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Low Oxygen Tension Inhibits Senescence in Bone Marrow Mesenchymal Stem Cells (BMSCs)

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Abstract

Stem cells have been used in regenerative medicine but are so few in the body that they require cell culture. Stem cell culture was performed under normal oxygen tension, and passage was carried out until the number of cells was sufficient for therapy. Stem cell cultures under normal oxygen tension do not match the stem cell microenvironment, leading to premature senescence. This study aims to determine the association of low oxygen tension with premature senescence of mesenchymal stem cells (MSCs) through inhibition of p21 expression by HIF-1α. The research method used rabbit bone marrow in New Zealand as a source of MSCs. The results of isolation of MSCs were divided into two groups cultured on normal and low oxygen tension until 10 passages. Cells were identified using flow cytometry cd105 and cd34. At early and late passage, the expression of p21 and HIF-1α were examined using immunofluorescence while senescence was examined using β-galactosidase assay. The results showed that in low oxygen cultures HIF-1α expression increased significantly (p <0.05) while p21 expression decreased significantly (p <0.05) as did the β-galactosidase assay. The conclusion of this research is low oxygen tension culture able to decrease premature senescence culture of invitro stem cells mesenchymal through obstacles p21 by HIF-1α.

Keywords*: p21 expression, HIF-1α expression, late passage, premature senescence.*

Introduction

Mesenchymal stem cells have been used in regenerative medicine because they can differentiate and secretory activity (Richardson et al., 2012). The number of MSCs is minimal (0.01-0.001%) in the bone marrow so it needs to be cultured in vitro. Mesenchymal stem cells have a limited life span during in-vitro culture, resulting in senescence characterized by enlarged and irregular cell form (Wagner et al., 2008). The passage of MSCs also resulted in decreased nucleocytoplasmic ratio, increased cytoplasmic granulation, and debris. Repeated passage during culture will inhibit the growth of MSCs. Aging can lead to changes in the ability to differentiate of MSCs into osteoblasts or adipocytes, resulting in ineffective cell therapy (Zhuang et al., 2015).

The delay of senescence of MSCs while in vitro culture was necessary for improving the success of clinical therapy (Kim et al., 2012). Several studies have been made to inhibit senescence in in-vitro culture with isolation techniques, growth media, and culture conditions (Tsai et al., 2011). The caloric restriction on the culture medium can inhibit aging and prolong the life span of MSCs (Kim et al., 2012). The Culture conditions with various culture conditions by multiple concentrations have been performed (Yan et al., 2012; Yamamoto et al., 2013). Low oxygen tension cultures with a 1-7% O_2 concentration were physiologic conditions for both embryonic stem cells and adult stem cells, whereas the standard oxygen tension culture of 21% O2 concentrations was incompatible with the stem cell microenvironment.

Normal oxygen tension cultures can induce oxidative stress to increase ROS (reactive oxygen species) in cells. ROS is capable of causing DNA damage resulting in early senescence through p53, p21, and p16 proteins (Zhang et al., 2005). The inhibition of ROS can decrease senescence through the down-regulation protein of p21 (Macip et al., 2002). Aging is associated with a decrease in the ability of MSCs for osteogenesis, chondrogenesis, and myogenesis and increasing adipogenesis. The p53/p21 decreases in MSCs originating from old donors or passage of cell culture over 30. This suggests the possibility of cancer stem cell form (Han et al., 2012).

Low oxygen tension induced HIF-1α which plays an essential role

in hypoxic transduction signaling and regulates the development and differentiation (Yamamoto et al., 2013). HIF-1α is regulated by cellular O_2 concentration and determines the transcriptional activity of HIF-α. The HIF-1α research is mainly on cancer cells. The HIF-1α induced during ischemia in tumor progression can induce proliferation, VEGF, and angiogenesis (Ziello et al., 2007; Kakudo et al., 2015; Mathieu et al., 2011). Low oxygen tension culture's effect on the nature of stemness, differentiation, and function in the in vitro culture mechanism is not yet clear because there are differences in the results of some studies that have been done. This difference depends on the concentration of O_2 and stem cell type. Low oxygen tension culture stimulates proliferation and maintains its multipotent state, while other studies show different results in which hypoxia has the potential to promote stem cell differentiation into more specific cells (Csete, 2005; Grayson et al., 2007; Koay et al., 2008; Khan et al., 2007). Therefore, the researchers wanted to analyze whether there was a decrease in p21 expression in stem cell cultures with low oxygen tension so that it could reduce premature cell aging.

