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Abrogation of age-induced microRNA-195 rejuvenates the senescent mesenchymal stem cells by reactivating telomerase

Motoi Okada¹, Ha Won Kim¹, Kaoru Matsu-ura, Yi-Gang Wang, Meifeng Xu, and Muhammad Ashraf²

Department of Pathology and Lab of Medicine, University of Cincinnati Medical Center, 231 Albert Sabin Way, Cincinnati, OH 45267

Abstract

Previously, we reported that a novel sub-population of young mesenchymal stem cells (YMSCs) existed in old bone marrow, which possessed high anti-aging properties as well as excellent efficacy for cardiac repair. MicroRNAs (miRNAs) have emerged as key regulators in post-transcriptional gene expression programs, and however, it is unknown whether miRNAs directly control stem cell senescence. Here we present the first evidence that miR-195 overexpressed in old MSCs induces stem cell senescence deteriorating their regenerative ability by directly deactivating *telomerase reverse transcriptase (Tert)*, and abrogation of miR-195 can reverse stem cell aging. MiRNAs profiling analysis in YMSCs and OMSCs by microarray showed that miR-140, miR-146a/b and miR-195 were significantly upregulated in OMSCs, which led us to hypothesize that these are age-induced miRNAs involved in stem cell senescence. Of these miRNAs, we found miR-195 directly targeted 3'-untranslated region of *Tert* gene by computational target prediction analysis and luciferase assay, and knockdown of miR-195 significantly increased *Tert* expression in OMSCs. Strikingly, miR-195 inhibition significantly induced telomere re-lengthening in OMSCs along with reduced expression of senescence-associated β -galactosidase. Moreover, silencing miR-195 in OMSCs by transfection of miR-195 inhibitor significantly restored anti-aging factors expression including *Tert* and *Sirt1* as well as phosphorylation of *Akt* and *FOXO1*. Notably, abrogation of miR-195 markedly restored proliferative abilities in OMSCs. Transplantation of OMSCs with knocked out miR-195 reduced infarction size and improved LV function. In conclusion, rejuvenation of aged stem cells by miR-195 inhibition would be a promising autologous therapeutic strategy for cardiac repair in the elderly patients.

²Correspondence should be addressed to: Current address: Muhammad Ashraf, PhD, Department of Pharmacology, University of Illinois at Chicago, 835 S Wolcott Ave, Chicago, IL 60612, (Tel) 312-355-1905, ashrafm2@uic.edu.

¹These authors equally contributed to this work.

DISCLOSURES

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AUTHOR CONTRIBUTIONS:

MO, HWK: Experimental design, performed experiments, collection and analysis of data; MS writing and final approval of Ms. KM: Performed experiments and collection of data; YGW, MX: Interpretation of data and Ms writing and final approval of Ms; MA: conception and design, financial support, Ms writing and final approval of Ms.

Keywords

Aging; stem cells; miR-195; telomerase; heart

Introduction

Stem cells regenerate multiple adult tissues including heart, brain, nerves, blood, skin and skeletal muscle [1]. Because there is a decline in tissue regenerative ability with aging is likely that age-induced alterations in the function of stem cells may impair regeneration in age-related diseases [2]. However, it remains largely unknown whether a decline in stem cell function with aging influences mammalian life span. The most significant phenotype in mammalian aging is telomere shortening which induces disruption of chromosome integrity by DNA damage and apoptosis [3]. Telomerase lengthens telomeres in DNA strands by adding DNA sequence repeats (“TTAGGG” in all vertebrates) to the 3' end of DNA strands in the telomere regions, which are found at the ends of eukaryotic chromosomes, thereby preventing senescent cells from postmitotic condition and apoptosis [4]. However, telomerase activity tends to decline with aging and loss of telomerase function in mice reduces the regenerative capacity of proliferative organs, accelerates the development of aging phenotypes, reduces life span, and increases cancer incidence [5]. Telomerase-deficient mice exhibit defects in stem cell function in the forebrain, epidermis, intestinal epithelium, and hematopoietic system [6]. Telomerase knockout mice severely suffer from severe left ventricular (LV) failure [9] and telomerase expression and activity are also markedly decreased in the adult heart [7]. Patients with chronic heart failure also have 25% shorter cardiac telomeres compared to healthy age controls [8]. Furthermore, shorter cardiac telomeres were found in aged hearts with mild hypertrophy and biventricular failure [10], suggesting that telomere length is associated with the severity of heart failure.

Bone marrow stem cells (BMSCs) are heterogeneous population including small cells and large cells, and generally, BMSC population in aged animals contains more senescent large cells than that from young animals [14]. Previously, Kucia *et al* isolated a population of rare (~0.01%) Sca-1⁺/Lin⁻/CD45⁻ cells from bone marrow mononuclear cells (termed as very small embryonic like stem cells; VSELs) [15]. VSELs express pluripotency markers, SSEA-1, Oct-4, Nanog, and Rex-1 as well as telomerase protein Rif-1. Recently, we have also discovered that a sub-population of small young mesenchymal stem cells (YMSCs) exists in the old BMSCs [16]. These cells highly express anti-aging genes, pluripotency genes and cardiac differentiation markers. Furthermore, transplantation of YMSCs into the infarcted murine heart exhibited increased neovascularization and cardiomyogenesis and improved cardiac functions, as compared to OMSCs. Although it remained unclear whether or not these small cells are pluripotent stem cells present in the adult. Therefore, the juvenile sub-population of MSCs which exists in the aged bone marrow possesses anti-aging properties of YMSCs and can be exploited for stem cell-based therapy in elderly patients.

MicroRNAs (miRNAs) have emerged as key regulators in the gene expression program associated with cellular senescence [11]. Hackl *et al* have reported that miR-17 family was downregulated in aged tissues and cells [12], and another study by Faraonio *et al* showed

that replicative senescence in normal human diploid fibroblasts is accompanied by altered expression of a set of miRNAs, with 14 and 10 miRNAs being either up or downregulated [13]. These studies suggest that some miRNAs contribute to cellular senescence at least in part. However, it is unclear whether changes in miRNA profiles are directly involved in the onset of pathophysiological changes or rather outcome of the senescence process resulting from tissue aging and dysfunction.

In this study, we investigated the role of miRNAs involved in stem cell aging and their impact on cardiac repair. Here we report our novel findings that regenerative ability of aged stem cells are deteriorated by increased expression of miR-195 which targets *telomerase reverse transcriptase (Tert)* gene, and abrogation of miR-195 in aged stem cells markedly induces *Tert* reactivation, resulting in significant telomere re-lengthening. Additionally, we show that transplantation of aged stem cells lacking miR-195 significantly reduced infarction size and concomitant improvement of cardiac function. These findings demonstrate that silencing miR-195 reverses the senescence clock in aged stem cells by telomerase reactivation and this strategy holds a great promise for stem cell based repair of aging myocardium.

