Master of Science

miRNA-34a as a Senescence-Associated Factor via *p21* in Liver-Derived Mesenchymal Stem Cells

간 유래 중간엽 줄기세포에서 p21을 통한 miR-34a의 노화 조절 연구

The Graduate School

of the University of Ulsan

Department of Medical Science

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강서온의 이학석사학위 논문을 인준함

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감사의 글

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힘들 때마다 시 한 편을 곱씹습니다. 그 시에서 가장 좋아하는 구절을 끝으로 감사 말씀을 마칩니다.

“그러나 나는 아직 당신 앞에 한 그루 나무처럼 서 있다.”

강서온 올림

Abstract

Mesenchymal stem cells (MSCs) are ideal for cell therapy since they possess the characteristics of stem cells and immunomodulatory properties. However, there is a disadvantage in that it is difficult to maintain function for a long time compared to its potential, so many researchers are continuing their efforts to understand the aging of MSCs. In this study, we investigated microRNAs (miRNAs) that may play significant roles in the aging process by monitoring cell senescence in liver-derived MSCs (LD-MSCs).

To identify the characteristics of senescent cells, this study examined mRNA expression of senescence-associated factors and their related miRNA expression level using qRT-PCR in early and late passage LD-MSCs. A morphological difference in early and late passage cells was observed through optical diffraction tomography (ODT).

As a result, differences between early passage and late passage cells were observed. In comparing the morphology of early and late passaged cells, alterations in the structure of the cells were observed. To analyze the internal structure of cells using ODT, an increase in cell size, multinucleation, and vacuoles were observed. In addition, lipid droplets known to regulate cell metabolism were found to be reduced in late passage cells. P21 is known to be a senescence-related factor that induces cell cycle arrest. Late passage LD-MSCs expressed higher levels of *p21*, and differences in the expression of several miRNAs were identified to select miRNAs capable of regulating *p21*. MiR-106b, a representative *p21* suppressor, observed no significant differences over time. While miR-34a, known to induce *p21*, is more abundant in late passage LD-MSCs. The miR-34a has the potential to regulate senescence of LD-MSCs.

Taken together, LD-MSCs in late passage showed a distinct morphological difference, and p21 regulates cellular senescence by arresting the cell cycle. Through the induction of the expression of p21, miR-34a has the potential to promote cellular

senescence. This suggests that miR-34a can be a potential regulator of cellular senescence via p21 expression induction.

Keywords: Mesenchymal stem cells, liver-derived mesenchymal stem cells, senescence, miRNA, p21, miR-34a

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Introduction

Stem cells refer to a population with the ability to self-renew and differentiate into multiple cell lineages, replacing diseased or aged cells. Due to these characteristics, it has been actively studied for the regeneration of damaged tissues and the treatment of incurable diseases since the discovery of stem cells. Stem cells can be classified into embryonic stem cells (ESCs), adult stem cells (ASCs), and induced pluripotent stem cells (iPSCs)[1]. ESCs have a high level of differentiation potential and can generate cells of all lineages. However, they are hard to control differentiation and are not free from ethical issues since they are induced from woman's fertilized eggs[2]. As iPSCs are derived from somatic cells, ethical concerns can be avoided, and their differentiation potential is comparable to ESCs, but there is uncertainty as to whether they are capable of developing cancer[3]. ASCs are found in almost all mammalian tissues and organs. In spite of their stable differentiation, they are rare and are difficult to mass-proliferate. In particular, mesenchymal stem cells (MSCs), a type of ASCs, are considered valuable in the clinical field for their unique characteristics[2, 4].

Since Friedenstein et al. isolated MSCs in 1970[5], researchers have focused on exploring their properties, including their immunomodulatory capacities. It is essential to note that mesenchymal stem cells have not only stem cell characteristics but also immunomodulatory capacity [6]. In cell-based therapy, this is a very significant aspect, as it suggests the possibility of safe transplantation and indicates the application potential for various diseases. Several studies have demonstrated that MSCs are clinically useful under certain conditions, but the age of the donor is a significant factor that affects MSCs' quality. With age, the cells become less capable of differentiating and proliferating, which causes them to perform worse than cells derived from younger donors. Moreover, when MSCs are cultured in vitro for an extended period of time, their aging progresses together regardless of the donor's age. Therefore, in light of the wide range of applications that MSCs may be

applied to, it is essential to monitor senescence and to understand the molecular mechanisms of aging in the cells. This is to better comprehend and utilize MSCs for therapeutic purposes.

MicroRNAs (miRNAs) are non-coding RNAs that have an average length of 20 to 30 nucleotides. Typically, miRNAs are synthesized from DNA sequences into primary miRNAs (pri-miRNAs) and then further processed to form precursor miRNAs (pre-miRNAs) and mature miRNAs[7]. To regulate expression, it inhibits protein translation or induces the cleavage of the target mRNA. Typically, it inhibits the expression of the target gene by interacting with the 3' UTR of the mRNA[8, 9]. However, it has been reported that interactions have also been observed with other regions, such as 5' UTRs, coding sequences, and gene promoters. In addition, miRNAs have been shown to stimulate gene expression in certain conditions[10]. The roles played by miRNAs in various biological processes are important for normal animal development. The miRNAs play an important role in translational regulation and affect stem cells' fate and behavior[11, 12]. Moreover, the miRNA regulates the expression of stemness factors[13, 14]. MiR-145 inhibits stem cell self-renewal and induces differentiation by directly repressing *OCT4, SOX2*, and *KLF4*. Through a regulatory feedback loop, *OCT4* represses miR-145 expression[14].

In a previous study, we identified that human liver-derived mesenchymal stem cells (LD-MSCs) have beneficial properties for liver recovery[15]. These results are consistent with the hypothesis that MSCs derived from specific tissues may be more suitable for repair and regeneration in the same organ due to their innate adaptability to the microenvironment[16-18]. Although LD-MSCs have proven to be promising for liver regenerative application, functional degradation is inevitable because of the nature of MSCs, therefore LD-MSCs require means for maintaining an appropriate state[19].

In my study, cells were examined over time to identify alterations in genes and miRNAs associated with potency and proliferation. This suggests that LD-MSCs should be maintained in a cell state suitable for therapeutic purposes. Consequently, this study aims to expand the functionality of LD-MSCs as a therapeutic source by controlling and maintaining them under the most suitable conditions for treating diseases, thereby improving treatment efficacy. The selected miRNAs are expected to regulate the cellular senescence of LD-MSCs.

Material and Methods

Characterization of MSCs

According to the protocol in previous study[15], after dissociating cells with trypsin/EDTA, cells were collected and immunostained with fluorescence-conjugated primary antibodies for 30 minutes at 4°C. Several antibodies were used: PE-CD34, FITC-CD90 and FITC-CD105 (BD Biosciences Pharmingen). Following immunostaining, the cells were washed 3 times with PBS and resuspended in 0.2 ml PBS for analysis. Fluorescence measurements were performed using a FACS Calibur (Becton Dickinson and Company) and analyzed with FlowJo software (ver 10.8.2; Treestar).

Cell culture

Human Wharton's jelly-mesenchymal stem cells (WJ-MSCs) were provided by Center for Cell Therapy (Asan Medical Center, Seoul, Korea). WJ-MSCs were incubated with culture medium which was based on MEM-α supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% Antibiotic-Antimycotic (A/A; Gibco. The LD-MSCs were obtained in a previous study. LD-MSCs were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS; Gibco), 10 ng/ml fibroblast growth factor 2 (FGF2; Peprotech), 1% Non-Essential Amino Acid (NEAA; Gibco), 1% Antibiotic-Antimycotic (A/A; Gibco) and 0.1 mM 2-mercaptoethanol (2-mer; sigma). Both types of cells were seeded on 0.1% gelatin-coated culture plate. The growth medium was changed every 2-3 days and maintained at 37 °C in a 5% CO_2 humidified incubator. At 90% confluence, cells were passaged using 0.1% trypsin/EDTA (T/E; Gibco).

Senescence-associated β-galactosidase (SA-β-gal) staining

SA-β-gal activity was analyzed in different passages (P8 and P16) of LD-MSCs using a

Senescence Cells Histochemical Staining Kit (sigma-aldrich), according to the manufacturer's protocol. In brief, the cells were cultured at $1.5x10^5$/well on 0.1% gelatin-coated 6-well plates. After cultured for 2 days, cells were rinsed with PBS and fixed for 6-7 min with the fixation solution in the kit. Subsequently, the cells were washed 3 times with PBS and incubated overnight at 37°C in a CO_2-free chamber with freshly prepared SA-β-gal stain solution. SA-β-gal-positive cells exhibited a blue color.

Determining the cell growth rate

To evaluate the degree of cell growth rate over time, Cells were seeded on 0.1% gelatin-coated plates at $1.5x10^4/cm^2$ and passaged every 3-4 days. Cell numbers were measured by a hemocytometer. The amount of cell number was calculated as the cell growth rate using the formula below.

(Total cell number/Initial cell number)/Cultivation period.

Optical diffraction tomography (ODT) imaging

As described in previous studies[20], ODT was used to reconstruct a 3D refractive index tomography of a cell specimen by using several 2D holographic images of the sample. A commercial ODT microscope (HT-2H; Tomocube) with a 532 nm laser light source was used to measure single living cells under conditions of 37°C temperature and an atmosphere consisting of 5% CO_2.

Adipogenesis and osteogenesis of LD-MSCs *in vitro*

Differentiation of LD-MSCs was accomplished using the Adipogenesis and Osteogenesis differentiation kit (Thermofisher Scientific). To differentiate adipogenesis and osteogenesis, cells were plated on 0.1% gelatin-coated 6-well plate at $7x10^4$/well and $5x10^4$/well, respectively. The differentiation medium was replaced every two days. Based on the

recommendations provided by the kit, differentiation protocols were followed.