Methods

This study used experimental laboratory explorative studies with normal oxygen tension (O_2 21%) and low oxygen tension (O_2 1%) in MSCs cultures taken from the bone marrow of three male New Zealand rabbits aged six months and weight 900 grams to 10 passages. The research was conducted at the Institute of Tropical Disease Science of Airlangga University. This research has obtained a certificate of ethical research from the Faculty of Veterinary Medicine, Airlangga no. 154-KE.

Isolation and Characterization of Bone Marrow MSCs

Briefly, three rabbits were performed in general anesthesia using ether. Bone marrow was taken in the lateral femoral condyle area using a 10 mL syrup that had been given heparin. The aspirate obtained was transferred into a 15 mL sterile tube and diluted with sterile 1x PBS, of the same ratio then centrifuged at 3000 rpm for 15 minutes. The resuspension was transferred to a 15 mL tube which was first given Ficoll-hypaque and then centrifuged at 1,600 rpm for 30 minutes at room temperature. The buffy coat layer formed adds PBS 1x to 15 mL volume. The tube was centrifuged at 1600 rpm for 10 minutes. The pellets were resuspended using a 6 mL culture medium then put into 2-disc culture (10 cm) each 3 mL then added culture medium each 7 mL. One disk culture was fed into a 37 °C temperature incubator of 5% CO_2 and another disc culture was incorporated

into a hypoxic chamber (Biospherix) with a 1% O_2 concentration. Cells were splitting if their growth 80% (confluent). Mesenchymal stem cells identified cd105 positive and cd34 negative using flow cytometry. At early and late passage, stem cell cultures were fixation using 10% formaldehyde for 15 min.

β-galactosidase assay

Examination of senescence of MSCs was done using the β-galactosidase assay. The stem cells in the round glass object were examined using the β-galactosidase enzyme assay kit (Promega) according to the given manual instructions. Cell lysate is prepared by means of cells in glass objects washed using PBS 1x 2 times. The 1x 400-900 µL lysis buffer (RLB) reporter was added to cover the entire surface of the cell and then incubated

at room temperature for 15 minutes. The entire surface of the plate in scrape and cell lysate is inserted into a centrifuge micro tube and then placed in the ice. The vortex tube for 10-15 seconds is then centrifuged at a maximum temperature of 4 °C for 2 minutes and then the supernatant is transferred to another tube. Cell lysate 100 µL was dissolved in RLB 1x 50 µL. The 150 µL cell lysate solution plus 150 µL 2x buffer assay (o-nitrophenyl-β-D-glucopyranoside / ONPG) was then mixed using a vortex. The cell lysate was incubated at 37 °C for 30 min until β-Galactosidase and then hydrolyzed to yellow o-nitrophenol then the reaction was discontinued by adding 500 µL of 1M sodium carbonate. The absorbance result is read on 420 nm using an Elisa reader.

Expression of p21 and HIF-1α

The primary antibody was using anti-p21 rabbit labeled TRITC and anti-HIF-1α rabbit labeled FITC so that cells expressing p21 would be red and that HIF-1α would be green in its cytoplasm. Immunofluorescence was performed by means of the cells on the washed slipcover 3x using 1x PBS 0.1% tween 20 (PBST) for 5 minutes. Cells added 1% BSA in PBST and incubated for 30 min. The anti-p21 antibody labeled TRITC dissolved in 1% BSA in PBST was added to the cell then incubated in humidified chamber temperature of 37 °C for 1 hour. Cells were washed 3x using PBST for 5 minutes and then the cells were seen using a fluorescence microscope (FSX-100 Olympic). Photos obtained are processed using ImageJ2x.

Statistics Analysis

The data obtained in this study is data ratio, normal distribution, and homogeneous so using parametric analysis (ANOVA).