Materials and Methods

Isolation of OMSCs and YMSCs from bone marrow

Our study followed the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication #85-23, revised 1985) and protocol approved by the Institutional Animal Care and Use Committee, University of Cincinnati. BMSCs were isolated from bone marrow of C57BL/6 mice (young; 2 months, old; 24 months, respectively) as described previously [16]. Briefly, BMSCs were cultured in 100 mm dishes with low glucose DMEM (HyClone Laboratories) supplemented with 10% fetal bovine serum (FBS) for 6–10 days. The non-adherent hematopoietic bone marrow cells were discarded during the routine fresh medium replacement. YMSCs were isolated from old bone marrow by using 3 μ m pores (cell culture insert; BD Bioscience). The homogeneity of YMSC population was confirmed with surface marker expression by FACS analysis as described previously [16].

Quantitative fluorescence *in situ* hybridization (Q-FISH) for telomere length measurement

Telomere was detected by FISH with PNA Telomere Cy3-labeled probe (F1002; TelC-Cy3, PNA Bio, Inc) according to manufacturer's instructions. Briefly, cells were fixed with 4% paraformaldehyde for 1 hr. After washing with PBS, cells were incubated with RNase 100 ng/ μ L for 20 min and 0.005% pepsin for 5 min at 37°C. After dehydrated by incubating with 70%, 85% and 100% of cold ethanol, cells were incubated with 200 nM PNA Telomere probe for 10 min at 85°C and incubated for 2 hr at room temperature. Then, cells were incubated with 2 \times saline-sodium citrate (SSC) buffer for 10 min at 60°C. DAPI and Cy3 signals were acquired simultaneously into separate channels using a confocal microscope (Fluoview FV1000, Olympus) and maximum projection from image stacks (10 sections at steps of 1 μ m) were generated for image quantification. Telomere length was analyzed by using TFL-TeloV2-2 free software (Vancouver, Canada). With the program, the integrated

fluorescence intensity value for each telomere, which is proportional to the number of hybridized probes, is calculated and presented. TFL-Telo is an application program used to estimate the length of telomeres from captured images of metaphases that have been stained for Q-FISH analysis [17].

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from various treatment groups of the cells with RNeasy Mini Kit (Qiagen, MD, USA) and cDNA was prepared using Omniscript-RT Kit (Qiagen, MD USA), according to the manufacturer's instructions. For PCR amplification, 1 µg of the cDNA from the reverse transcription reaction was then added to a PCR mix containing the suggested quantity of QIAGEN® PCR buffer, Q-Solution, dNTP mix, reverse and forward primers, Taq DNA polymerase and distilled water. Each PCR reaction was performed with specific primers.

Isolation and detection of miRNA

Extraction of miRNAs was performed by using mirVana™ miRNA Isolation Kit, and miR-195 expression was detected by using mirVana™ qRT-PCR miRNA Detection Kit (Ambion, Life Technologies) and QuantiTect SYBR green PCR kit (Qiagen) as previously described [18]. Specific miRNA primers were purchased from Ambion.

miRNA microarray

Total RNA samples obtained from OMSCs and YMSCs were sent to Exiqon, Inc (Denmark) for miRNA microarray profiling. Data were analyzed by Exiqon with in-house developed computer programs. Intensity values were transformed into log₂ scale, and fold changes were given in log₂ scale. A t-test was performed between OMSCs and YMSCs profiling, and statistically significant difference was considered at $p < 0.01$.

FISH to detect miR-195

In situ detection of miR-195 was performed in OMSCs and YMSCs plated on chamber slide. Samples were fixed in 4% paraformaldehyde for cell culture at room temperature for 20 min followed by two washes in PBS. Fixed cells were then pre-hybridized in hybridization solution (BioChain Inc., CA) for 3 hr at room temperature before hybridization. Probe (3 pmol; LNA-modified and FITC-labeled oligonucleotide; purchased from Exiqon) complementary to miR-195 was hybridized to the cells for 13–16 hr at 22°C lower than predicted T_m of the probe. Subsequent to post-hybridization washes with SSC buffer, *in situ* hybridization signals were detected with confocal microscope (Fluoview FV1000, Olympus).

Luciferase activity assay

Precursor miR-195 expression vector was constructed in a feline immunodeficiency virus-based lentiviral vector system (Genecopoeia, Inc). Luciferase reporter constructs containing 3'-untranslational region (UTR) of mouse *Tert* was designed to encompass mmu-miR-195 binding sites. Cells were plated into 24 well plates in triplicate and co-transfected with miR-195 vector (or scramble vector) and reporter construct with Lipofectamine 2000™

(Invitrogen). Firefly luciferase activities were measured by using Dual Luciferase Reporter Assay System kit (Promega) per manufacturer's instructions. Transfection efficiency was normalized by Renilla luciferase activity.

Lentivirus-mediated miR-195 inhibition in OMSCs

Lentivirus containing miR-195 inhibitor-expressing vector was generated by using Lenti-Pac HIV Expression Packaging System (GeneCopoeia, Inc) per manufacturer's protocol. Briefly, 2.5 μ l of lentiviral miR-195 inhibitor expression plasmid or scramble, 5.0 μ l of EndoFectin Lenti and EndoFectin Lenti reagent were added in Opti-MEM I, and formed the DNA-EndoFectine complex. 10–25 min after incubating the complex at room temperature, the DNA-EndoFectine complex was added to 293Ta cells in DMEM with 10% FBS, and then, incubated in 5% CO₂ at 37°C overnight. The culture medium was replaced with fresh DMEM with 5% FBS and 1/500 volume of the TiterBoost reagent to the culture medium. The virus pseudovirus-containing culture medium was collected at 48 hr post transfection and concentrated after filtration. For the transduction of OMSCs with lentivirus, 10 \times 10⁶ of OMSCs was plated and 20 μ l of virus suspension were added. To enhance lentiviral transduction efficiency, cells were placed at 4°C for 2 hr and then incubate in a 5% CO₂ at 37°C for 48 hr.

Senescence-associated β -galactosidase (gal) staining

Senescence-associated β -gal was detected by Senescence Detection Kit (BioVision, CA) per manufacturer's instruction.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Apoptotic cell death was detected by TUNEL according to instructions of the manufacturer (TMR Red; Roche Applied Science). For quantification, the numbers of TUNEL positive cells were counted in at least 5 randomly selected high-power fields (magnification \times 200) with 3 independent samples.

Western blot analysis

Western blot was carried out as previously described [19]. Briefly, cells were lysed in lysis buffer, pH7.4 [(in mM) 50 HEPES, 5 EDTA, 50 NaCl], 1% Triton X-100, protease inhibitors [(10 μ g/mL aprotinin, 1mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin) and phosphatase inhibitors [(in mM) 50 sodium fluoride, 1 sodium orthovanadate, 10 sodium pyrophosphate]. The protein samples (40 μ g) were electrophoresed using SDS-polyacrylamide gel and electro-immunoblotted. The specific antibodies used for the detection of Tert (sc-68720) was purchased from Santa Cruz, p53 (#2524), Sirt-1 (#2028), Phospho-FoxO1 (#2599), Bcl-2 (#3498), Cleaved caspase-3 (#9661), Akt (#2920), Phospho Akt (#4060) and Actin (#4968) were purchased from Cell Signaling. All antibodies were used as diluted with 1:1000.

Determination of cell proliferation rate

Cell proliferation rate was determined by cell growth assay and colony formation assay as previously reported [16].