Total RNA extraction and quantitative RT-PCR

The total RNA of all stem cells was extracted using a Qiagen RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's instructions. A sample of approximately $1x10^6$ cells was prepared, and QIAzol lysis solution was applied to extract mRNA and miRNA from samples (Qiagen). RNA concentration and purity were quantified spectrophotometrically using the NanoDrop ND-2000 (NanoDrop Technologies).

The complementary DNA (cDNA) was synthesized with a ReverTra Ace qPCR RT Master Mix (Toyobo), and qRT-PCR was carried out with 5× HOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne) utilizing the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories). Based on the delta-delta Ct method, relative expression values were calculated and normalized to GAPDH expression.

MiRNA cDNA was generated with a miRCURY LNA RT Kit (Qiagen), and qRT-PCR was executed with miRCURY LNA SYBR Green using the same detection system as mRNA. To determine the relative amount of RNA in each sample, miRNA expression was normalized to small nuclear RNA (snRNA) U6. It is also based on delta-delta Ct method. All processes were followed according to the protocols supplied by the manufacturers. Each sample was examined in triplicate. The primer sequences are presented in Table 1.

Table 1. Primer information

Name	Primer sequence (5’ to 3’)
mRNA	
GAPDH	F: GAGTCAACGGATTTGGTCGT R: TTGATTTTGGAGGGATCTCG
OCT4	F: GTGGAGGAAGCTGACAACAA R: GCCGGTTACAGAACCACACT
NANOG	F: CAAAGGCAAACAACCCACTT R: TCTGCTGGAGGCTGAGGTAT
SOX2	F: AACCCCAAGATGCACAACTC R: CGGGGCCGGTATTTATAATC
c-MYC	F: GCTGCTTAGACGCTGGATTT R: CACCGAGTCGTAGTCGAGGT
CCNA2	F: CCTGCATTTGGCTGTGAACTAC R: TGCCCACAAGCTGAAGTTTTC
CCNE1	F: CCACACCTGACAAAGAAGATGATGAC R: GAGCCTCTGGATGGTGCAATAAT
CDK2	F: GCTTTTGGAGTCCCTGTTCG R: GGTCCCCAGAGTCCGAAAGA
p21	F: ATGAAATTCACCCCCTTTCC R: CCCTAGGCTGTGCTCACTTC
TP53	F: GTTCCGAGAGCTGAATGAGG R: TGAGTCAGGCCCTTCTGTCT
TGFB1	F: TCGCCAGAGTGGTTATCTT R: TAGTGAACCCGTTGATGTCC
PPARγ	F: AGAAGCCTGCATTTCTGCAT R: TCAAAGGAGTGGGAGTGGTC

Name	Sequence (5’ to 3’)
C/EBPα	F: AACCTTGTGCCTTGGAAATG
	R: CCCTATGTTTCCACCCCTTT
RUNX2	F: TTTGCACTGGGTCATGTGTT
	R: TGGCTGCATTGAAAAGACTG
COL1A1	F: CCAAATCTGTCTCCCCAGAA
	R: TCAAAAACGAAGGGGAGATG
miRNA	
U6	F: GCTTCGGCAGCACATATACTAAAAT
	R: CGCTTCACGAATTTGCGTGTCAT
miR-10a	F: GCAGTACCCTGTAGATCCGA
	R: GGTCCAGTTTTTTTTTTTTTTCAC
miR-16	F: CGCAGTAGCAGCACGTA
	R: CAGTTTTTTTTTTTTTTCGCCAA
miR-145	F: GTCCAGTTTTCCCAGGAATC
	R: AGGTCCAGTTTTTTTTTTTTTTAGG
miR-106b	F: GCAGTAAAGTGCTGACAGTG
	R: GGTCCAGTTTTTTTTTTTTTTATCTG
miR-34a	F: GCAGTGGCAGTGTCTTAG
	R: GGTCCAGTTTTTTTTTTTTTTACAAC

Cell cycle analysis

Cells were seeded at $1.5x10^5$/well at 0.1% gelatin-coated 6-well plate. After culturing the cells for 2 days, cells were washed twice with PBS and fixed with chilled 70% ethanol for 10 min at 4°C, and then washed with PBS and resuspended with PI solution (0.05 mg/ml) containing RNase (0.1 mg/ml), and incubated at 4°C in the dark for 1 h. DNA content was then measured using the FACS Calibur (Becton Dickinson and Company) and analyzed with FlowJo software (ver 10.8.2; Treestar).

Statistical analysis

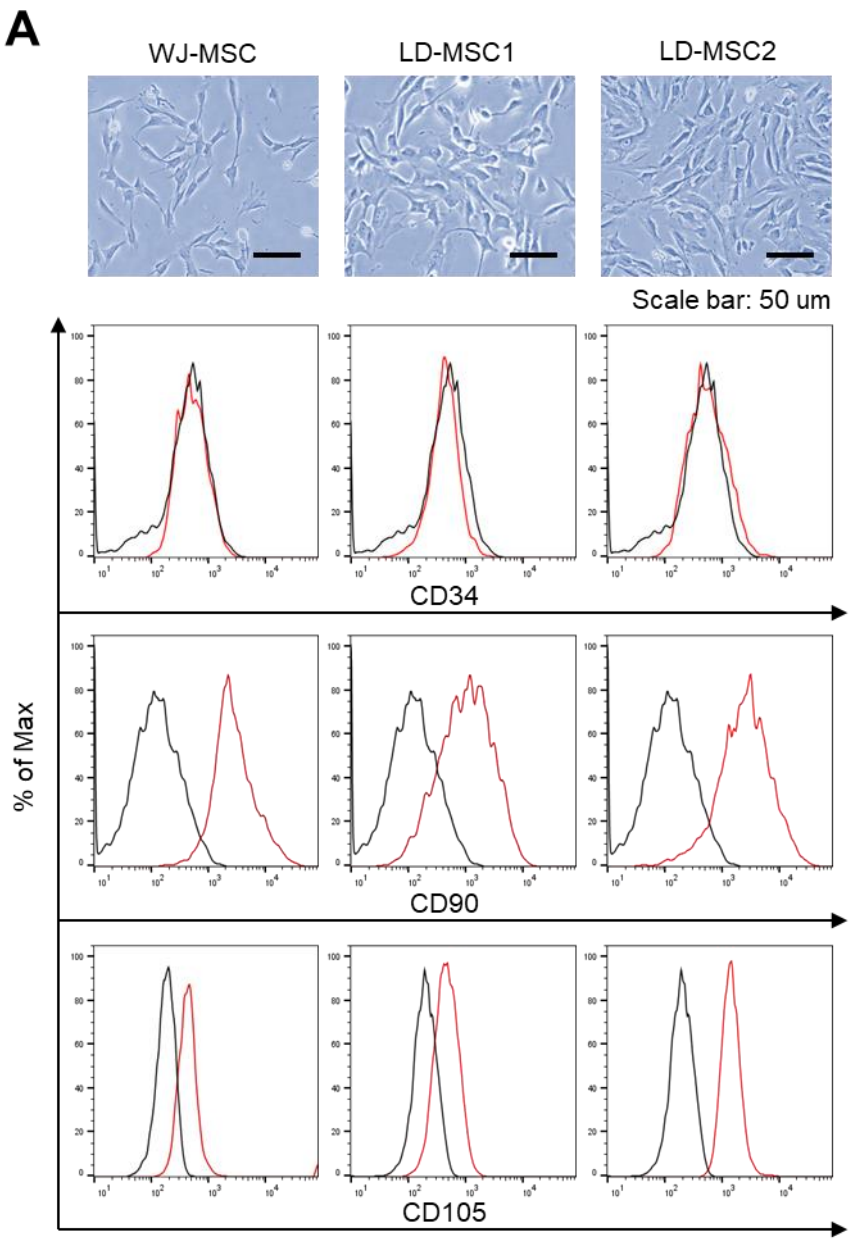
All experimental data were collected from at least three (n = 3) independent biological samples, and the data are presented as means ± standard deviations (SD). A statistical analysis was conducted using GraphPad Prism 8.0.1 software (GraphPad Software). Three or more data sets were compared by one-way or two-way ANOVA followed by Bonferroni's multiple comparison tests. Comparisons between the two groups were made using the two-tailed Student's t-test. $P < 0.05$ was considered statistically significant.

Results

Characterization of Mesenchymal Stem Cells Derived from Different Tissues

To define the liver-derived cells as stem cell, first, I characterized the cells compared with WJ-MSCs which have been characterized by Center for Cell Therapy (Asan Medical Center, Seoul, Korea). Both LD-MSCs and WJ-MSCs displayed morphological similarities (Figure 1A), which were spindle-like shapes with directionality and regularity. MSCs were assessed for MSC characteristics and identification criteria, as established by the International Society for Cellular Therapy (ISCT)[21]. The results were presented by surface marker expression through FACS analysis. CD90 and CD 105, MSC surface markers, were expressed. In contrast, CD34, a marker of hematopoietic stem cells, was negative. This is consistent with previous studies[15, 22]. WJ-MSCs and LD-MSCs both expressed representative stem cell markers such as *OCT4*, *NANOG*, *SOX2*, and *c-MYC*. As compared to WJ-MSCs, all markers were relatively highly expressed in LD-MSCs (Figure. 1B). Moreover, to demonstrate their differentiation potential, LD-MSCs were differentiated into adipogenic and osteogenic cells. Both LD-MSCs-derived differentiated cells were more expressed adipogenic markers (*PPARγ*, *C/EBPα*) and osteogenic markers (*RUNX2*, *COL1A1*) than undifferentiated LD-MSCs; (Figure 1C).