Results

Identification of MSCs

The results of cell identification using flow cytometry showed that cells had cd105 positif and cd34 negative both on normal and low oxygen tension culture with a CD105 value of 47.1% and 28.7%, respectively (Figure 1). This indicates that the isolated cells were mesenchymal stem cells.

Expression of HIF-1α, p21, and Senescence

The expression of HIF-1α of BMSCs rabbit on the normal oxygen tension culture appears in early and late passages although the number of expressions is small whereas in low oxygen tension culture. The intensity of HIF-1α expression in early and late BMSCs in normal oxygen tension culture was 0.4% and 1.3% while in low oxygen pressure culture respectively 5.9% and 16.7% (Figure 2). From the statistical analysis, it was found that the passage significantly ($p < 0.05$) was able to increase HIF-1α expression as well as O_2 concentration (Table 1).

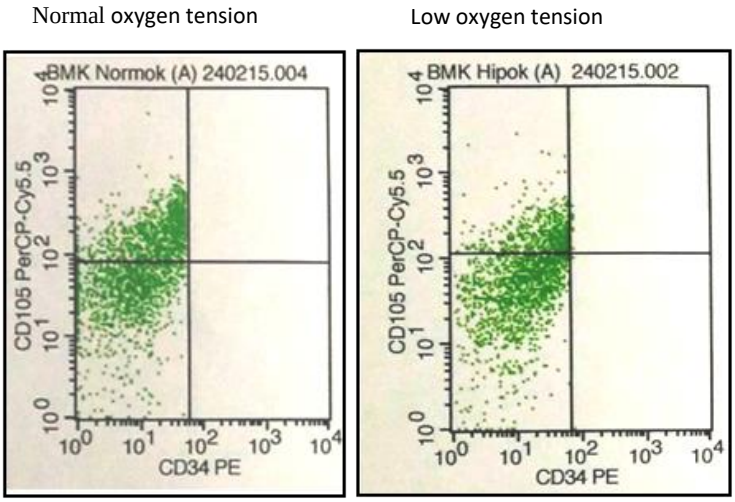

Figure 1. Levels of BMSCs surface antigen of New Zealand rabbits in normal and low oxygen tension cultures using flow cytometry.

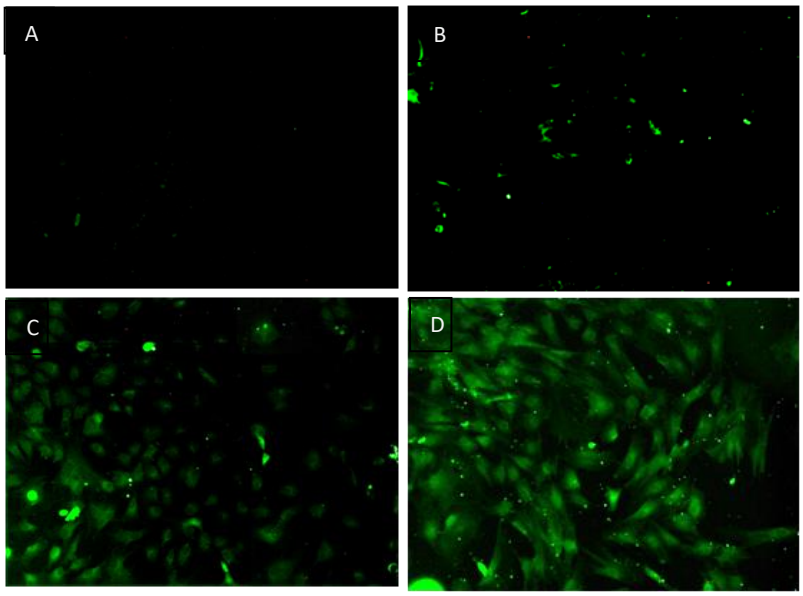

Figure 2. The intensity of HIF-1α expression in early and late BMSCs in normal oxygen tension culture was 0.4% and 1.3% (A and B) while in low oxygen tension culture respectively 5.9% and 16.7% (C and D). Immunofluorescent immune cells were seen using an Olympic microscope FSX-100 at 40x magnification

The enzyme β-galactosidase can identify mesenchymal stem cells with senescence. In this study, apart from identifying β-galactosidase, we also looked at the expression of p21 protein in MSCs in the cytoplasm. The p21 anti-rabbit antibody was labeled TRITC so that cells expressing p21 would glow red. Immunofluorescent immune cells were seen using an Olympic microscope FSX-100 at 40x magnification (Figure 3).

