MicroRNA-128 prevents apoptosis of mesenchymal stem cells by targeting Apaf-1

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Department of Medical Science

The Graduate School, Yonsei University

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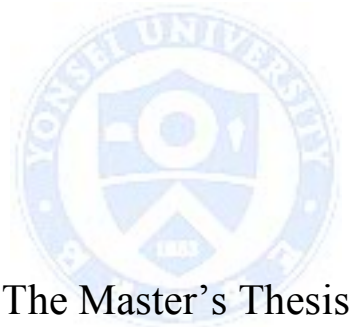
Directed by Professor Jong-Chul Park



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Master of Medical Science

Minji Seung

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This certifies that the Master's Thesis of Minji Seung is approved.

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June, 2015

Minji Seung

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ABSTRACT

MicroRNA-128 prevents apoptosis of mesenchymal stem cells by targeting Apaf-1

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Mesenchymal stem cells (MSCs) are highly specialized cells that can be derived from bone marrow. The unique qualities of differential and regenerative characteristics they possess make them a great tool for heart regeneration after injury. However, numerous barricades are lain ahead to pass preceding the pervasive application of their clinical use. The steep decrease in their viability upon encountering the hostile environment, the abatement of cell proliferation, and the augmentation of cell apoptosis occur and jeopardize the attachment and survival of MSCs after transplantation. Before MSCs can flaunt their regenerative qualities, the guarantee of their survival needs to be ensured. The apoptotic protease activating

factor 1 (Apaf-1), a monomer residing in the cytosol in its inactive form, is a molecule that is activated for the assembly of the apoptosome. The apoptosome is known to recruit and cleave the initiator caspase-9 to begin the pro-apoptotic caspase cascade; thus the abatement of the apoptosome formation was hypothesized to be pivotal. To target Apaf-1, we used microRNAs (miRNAs), post-transcriptional regulators that bind to complementary sequences in the 3'UTR of mRNAs typically resulting in inhibition of mRNA translation or the degradation of mRNAs. *In vitro* results using bone marrow derived human MSCs (hMSCs) showed that when hypoxia-stimulated intrinsic apoptosis occured, expression levels of apoptotic signals amplified despite no change in Apaf-1. Contrastingly, once hMSCs were transfected with specific microRNA-128 (miR-128), expression levels of Apaf-1 and apoptosis declined while survival percentages increased indicating that miR-128 can regulate Apaf-1 to enhance survival. In accordance to the results, decreased detection of Annexin V/PI-labeled cells showed that miR-128 transfected hMSCs had lower apoptotic characters signifying higher survival efficacy. In conclusion, downregulation of Apaf-1 via miR-128 regulation established a significant decrease in apoptosis hence promoted the increase of MSC survival after transplantation.

Key words: mesenchymal stem cells, apoptosis, Apaf-1, miRNA

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I. INTRODUCTION

Despite advancement of medical technologies and increasing medical knowledge, it is a well-established fact that heart diseases are still one of the significant causes of mortality worldwide. [1] Amongst them, ischemic heart disease is the most typical type of heart disease and the primary cause of heart attacks. [2] When ischemia occurs in the heart, it confines blood circulation to tissues following the restricted pathway, causing impediment of oxygen and nutrients the heart needs in

order to function while also creating a favorable environment for accumulation of toxins. [3] Progressively, the insufficient blood delivery resulting in the limited oxygen and nutrient supply and excess toxins, advance to damage or dysfunction of the surrounding muscle and tissue. [3]

In conjunction with everyday expansion of de novo treatments and pharmaceutical developments, bone marrow-derived mesenchymal stem cells (MSCs) have freshly emerged as an advantageous therapeutic method for heart diseases. Because of their secretome secreting abilities [4] and multipotency to differentiate into various cell types [5] such as chondrocytes, neurons, and also cardiomyocytes, [6] MSCs have promising features as aiding devices to regenerative medicine. However, limitations hinder their use in heart therapy as a consequence of the hypoxic environmental conditions created by the lack of blood flow to the injury region. Poor viability and inefficient engraftment rates after transplantation are the biggest constraints for applying the stem cells' regenerative capability for sufficient clinical treatment. [7]

As mentioned above, a major destructive disclosure to regenerative therapy involving stem cells is its survival. Apoptosis, a type of programmed cell death, is brought on by the drastic change in environment. Several apoptotic signaling pathways cooperatively induce cell apoptosis, leaving only a small percentage of the

transplanted MSC to survive. There are two different types of signaling pathways that drive cells to apoptosis: the extrinsic apoptotic pathway and the mitochondrial intrinsic apoptotic pathway. [8] While the extrinsic apoptotic pathway, also known as the death receptor pathway, involves the binding of death ligands to its equivalent cell surface receptors, [8] the mitochondrial intrinsic apoptotic pathway involves activation of pro-apoptotic proteins such as Bax, cytochrome c and caspases. [8] Apoptotic protease activating factor 1 (Apaf-1) is a protein involved in the mitochondrial intrinsic apoptotic signaling pathway that exists in a monomeric form in the cytoplasm in normal conditions. [9] When the mitochondria is stimulated by apoptosis initiators such as hypoxia and reactive oxygen species (ROS), its membrane potential decreases causing the pores to expand, ultimately releasing cytochrome c. [10,11] Originally confined to the mitochondrial intermembrane space, once released into the cytoplasm, cytochrome c binds to the monomeric Apaf-1 to form the oligometric apoptosome that recruits the initiator caspase, pro-caspase-9. [12-14] The pro-caspase-9 is then activated, initiating the caspase cascade, resulting in cell apoptosis via effector caspase-3 cleavage. [13] We hypothesize that silencing of the monomeric form of Apaf-1 will result in a domino effect to lower apoptosis. Decrease in Apaf-1 will decrease the binding of cytochrome-c despite its release from the mitochondria. This will in turn constrict the formation of the apoptosome, reducing the onset of the caspase cascade, and postponing apoptosis overall giving MSCs a chance to optimize their therapeutic potentials.

Recently, microRNAs (miRNA), single stranded noncoding RNA molecules of 19-23 nucleotides in size, emerged as tools to mechanically fine-tune a variety of cellular phenotypes via inhibition of translation or degradation of target messenger RNAs (mRNAs). [15] It accomplishes its function by base-pairing with complementary sequences within the 3'-untranslated region (3'UTR) of specific target mRNAs. Because some miRNAs are known to specifically target cellular processes such as apoptosis, [16-19] proliferation, [20,21] differentiation, [22-24] and other pivotal regulations, it has become a prevalent molecule in relation to heart diseases and stem cell therapy. [19,25,26] In this study, miRNAs will be used to regulate the expression level of Apaf-1 in hMSCs to decrease apoptosis and in due course, promote survival.

II. MATERIALS AND METHODS

1. Materials

Low glucose Dulbecco's modified eagle's medium (DMEM), fetal bovine serum, phosphate-buffered saline, and penicillin-streptomycin were obtained from Gibco by Life Technologies (Seoul, Korea). Apaf-1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Caspase-9 antibody was purchased from Cell Signaling (Cell Signaling Technology, Danvers, MA, USA). Horseradish peroxidase HRP-conjugated secondary antibodies of mice was obtained from Enzo Life Sciences (Farmingdale, NY, USA) while goat was obtained from Thermo Scientific (Rockford, IL, USA).

2. Cell culture

hMSCs were cultured in DMEM supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin in 100mm culture plates in 37 °C humidified incubators with 5% CO_2.