As mentioned above, although there may be differences depending on the organism from which the cells originate, the characteristics of LD-MSCs are similar to those of WJ-MSCs. The characteristics of senescent MSCs were observed after verifying the general features of MSCs[23, 24].


A
WJ-MSC
LD-MSC1
LD-MSC2
Scale bar: 50 um
% of Max
CD34
CD90
CD105

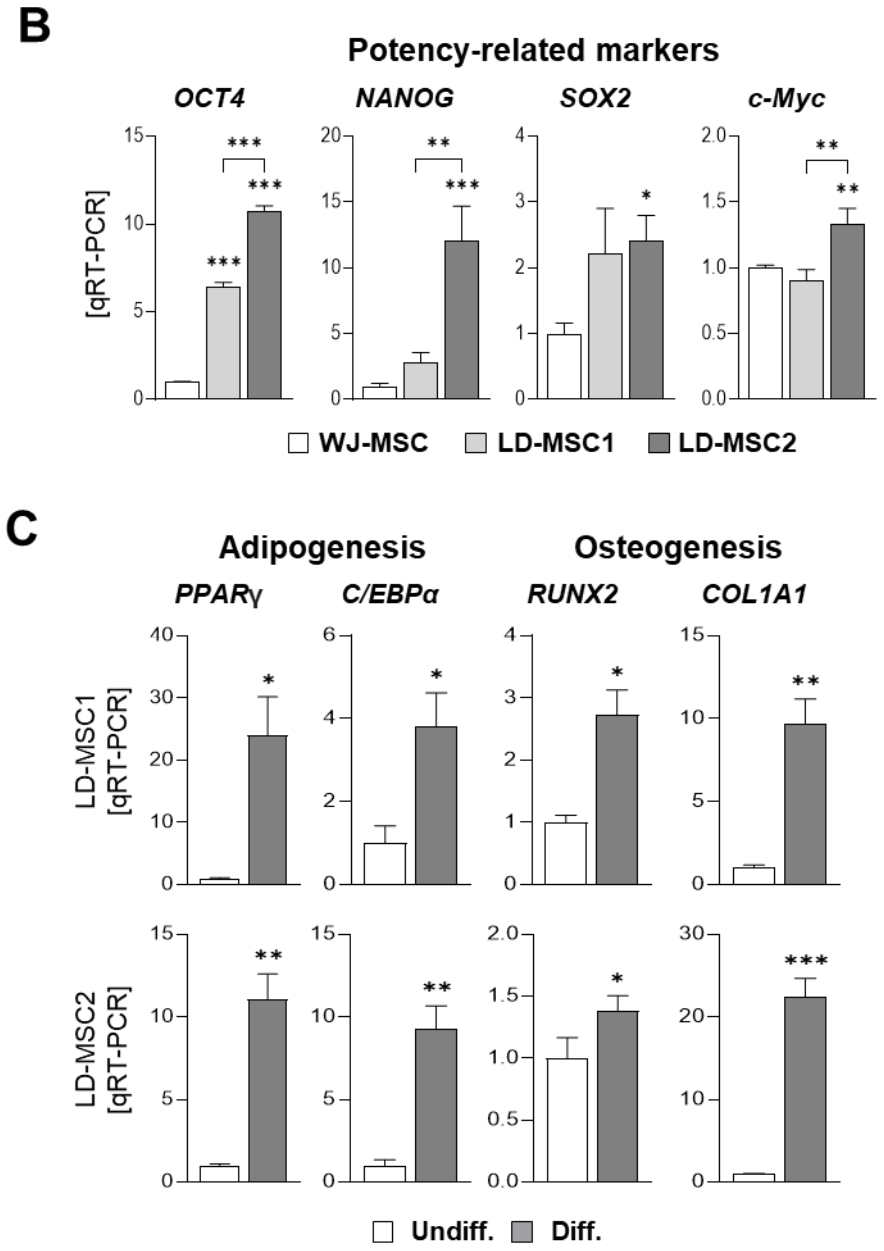

B
Potency-related markers
OCT4
NANOG
SOX2
c-Myc
[qRT-PCR]
WJ-MSC
LD-MSC1
LD-MSC2
C
Adipogenesis
Osteogenesis
PPARγ
C/EBPα
RUNX2
COL1A1
LD-MSC1 [qRT-PCR]
LD-MSC2 [qRT-PCR]
Undiff.
Diff.

Figure 1. Comparison of the characteristics between human liver-derived stem cells (LD-MSCs) and Wharton's jelly -derived mesenchymal stem cells (WJ-MSCs).

(A) Microscopy imaging of human Wharton's jelly derived stem cell (WJ-MSCs) and human liver-derived stem cells (LD-SCs) isolated from three different individuals. Scale bar, 50 μm. Flow cytometry measurement of WJ-MSCs, and two LD-SCs. CD34 was labeled as a hematopoietic stem cell marker. CD90 and CD105 were labeled as mesenchymal stem cell markers. (B-C) Quantitative real-time PCR (qRT-PCR) analysis of (B) pluripotency markers *(OCT4, NANOG, SOX2* and *c-Myc*) and (C) adipogenesis-related (*PPARγ, C/EBPα)* and osteogenesis-related (*RUNX2, COL1A1)* genes. P-values < 0.05 were considered significant. n = 4, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Cell properties alteration of senescent LD-MSCs

Senescence of MSCs is characterized by a series of distinct phenotypic changes, such as enlarged and flattened shapes, and loss of proliferative capacity[23, 24]. In LD-MSCs, the same characteristics were identified. As the culture progressed, the small spindle-shaped cells at the beginning grew larger (Figure. 2A). I also confirmed a cellular senescence in LD-MSCs using β-galactosidase staining. Senescence-Associated β-galactosidase (SA-β-gal) is activated in senescent cells[25], and they can be appeared as a blue precipitate when β-galactosidase is combined with a substrate, such as X-gal. In the result, SA-β-gal was not stained in the early passage, whereas SA-β-gal was expressed in the late passage cells (Figure. 2B).

Moreover, we observed the cells using optical diffraction tomography (ODT) to identify changes in the internal structure of cells. A non-invasive quantitative phase imaging (QPI) technology, ODT is widely used in biology and medicine. ODT reconstructs phase difference images of cells using digital holographic information and a backlight scattering principle to provide details about living cells as well as fixed cells, such as their refractive indexes and volumes. With this principle, cell structures can be separated by different RI values[20, 25]. In late passage cells analyzed using ODT, the enlargement of the nucleus and multinucleation (red arrow), the reduction of lipid droplets (green arrow), which regulate cell metabolism, and the number of vacuoles (blue arrow) can be observed (Figure. 2C). All of these changes are characteristic of senescent cells[23, 24]. The above changes are characteristic of senescent cells and clearly distinguish them from the cells of the early passages. As well as the morphological changes of the cells, the expression levels of *OCT4* and *c-Myc* were also significantly reduced in late passage cells (Figure. 2D).

These findings suggest that senescent cells may have lost their stem cell-related function and differentiation potential. In addition, it can be inferred that cell morphology

and stem cell function are related.

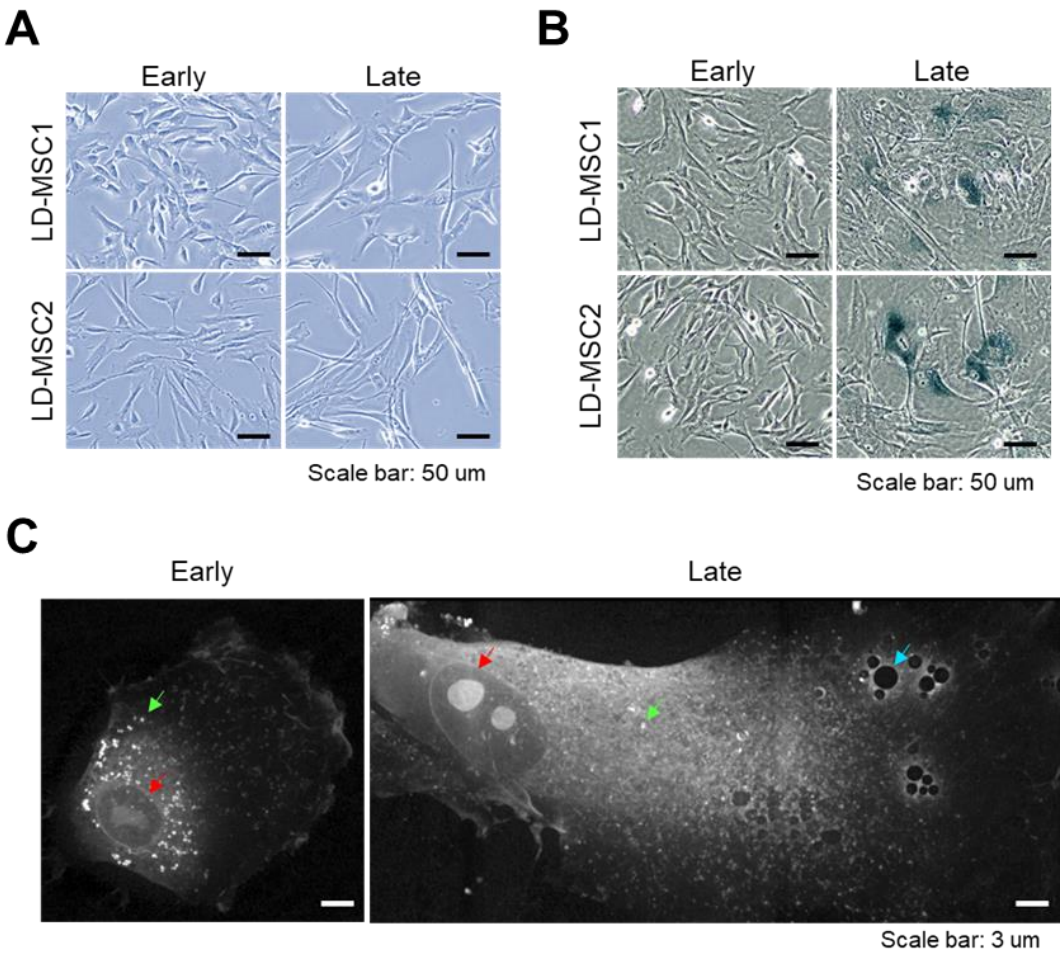

A
Early
Late
LD-MSC1
LD-MSC2
Scale bar: 50 um
B
Early
Late
LD-MSC1
LD-MSC2
Scale bar: 50 um
C
Early
Late
Scale bar: 3 um
D
Potency-related markers
OCT4
NANOG
SOX2
c-Myc
LD-MSC1 [qRT-PCR]
LD-MSC2 [qRT-PCR]
1.5
1.0
0.5
0.0
*
**
Early
Late

Figure 2. Morphological changes and measurement of differentiation capacity-related genes in senescent LD-MSCs.