Experimental model of acute myocardial infarction (AMI) and cell transplantation

In vivo cell transplantation using mouse AMI model was performed in accordance with our previous report [20]. Briefly, C57BL/6 mice (male 24 months old, 30~35 g body weight; n=10 animals/group) were prepared for left anterior descending (LAD) coronary artery ligation. Intraperitoneal anesthesia was administered with 0.1% of ketamine and 0.02% of xylene per body weight (g) for anesthesia. After endotracheal intubation and mechanical ventilation using Rodent Ventilator (Harvard Apparatus Model 683), the heart was exposed by left-sided minimal thoracotomy and LAD coronary artery was ligated with 6-0 silk. Immediately after ligation, 20 μ l DMEM without cells or containing 2×10^5 cells (scr OMSCs or $^{anti-195}$ OMSCs) were injected into infarction and border zones. Following injection, the opened chests of mice were sutured and all mice were allowed to recover.

Physiologic assessment of heart function

Transthoracic echocardiography was performed to study change in the heart function at 4 weeks after transplantation by using iE33 equipped with 15 to 7 MHz broadband transducer (Philips Ultrasound, Bothell, WA) as previously described [20]. Heart was imaged in the two-dimensional mode in the parasternal long-axis and/or parasternal short-axis views which were subsequently used to position the M-mode cursor perpendicular to the ventricular septum and LV posterior wall, after which M-mode images were obtained. Measurements of ventricular anterior thickness, LV internal dimension, and LV posterior wall thickness were made from two-dimensionally directed M-mode images of the LV in both systole and diastole. Ejection fraction was measured based on area-length method after measurement of LV internal area and distance from apex to basal LV in both systole and diastole. All the scoring system was patterned after the American Society of Echocardiography's scoring system used conventionally in interpreting clinical echocardiographic studies.

Infarction size determination

For measurement of infarction size and area of fibrosis, the hearts were arrested in diastole by intravenous injection of cadmium chloride and fixed in 10% buffered formalin. The heart was excised, cut transversely, and embedded in paraffin for Masson's trichrome staining. Infarct size was defined as sum of the epicardial and endocardial infarct circumference divided by sum of the total LV epicardial and endocardial circumferences using computer based planimetry with Image-J analysis software (version 1.6; NIH).

Statistical analysis

All values were expressed as mean \pm SE. Comparison between 2 mean values was evaluated by an unpaired Student 2-tailed t-test, and between 3 or more groups was evaluated by one way ANOVA followed by Bonferroni post-hoc analysis. Statistical significance was accepted at $p < 0.05$.

RESULTS

OMSCs exhibit the shorter telomeres along with impaired expression of anti-aging genes

We have previously reported that both YMSCs and OMSCs are positive for mesenchymal stem cell markers, CD29, CD44 and CD90, but negative for CD45 and CD119. We also showed that expression of pluripotency markers including Oct-4, Nanog, Sox-2 and Klf-4 was significantly upregulated in YMSCs as compared to OMSCs. [16] To validate our previous data that OMSCs expressed characteristics specific for senescence, we compared telomere length as well as anti-aging genes expression between OMSCs and YMSCs. As compared to YMSCs, OMSCs showed markedly short telomeres as examined by Q-FISH analysis (Fig. 1A). In addition, we observed that average nucleus size of OMSCs was bigger than that of YMSCs (~12 μm vs ~5 μm). Expressions of representative anti-aging genes including Sirt1, Tert and anti-apoptotic genes Bcl-2 were markedly reduced in OMSCs as compared to YMSCs (Fig. 1B). These results are in agreement with our previous report that OMSCs exhibited the impaired expression of cardiac lineage markers (Gata-4 and Mef2C) and pluripotency markers (Oct-4, Sox-2, SSEA-1 and Nanog) as well as senescence-associated β -gal expression, as compared to YMSCs [16].

miR-195 expression is markedly upregulated in OMSCs

To profile miRNA expression induced with aging, we isolated total RNA from OMSCs and YMSCs, and performed microarray analysis in OMSCs and YMSCs (Fig. 2A). Per our results, the expression of miR-140, miR-146a/b and miR-195 was significantly upregulated in OMSCs whereas expression of miR-29b, miR-205, miR-378 and miR-542-3p was downregulated in OMSCs (Fig. 2B&C). RT-PCR and real time PCR confirmed microarray result that miR-195 expression was significantly upregulated in OMSCs as compared to YMSCs (Fig. 2D&E). FISH analysis for miR-195 visualization further confirmed that miR-195 was highly expressed in OMSCs (Fig. 2F). This result led us to hypothesize that miR-195 was induced with aging, and its abrogation in OMSCs may restore the regenerative capacity of OMSCs. We transfected OMSCs with anti-miR-195 and confirmed that transfected OMSCs showed reduced expression of miR-195 while scramble did not affect it, as shown by real time RT-PCR (Figure 2G), indicating that miR-195 inhibitor we used was specific for reduction of miR-195 expression in stem cells.

Tert is a direct target of miR-195 in aging stem cells

To investigate the biological relevance of miR-195 induction during stem cell aging and its participation in OMSC senescence, we first carried out target prediction analysis by *in silico* search to find potential target genes of miR-195 responsible for aging and senescence. Interestingly, computational analysis predicted that mmu-miR-195 directly binds to 3'-UTR of mouse *Tert* gene (Fig. 3A). To experimentally validate this result, we performed RT-PCR and Western blot analysis to examine whether Tert expression is altered by miR-195 knockdown in OMSCs. Interestingly, expression of both mRNA and protein of Tert was significantly increased by miR-195 abrogation in OMSCs (Fig. 3B&3C), indicating that Tert is a putative target of miR-195. *Tert* as a target gene of miR-195 was confirmed by luciferase activity assay which showed that co-transfection of a miR-195 expression vector (pEZX-miR-195) with the vector containing 3'UTR of *Tert* gene significantly reduced

luciferase activity in comparison to co-transfection with miR-scramble vector (pEZX-miR-Sc) (Fig. 3D). These results demonstrated that Tert is a direct target of miR-195 in stem cell aging.

Abrogation of miR-195 rejuvenates OMSCs through telomere re-lengthening and anti-aging markers reactivation

To examine the mechanistic participation of miR-195 in stem cell aging, we knocked down miR-195 expression by using a lentiviral miR-195 inhibitor vector (Lenti-anti-miR-195) which could successfully transfect OMSCs to achieve abrogation of miR-195 (Fig. 4A). mCherry signal (red fluorescence) in this vector system allowed us to recognize the transfected OMSCs with miR-195 inhibitor or scramble vector (Fig. 4B, upper panel). Interestingly, abrogation of miR-195 significantly reduced senescence-associated β -gal expression in OMSCs as compared to scramble transfected OMSCs (Fig. 4B, 20.6 \pm 4.9% vs 54.5 \pm 8.1%, p <0.01). More importantly, OMSCs transfected with miR-195 inhibitor induced significant telomere re-lengthening (2.9 fold higher as compared to scramble transfection, Fig. 4C). Furthermore, inhibition of miR-195 reduced TUNEL-positive apoptotic cell death in OMSCs (Fig. 4D, 19.7 \pm 4.5% vs 60.8 \pm 2.7%, p <0.01), suggesting that rejuvenated OMSCs by miR-195 abrogation survive better under apoptotic condition such as ischemia. Furthermore, expression of anti-aging makers (Tert and Sirt-1) and pro-survival markers (p-Akt and Bcl-2) were significantly increased by transfection of anti-miR-195 in OMSCs whereas expression of senescence-associated markers (p53) and pro-apoptotic marker (cleaved caspase-3) was markedly reduced by miR-195 abrogation (Fig. 4E), supporting our hypothesis that inhibition of age-induced miR-195 can rejuvenate OMSCs by reactivation of anti-aging factors and suppression of senescence-associated markers. It is important to note that knockdown of miR-195 significantly restored the impaired cell proliferative abilities in OMSCs as evaluated by cell proliferation assay and colony formation assay (Fig. 4F&G). These results demonstrated that miR-195 induced during stem cell aging plays critical roles in their fate and behaviors.