3. Cell counting assay

hMSCs seeded accordingly, were re-suspended in DMEM containing 10% FBS and transferred into new 1.5ml tubes containing 0.4% trypan blue staining solution. hMSCs were allowed to stand for 5-15 minutes before 10 uL of trypan blue

stained cell suspension was transferred to both chambers of a hemocytometer. The live cells were unstained and appeared bright while the dead cells were stained blue. The live and dead cells were counted separately and cell viability was calculated.

4. Hypoxic stimulation

To hMSCs seeded accordingly, DMEM was changed to de-gassed serum free DMEM before stimulus was given. The airtight humidified hypoxic chamber (ThermoForma) was maintained at 37 °C and continuously gassed with 1% O_2, 5% CO_2, and 94% N_2.

5. MicroRNA transfection

MiRNA was purchased from Genolution Pharmaceuticals (Genolution Inc., Korea). Transfections of miRNAs were performed using TransIT-X2 Dynamic Delivery System reagent (Mirus). Briefly, hMSCs were seeded at the density of 7×10^5 cells per 60mm culture plate. TransIT-X2 Dynamic Delivery System reagent was diluted with Opti-MEM and combined accordingly with indicated miRNA mimic for each plate and incubated in room temperature for 15-30 minutes to allow sufficient formation of complexes. It was then added to each cell plate containing fresh 10% FBS-DMEM without antibiotics. After 24h incubation in a 5% CO_2 incubator at 37 ℃, appropriate experimentation was carried out.

6. Western blot analysis

hMSCs were washed once in PBS and lysed with lysis buffer (cell signaling, Beverly, MA, USA) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 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 PMSF. Protein concentrations were determined using the BCA protein assay kit (Thermo, Rockford, IL, USA). Proteins were separated in 8%-12% SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore Co, Bedford, MA, USA). After transfer of the proteins onto the membrane, the membrane was blocked with Tris-buffered saline-Tween 20 (TBS-T, 0.1% Tween 20) containing 10% non-fat dried milk overnight in 4 ℃ or 1h in room temperature. The membrane was then washed two times with TBS-T and incubated with primary antibodies overnight at 4 ℃. The membrane was washed three times with TBS-T for 5 min each and incubated for 1 h at room temperature with HRP-conjugated secondary antibodies. After extensive washing, the bands were detected with enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The band intensities were quantified using NIH Image J version 1.44p software.

7. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using 500 μL of RNAiso Plus Total RNA Extraction Reagent (Takara) per 60 mm culture plate. In brief, complementary DNA

(cDNA) was synthesized using the Reverse Transcription System (Promega, Madison, WI, USA). The single stranded cDNA was synthesized from the isolated total RNA via Avian Myeloblastosis virus (AMV) reverse transcriptase. A 20 μl reverse transcription reaction mixture containing 1 μg of total RNA, 1X reverse transcription buffer (10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% TritonX-100), 1 mM deoxynucleoside triphosphates (dNTPs) 0.5 U of RNase inhibitor, 0.5 μg of oligo(dT), and 15 U of AMV reverse transcriptase was incubated at 42 ℃ for 15 min, heated to 99 ℃ for 5 min, and then incubated at 0–5 ℃ for 5 min. PCR was performed for 30 cycles with 3′ and 5′ primers based on the sequences of Apaf-1 gene. GAPDH was used as the internal standard. The signal intensity of the amplification product was normalized to its respective GAPDH signal intensity.

8. Real-time polymerase chain reaction (real-time PCR)

Total RNA was isolated using RNAiso Plus Total RNA Extraction Reagent (Takara). In brief, 10 ng of purified total RNA was used for reverse transcription (Taqman® MicroRNA Reverse Transcriptase Kit, Applied Biosystems). The reverse transcribed products were then used for Taqman® MicroRNA Assays for quantification of specific miRNAs and U6 control transcripts, according to the manufacturer's conditions. Amplification and detection of specific products were performed in a Step One Plus Real Time PCR System (Applied Biosystems) at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec, and 60 °C for 60 sec. The

threshold cycle (Ct) of each target sample, located in the linear amplification phase of the PCR, was automatically defined and normalized to the corresponding U6 value (ΔCt value). The relative difference in expression levels of each miRNA in apoptotic hMSCs (ΔΔCt) was calculated and presented as fold inductions ($2^{-\Delta\Delta Ct}$).

9. Immunoprecipitation analysis

hMSCs were washed once with PBS and lysed in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, and 1 mM Na_2EDTA. Protein concentrations were determined using the BCA protein assay kit (Thermo, Rockford, IL, USA). Quantified proteins were then bound to Dynabeads Protein G (Novex by Life Technologies) pre-bound with Apaf-1 antibody in antibody binding buffer, PBS with 0.02% Tween 20, for 2 h in room temperature with rotation on rotators. Proteins were eluted and denatured using 2X elution buffer containing, 0.2M Tris, pH 6.8, 2% SDS, 0.04% Coomassie Blue G250, 40% Glycerol, and β-Mercaptoethanol, then separated on 8% SDS-polyacrylamide gel and transferred to the PVDF membrane (Millipore Co, Bedford, MA, USA). After, the membrane was blocked with Tris-buffered saline-Tween 20 (TBS-T, 0.1% Tween 20) containing 10% non-fat dried milk overnight at 4 ℃. The membrane was then washed two times with TBS-T and incubated with primary antibodies overnight at 4 ℃. The membrane was then washed five times with TBS-T for 5 min each and incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary

antibodies. After extensive washing, the bands were detected with enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The band intensities were quantified using NIH Image J version 1.44p software.

10. Cell cytotoxicity assay

Membrane integrity was measured using CellTox Green Cytotoxicity Assay kit (Promega Corporation, Madison, WI, USA). In brief, hMSCs were plated at 5×10^3 cells per well. Once attached, 100 μl of 2X reagent containing Assay Buffer with 2X CellTox Green Dye was distributed to each well and incubated at room temperature for 15 minutes, shielded from ambient light. Fluorescence was measured at $488nm_{Ex}/525nm_{Em}$ with Varioskan Flash Multimode Reader (Thermo Scientific, USA). Each assay was repeated 3 times.

11. Annexin V/PI apoptosis analysis

Apoptosis was measured using an FITC Annexin V Apoptosis Detection Kit I (BD Biosciences). Cells were pelleted and analyzed in the BD Accuri C6 Flow Cytometer system. The excitation frequency was 488 nm. The green fluorescents emitted by Annexin V (FL1) and the red fluorescents emitted by PI (FL3) were measured using 525 nm and 575 nm band pass filters, respectively. A total of at least 2×10^4 cells were analyzed in each sample. The levels of viable cells, early

apoptotic cells and non-viable cells were measured as percentages of Annexin V-/PI-, V+/PI- and Annexin V+/PI+ cells, respectively.

12. Statistical analysis

All quantified data are of means of triplicate samples and the error bars represent the standard deviation (STDEV) of the mean. Statistical significance was determined by Student's t-test. Assessments considering more than two groups were performed by one-way ANOVA using Bonferroni's correction. Statistics with $P<0.05$ were considered significant.

III. RESULTS

1. Change in hMSC viability and expression levels of Apaf-1 in correspondence to hypoxic stimulation

To determine the optimal time for relevant hypoxic stimulation, hMSC viability was determined under time dependent hypoxic conditions. Compared to the 0 h control sample where no hypoxic stimulation was given, there was a significant decrease in % cell viability during 3 h, 6 h, 9 h, 12 h, and 24 h of hypoxic stimulation (Figure 1). Apaf-1 protein expression levels in hMSCs were also determined under time dependent hypoxic stimulation. No significant alterations to Apaf-1 protein expression levels were observed (Figure 2).