In early in vitro cultures, senescence may be because of cultural passage. This study showed increased p21 expression in the early and late passages. This situation occurs in all normal and low oxygen tension culture conditions. This indicates that the passage of this study did not increase the expression of p21 except in the transition from the early passage to the late passage and low oxygen pressure culture could decrease the expression of p21. The intensity of p21 expression in early and late passages BMSCs in normal oxygen tension culture was 5.5% and 11.2% while in low oxygen pressure culture respectively 1.7% and 4.2% (Figure 3). From the statistical analysis results obtained, passage and O2 tension increased the expression of p21 which is statistically significant p<0,05 (Table 1). The interaction between the passage and O_2 pressure was also statistically significant to increase the expression of p21 BMSCs rabbit.

The level β-galactosidase in this study shows a pattern like p21 expression. This indicates that the change in p21 expression will correspond to β-galactosidase levels. Low oxygen tension cultures can decrease the occurrence of senescence in BMSCs rabbits. The β-galactosidase levels in early and late passages BMSCs in normal oxygen tension culture were 0.98 U/g and 1.09 U/g, while in low oxygen pressure culture, respectively, 0.14 U/g and 0.18 U/g (Table 1). The statistical test results show that the passage and O2 tension can increase the level of β-galactosidase, which is statistically significant (p <0.05 (Table 1). The interaction between the passage and O_2 pressure also

increased the level of β-galactosidase BMSCs rabbit.

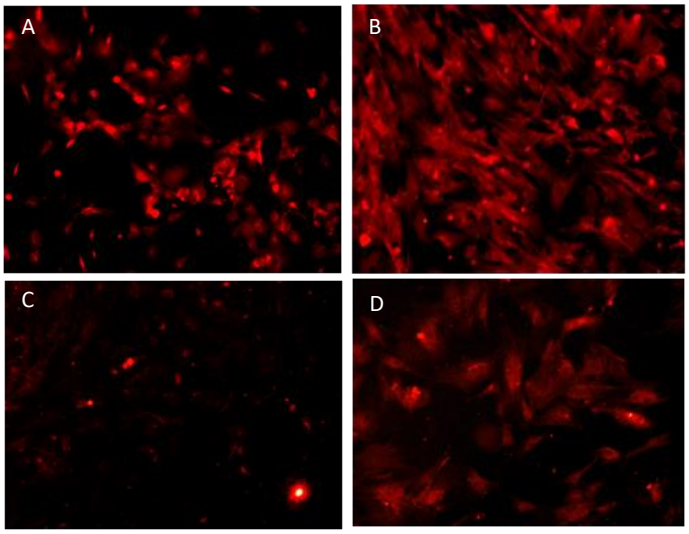

Figure 3. The intensity of p21 expression in early and late BMSCs in normal oxygen tension culture was 5.5% and 11.2% (A and B) while in low oxygen tension culture respectively 1.7% and 4.2% (C and D). Immunofluorescent immune cells were seen using an Olympic microscope FSX-100 at 40x magnification.

Table 1. Mean and analysis result of expression of HIF-1α, p21 and the level of β-galactosidase BMSCs rabbit on early and late passages in normal and low oxygen tension culture.

	Early Passages			Late Passages			p
	HIF-1α	P21	B-galactosidase	HIF-1α	P21	B-galactosidase	
Normal O2 Tension	0,40 ± 0,025^{a}	5,45 ± 0,49^{cd}	0,98 ± 0,05^{d}	1,30 ± 0,19^{b}	11,23 ± 1,43^{e}	1,09 ± 0,09^{de}	0.001
Low O2 tension	5,87 ± 0,89^{d}	1,71 ± 0,23^{a}	0,14 ± 0,02^{a}	16,67 ± 2,01^{f}	4,20 ± 0,39^{b}	0,18 ± 0,02^{ab}	
p				0,001			

Note: The same letter notation shows no significant difference (p> 0.05).