Transplantation of OMSCs deleting miR-195 shows the improved efficacy for cardiac repair

To investigate functional consequence and therapeutic relevance of age-induced miR-195 in ischemic heart repair, we transfected OMSCs with miR-195 inhibitor (^{anti-195}OMSCs) or scramble (^{scr}OMSCs), and then, transplanted into the infarcted mouse heart. All animals survived full length of studies and there were no deaths related to cell transplantation. The animals (n=10 per group) were harvested at 4 weeks after respective treatment. Notably, transplantation of ^{anti-195}OMSCs significantly reduced infarction size with better efficacy when compared to that of ^{scr}OMSCs (^{anti-195}OMSCs: 23.6 \pm 10.2%, ^{scr}OMSCs: 41.8 \pm 13.1%, p <0.05) (Fig. 5A). To further investigate whether transplantation of ^{anti-195}OMSCs improves the therapeutic potential of aged stem cells for cardiac functions, left ventricle (LV) function was assessed by echocardiography after cell transplantation. The indices of LV contractile function were well-preserved in ^{anti-195}OMSC injection group compared to ^{scr}OMSC injection group (Fig. 5B–E). Indices of global heart functions were well preserved in ^{anti-195}OMSC transplanted groups including LV diastolic volume (^{scr}OMSC: 132.7 \pm 37.1 mm³, ^{anti-195}OMSC: 94.5 \pm 17.2 mm³, p <0.05), LVEF (^{scr}OMSC:

28.5±12.2%, anti-195OMSC: 46.2±12.7%, $p<0.05$) and LVFS (^{scr}OMSC: 12.9±3.5%, anti-195OMSC: 24.8±4.6%, $p<0.05$). We also analyzed LV functions by TEI (myocardial performance index, Fig. 5D) and E/E' (strong predictor of LVEDP, Fig. 5E) since TEI is useful index for assessing cardiac systolic and diastolic function, and E/E' is well correlated with the prognosis of heart failure. TEI and E/E' also indicated that anti-195OMSC has better efficacy to improve cardiac functions as compared to ^{scr}OMSC. Taken together, these results demonstrated that impaired regenerative abilities of the senescent stem cells can be remarkably reversed by abrogation of age-induced miR-195.

DISCUSSION

Elderly patients are more likely to have cardiac arrest following MI [21]. Myocardial ischemia results in massive death of cardiomyocytes, and acceleration of cell death in the aged myocardium has been proposed as a mechanism for increased risk of heart failure in the elderly. Tissue-resident adult stem cells including cardiac stem cells maintain normal tissue homeostasis and participate in tissue repair and regeneration in response to damage [22]. Aging is accompanied by a decline in regenerative capacity including myocardial regeneration, wound healing, skeletal muscle mass and strength and neurogenesis [23]. Since the regenerative activities of the damaged tissues are attributable to the resident adult stem cells, these age-related deterioration of organ functions may be due to the impaired functionality of stem cells [2, 24]. Therefore, it is important to understand the relationship between stem cell function and their ability to treat age-related degenerative diseases including cardiovascular diseases.

The main hypothesis of this study was that rejuvenation of the senescent stem cells with antiaging miR can improve their repair capacity of the aging myocardium following MI. Transplantation of these MSCs results in significant improvement of cardiac function and reduction of infarct size. The effects of MSCs could be through their direct differentiation into cells of cardiac lineage alone or together with paracrine effects. Previously, we reported that a novel sub-population of YMSCs with high proliferative and differentiation capacity existed in the aged bone marrow, and transplantation of YMSCs into the infarcted heart was more effective to reduce the infarction size and to improve LV functions than OMSCs transplantation [16]. These findings led us to search for the key factors responsible for progression of stem cell aging and to hypothesize that maintaining juvenile properties, *e.g.* anti-aging, pluripotency, high proliferation and differentiation rates, would offer a great therapeutic strategy for repair of aging myocardium.

The major findings of this study are that 1) miR-195 expression is highly upregulated in OMSCs, 2) miR-195 directly targets 3'-UTR of *Tert* gene, thereby promoting telomere shortening in OMSCs, 3) Abrogation of age-induced miR-195 rejuvenates OMSCs by reactivating *Tert* as well as anti-aging factors, 4) Genetic deletion of miR-195 in OMSCs can reverse their impaired regenerative abilities for cardiac repair. In the present study, we focused on age-induced miRNAs, especially for miR-195 to improve cell functional capacity of aging stem cells since miRNAs are key regulators in the gene expression program associated with cellular senescence [11]. Several studies have reported that miR-17 family was downregulated in aged tissues and cells in human [12], and senescence in human

lung fibroblasts was accompanied by upregulation of miR-152, miR-410, miR-431, and miR-493, and down-regulation of miR-155, miR-20a, miR-25, and miR-15a [25]. Another study showed that replicative senescence in normal human diploid fibroblasts is accompanied by altered expression of miRNAs [13]. Recently, Boon and Dimmeler *et al.* have reported that expression of age-induced miR-34a, targeting PNUM, is a key mechanism that regulates cardiac contractile function during aging and after AMI [26]. MiR-34a inhibition reduced telomere shortening, DNA damage and cardiomyocyte apoptosis, and improved functional recovery after MI. Taken together, these studies suggested that altered expression of miRNAs with aging is a key factor in organ dysfunctions, and modulation of age-induced miRNAs can reduce the effects of cellular senescence. In the present study, we demonstrated for the first time that miR-195 expression was significantly induced with aging in bone marrow-derived stem cells and abrogation of age-induced miR-195 can rejuvenate the senescent MSCs by inducing telomerase reactivation, resulting in telomere re-lengthening. In addition, rejuvenation of OMSCs by knockdown of miR-195 resulted in increased cell proliferation and colony formation.