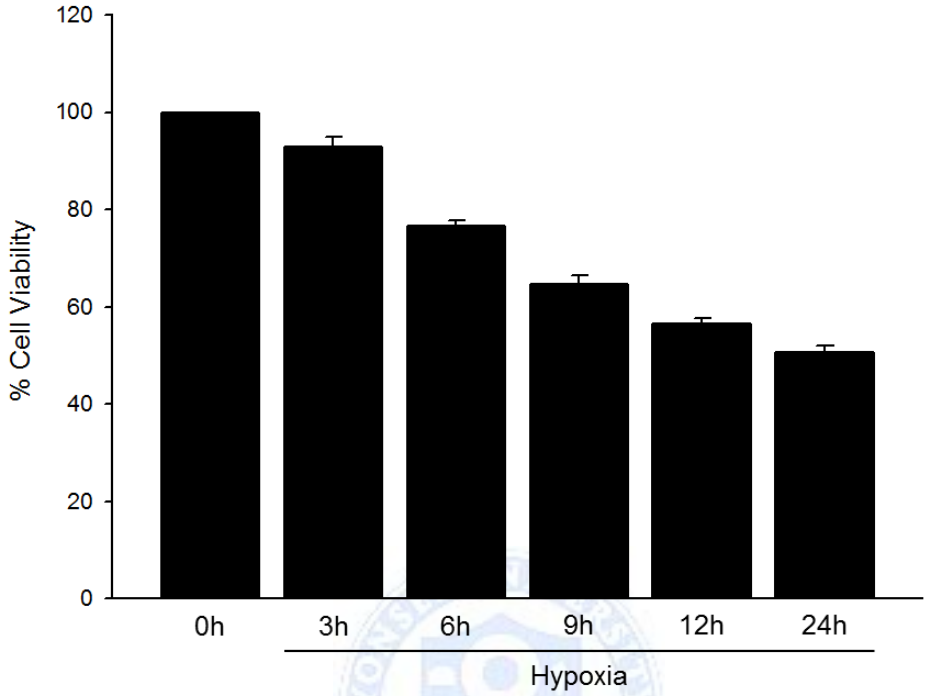


Figure 1. The effect of hypoxic stimulation on hMSC viability. The effects of hypoxic stimulation on hMSCs were determined by cell counting. hMSCs were stimulated with no hypoxic stimulation under growth conditions of 37 °C in 5% CO_2 incubator or 3 h ~ 24 h hypoxic stimulation under growth conditions of 37 °C, continuously gassed with <1% O_2, 5% CO_2, and 94% N_2. Data are normalized by 0h control. Data are presented as the mean value ± STDEV of three separate experiments. hMSCs: human mesenchymal stem cells.

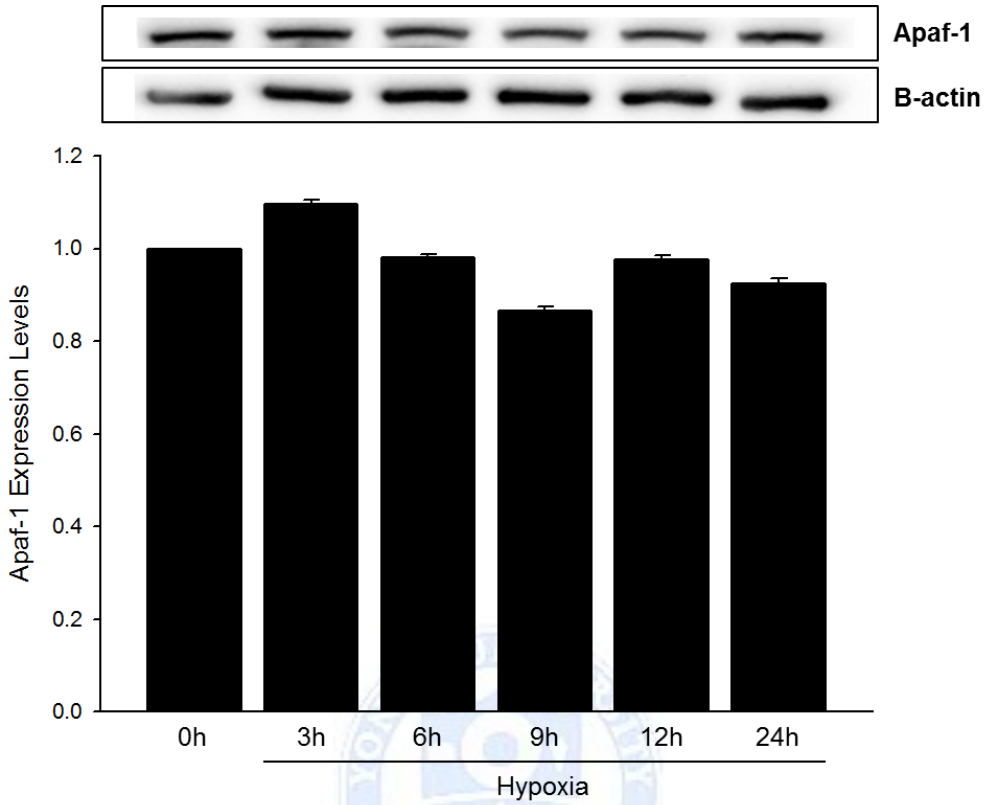
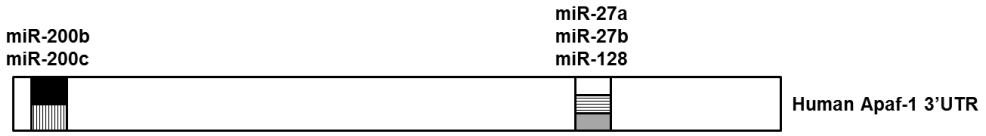


Figure 2. Alteration of hMSC Apaf-1 expression levels in response to hypoxic stimulation. The effects of hypoxic stimulation on Apaf-1 protein expression levels were determined by western blot analysis. hMSCs were stimulated with no hypoxic under growth conditions of 37 °C in 5% CO_2 incubator stimulation or 3 h ~ 24 h hypoxic stimulation under growth conditions of 37 °C, continuously gassed with <1% O_2, 5% CO_2, and 94% N_2. Data is normalized by β-actin. Data are presented as the mean value ± STDEV of three separate experiments.

2. Identification of specific miRNA targeting Apaf-1

Considering that there were no significant changes in the Apaf-1 protein levels when given hypoxic stimulation, hMSCs were transfected with candidate miRNAs in normal conditions with no hypoxic stimulation in order to determine their regulatory effects on Apaf-1. The candidate miRNAs were predicted and selected by their aggregate P_{CT} scores assessed through Targetscan online program (www.targescan.org) (Figure 3). hMSCs transfected with miR-128 showed significant decrease in Apaf-1 protein expression levels compared to the control while, on the contrary, miR-27a, miR-27b, and miR-200c showed an increase (Figure 4). In correlation to the decreased Apaf-1 protein expression levels, improvement of cell viability was observed after 12h of hypoxic stimulation for hMSCs transfected with miR-128 (Figure 5). mRNA level detection of Apaf-1 showed most significant regulatory effects on hMSCs transfected with miR-128 (Figure 6).