Discussion

The identification of BMSCs showed that the levels of cd105 were higher in normal oxygen tension cultures. This is in accordance with the research done, namely in normal oxygen tension culture of BMSCs. This result is consistent with study conducted by Lapi, 2008 that BMSCs rabbit does not express CD90, whereas BMSCs mice and humans express CD90 (Lapi et al., 2008). In another study , BMSCs in rabbits did not express CD13, CD29, CD59, CD73, CD90, CD105, or CD166 (Lee et al., 2014).

The expression of HIF-1α of BMSCs rabbit on the normal oxygen tension culture was lower than in low oxygen tension culture. The expression of HIF-1α was increased and up-regulation when BMSCs were cultured in low oxygen tension (1% O_2). The passage can increase HIF-1α expression in BMSCs (Gao et al., 2013). The state of low oxygen tension culture can induce HIF-1α expression. The increased expression of HIF-1α is capable of up-regulation of VEGF and FGF-2 proteins (Kakudo et al., 2015). The increased expression of HIF-1α can also induce TWIST expression. Low oxygen tension can prevent premature senescence via HIF-1α in fibroblast cells. The TWIST binds the promoter of E2A so that it inhibits the expression of p21. The barriers to p21 prevented the occurrence of senescence (Tsai et al., 2011).

Factors that play a role in premature stem cell senescence are cell damage during isolation and injection, hypoxia, absence of growth factors, impaired cell-to-cell contact, low vascularity, and pro-inflammatory cytokines. Cell death can be in the form of apoptosis or necrosis, which can be triggered by ischemia, free radicals, and calcium overload, which can be inhibited by growth factors (IGF-1, HGF, GCSF, VEGF), caspase inhibitors, antioxidants, overexpression of Bcl-2, Akt, and HIF-1α (Abraham and Gerstenblith, 2007).

Senescence causes stem cells to lose their ability to stemness and self-renewal, but senescence also protects against uncontrolled growth and cancer. The senescence function is controlled by genes that regulate the cell cycle, namely RB1, RB2/P130, and P107, thereby controlling the expression of p53, p21, and p27. In the human bm MSCs study, it was found that in the absence of the RB1 gene, there would be a decrease in the expression of p53, p21, and p27 which resulted in cell cycle

arrest and DNA damage, while p16 did not experience any changes. This is different from several other studies which state that p16 plays a role in the occurrence of senescence (Alessio et al., 2013).

Premature senescence will result in decreased proliferation, altered chromatin structure, epigenetic changes, and high acid galactosidase activation caused by telomere shortening and oxidative stress. In this study, passage causes telomere shortening because cells are triggered to do mitosis while normal oxygen tension causes oxidative stress (Hernandez-Segura, Nehme, & Demaria, 2018; Herranz & Gil, 2018).

In this study, p21 could be reduced by low oxygen tension culture so as t to inhibit senescence, which was indicated by low levels of β-galactosidase 21 is an important protein for cell survival because p21-deficient mice cause DNA damage and trigger apoptosis (Yosef et al., 2017). In addition, p21 is also able to inhibit senescent cells in the lungs and epidermis (Yosef et al., 2016). HIF1α can directly modulate the cell cycle by targeting the gene from p21. Previous studies have shown that HIF1α can increase hTERT expression in the presence of mitochondrial ROS (Bell, Klimova, Eisenbart, Schumacker, & Chandel, 2007).

Conclusion

Low pressure oxygen culture was associated with decreased senescence in BMSCs through HIF-1a which could regulate the decrease in p21 expression so that senescence could be prevented.

Conflict of Interest

The authors declare no conflict of interest.

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References

Augello, A., Kurth, TB., Bari, C. (2010). Mesenchymal stem cells: A perspective from invitro cultures to invivo migration and niches. *Europan Cells and Materials*, *20*, 121–133.