One of the important mechanisms for cellular senescence is downregulation of telomerase and the resulting loss of its activity. Telomere shortening induces disruption of chromosome integrity because uncapped telomeres activate signaling pathways associated with DNA damage and apoptosis [3]. Human adult hematopoietic and non-hematopoietic stem cells (HSCs) showed a decline in telomerase activity as well as immune response with aging [27, 28], and this could be correlated with telomere shortening in T- and B-lymphocytes [29, 30]. Cardiac stem cells (CSCs) play important roles in delaying cardiac aging and dysfunction through differentiation into cardiomyocytes, and lack of telomerase activity and short telomeres can induce CSCs senescence. Similarly, our previous study showed that MSCs obtained from the aged mice (20–24 months old) showed the impaired telomerase expression along with shorter telomeres, resulting in the impaired proliferation rate and differentiation ability as compared to that from young animals (2 months old) [31]. Other studies also showed that overexpression of telomerase in human MSCs increases proliferation and differentiation capacity [32], and the telomerase knockout MSCs did not differentiate into adipocytes or chondrocytes even at earlier passages [33]. Therefore, it is important to upregulate telomerase in the aged stem cells to promote regenerative potential as well as longevity of organ. In this study, we showed that expression of Tert was impaired in OMSCs, and inhibition of age-induced miR-195 reactivated Tert expression in OMSCs, thereby lengthening telomeres and improving regenerative functions of the aged stem cells. There are many other target genes of miR-195. However, we found that Tert is highly downregulated in aging stem cells which is one of the putative target of miR-195. Since telomerase deactivation with aging is a most commonly accepted process for cellular senescence, we reasoned Tert as a major target of miR-195 for stem cell aging. However, we do not rule out the possibility that miR-195 targets other genes for aging. As an additional mechanism, Bcl-2, an anti-apoptotic gene, was also found to be a target of miR-195, and our preliminary data revealed that knockdown of miR-195 increased Bcl-2 expression, thereby reducing apoptosis of stem cells upon H₂O₂ stimulation (unpublished data). It also remains to be investigated whether overexpression of miR-195 in other stem cell types such as HSCs, CSC or other tissue-resident stem cells impairs their function. It is also undetermined

whether miR-195 expression is induced in human stem cell types and inhibits Tert expression in human cells. Furthermore, the role for the other miRNAs found in the microarray-based profiling in this study (miR-29b, miR-205, miR-378 and miR-542-3p; downregulated with aging, Fig. 2C), is not known. Although our data indicated that age-induced miR-195 alone can partially reverse stem cell aging and restore normal function, it is possible that combination with the other age-regulated miRNAs (upregulated or downregulated with aging) may contribute to full restoration of stem cell functions. We have found that at least three miRNAs, including miR-140, miR-146a/b and miR-195, were significantly upregulated in OMS. The combination of miR-195 inhibition and involvement of other miRNAs may have a synergistic effect on --rejuvenation of stem cells as well as improvement of heart function in aged hearts.

Further in depth studies are needed to understand the mechanistic participation of age-regulated miRNAs in age related abnormalities of stem cells. We also observed that the effects of YMSCs and modified OMSCs were similar on cardiac repair (data not shown). Infarction size was not significantly different between the use of YMSCs and modified OMSCs. Furthermore, we previously reported that proliferation and differentiation abilities of modified OMSCs were also improved to the level of YMSCs (16). However, additional studies are required to further understand rejuvenation of stem cells and its impact on repair of aging heart.

CONCLUSION

This study demonstrated that age-induced miR-195 is a key regulator of bone marrow-derived stem cell aging by targeting telomerase, and abrogation of miR-195 is a novel promising strategy to reverse aging clock in the senescent stem cells for repairing the old ischemic myocardium.

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Significance Statement

In this study, we investigated the role of miRNAs involved in stem cell aging and their impact on cardiac repair. Here we report our novel findings that regenerative ability of aged stem cells is deteriorated by increased expression of miR-195 which targets telomerase reverse transcriptase (Tert) gene, and abrogation of miR-195 in aged stem cells markedly induces Tert reactivation, resulting in significant telomere re-lengthening. Additionally, we show that transplantation of aged stem cells lacking miR-195 significantly reduced infarction size and concomitant improvement of cardiac function. These findings demonstrate that silencing miR-195 reverses the senescence clock in aged stem cells by telomerase reactivation and this strategy holds a great promise for stem cell based repair of aging myocardium.

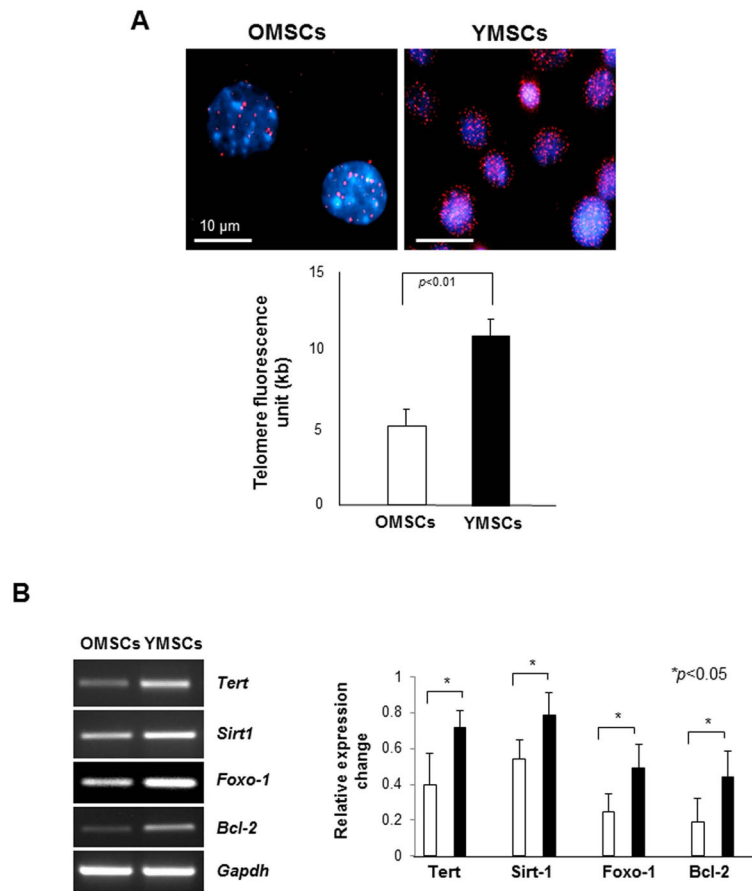


Figure 1. OMSCs exhibit the shorter telomeres with impaired expression of anti-aging genes
(A) Q-FISH analysis showed that OMSCs have shorter telomeres as compared to YMSCs.
(B) Expressions of anti-aging genes (*Tert*, *Sirt1*) and anti-apoptotic gene (*Bcl2*) were impaired in OMSCs when compared to YMSCs.