Position	miRNA	Sequence (5′ →3′)	Aggregate P_{CT}
Position 190-196 of Apaf-1 3′UTR	miR-200b	UAAUACUGCCUGGUAAUGAUGA	0.72
Position 190-196 of Apaf-1 3′UTR	miR-200c	UAAUACUGCCGGGUAAUGAUGGA	0.72
Position 2236-2242 of Apaf-1 3′UTR	miR-27a	UUCACAGUGGCUAAGUUCCGC	0.70
Position 2236-2242 of Apaf-1 3′UTR	miR-27b	UUCACAGUGGCUAAGUUCUGC	0.70
Position 2236-2242 of Apaf-1 3′UTR	miR-128	UCACAGUGAACCGGUCUCUUU	0.70

Figure 3. Potential miRNAs targeting Apaf-1. Candidate miRNAs were found using the Targetscan program. 5 miRNAs were selected using their aggregate P_{CT}. The location of the 5 miRNAs on the human Apaf-1 3'UTR and their sequences are listed.

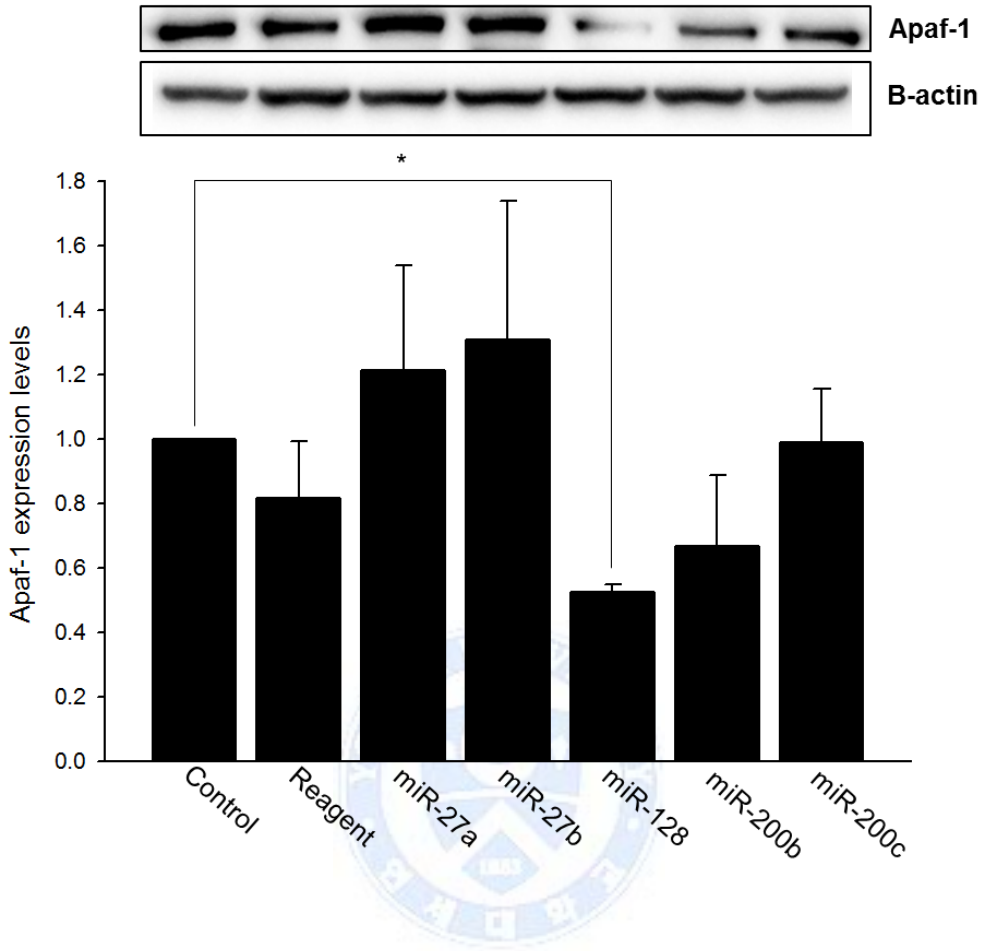


Figure 4. Regulatory effects of candidate miRNAs on Apaf-1 expression levels. Effects of miRNA transfection on Apaf-1 protein expression level were determined by western blot analysis. Data was normalized by β-actin. Data are presented as the mean value ± STDEV of three separate experiments (*P < 0.05).

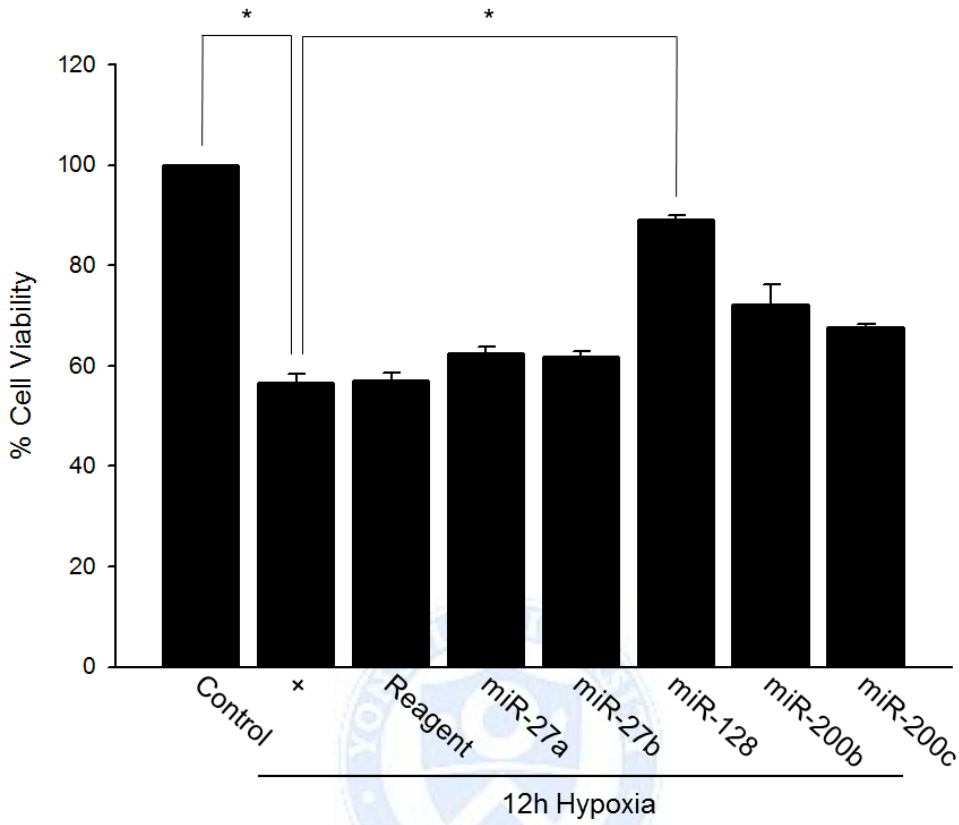


Figure 5. Selective enhancement of hMSC viability after hypoxic stimulation following candidate miRNA transfection. Effects of hypoxic stimulation on candidate miRNA transfected hMSC survival were determined by cell viability test. Data was normalized by control. Data are presented as the mean value ± STDEV of three separate experiments (*P < 0.05).