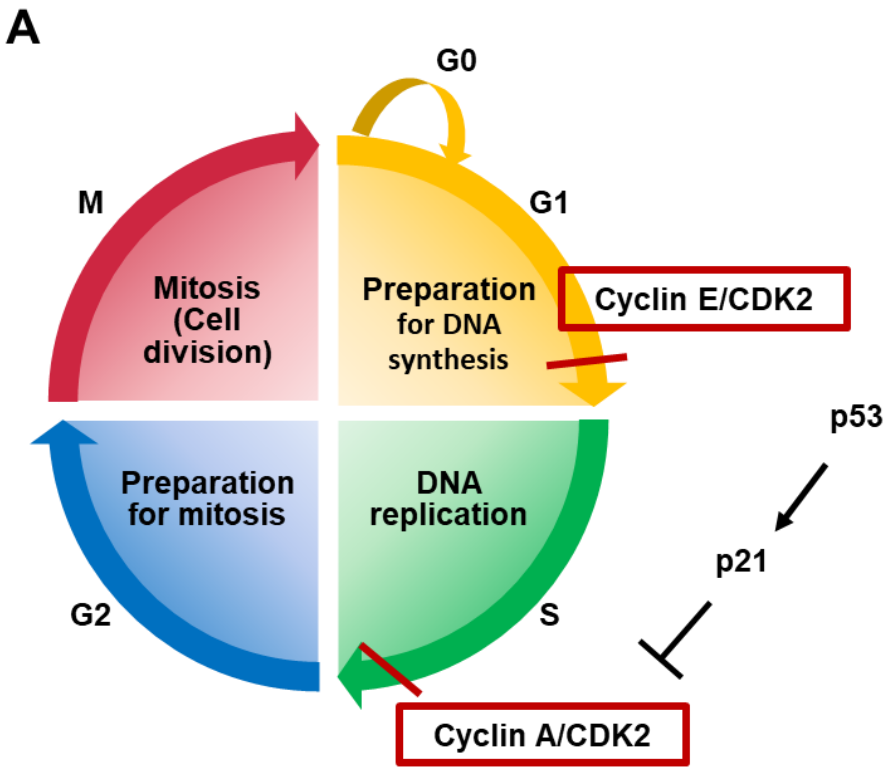
(A) Microscopy imaging of two human liver-derived stem cells (LD-MSC1 and LD-MSC2) over time. Scale bar, 50 μm. (B) SA-β-gal-positive cells could be observed in late passage cells. Scale bar, 50 μm. (C) Cross-sectional slices of the 3D image in the x-y planes of a LD-MSC2 obtained using HT-2 ODT microscope. Scale bar = 3 μm. (D) mRNA expression analysis of pluripotency-related genes according to the passaging. P-values < 0.05 were considered significant. n = 4, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

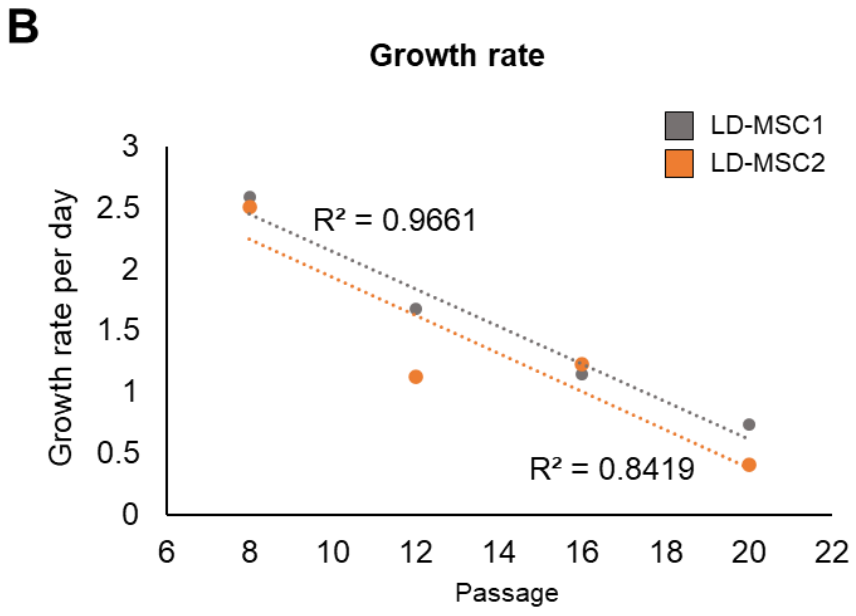
In biology, senescence refers to the process of cells aged and permanently stopped dividing, but not dying. Therefore, the measurement of cell proliferation rate is a good indicator to evaluate the degree of aging. To assess the proliferative capacity of the cells, growth rates were measured. The proliferation rate of both LD-MSCs gradually decreased with passages. In particular, LD-MSC2 was appeared the worst proliferation rate at passage 20 (proliferation rate = 0.4-fold increase, Figure 3B). Decreasing proliferative capacity is associated with changes in the cell cycle[26]. Cell cycle is distinguished by 4 stages, each of which is as the following process: G1, preparation for DNA synthesis; S, DNA replication; G2, preparation for mitosis; M, mitosis (cell division) (Figure. 3A). All cells undergo the cell cycle and finally divide. In the absence of normal passage through each stage, it cannot divide, which naturally lowers its proliferation rate. We investigated the cause of decreased proliferation rates by cell cycle analyzing in the early and late LD-MSCs. The results of cell cycle analysis using flow cytometry revealed alterations in the cell cycle distribution of late passage cells. In the G0/G1 phase, the percentage of cells decreased, whereas the percentage of cells in the S phase increased. It means that an S arrest has occurred. This was confirmed through an increase in the population of cells with increased amounts of DNA contents (Figure. 3C). Additionally, it was observed that the size of late passage cells was larger than that of early passage cells in the same phase (Figure. 3D). These results are also associated with the morphological changes described above in the cells.

Next, cell cycle-related markers were assessed at the gene level. Cyclins and CDKs are key components of the cell cycle machinery that regulates the cell cycle[26]. They are regulated by proteins such as p21 and p53, which are known as cancer suppressors. *Cyclin A* (*CCNA*) and *CDK2*, which are involved in the S/G2 phase, were significantly reduced in late passage cells. P21 is a regulatory protein that inhibits the CCNA/CDK2 complex and is induced by p53[26, 27]. There was a marked difference in the expression level of *p21* compared to the early passage cells, however, there was no significant difference in the

expression level of the *TP53* gene encoding p53 (Figure. 3E).

In summary, A decrease in proliferation of late passaged LD-MSCs was associated with an arrest in the S phase. Examining genes involved in regulating the S/G2 phase process, p21 appears to be directly implicated in S phase arrest. Suggesting that cell cycle regulation is necessary for enhancing cell proliferation, and p21 might be an important candidate.


A
G0
M
G1
Mitosis (Cell division)
Preparation for DNA synthesis
Cyclin E/CDK2
p53
p21
Preparation for mitosis
DNA replication
G2
S
Cyclin A/CDK2


B
Growth rate
LD-MSC1
LD-MSC2
R² = 0.9661
R² = 0.8419
Growth rate per day
3
2.5
2
1.5
1
0.5
0
6
8
10
12
14
16
18
20
22
Passage