Bell, E. L., Klimova, T. A., Eisenbart, J., Schumacker, P. T., & Chandel, N. S. (2007). Mitochondrial Reactive Oxygen Species Trigger Hypoxia-Inducible Factor-Dependent Extension of the Replicative Life Span during Hypoxia. *Molecular and Cellular Biology*, *27*(16), 5737–5745. https://doi.org/10.1128/mcb.02265-06

Csete, M. (2005). Oxygen in the cultivation of stem cells. *Ann NY Acad Sci*, *1049*, 1–8. https://doi.org/10.1196/annals.1334.001

Gao, YS., Ding, H., Xie, XT., Zhang, C. (2013). Osteogenic induction protects rat bone marrow e derived mesenchymal stem cells against hypoxia-induced apoptosis in vitro. *Journal of Surgical Research*, 1–7. https://doi.org/10.1016/j.jss.2013.03.082

Grayson, WL., Zhao, F., Bunnell, B., Ma, T. (2007). Hypoxia enhances proliferation and tissue formation of human mesenchymal stem cells. *Biochemical and Biophysical Research Communications*, *358*, 948–953. https://doi.org/10.1016/j.bbrc.2007.05.054

Han, J., Mistriotis, P., Lei, P., Wang, D., Liu, S., Andreadis, S. (2012). Nanog Reverses the Effects of Organismal Aging on Mesenchymal Stem Cell Proliferation and Myogenic Differentiation Potential. *Stem Cells*, *30*, 2746–2759. https://doi.org/10.1002/stem.1223

Hernandez-Segura, A., Nehme, J., & Demaria, M. (2018). Hallmarks of Cellular Senescence. *Trends in Cell Biology*, *28*(6), 436–453. https://doi.org/10.1016/j.tcb.2018.02.001

Herranz, N., & Gil, J. (2018). Mechanisms and functions of cellular senescence. *Journal of Clinical Investigation*, *128*(4), 1238–1246. https://doi.org/10.1172/JCI95148

Kakudo, N., Morimoto, N., Ogawa, T., Taketani, S. (2015). Hypoxia Enhances Proliferation of Human Adipose-Derived Stem Cells via HIF-1 α Activation. *Plos One*, 1–14. https://doi.org/10.1371/journal.pone.0139890

Khan, WS., Adesida, AB., Hardingham, T. (2007). Hypoxic conditions increase hypoxia-inducible transcription factor 2 α and enhance chondrogenesis in stem cells from the infrapatellar fat pad of osteoarthritis patients. *Arthritis Research & Therapy*, *9*(3), 1–9. https://doi.org/10.1186/ar2211

Kim, HJ., Ji, BR., Kim, JS., Lee, HN., Ha, DH., Kim, C. (2012). Proteomic analysis of proteins associated with cellular senescence by calorie restriction in mesenchymal stem cells. *Invitro Cell Dev Biol*, *48*, 186–195. https://doi.org/10.1007/s11626-012-9485-0

Koay, EJ., Athanasiou, P. (2008). Hypoxic chondrogenic differentiation of human embryonic stem cells enhances cartilage protein synthesis and biomechanical functionality. *Osteoarthritis and Cartilage*, *16*(12), 1450–1456. https://doi.org/10.1016/j.joca.2008.04.007

Lapi, S., Nocchi, F., Lamanna, R., Passeri, S., Iorio, M., Paolicchi, A., Urciuoli, P., Coli, A., Abramo, F., Miragliotta, V., Giannessi, E., Stornelli, M. (2008). Different media and supplements modulate the clonogenic and expansion properties of rabbit bone marrow mesenchymal stem cells. *BMC Res Note*, *7*, 1–7. https://doi.org/10.1186/1756-0500-1-53

Lee, TC., Lee, TH., Huang, YH., Chang, NK., Lin, YJ., Chien, C., Yang, WH., Lin, M. (2014). Comparison of Surface Markers between Human and Rabbit Mesenchymal Stem Cells. *Plos One*, *9*(11), 1–10. https://doi.org/10.1371/journal.pone.0111390

Macip, S., Igarashi, M., Fang, L., Chen, A., & Pan, ZQ., Lee, SW., Aaronson, S. (2002). Inhibition of p21-mediated ROS accumulation can rescue p21-induced senescence. *The*

EMBO Journal, *21*(9), 2180–2188.