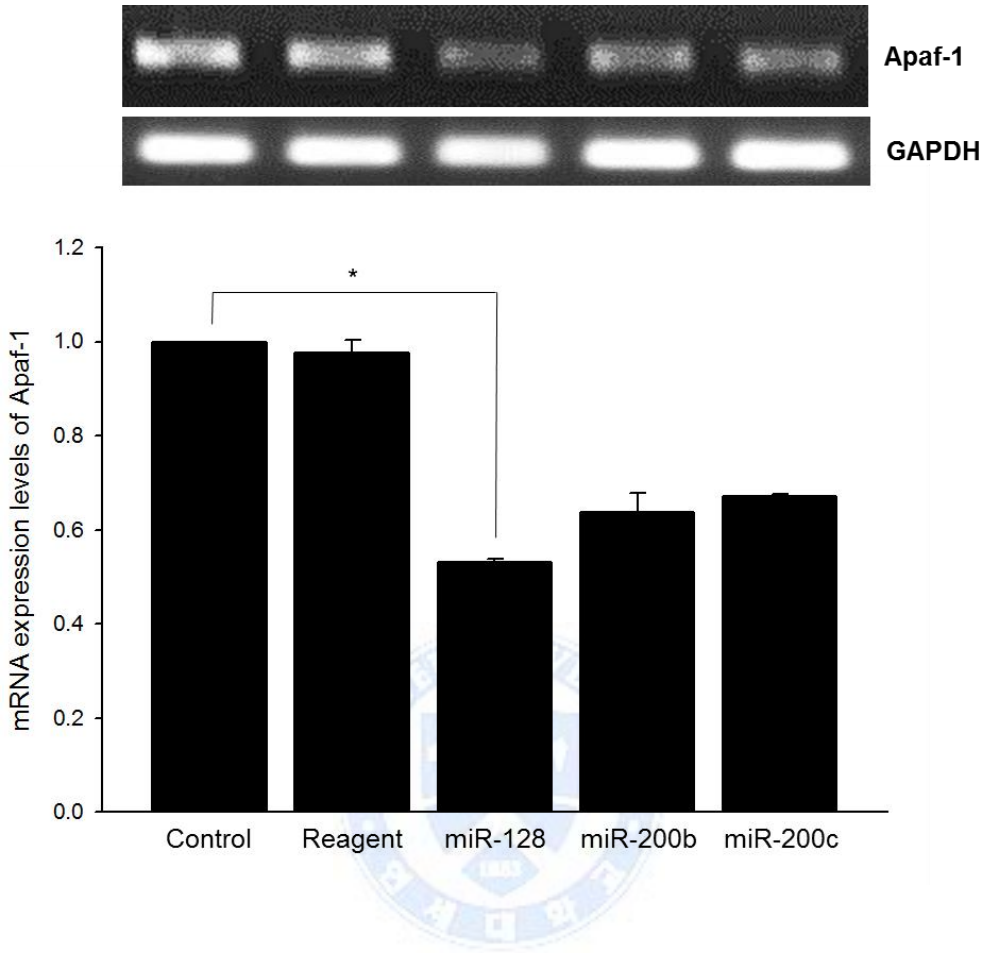


Figure 6. Possible Apaf-1 mRNA degradation of hMSCs transfected with selective candidate miRNAs. Effects of miRNA transfection on Apaf-1 mRNA expression levels were determined by RT-PCR. Data was normalized by GAPDH. Data are presented as the mean value ± STDEV of three separate experiments (*P < 0.05).

3. microRNA-128 directly targets Apaf-1 in hMSCs

With intention to link the Apaf-1 protein levels and its regulation via miR-128, the unchanging protein levels of Apaf-1 in hypoxic conditions was compared to the miR-128 levels. Real-time PCR was performed to determine the relative expression levels of miR-128 in a time dependent matter. As predicted, the relative expression levels of miR-128 did not change in correspondence to the protein expression levels of Apaf-1. The changes only conferred an auto-regulatory fluctuation (Figure 7).

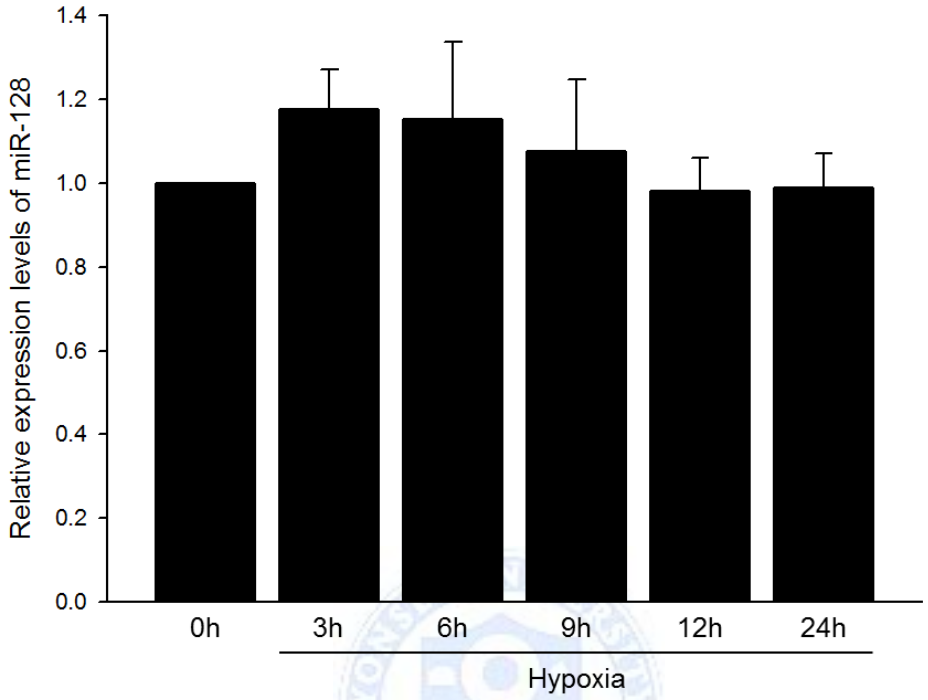


Figure 7. The effect of hypoxic stimulation on relative expression levels of miR-128. Effect of hypoxic stimulation on relative expression levels of miR-128 was determined by real-time PCR. Samples were prepared under time dependent hypoxic stimulation. Data are presented as the mean value ± STDEV of three separate experiments.

4. Particular microRNA-128 attenuation of apoptosome formation through Apaf-1 regulation

Along with confirmation of direct regulation of Apaf-1 via miR-128, apoptosome formation control was also established. hMSCs transfected with miR-128 were given hypoxic stimulation for 12h. Caspase-9 was detected after being immunoprecipitated by Apaf-1 via Dynabead extraction. In the immunoprecipitated samples, distinct amount of caspase-9 bound to Apaf-1 decreased in miR-128 transfected hMSCs stimulated with 12h of hypoxia (Figure 8). The whole cell lysate western blot samples and immunoprecipitated samples correlated in that the active form of caspase-9, more distinct in the immunoprecipitated sample, decreased in hMSCs transfected with miR-128. The decreased overall caspase-9 suggests that there were less protein-protein bound complexes formed between Apaf-1 and caspase-9 signifying that the Apaf-1 / cytochrome c conformed apoptosome formation was interrupted by the decrease in Apaf-1 via miR-128 regulation, thus lessening the recruitment of pro-caspase-9 (Figure 8).

