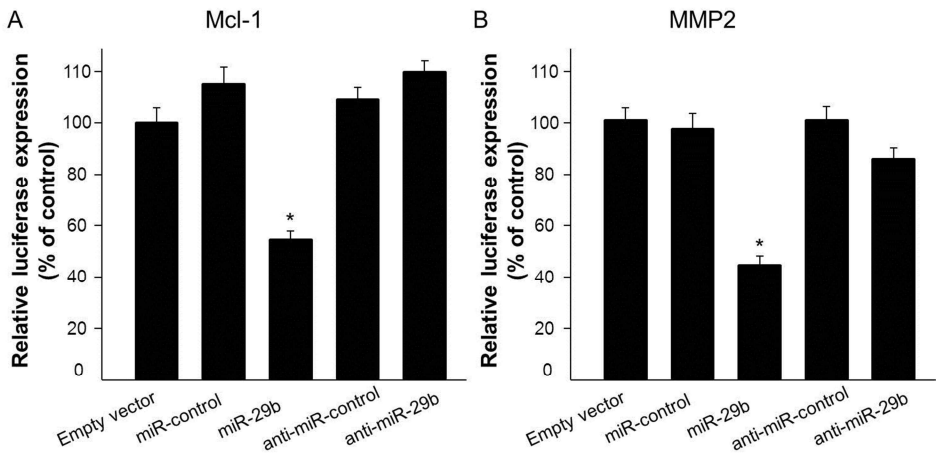

Figure 10. The targets of microRNA-29b: Mcl-1 and MMP2 3'-UTR expressions of Mcl-1 and MMP2 were detected by the luciferase assay. The control, Mcl-1, or MMP2 3'-UTR vectors were transfected with microRNA-control or microRNA-29b. Luciferase activities were measured and normalized to Renilla activity. *P< 0.05 vs. empty vector.

IV. DISCUSSION

Interleukins are cytokines that can regulate cellular function including cellular stimulation, growth, and differentiation through gene activation. Interleukin-3 (IL-3) is mainly released from activated T lymphocytes infiltrating the inflammatory tissue [28] and is known to stimulate the proliferation and migration of SMCs [6]. It is also known that IL-3 is increased in patients with coronary artery disease [5]. As a result, IL-3 may be used as a parameter for the prediction of restenosis. However, there are some other views about the functions of IL-3. Based on some studies, IL-3 also exhibits an independent pathway followed by different stimuli for inflammation [31]. Our data showed that IL-3 acts as a stimulus to investigate the mechanism of SMC proliferation and migration and successfully increased SMC proliferation and migration (Figures 1 and 5).

IL-3 signaling is mainly related to the signaling pathways of proliferation and survival of kinases such as Janus kinase (JAK), phosphotidylinositol-3 kinase (PI-3K)/Akt, and the MAPK kinase (MEK)/mitogen-activated protein kinase (MAPK) pathway [32-33]. In addition, recent studies have revealed that IL-3 contributes to the stabilization of transcripts to increase cell growth in patients with leukemia [34]. Involved in the downstream signaling of IL-3 stimulation,

Mcl-1 is examined in other tissues related to proliferation. The increased expression of Mcl-1 has been shown in several tissues, such as lymphoma and human neutrophils, to be related to vascular endothelial growth factor and cytokines [35-36]. IL-3 is also known to induce Mcl-1 expression thorough MAPK and AKT signaling for proliferation and migration [37-39]. Ru et al. showed that microRNA-29b directly down-regulated the translation of Mcl-1 protein in prostate cancer cells and cholangiocarcinoma cell lines [40]. IL-3 is also known to induce matrix metalloproteinases (MMPs), which are well known for their direct involvement in SMC migration and proliferation in experiments using MMP inhibitors and several surgical models [32-33]. In hepatocellular carcinoma, Fang J.H. et al. showed a correlation between microRNA-29b and MMP2 by demonstrating that microRNA-29b significantly suppressed angiogenesis, invasion, and metastasis in cancer by regulating MMP2 expression [41]. Maegdefessel L. et al. also showed that microRNA-29b can down-regulate MMP2 activity and expression related to fibrosis in human aortic smooth muscle cells through TGF-β1 stimulation [42]. Several papers show that the microRNA-29 family can inhibit proteins that are related to migration and proliferation such as collagen, MMPs, and Mcl-1 in cancer cells [8, 43]. In addition to these molecular signaling pathways, IL-3 is also known to play an important