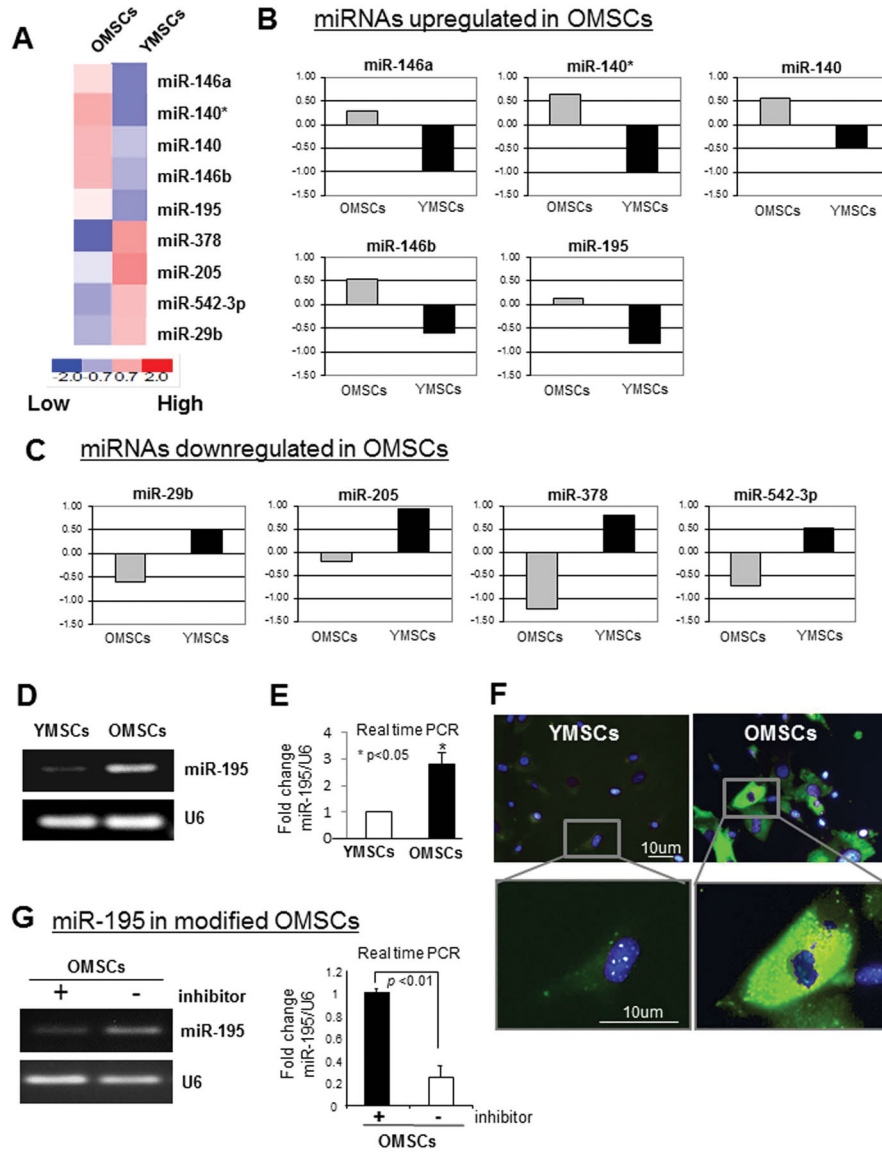


Figure 2. miR-195 expression is highly upregulated in OMSCs

(A–C) miRNA microarray analysis was performed to profile miRNAs expression in OMSCs and YMSCs. Expression of miR-140, 146a/b and 195 was significantly upregulated in OMSCs while miR-29b, miR-205, miR-378 and miR-542-3p were downregulated. (D, E) Higher expression of miR-195 in OMSCs was confirmed by RT-PCR. (F) FISH analysis also indicated higher expression of miR-195 in OMSCs as compared to YMSCs. FISH was performed by using miR-195 specific 5'-fluorescein labeled miRCURY LNATM detection probe. (G) The expression of miR-195 in OMSCs transfected with anti-miR-195 and that transfected with scramble miR.

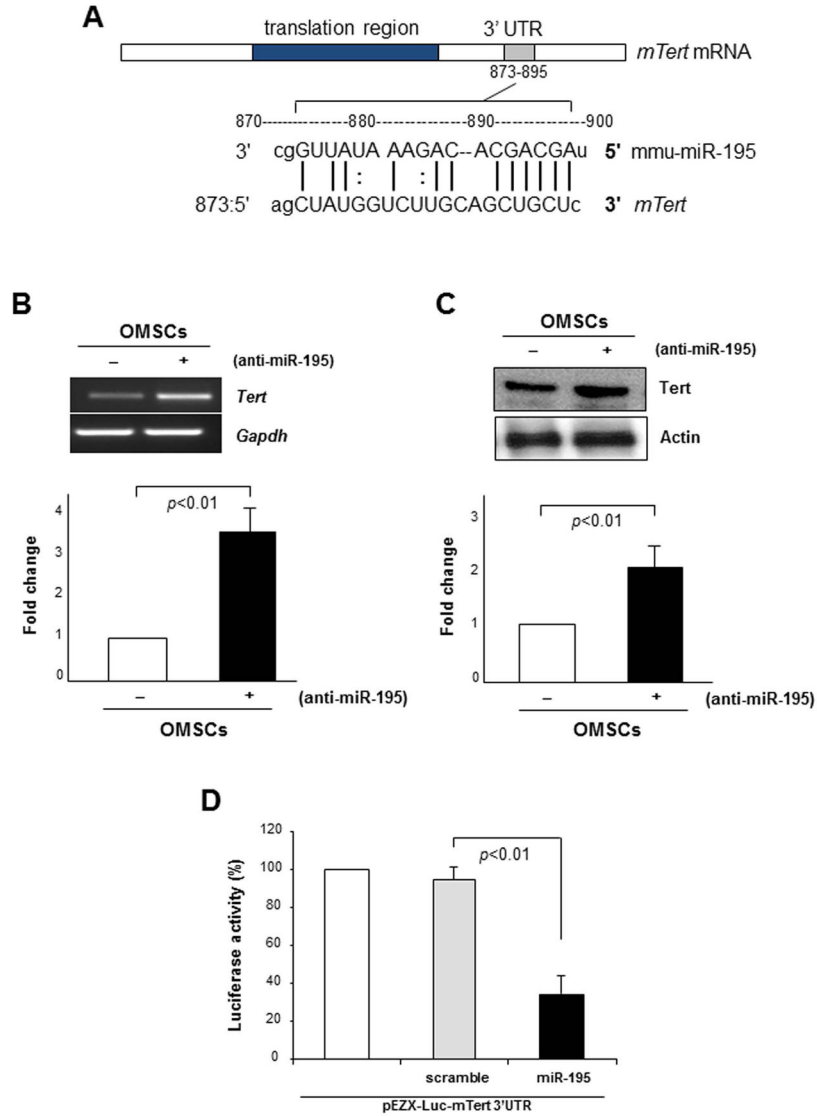
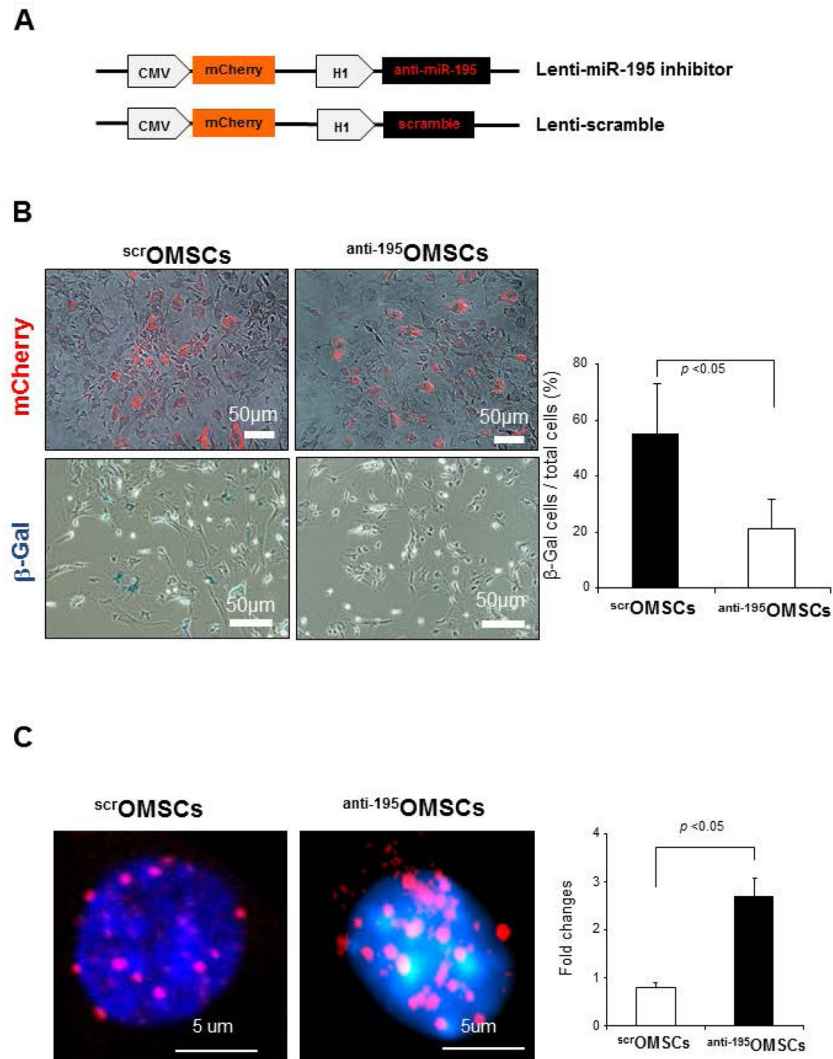