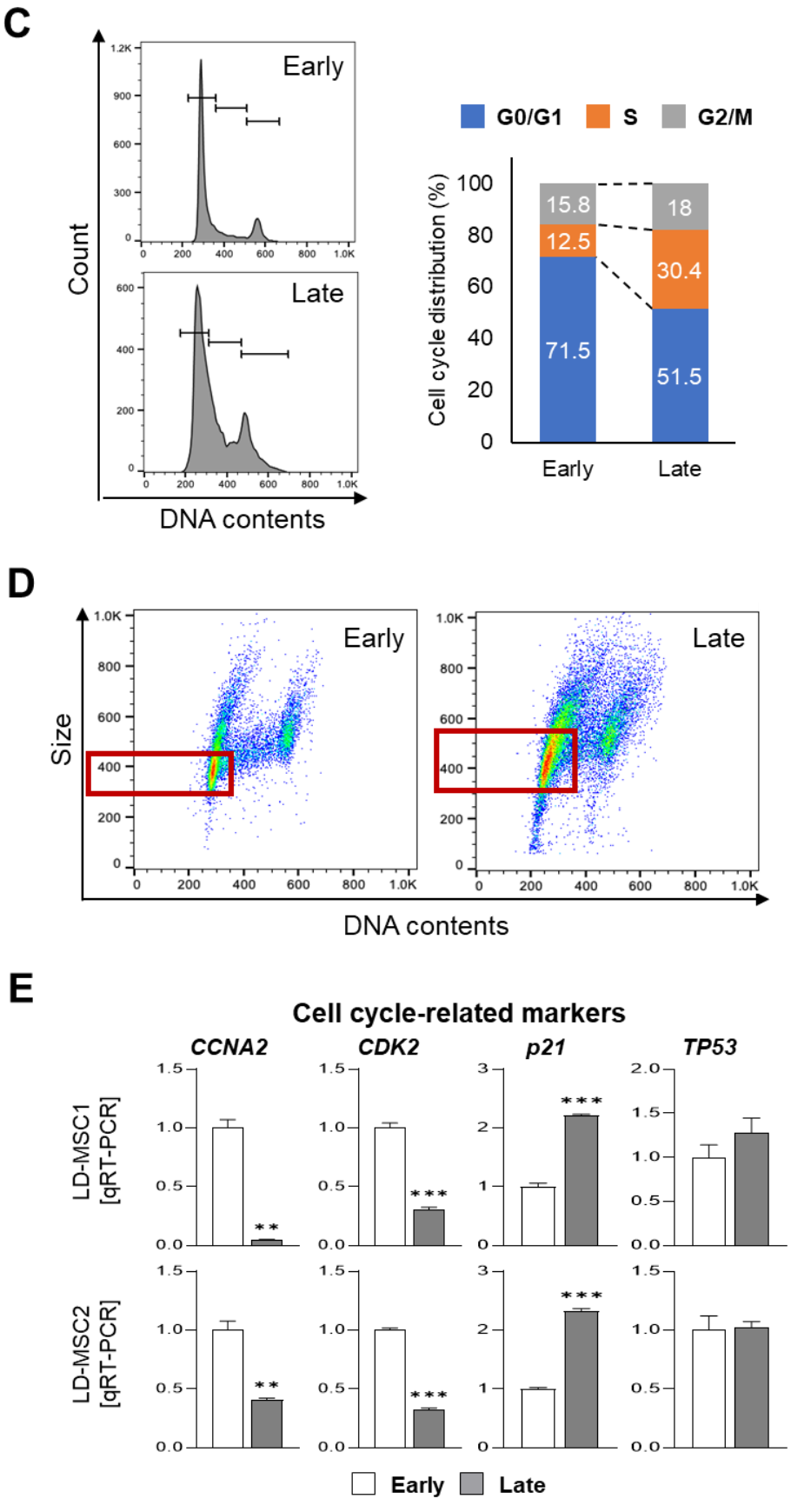

C
Early
Late
Count
DNA contents
G0/G1
S
G2/M
Cell cycle distribution (%)
15.8
12.5
71.5
18
30.4
51.5
Early
Late
D
Early
Late
Size
DNA contents
E
Cell cycle-related markers
CCNA2
CDK2
p21
TP53
LD-MSC1 [qRT-PCR]
LD-MSC2 [qRT-PCR]
Early
Late

Figure 3. Cell growth rate reduction and cell cycle analysis in senescent cells.

(A) Graphical summary of cell cycle phases and involved proteins. (B) Growth rate of LD-MSCs based on passages. (C) DNA content histogram of LD-MSCs, stained by Propidium iodide (PI) in flow cytometry (left panel). Bar graph of percentage of cells in each cell cycle phase (right panel). (D) Pseudo-color plot of early and late passage LD-MSCs. The y-axis represents cell size. (E) qRT-PCR analysis of cell cycle-related (*Cyclin A; CCNA2, CDK2*) genes and regulate (*p21, TP53)* genes. P-values < 0.05 were considered significant. n = 4, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Time-dependent differences in miRNA expression levels

MiRNAs are small non-coding RNA molecules that regulate cellular activity. Through the regulation of mRNA expression, it affects a wide range of biological processes (Figure 4A). MiRNA-induced silencing complex (RISC) is formed by loading the processed miRNA onto the Argonaute (AGO) family of proteins, an essential component of RISC. RISC regulates gene expression by degrading target mRNA or suppressing transcription in accordance with miRNA complementarity.

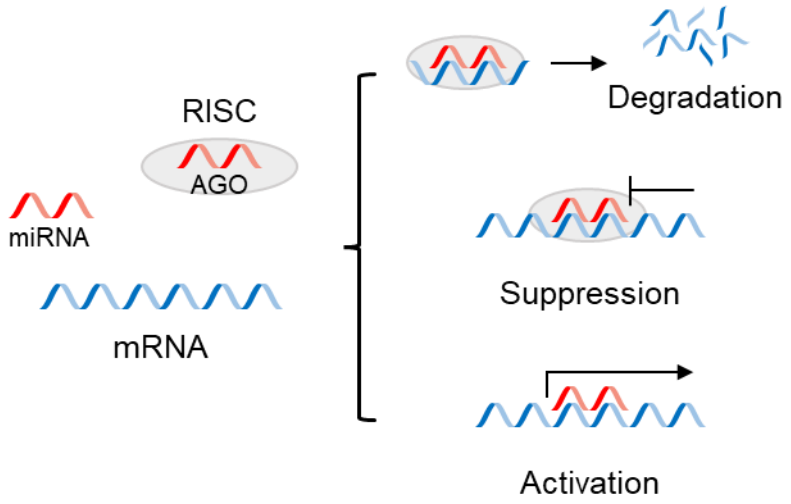
The expression of several miRNAs known to be associated with stem cell potency and p21 was investigated in order to select miRNAs that could regulate this feature in LD-MSCs. MiR-10a improves the differentiation capabilities of MSCs and miR-16 enhances the expression of potency-related genes.[28, 29] Moreover, previous studies have demonstrated that miR-145 inhibits *OCT4*, *NANOG*, and *SOX2*[14]. In particular, miR-106b is a well-known *p21* inhibitory regulator and, conversely, miR-34a induces *p21*[14, 28, 30-32]. These descriptions make it possible to predict the expression level of miRNAs in early and late passage cells (Figure. 4B). To be able to compare clearly, miRNA expression was also examined in hiPSC. Despite numerous passages and long-term culture, hiPSC remain undifferentiated and constantly divide. Therefore, functional differences in MSCs can be assessed relatively.

In the case of miR-10a, it was measured that the expression level was relatively higher in LD-MSCs than in WJ-MSCs, and miR-145, which suppresses the expression of potency-related genes, was found to be low in LD-MSCs (Figure. 4C). These results showed the same tendency as the mRNA expression level of potency-related markers (Figure. 1B). MiR-106b, miR-302 family, and miR-34a regulate *p21*, which inhibits cyclin/CDK complex[33]. As compared to WJ-MSCs, the expression levels of miR-106b and miR-34a were higher in LD-MSCs, however, there were significant differences between

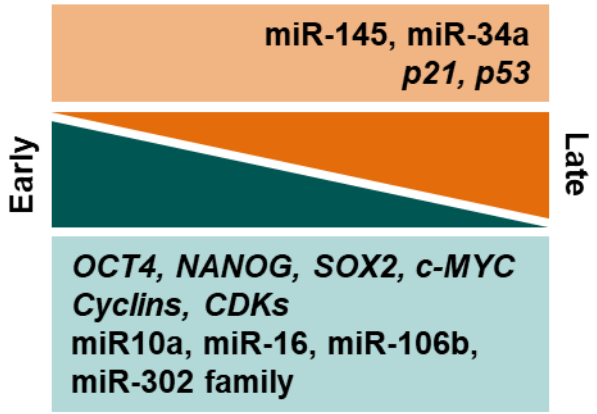
LS-MSC1 and LD-MSC2. Interestingly, the miR-302 family is rarely expressed in LD-MSCs. This suggests the existence of specific miRNAs that can regulate LD-MSCs (Figure 4C). As we were interested in miRNAs present in LD-MSCs, we excluded miR-302 from the list of miRNAs that can regulate *p21*. In examining the expression level of potency-related miRNAs over time, it was generally determined that there was a difference between early and late passage cells, however, it was difficult to specify a tendency since the expression status varied for each type of cell. In contrast to what was expected, miR-106b was not substantially decreased, although miR-34a increased (Figure 4D). The findings suggested that miR-34a can regulate p21 in senescent LD-MSCs.

Taken together, the expression patterns of MSCs and hiPSC are clearly distinctive, and even in cells of the same type, there are differences depending on the individual. The miR-34a is more abundant in late passage LD-MSCs and has the potential to regulate senescence of LD-MSCs.