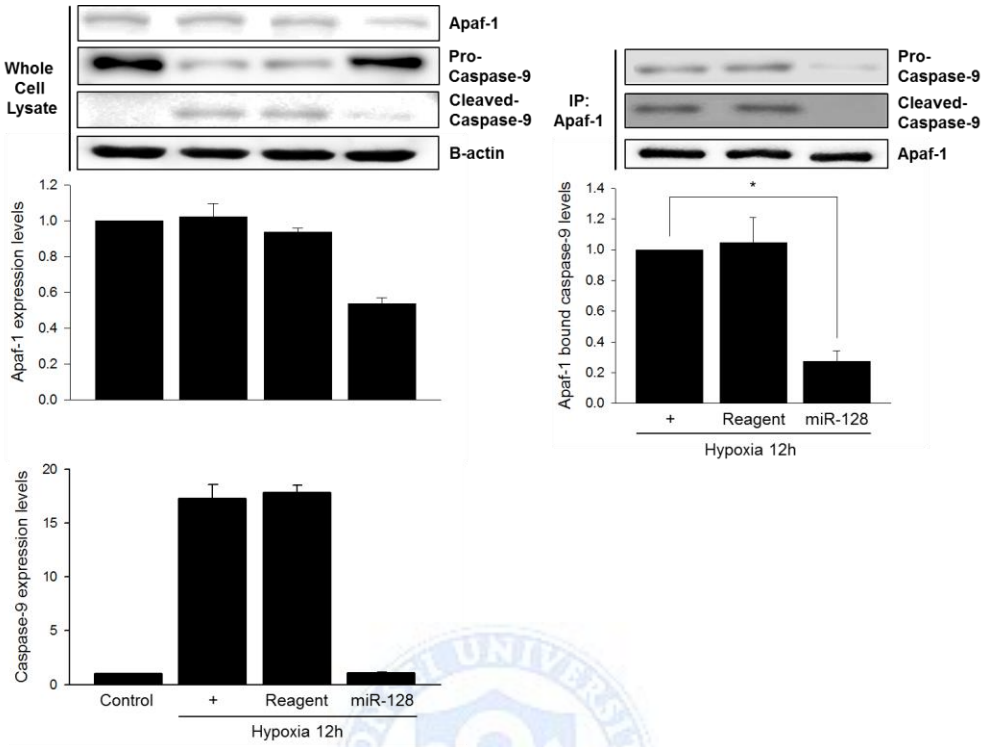


Figure 8. Specific inhibition of apoptosome formation after hypoxic stimulation via miR-128 transfection. Immunoprecipitation results showing decreased levels of caspase-9 interacting with Apaf-1. Samples were immunoprecipitated with Apaf-1 specific antibodies and blotted with caspase-9 antibody using Dynabeads. Immunoprecipitation data was normalized by Apaf-1 while whole cell lysate data was normalized by β-actin. Data are presented as the mean value ± STDEV of three separate experiments (*$P < 0.05$).

5. miRNA-128 suppresses apoptosis in hypoxia stimulated hMSCs

In consideration of apoptosis regulation via suppressing Apaf-1 with miR-128, cell cytotoxicity was validated. The dramatically increased change in membrane integrity was observed when given hypoxic stimulation but only small changes to the membrane integrity was observed in hMSCs transfected with miR-128 (Figure 9). In equivalence to the decreased change in membrane integrity, reduction of cells showing late apoptotic characters confirmed that targeting Apaf-1 with miR-128 transfection ultimately reduced overall apoptosis (Figure 10).

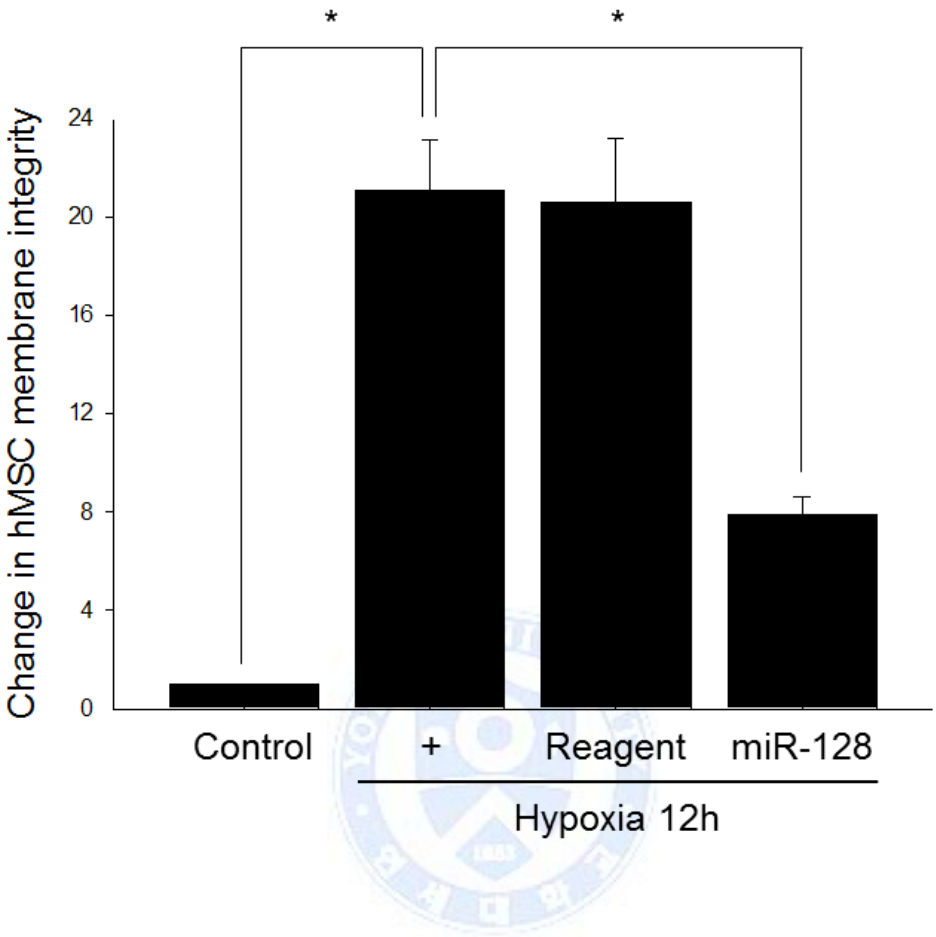


Figure 9. Sequential downregulation of cell cytotoxicity of miR-128 transfected hMSCs with hypoxic stimulation. Effects of miR-128 transfection on hMSC membrane integrity due to toxin accumulation after hypoxic stimulation. Data are presented as the mean value ± STDEV of three separate experiments (*P < 0.05).

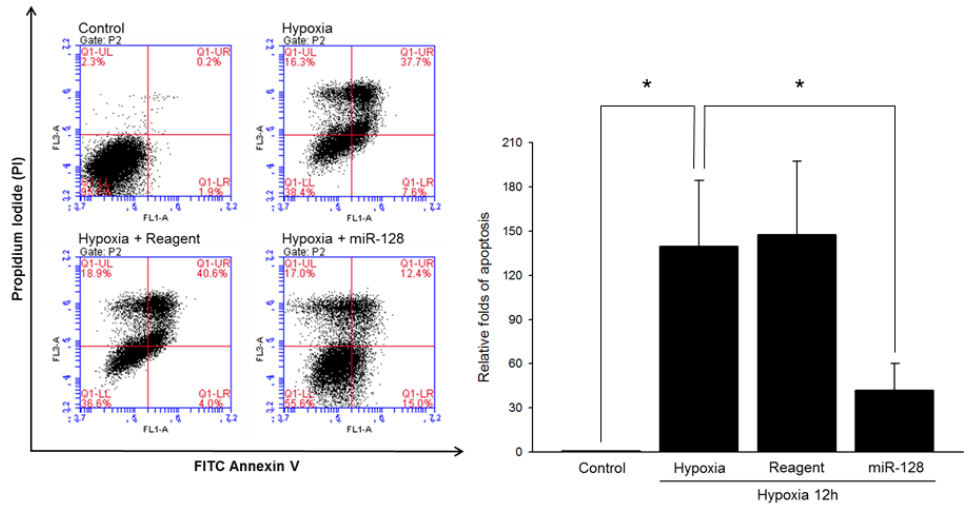


Figure 10. Inhibition of hMSC death via miR-128 trnasfetion. Effects of miR-128 transfection on overall apoptosis after hypoxic stimulation. Graph shows quantification of late apoptotic cells that are Annexin V+, PI+. Data was normalized by control. Data are presented as the mean value ± STDEV of three separate experiments (*P < 0.05).