role in collagen-induced arthritis. Interestingly, IL-3 was increased during the onset of arthritis, whereas IL-3 was down-regulated in late phage inflammation. Therefore, the authors stated that arthritis exhibited a significant improvement only when IL-3 was blocked in early phase inflammation [44]. Even though the condition of this study is not exactly matched to our experimental condition, it suggests appropriate treatment timing for patients with coronary artery disease. Here, we investigated the signaling pathway stimulated by IL-3 to determine whether microRNA-29b can regulate proliferation and migration through selectively inhibiting its own targets and demonstrated that microRNA-29b specifically decreased Mcl-1 and MMP2 (Figures 3 and 8).

Although it has been discovered that many microRNAs such as microRNA-26, -143, or -145 contribute to smooth muscle cell proliferation and migration in restenosis or atherosclerosis [45-46], the roles and mechanisms of many microRNAs in cardiovascular diseases remain unknown. In addition, there have been no direct reports indicating a relationship between microRNA-29b and smooth muscle cells with regard to proliferation and migration. Since several cancer studies showed that microRNA-29b can regulate migration, proliferation, and apoptosis, we chose and used microRNA-29b to reduce the proliferation and migration processes of SMCs. MicroRNA-29b indeed significantly reduced

proliferation and migration in IL-3 stimulation compared to Mcl-1 and MMP2 reductions.

In summary, our findings demonstrated that microRNA-29b successfully suppressed the proliferation and migration of SMCs induced by IL-3 treatment by targeting Mcl-1 and MMP2.

V. CONCLUSION

These results suggest that microRNA-29b could be useful alone or in combination with conventional therapies to treat cardiovascular diseases such as atherosclerosis and restenosis.

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ABSTRACT (IN KOREAN)

Interleukin-3에 의해 자극된 혈관 평활근 세포의 이동과 증식에서 microRNA-29b의 역할

<지도교수 황 기 철>

연세대학교 대학원 의과학과

이 지 윤

혈관의 손상과정에서 발생하는 Interleukin-3(IL-3)의 자극으로 인하여 Erk1/Erk2 MAPkinase pathway 와 PI-3K/Akt pathway 를 통해 혈관 평활근 세포의 Mcl-1 과 MMP2 의 발현이 증가하게 된다. 이는 혈관 평활근 세포의 이동 및 증식 능력을 증가시키는데 주요한 신호전달 체계로 알려져 있다. 본 실험에서는 IL-3 을 처리하여 혈관 평활근 세포의 이동과 증식을 강화하여, IL-3 가 Mcl-1 과 MMP2 를 자극함을 확인하고, 같은 target 을 갖는 microRNA-29b 를 Targetscan computer prediction system 을 이용해 선정하고, 이를 cell 내로 과발현하여 혈관 평활근 세포의 이동과 증식에 미치는 영향을 확인하고자 하였다. 또한 이에 관련한 Erk1/Erk2 MAPkinase pathway 와 PI-3k/Akt pathway 신호 관련

기전도 함께 분석하고자 하였다. IL-3 의 처리를 통한 혈관 평활근 세포의 이동과 증식은 다양한 시약과 migration assay 를 통하여 증가를 확인하였고 microRNA-29b 에 의한 유의한 감소효과 역시 같은 방법을 통하여 확인하였다. 중요한 신호전달 분자로 예측되는 Mcl-1 와 MMP2 의 활성 혹은 발현 정도가 IL-3 에 의해서 증가된 것을 확인하였으며 microRNA-29b 처리에 의한 감소를 RT-PCR 및 Immunoblot 등의 실험 방법을 통하여 확인하였으며 Luciferase assay system 을 이용하여 microRNA-29b 가 특이적으로 Mcl-1 와 MMP2 을 저해함을 밝혔다. 이상의 결과에서 microRNA-29b 가 atherosclerosis 나 restenosis 와 같은 심혈관 질환에 대한 치료에 있어서 단독으로 혹은 기존 치료법과의 복합처치 등의 관점에서 유용한 관점을 제시할 수 있을 것이라 기대된다.

핵심 되는 말: 혈관평활근 세포, 세포 증식, 세포 이동, microRNA-29b