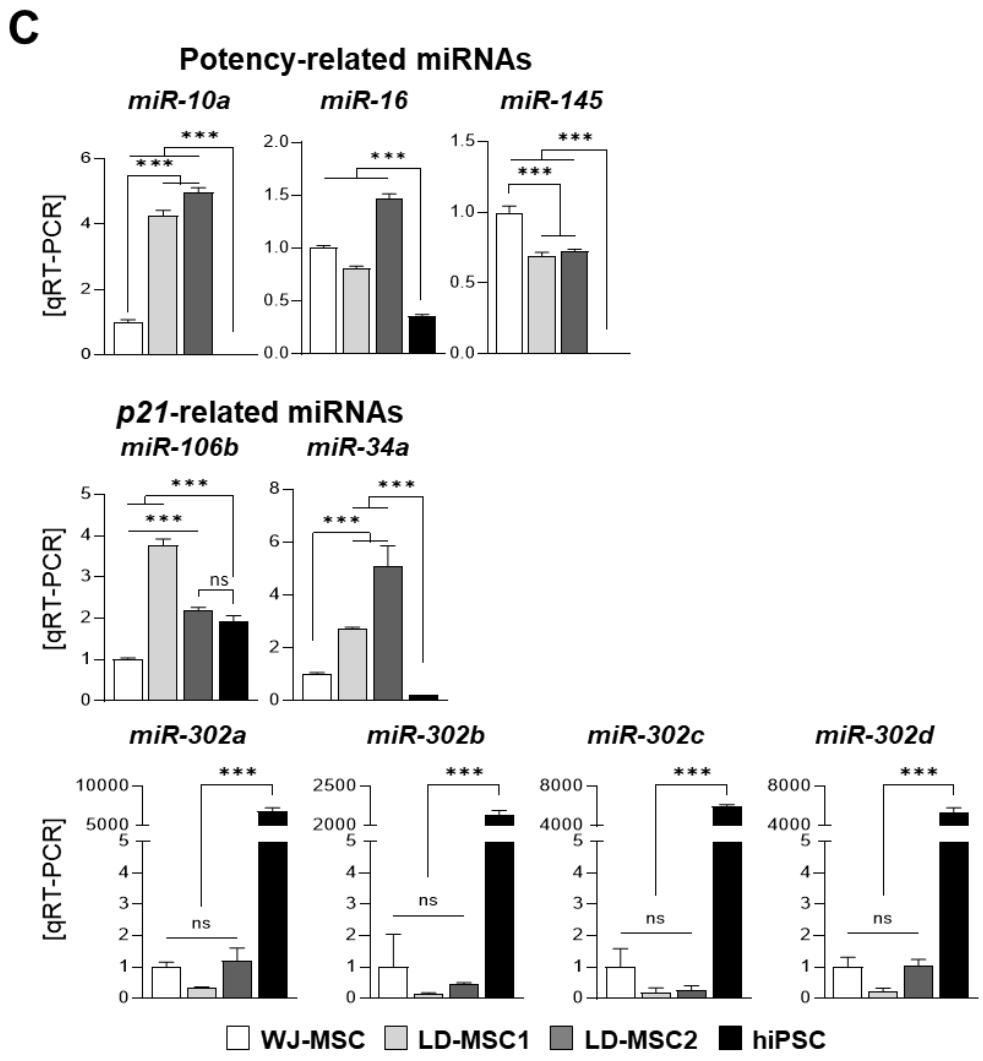
A



B



- miR-10a: MSC potential upregulation
- miR-16: Induce the expression of potency-related genes
- miR-145: *OCT4, NANOG, SOX2* suppression
- miR-34a: *p21* induction
- miR-106b, miR-302 family: *p21* suppression


C
Potency-related miRNAs
miR-10a
miR-16
miR-145
[qRT-PCR]
p21-related miRNAs
miR-106b
miR-34a
miR-302a
miR-302b
miR-302c
miR-302d
***
ns
WJ-MSC
LD-MSC1
LD-MSC2
hiPSC

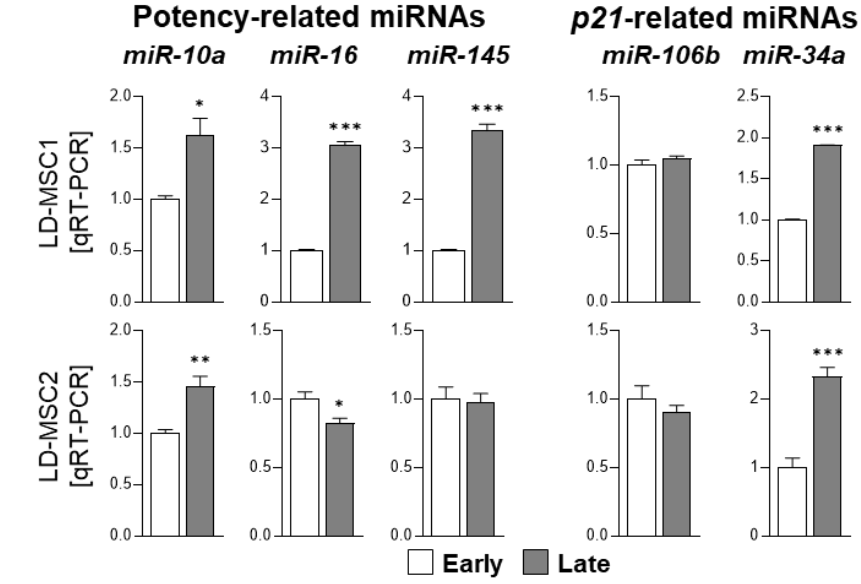

D
Potency-related miRNAs
p21-related miRNAs
miR-10a
miR-16
miR-145
miR-106b
miR-34a
LD-MSC1 [qRT-PCR]
LD-MSC2 [qRT-PCR]
Early
Late

Figure 4. miRNA expression levels in time.

(A) miRNA regulatory mechanism. (B) Gene expression prediction based on culture time. (C) miRNA expression analysis of pluripotency-related (miR-10a, miR-16, miR-145) and p21-related (miR-106b, miR-34a, miR-302 family) miRNA according to the cell types. (D) miRNA expression analysis of pluripotency-related and p21-related miRNA according to the time-dependent. P-values < 0.05 were considered significant. n = 4, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

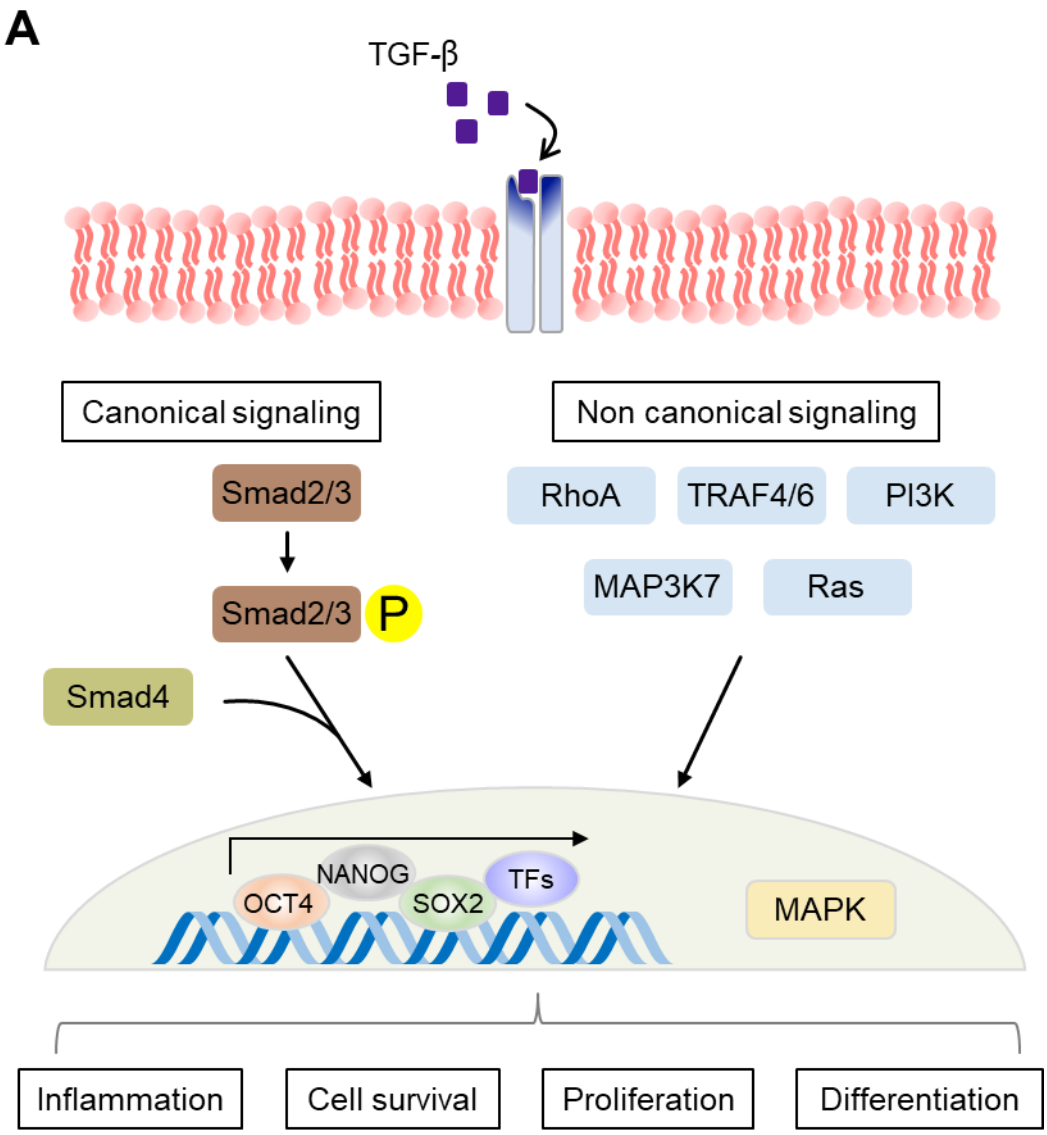
TGF-β1 treatment induces cellular activity