IV. DISCUSSION

Apaf-1 is a molecule that is associated with the assembly of the pro-apoptotic apoptosome formation. [9] The importance of Apaf-1 and its role in apoptosis regulation via formation of the apoptosome, have been intensely studied in the past. [27,28] It normally resides in the cytosol in its monomeric form until it is coupled with the mitochondria secreted cytochrome c to form the caspase-9 recruiting apoptosome. [13] Once the apoptosome is formed, it initiates the caspase cascade via activation of initiator caspase-9. [29] In order to decrease hMSC apoptosis, we targeted the Apaf-1 molecule with miRNA regulation. In this study, our research suggests that expression levels of Apaf-1 were successfully decreased by miR-128 regulation and that overall apoptosis decreased consequentially.

Although Apaf-1 is known to be a key regulatory molecule of apoptosis, limited amount of studies are done on hMSC Apaf-1 regulation. Apaf-1 is solely related to the intrinsic mitochondria mediated apoptotic pathway, and is a naturally existing cytosolic molecule. [13] Because of its original existence, its expression levels may not show variations with hypoxic stimulations but only conform to its active form. Accordingly, despite no apparent increase to its expression levels under time dependent hypoxic stimulation, the degradation of Apaf-1 mRNA resulted in the decrease of ultimate apoptosis. Obstructing the apoptotic pathway midway increased

hMSC survival. This in turn gives hMSCs a chance to adapt to the hypoxic environment and carry out their initial purpose of regenerative therapy via differentiation, recruitment and differentiation of pre-existing cardiac stem cells, self-renewal, secretion of secretomes, etc. Another interpretation of the data can suggest that not a sufficient amount of apoptosis can be regulated with only targeting one molecule of the intrinsic apoptotic pathway. However, because miRNAs can be multi-targeting and because our research can contribute to future studies, the interpreted data may not be conclusive.

Many miRNAs have been discovered to regulate many cellular processes. Some miRNAs such as miR-17-92 cluster, miR-21, miR133, and miR-221/222 are well-known examples of anti-apoptosis regulators. [30] However, specific roles and mechanisms of many contributing to stem cell therapy and cardiovascular diseases still remain to be determined. MiR-128 is a well-known miRNA that plays an important role in tumor genesis and cancer development. [31] It is also considered to be an effective target for malignant tumor therapy. [31] However, there have not been any previous studies done in linking miR-128 and Apaf-1 in hMSCs altogether.

Although there has been studies done on caspase-9 activation through an endoplasmic reticulum stress specific apoptotic pathway independent of the apoptosome formation, [32] the percentage of its influence is still to be determined.

Meanwhile, pro-caspase-9 recruitment by the apoptosome remains to be the main mechanism proven to lead to cell apoptosis. In order to validate the decrease in the hallmark protein-protein interaction between Apaf-1 and caspase-9 and apoptosome formation, the interacting proteins were extracted as a whole through an immunoprecipiation technique. Results showed decreased pro-form of caspase-9 in samples of hMSCs transfected with miR-128 without any detection of the active caspase-9. While there are only just speculations about what the structure of the apoptosome might be, [29] no studies show visual data of the actual detection of the apoptosome structure, let alone the decrease of the complex iteself. Caspase-9's unique character that involves its recruitment by the Apaf-1/cytochrome c conformed apoptosome allows us to pin point the decrease in apoptosome formation indirectly without physically visualizing the decrease in the structure.

Another major type of programmed cell death that cannot be ignored when it comes to hMSC survival after it encounters hypoxic conditions is necrosis. Although necrosis is known to occur after physicochemical or physical cellular stress stimuli, [33] hypoxia can also bring on necrosis. [34,35] However, because our study focuses on apoptosis and its regulations via targeting the Apaf-1 molecule, we quantified cells that were only in the late apoptotic stage: Annexin V+, PI+ cells. Further studies can be based on finding multi-targeting miRNAs that target multiple factors that is involved in necrosis, apoptosis, and even pyroptosis in order to

effectively reduce cell death in the future.

Overall, our research demonstrates promising outcomes of miR-128 on hMSC apoptosis. With further elaborative studies regarding this current study, disclosure of miR-128 as a beneficial therapeutic tool for future heart regenerative stem cell therapy can be expected.

V. CONCLUSION

The cumulating results suggest that microRNA-128 can be incorporated alone or in combination with other factors to regulate Apaf-1. The overall results of this study validates that Apaf-1 is a key regulator of the intrinsic mitochondria-mediated apoptotic pathway that is responsible for the survival of hMSCs in face of hypoxic environment. Repression of Apaf-1 using transfection of miR-128 promoted hMSC survival. Hence, miR-128 can be used as a novel treatment method for injured hearts via augmentation of hMSC survival.

REFERENCE

1. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, et al. Executive summary: heart disease and stroke statistics--2014 update: a report from the American Heart Association. Circulation 2014;129:399-410.
2. Moran AE, Forouzanfar MH, Roth GA, Mensah GA, Ezzati M, Murray CJ, et al. Temporal trends in ischemic heart disease mortality in 21 world regions, 1980 to 2010: the Global Burden of Disease 2010 study. Circulation 2014;129:1483-92.
3. Frost RJ, van Rooij E. miRNAs as therapeutic targets in ischemic heart disease. J Cardiovasc Transl Res 2010;3:280-9.
4. Ranganath SH, Levy O, Inamdar MS, Karp JM. Harnessing the mesenchymal stem cell secretome for the treatment of cardiovascular disease. Cell Stem Cell 2012;10:244-58.
5. Miyahara Y, Nagaya N, Kataoka M, Yanagawa B, Tanaka K, Hao H, et al. Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. Nat Med 2006;12:459-65.
6. Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation 2002;105:93-8.
7. Ren G, Chen X, Dong F, Li W, Ren X, Zhang Y, et al. Concise review:

mesenchymal stem cells and translational medicine: emerging issues. Stem Cells Transl Med 2012;1:51-8.

8. Crow MT, Mani K, Nam YJ, Kitsis RN. The mitochondrial death pathway and cardiac myocyte apoptosis. Circ Res 2004;95:957-70.

9. Yuan S, Topf M, Reubold TF, Eschenburg S, Akey CW. Changes in Apaf-1 conformation that drive apoptosome assembly. Biochemistry 2013;52:2319-27.

10. Zhou J, Xi C, Wang W, Fu X, Jinqiang L, Qiu Y, et al. Triptolide-induced oxidative stress involved with Nrf2 contribute to cardiomyocyte apoptosis through mitochondrial dependent pathways. Toxicol Lett 2014;230:454-66.

11. Kumar S, Kain V, Sitasawad SL. Cardiotoxicity of calmidazolium chloride is attributed to calcium aggravation, oxidative and nitrosative stress, and apoptosis. Free Radic Biol Med 2009;47:699-709.