Figure 3. *Tert* is a direct target of miR-195 in stem cell aging

(A) Computational analysis predicted *mTert* as a potential target of mmu-miR-195. (B, C) Transfection of OMSCs with anti-miR-195 restored expression of *Tert* mRNA as well as telomere specific protein TRF2, as examined by RT-PCR and Western blot, respectively. (D) Co-transfection with pEZX-Luc vector containing *mTert* 3' UTR and a plasmid encoding miR-195 showed decreased luciferase activity ($p < 0.01$ vs pEZX-miR-SC transfected cells). Transfection efficiency was normalized by Renilla luciferase activity.



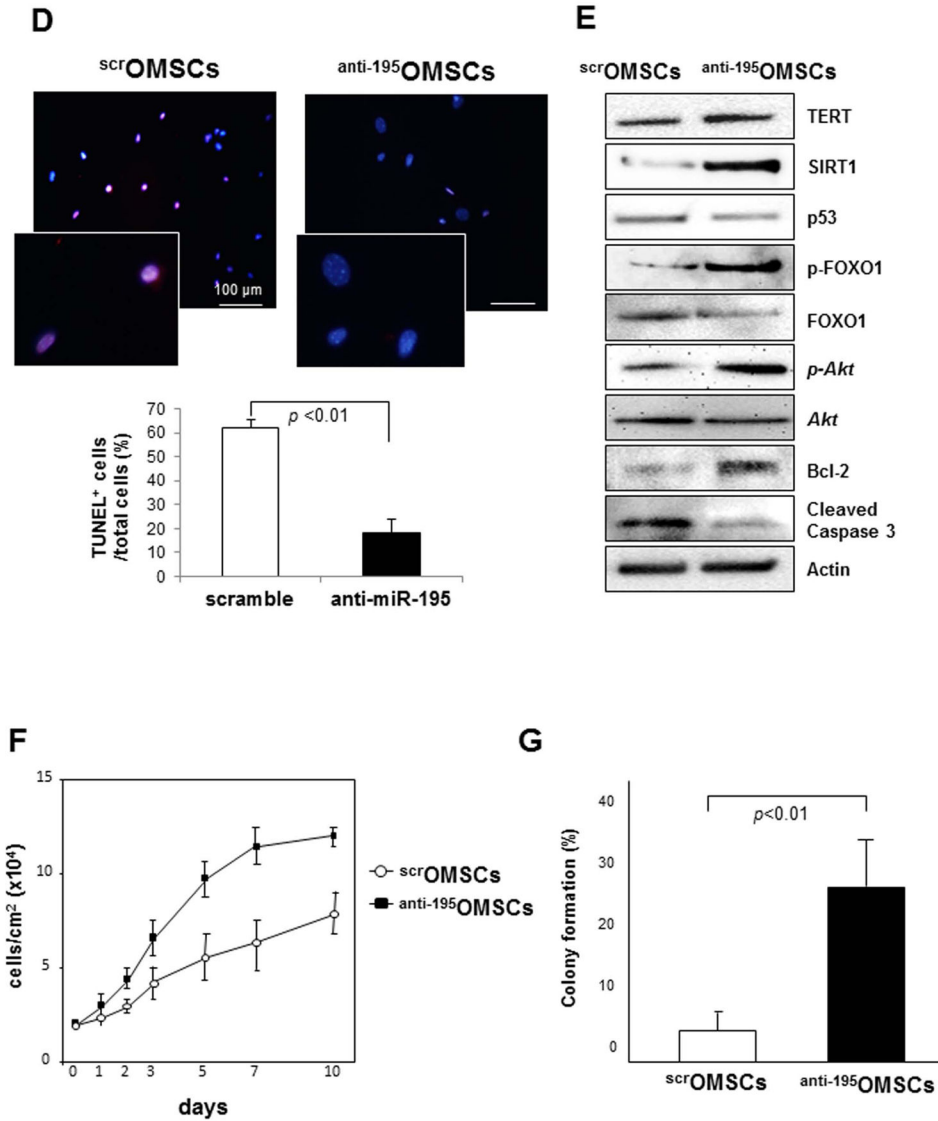
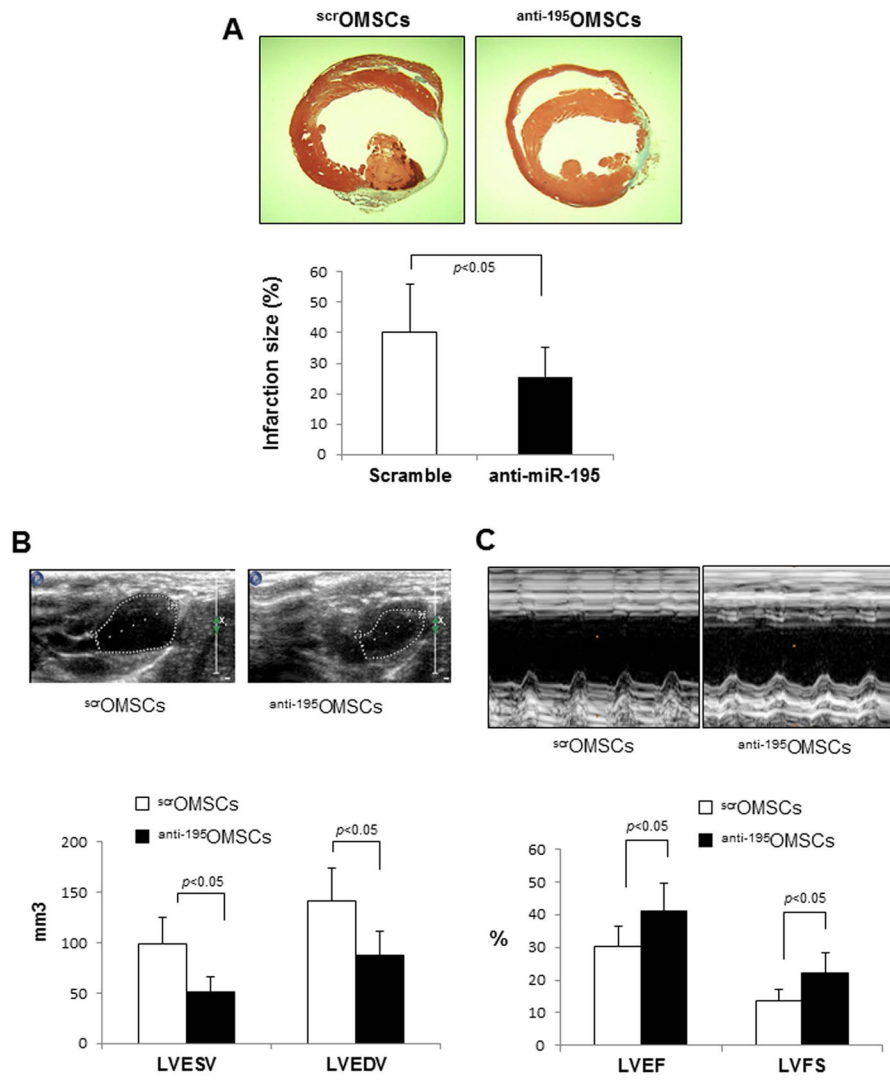