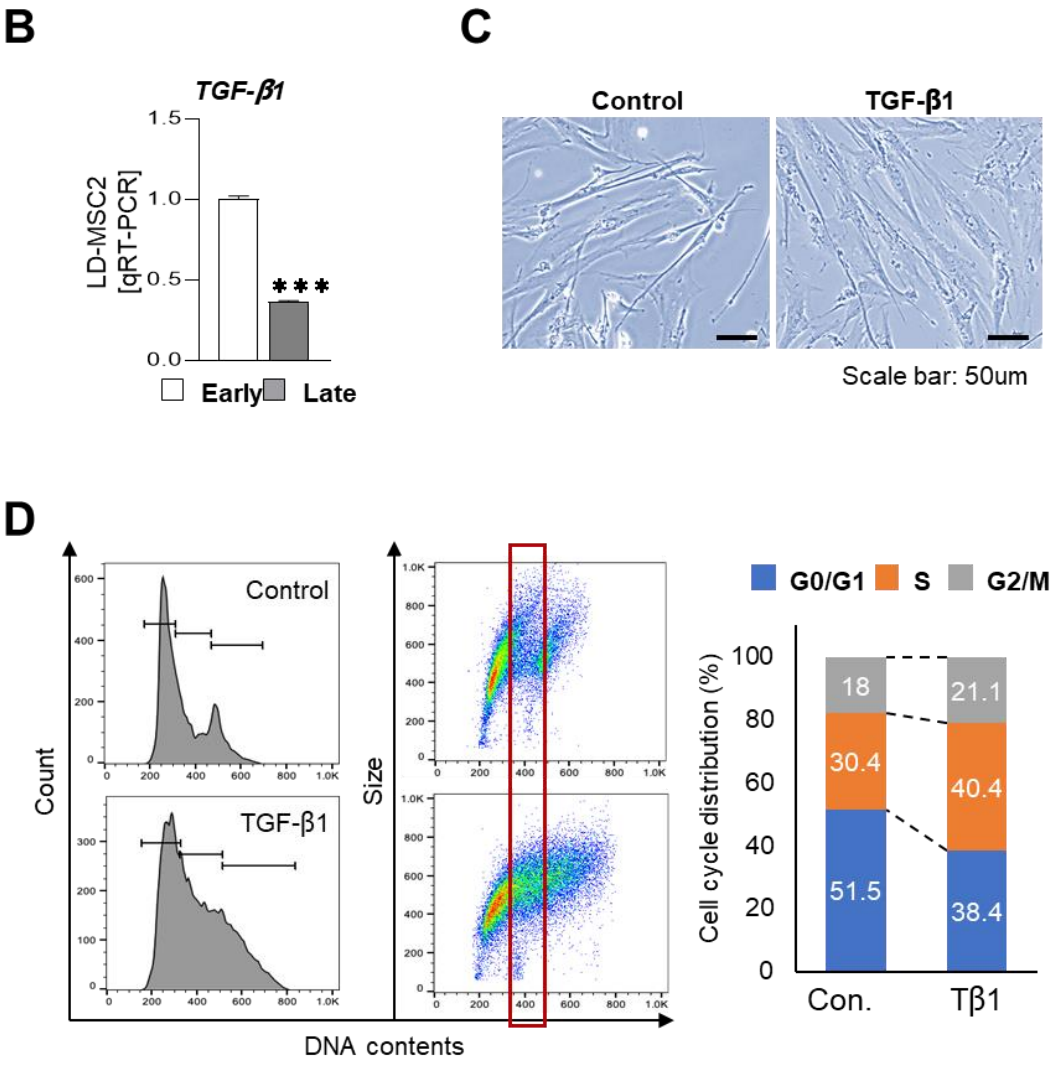
In a wide range of biological systems, TGF-β signaling is known to play a critical role in the regulation of cell growth, differentiation, and development. In particular, it regulates a network of transcription factors (TFs) that are related to undifferentiated cells, such as OCT4, NANOG, and SOX2, and stimulates the proliferation of cells (Figure 5A)[34]. For this reason, the mRNA expression of TGF-β1 in aged LD-MSC2 was first investigated (Figure. 5B). Late passage cells showed significant reductions in the expression of TGF-β1, as expected. When cultured in a culture medium supplemented with TGF-β1, morphological changes such as flattening of the cells were observed compared to before treatment (Figure. 5C). Cell cycle analysis exhibited a decrease in the number of cells in G0/G1 phase and an increase in the number of cells in S phase (Figure 5D).

In addition, mRNA and miRNA expression differed. As compared to the late passage cells, the TGF-β1 treated cells exhibit higher expressions of *OCT4*, *NANOG*, and *c-Myc* (Figure. 5E). A shift in cell cycle distribution coincided with the increase in *CCNE1*, which is involved in the G1/S phase. In spite of the significant increase in *CCNA2* expression in the S/G2 phase and a decrease in *p21* expression as much as the expression level in the early passage, the number of cells in the G2/M phase did not increase (Figure 5F). It appears that the cell cycle is regulated by other factors besides cyclins/CDKs. MiRNAs involved in these mRNAs also lost their directionality. Particularly, potency-related miRNAs showed opposite levels of expression of mRNA. Furthermore, miR-34a, which induces *p21*, and miR-106b, which inhibits *p21*, both decreased, indicating conflicting results.

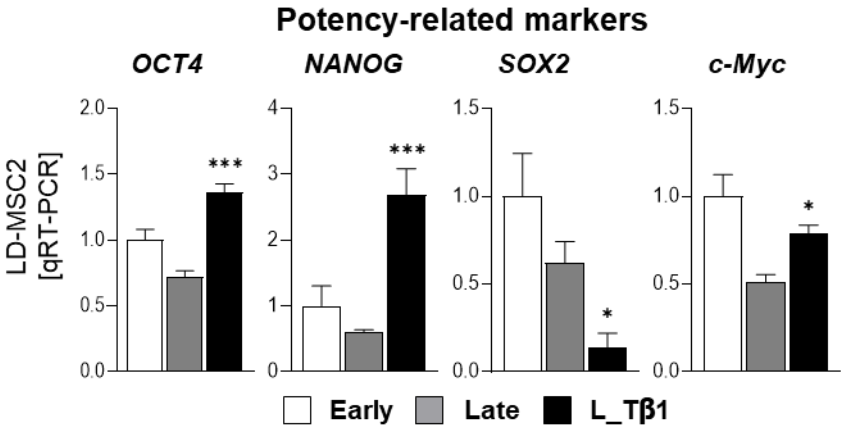
In conclusion, the effect of TGF-G1 on cell function appears to be more direct and more effective than expected. Considering the combined synergistic effects of other small molecules and miRNAs, TGF-β1 is not considered suitable for regulating cellular functions

in aged LD-MSCs.

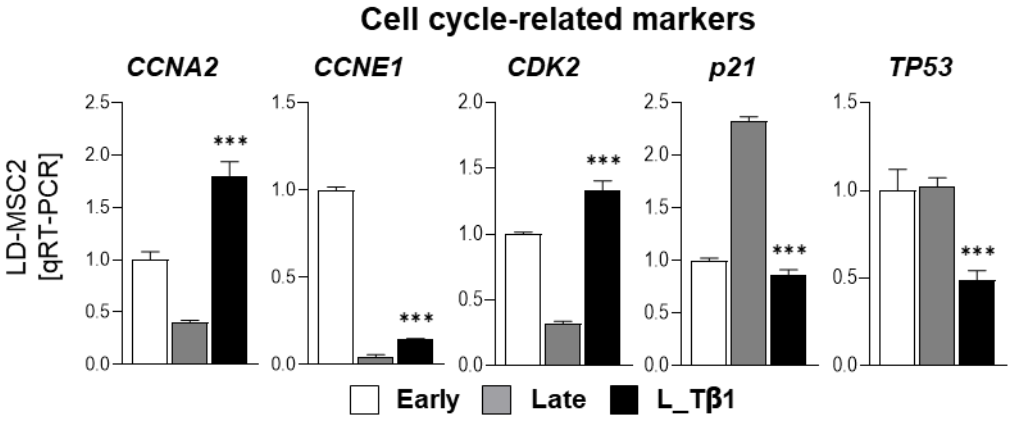

A
TGF-β
Canonical signaling
Non canonical signaling
Smad2/3
Smad2/3
P
Smad4
RhoA
TRAF4/6
PI3K
MAP3K7
Ras
NANOG
OCT4
SOX2
TFs
MAPK
Inflammation
Cell survival
Proliferation
Differentiation


B
TGF-β1
LD-MSC2 [qRT-PCR]
1.5
1.0
0.5
0.0
***
Early
Late
C
Control
TGF-β1
Scale bar: 50um
D
Control
TGF-β1
Count
Size
DNA contents
G0/G1
S
G2/M
Cell cycle distribution (%)
100
80
60
40
20
0
18
30.4
51.5
21.1
40.4
38.4
Con.
Tβ1

E


Potency-related markers
OCT4
NANOG
SOX2
c-Myc
LD-MSC2 [qRT-PCR]
Early
Late
L_Tβ1

F


Cell cycle-related markers
CCNA2
CCNE1
CDK2
p21
TP53
LD-MSC2 [qRT-PCR]
Early
Late
L_Tβ1

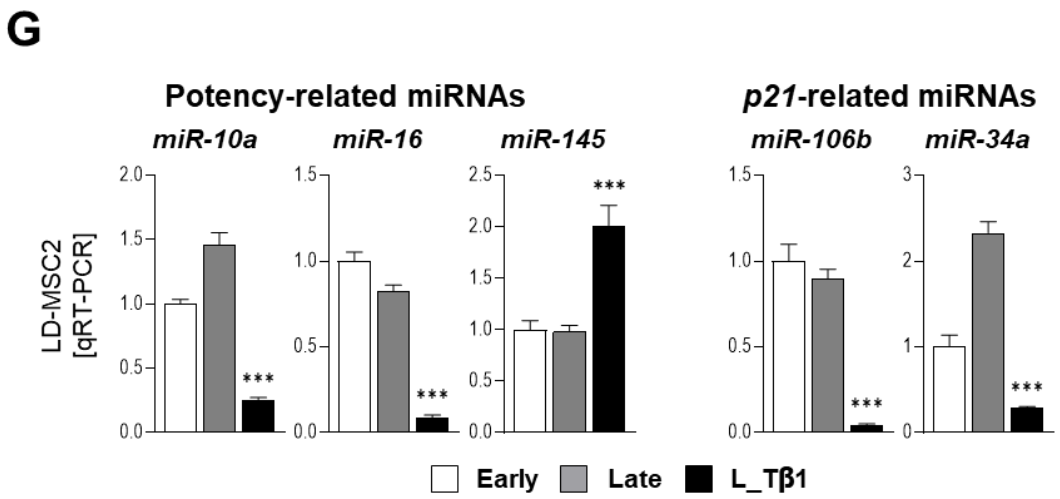


Figure 5. Cell features changes after TGF-β1 treatment.