Mathieu, J., Zhang, Z., Zhou, W., Wang, AJ., Heddleston, JM., Pinna, CMA., Hubaud, A., Stadler, B., Choi, M., Bar, M., Tewari, M., Liu, A., Vessella, R., Rostomily, R., Born, D. (2011). HIF Induces Human Embryonic Stem Cell Markers in Cancer Cells. *Cancer Res*, *71*(13), 4640–4652. https://doi.org/10.1158/0008-5472.CAN-10-3320

Richardson, JD., Nelson, AJ., Zannettino, ACW., Gronthos, S., Worthley, SG., Psaltis, P. (2012). Optimization of the Cardiovascular Therapeutic Properties of Mesenchymal Stromal/Stem Cells–Taking the Next Step. *Stem Cell Rev and Rep*, (April), 1–22. https://doi.org/10.1007/s12015-012-9366-7

Tsai, CC., Chen, YJ., Yew, TL., Chen, LL., Wang, JY., Chiu, C. (2011). Hypoxia inhibits senescence and maintains mesenchymal stem cell properties through down-regulation of E2A-p21 by HIF-TWIST. *Blood*, *117*(2), 459–469. https://doi.org/10.1182/blood-2010-05-287508.The

Wagner, W., Horn, P., Castoldi, M., Diehlmann, A., Bork, S., Benes, V., Blake, J., Pfister, S., Eckstein, V., Ho, A. (2008). Replicative Senescence of Mesenchymal Stem Cells : A Continuous and Organized Process. *Plos One*, *3*(5), 1–12. https://doi.org/10.1371/journal.pone.0002213

Yamamoto, Y., Fujita, M., Tanaka, Y., Kojima, I., Kanatani, Y., Ishihara, M. (2013). Low Oxygen Tension Enhances Proliferation and Maintains. *Biores*, *2*, 199–205. https://doi.org/10.1089/biores.2013.0004

Yan, F., Yao, Y., Chen, L., Li, Y., Sheng, Z., Ma, G. (2012). Hypoxic Preconditioning Improves Survival of Cardiac Progenitor Cells : Role of Stromal Cell Derived Factor-1alfa – CXCR4 Axis. *Plos One*, *7*(7), 1–9. https://doi.org/10.1371/journal.pone.0037948

Yosef, R., Pilpel, N., Papismadov, N., Gal, H., Ovadya, Y., Vadai, E., … Krizhanovsky, V. (2017). p 21 maintains senescent cell viability under persistent DNA damage response by restraining JNK and caspase signaling. *The EMBO Journal*, *36*(15), 2280–2295. https://doi.org/10.15252/embj.201695553

Yosef, R., Pilpel, N., Tokarsky-Amiel, R., Biran, A., Ovadya, Y., Cohen, S., … Krizhanovsky, V. (2016). Directed elimination of senescent cells by inhibition of BCL-W and BCL-XL. *Nature Communications*, *7*. https://doi.org/10.1038/ncomms11190

Zhang, X., Li, J., Sejas, DP., Pang, Q. (2005). The ATM / p53 / p21 Pathway Influences Cell Fate Decision between Apoptosis and Senescence in Reoxygenated Hematopoietic Progenitor Cells. *The Journal of Biological Chemistry*, *280*(20), 19635–19640. https://doi.org/10.1074/jbc.M502262200

Zhuang, Y., Li, D., Fu, J., Shi, Q., Lu, Y., Ju, X. (2015). Comparison of biological properties of umbilical cord - derived mesenchymal stem cells from early and late passages : Immunomodulatory ability is enhanced in aged cells. *Molecular Medicine Reports*, *11*, 166–174. https://doi.org/10.3892/mmr.2014.2755

Ziello, JE., Jovin, IS., Huang, Y. (2007). Hypoxia-Inducible Factor (HIF)-1 Regulatory Pathway and its Potential for Therapeutic Intervention in Malignancy and Ischemia. *Yale Journal of Biology and Medicine*, *80*, 51–60.