Figure 4. Abrogation of miR-195 rejuvenates OMSCs by telomere re-lengthening and anti-aging markers reactivation

(A) Structures of Lenti-miR-195 inhibitor and Lenti-scramble which contain mCherry reporter gene (red signal). (B) Abrogation of miR-195 significantly reduced senescence-associated β -gal expression in OSMCs as compared to scramble transfected OMSCs. (C) OMSCs showed significant telomere re-lengthening when they were transfected with miR-195 inhibitor. (D) miR-195 inhibition markedly reduced TUNEL-positive apoptotic cell death in OMSCs. (E) Expressions of TRF2, SIRT1 and Bcl-2 as well as phosphorylation of FOXO1 and Akt were significantly increased by transfection of anti-miR-195 in OMSCs whereas expression of p53 and cleaved caspase 3 was reduced by miR-195 abrogation. (F, G) Knockdown of miR-195 significantly restored cell proliferative abilities in OMSCs as examined by cell proliferation assay and colony formation assay.



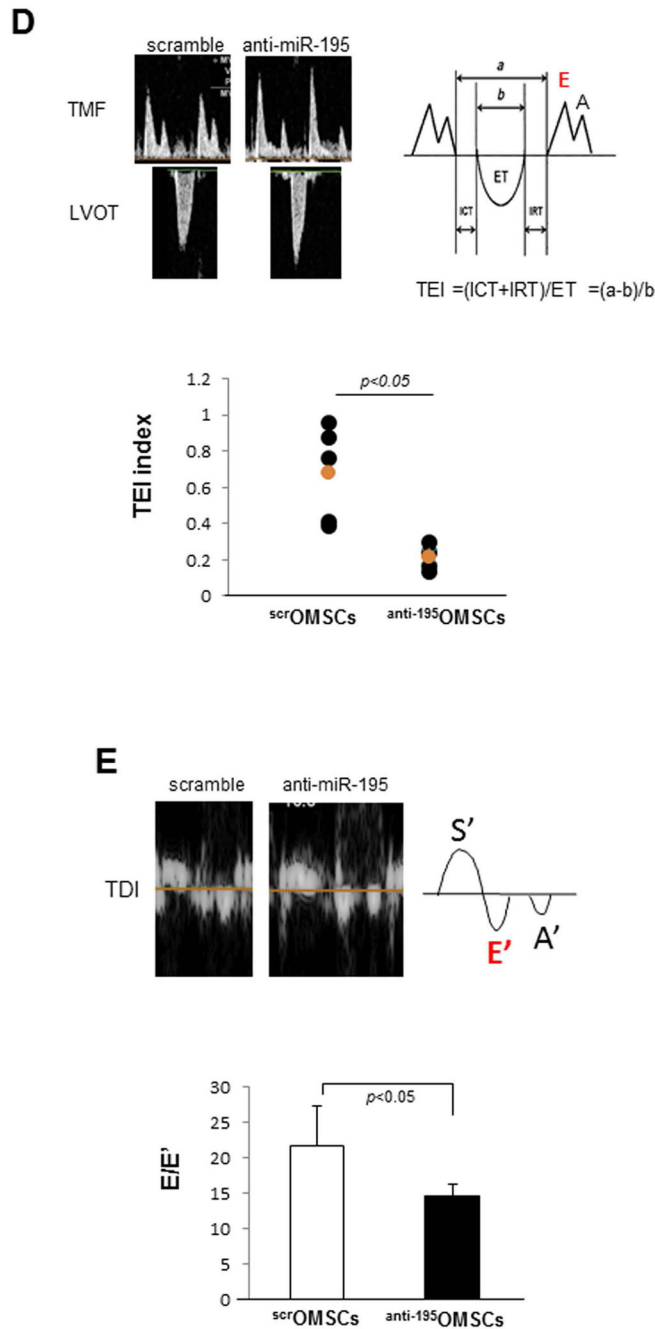


Figure 5. Transplantation of miR-195 deleted OMSCs shows the improved efficacy for cardiac repair

(A) Transplantation of OMSCs lacking miR-195 into the infarcted heart significantly reduced infarction size as determined by Masson trichrome staining and Image-J analysis software. Transplantation of OMSCs transfected with anti-miR-195 improved systolic functions (LVEDV, LVESV, LVEF and LVFS) of the infarcted heart. (B) Representative figure of left ventricular long axis view. LVESV; left ventricular endo systolic volume, LVEDV; left ventricular endo diastolic volume. (C) Representative figure of left ventricular

M-mode image. LVEF; left ventricular ejection fraction, LVFS; left ventricular fractional shortening. Transplantation of OMSCs transfected with anti-miR-195 improved diastolic (TEI index and E/E') functions of the infarcted heart. **(D)** TEI index; isovolumic contraction time plus isovolumic relaxation time divided by ejection time, myocardial performance index. **(E)** E/E'; peak velocity of early diastole mitral inflow, E divided peak velocity of mitral annulus measure by tissue doppler, E'

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