(A) TGF-β signaling pathway in cells. (B) mRNA expression analysis of *TGF-β1* according to the passaging. (C) Microscope image of morphological changes in TGF-β1-treated cells. (D) DNA content histogram of control and TGF-β1-treated LD-MSCs in flow cytometry (left and middle panel). Percentage of cells in each cell cycle phase (right panel). (D) Dot plot of early and late passage LD-MSCs. The y-axis represents cell size. (E-G) qRT-PCR analysis of potency-related genes, cell cycle-related genes, and miRNAs. P-values < 0.05 were considered significant. n = 4, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Discussion

As with previous studies[15], this study is based on the widely regarded hypothesis that MSCs derived from a particular organ may be more suitable for tissue repair and regeneration in that organ because they are already attuned to their innate microenvironment[16-18]. The LD-MSCs used in this study were from a previous study, and they maintained their markers (CD90 and CD105), potential, and proliferative efficiency after freezing[15, 22]. It appears that LD-MSCs are capable of contributing as a cell therapy source indefinitely, provided they maintain a certain level of function. As previous studies have demonstrated that LD-MSCs are more likely to differentiate into hepatocyte-like cells, they can be a promising source of liver-specific stem cells[15]. There is, however, a problem of functional degradation of senescent MSCs, so methods and mechanisms are being investigated to rejuvenate senescent MSCs. Senolytic drugs with antioxidant properties are used to activate senescent cells[35], or to initialize MSCs by reprogramming[36]. The aim of the study was also to improve the function of MSCs by regulating the factors that contribute to aging.

In this study, LD-MSCs were evaluated as a potential source of cell therapy for liver regeneration as they had a higher potential than WJ-MSCs. The potential and proliferative efficiency of the cells decreased with long-term culture, and changes such as enlargement were observed, as would be expected with an aging phenotype of general MSCs[23, 24]. ODT imaging demonstrated that lipid droplets, which regulate cell metabolism, were significantly reduced when inspecting intracellular changes. These results allow us to speculate that cell function may have declined before examination through other molecular experiments. Even though it can be difficult to relate cell morphology to function, understanding structural changes within cells can lead to better comprehension of cell function, since all mechanisms for functioning occur within cells.

Cell proliferation is correlated with the cell cycle, and cell cycle analysis of LD-MSCs revealed that they were arrested at the S/G2 phase. *Cyclin A (CCNA2)/CDK2*, which are involved in the S/G2 process, were substantially decreased, whereas *p21*, which inhibits them, increased significantly[27]. Meanwhile, no significant changes were observed in *TP53*, which encodes a gene for p53 that induces p21, indicating that factors other than p53 are responsible for regulating p21. It also suggests that MSCs are capable of regulating the activation of oncogenes by senescence and ensuring their stability.

Induced pluripotent stem cells (hiPSC) were used to determine clear differences with MSCs. MiR-10a has been reported to restore the differentiation efficiency of MSCs by targeting *KLF4*, whereas miR-145 suppresses it by directly targeting *OCT4*, *SOX2*, and *KLF4*. Interestingly, even though the target of the two miRNAs is the same as KLF4, the results illustrate that the expression patterns of miRNAs differ according to the type of cell[14, 28, 30]. This result is also consistent with the expression status of cell cycle-related miRNAs. Despite Mir-106b being well known as a miRNA that inhibits *p21*, no difference was observed in this study. Instead, miR-34a, a miRNA that induces *p21* expression, was increased. This is linked to the results of TP53-independent *p21*, indicating the possibility that the cell cycle of LD-MSCs can be regulated by miR-34a.

Transforming growth factor β factors (TGF-β1, TGF-β2, and TGF-β3) are cytokines that play multiple roles in the regulation of a variety of biological processes across a wide range of cell types. Actin cytoskeleton reorganization is one of the earliest cellular responses to TGF-β signaling, and induces cell morphological changes as shown in this study through rapid actin polymerization. Also, when TGF-β signaling is activated, Smad3-mediated transcription is induced, and the expression of TFs such as *OCT4* and *NANOG* is increased, which is consistent with previous findings[34, 37]. However, the expression of *c-Myc*, a proto-oncogene, is also increased, which may decrease the stability of MSCs. TGF-β1 treatment increased the expression of genes involved in G1/S and S/G2 phases, and the

expression of *p21*, a S/G2 phase inhibitor, was also reduced to the level of early passage cells, which expected resumption of the cell cycle, but continued S phase arrest was observed. These results seem to show multiple effects because TGF-β is involved in several biological processes. Therefore, future studies will use miRNAs targeting the sub-pathway instead of direct TGF-β treatment to narrow down the affected range and identify more effective regulators.

Taken together, although the regulation of pluripotency-related genes through miRNAs has not been clearly elucidated, miR-34a regulates the senescence-related gene *p21*, suggesting that miR-34a could be a potential senescence regulator in future studies on senescence mechanisms. It is difficult to conclude that there is a direct correlation between efficacy and proliferation of LD-MSCs. However, according to research results that overexpression of cell cycle proteins or inactivation of inhibitors increase reprogramming efficiency during somatic cell reprogramming[26], it is considered efficient to first increase proliferation efficiency and increase differentiation potential in order to maintain MSCs function. In future research, we will discover candidates that can put the cell cycle on the right track and identify mechanisms that can control the potential based on the results. Providing patients with a source of cell therapy with strong regenerative potential could overcome existing limitations and enhance the use of cell-based therapies.

Conclusion

In this study, miR-34a was found to have the ability to regulate senescence within LD-MSCs by regulating p21 expression. This is meaningful for the development of MSCs-based cell therapy. Despite being recognized as a stable and promising cell therapy source, MSCs are difficult to culture for long periods of time, which is a chronic problem with MSCs. In light of the difficulty of isolating MSCs each time, senescence control may prevent functional degradation of MSCs due to senescence. Furthermore, miRNAs that are naturally present in cells can be used to produce safer raw materials.

Therefore, the discovery of LD-MSCs-specific miRNAs that regulate senescence will be an important indicator of the ability to maintain the unique characteristics of LD-MSCs and to effectively generate differentiated cells from LD-MSCs.

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국문요약

중간엽 줄기 세포 (mesenchymal stem cell)는 줄기 세포의 특성과 면역 조절 특성을 가지고 있기 때문에 다양한 질환에 광범위하게 활용될 수 있다. 그러나 잠재력에 비해 장기간 기능을 유지하기 어렵다는 단점이 있어 많은 연구자들이 MSC의 노화를 이해하기 위한 노력을 지속하고 있다. 세포 노화에서 마이크로 RNA (miRNA)의 역할을 조사하기 위해 많은 연구가 수행되었으나 MSC의 노화에서 이들이 수행하는 역할은 명백히 규명되지 않았다. 본 연구에서는 세포 노화를 모니터링하여 노화 과정에서 조절 역할을 할 수 있는 miRNA에 대해 살펴보고 조절 인자를 탐색하고자 하였다.

초기 계대 세포와 후기 계대 세포는 뚜렷한 형태학적 변화가 관찰된다. 세포의 내부 구조를 분석한 결과, 세포의 핵 크기 증가, 다핵화, 액포가 관찰되었고 세포 대사를 조절하는 것으로 알려진 lipid droplets이 후기 계대 세포에서 감소하는 것으로 나타났다. 또한 분화능 관련 유전자인 OCT4와 c-Myc의 발현 수준이 감소하여 노화 세포에서 세포의 기능이 저하 됐음을 알 수 있다. 분화능을 조절하는 miRNAs (miR-10a, miR-16, miR-145)는 계대 초기 세포와 후기 세포에서 차이를 보였지만 발현 패턴이 다양해 명확한 분석이 어려웠다. MiR-34a는 세포 주기의 S/G2 단계를 억제하는 p21을 유도한다. LD-MSCs에서 p21의 발현량도 miR-34a의 양에 따라 증감하는 것을 확인하였다.

종합하면, miRNA를 통한 다능성 관련 유전자의 조절이 명확하게 설명되지는 않았지만 miR-34a는 노화 관련 유전자 p21을 조절하여, miR-34a가 노화 메커니즘에 대한 향후 연구에서 잠재적인 노화 조절자가 될 수 있음을 시사한다.

Abbreviation

ASCs	Adult stem cells
c-Myc	MYC proto-oncogene
C/EBPα	CCAAT/enhancer binding proteins alpha
CCNA	Cyclin A
CCNE	Cyclin E
CDK2	Cyclin dependent kinase2
COL1A1	Collagen Type I Alpha 1 Chain
ESCs	Embryonic stem cells
iPSCs	Induced pluripotent stem cells
LD-MSCs	Liver-derived mesenchymal stem cells
miRNA	MicroRNA
MSCs	Mesenchymal stem cells
OCT4	Octamer-binding transcription factor 4
ODT	Optical diffraction tomography
PPARγ	Peroxisome Proliferator-Activated Receptor gamma
RUNX2	Runt-related transcription factor 2
SA-β-gal	Senescence-associated b-galactosidase
SOX2	Sex-determining region Y-box 2
TGF-β	Transforming growth factor-beta
TP53	Tumor protein53
WJ-MSCs	Wharton's jelly mesenchymal stem cells