12. Reubold TF, Wohlgemuth S, Eschenburg S. Crystal structure of full-length Apaf-1: how the death signal is relayed in the mitochondrial pathway of apoptosis. Structure 2011;19:1074-83.

13. Yuan S, Yu X, Topf M, Ludtke SJ, Wang X, Akey CW. Structure of an apoptosome-procaspase-9 CARD complex. Structure 2010;18:571-83.

14. Schafer ZT, Kornbluth S. The apoptosome: physiological, developmental, and pathological modes of regulation. Dev Cell 2006;10:549-61.

15. Salloum FN, Yin C, Kukreja RC. Role of microRNAs in cardiac

preconditioning. J Cardiovasc Pharmacol 2010;56:581-8.

16. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. Cancer Res 2005;65:6029-33.

17. Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A 2005;102:13944-9.

18. Xu C, Lu Y, Pan Z, Chu W, Luo X, Lin H, et al. The muscle-specific microRNAs miR-1 and miR-133 produce opposing effects on apoptosis by targeting HSP60, HSP70 and caspase-9 in cardiomyocytes. J Cell Sci 2007;120:3045-52.

19. Kukreja RC, Yin C, Salloum FN. MicroRNAs: new players in cardiac injury and protection. Mol Pharmacol 2011;80:558-64.

20. Kim MH, Ham O, Lee SY, Choi E, Lee CY, Park JH, et al. MicroRNA-365 inhibits the proliferation of vascular smooth muscle cells by targeting cyclin D1. J Cell Biochem 2014;115:1752-61.

21. Choe N, Kwon JS, Kim JR, Eom GH, Kim Y, Nam KI, et al. The microRNA miR-132 targets Lrrfip1 to block vascular smooth muscle cell proliferation and neointimal hyperplasia. Atherosclerosis 2013;229:348-55.

22. Gama-Carvalho M, Andrade J, Bras-Rosario L. Regulation of Cardiac Cell Fate by microRNAs: Implications for Heart Regeneration. Cells 2014;3:996-1026.

23. Jayawardena T, Mirotsou M, Dzau VJ. Direct reprogramming of cardiac fibroblasts to cardiomyocytes using microRNAs. Methods Mol Biol 2014;1150:263-72.

24. Wang YS, Li SH, Guo J, Mihic A, Wu J, Sun L, et al. Role of miR-145 in cardiac myofibroblast differentiation. J Mol Cell Cardiol 2014;66:94-105.

25. de Winter RJ, Koster RW, Sturk A, Sanders GT. Value of myoglobin, troponin T, and CK-MBmass in ruling out an acute myocardial infarction in the emergency room. Circulation 1995;92:3401-7.

26. Kane NM, Thrasher AJ, Angelini GD, Emanueli C. Concise review: MicroRNAs as modulators of stem cells and angiogenesis. Stem Cells 2014;32:1059-66.

27. Perez-Paya E, Orzaez M, Mondragon L, Wolan D, Wells JA, Messeguer A, et al. Molecules that modulate Apaf-1 activity. Med Res Rev 2011;31:649-75.

28. Riedl SJ, Salvesen GS. The apoptosome: signalling platform of cell death. Nat Rev Mol Cell Biol 2007;8:405-13.

29. Yuan S, Akey CW. Apoptosome structure, assembly, and procaspase activation. Structure 2013;21:501-15.

30. Subramanian S, Steer CJ. MicroRNAs as gatekeepers of apoptosis. J Cell Physiol 2010;223:289-98.

31. Li M, Fu W, Wo L, Shu X, Liu F, Li C. miR-128 and its target genes in

tumorigenesis and metastasis. Exp Cell Res 2013;319:3059-64.

32. Morishima N, Nakanishi K, Takenouchi H, Shibata T, Yasuhiko Y. An endoplasmic reticulum stress-specific caspase cascade in apoptosis. Cytochrome c-independent activation of caspase-9 by caspase-12. J Biol Chem 2002;277:34287-94.

33. Vanlangenakker N, Vanden Berghe T, Vandenabeele P. Many stimuli pull the necrotic trigger, an overview. Cell Death Differ 2012;19:75-86.

34. Shimizu S, Eguchi Y, Kamiike W, Itoh Y, Hasegawa J, Yamabe K, et al. Induction of apoptosis as well as necrosis by hypoxia and predominant prevention of apoptosis by Bcl-2 and Bcl-XL. Cancer Res 1996;56:2161-6.

35. Steinbach JP, Wolburg H, Klumpp A, Probst H, Weller M. Hypoxia-induced cell death in human malignant glioma cells: energy deprivation promotes decoupling of mitochondrial cytochrome c release from caspase processing and necrotic cell death. Cell Death Differ 2003;10:823-32.

ABSTRACT (in Korean)

Apaf-1을 타깃으로 하는 microRNA-128을 이용한 중간엽 줄기세포 사멸 억제

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승 민 지

중간엽 줄기세포는 골수에서 유래한 특화된 세포 중 뛰어난 분화능, 재생능력에 기여하는 secretome의 분비를 통한 주면 세포의 회복등과 같은 특성을 같고 있다고 알려져 있다. 이러한 중간엽 줄기세포의 특성들은 손상된 심장의 재생치료에 유용한 도구로 사용될 수 있다. 하지만 이를 보편적으로 응용, 또는 사용하기에 앞서 선결해야 할 여러 장애물이 있다. 심근경색과 같은 심장 질환의 경우, 일반적으로 가혹한 저산소 조건 하에서의 세포사멸을 방지할 수 있는 방법을 모색하였다. 세포사멸 과정에서 중요한 역할을 하는 인자 중 하나인 Apaf-1은 세포기질에 원래 존재하며 cytochrome c와 결합하여

apoptosome의 형성에 기여한다. 이렇게 형성된 apoptosome은 비활성상태의 pro-caspase-9을 불러들여 효소적 절단 과정을 통해 활성화 상태의 caspase-9을 만들게 되며 이는 추가적인 세포사멸 기전을 전파하게 된다. 따라서 본 연구에서는 Apaf-1의 생성을 이러한 신호전달 기전의 전파를 억제할 것이라는 가설을 세워 그를 검증하고자 하였다. Apaf-1의 발현 억제를 위해서는 microRNA를 사용하였는데 miRNA는 표적 유전자의 3'UTR부분에 상보적으로 결합해 표적 유전자의 발현을 억제하는 post-transcriptional regulator로 알려져 있다. 본 연구 결과에 따르면 인간 골수에서 채취된 중간엽 줄기세포에 저산소 자극을 주었을 때 Apaf-1에 의한 apoptosome 형성이 증가하여 세포 사멸이 증가하는 것이 관찰되었다. Apaf-1을 표적으로 하는 miRNA인 MIR-128을 전처리 할 경우 낮아진 Apaf-1의 발현과 apoptosome 형성에 의존하여 인간 중간엽 줄기세포의 생존율 역시 향상되는 것을 확인하였다. 본 연구의 결과는 세포사멸의 주요 인자 중 하나인 Apaf-1의 발현을 miRNA를 통해 억제하는 것이 저산소 조건 하에서 중간엽 줄기세포의 생존을 높이는데 기여한다는 것을 보여주며, 이러한 접근 방식은 기존 세포치료의 난제 중 하나인 이식 후 낮은 생존율을 높이는데 유용하게 사용될 수 있을 것으로 기대된다.

핵심 되는 말: 중간엽 줄기세포, 세포 사멸, Apaf-1, miRNA