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Cold Atmospheric Pressure Plasma as a Tool to Control the Proliferation of Various Mammalian Cells Including Human Mesenchymal Stem Cells for Regenerative Medicine

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ABSTRACT: Cell proliferation is one of the most critical processes for development, tissue regeneration, and wound healing, and is tightly linked with cell differentiation and migration. Also, the regulation of cell proliferation is essential for appropriate tissue regeneration and improved culture system, which requires an effective control tool for cell proliferation both *in vivo* and *in vitro*. Recently, cold atmospheric pressure plasma (CAP) has been shown to have considerable effects on cell proliferation that have been attributed to the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Many studies on CAP application suggest that CAP can be developed as an efficient tool to activate proliferation *in vitro* for personalized cell therapies and *in vivo* for wound healing. In this review, we discuss how CAP has been applied to control proliferation in various mammalian cells and its molecular mechanisms for biomedical applications. In our study, we demonstrated that nitric oxide (NO) from CAP is the major factor for activating the proliferation of human mesenchymal stem cells. When exposed to CAP, many different types of human cells displayed highly increased expression of cytokines and growth factors both *in vitro* and *in vivo*, strongly suggesting that common mechanisms and components are involved in CAP-induced cell proliferation. Furthermore, CAP induced epigenetic modifications in human mesenchymal stem cells to boost the expression of cytokines and growth factors, thereby promoting cell proliferation. Further studies are needed to verify that NO of the CAP and epigenetic modifications are the common mechanism of action of CAP in culture and in wounded tissues. In addition, the molecular mechanism of the epigenetic modifications induced by CAP treatment should be investigated to develop CAP as an applicable tool for regenerative medicine and wound healing.

KEY WORDS: cold atmospheric pressure plasma (CAP), cell proliferation, human mesenchymal stem cells, wound healing, nitric oxide, epigenetic modifications, regenerative medicine

I. THE REGULATION OF CELL PROLIFERATION IS CRITICAL FOR THE BIOLOGICAL SYSTEM *IN VIVO* AND *IN VITRO*

Cell proliferation is one of the most fundamental processes of all living organisms. It is crucial for the formation of multicellular organisms during development[1] and for the maintenance of the body by tissue regeneration and wound healing.

For proliferation, cells divide to produce two daughter cells through the cell division cycle, which is tightly regulated by cellular environments and extracellular stresses.[2,3] Uncontrolled cell proliferation often leads to tumor and cancer development.[4,5] In the process of development, tissue regeneration, and wound healing, cell proliferation is tightly linked with cell differentiation and migration, and their interactions are necessary for regulating the size and function of all tissues and organs.[1,2,6,7]

Control of cell proliferation *in vitro* is also important for the cell culture system, which provides an essential tool for studying cellular physiology. However, it is difficult to maintain the state of proliferation *in vitro* in many types of cells, especially in adult stem cells and normal cells taken from tissues, because they have a limited replication capacity,[8,9] and often fail to maintain their own characteristics in the culture.[10,11] Moreover, some cells easily undergo cellular senescence under replication stress[12,13] or minute environmental stimuli.[14,15] Thus, in order to maintain cell proliferation *in vitro*, high concentrations of a diverse combinatory mixture of cytokines and growth factors are added in the culture medium. However, this culture method is still controversial because the exact mechanisms, as well as the physiological effects of high doses of cytokines and growth factors, have not been defined.[16] The cost of cytokines and growth factors for the culture system is relatively high as well. Thus, an effective control tool for cell proliferation, both *in vivo* and *in vitro*, would be needed for tissue regeneration and improved culture system.

Recently, many studies including ours, have reported that cold atmospheric pressure plasma (CAP) is an efficient method to activate the proliferation of various mammalian cell types, including adult stem cells, progenitors, and normal cells. In this review, we summarize current knowledge regarding the use of CAP to control the proliferation of various mammalian cells including human mesenchymal stem cells, and its potential medical applications. We also discuss the molecular mechanisms by which CAP induces the expression of growth factors and cytokines to enhance the proliferation of various mammalian cells.

II. CAP AS A TOOL TO CONTROL CELL PROLIFERATION FOR BIOMEDICAL APPLICATIONS

Many applications of CAP were mainly focused on its cell death-inducing effect in various cancer cells, suggesting its potential use in anti-cancer therapy.[17–21] Recently, diverse physiological effects of CAP, such as tissue regeneration,[22–26] activation of immune responses,[27–30] cell differentiation,[31–33] and wound healing,[34–38] have been reported in various human and mouse cells. These physiological outcomes of CAP application are strongly associated with the activation of cell proliferation in normal, progenitor, and adult stem cells.

The various biological effects of CAP have been reported to mainly rely on reactive oxygen species (ROS) and reactive nitrogen species (RNS).[39] The variety and composition of ROS and RNS generated from CAP largely depends on plasma gas source,

exposure dosage, and device type. For example, air-based CAP mostly generates large amounts of hydrogen peroxide and ozone, which are powerful agents triggering DNA damage to induce cell apoptosis in various cancer cells.[17–19,21,40] In contrast, nitrogen (N_2), argon (Ar), or helium (He)-based CAP produce different combinations and concentrations of ROS and RNS that lead to the activation of proliferation in various mammalian cell types.[22,34,36–38,41,42]

There are two major types of atmospheric pressure plasma devices frequently used for biomedical applications: air dielectric barrier discharges (DBD) and plasma jets with He or Ar gas flow.[43] The plasma jet devices using a noble gas flow through the DBD have a lower breakdown voltage and afford a lower-level dose of ozone and OH than air-DBDs. Thus, they have been used for many biomedical applications of CAP to various cells and tissues, as they generate very stable plasma with low gas temperature.[24–26,35,36,38,42] There are also two types of operating conditions for the plasma jet. If the induced electric field becomes a parallel component to the jet axis, the ionization waves carrying charged particles are emitted from the nozzle together with ROS and RNS. Devices of this type have been used for direct exposure of plasma to specific targets in many biomedical applications. If the induced electric field is mainly perpendicular to the jet axis, the ionization waves are not propagating toward the target. This type of device has the advantage of more delicate control of the ROS and RNS without charge effect. We have applied this perpendicular type of jet in our studies to examine the cellular effect of the CAP.[22–26,44]

Cells can be exposed to CAP either directly or through a plasma-activated medium (PAM). The biological effects of a direct CAP exposure result from both short- and long-lived ROS and RNS, while long-lived species and secondary species produced by chemical reactions of ROS and RNS from CAP induce its effects in PAM.[45,46] Many physiological studies about the effects of CAP on cell proliferation and wound healing have used direct CAP exposures[22,24–26,35,36,38,42] although CAP-treated solutions have also been employed in limited cases.[23] Collectively, CAP may be a useful tool in a wide range of fields since different physiological outcomes are possible by manipulating the CAP-generating devices and conditions for its application.

III. CAP ACTIVATES THE PROLIFERATION OF HUMAN ADULT STEM CELLS AND PROGENITOR CELLS *IN VITRO*

Stem cells, in general, have the capacity to self-renew and to differentiate into multiple mature cell types.[47] Adult stem cells present in tissues and organs of the body are multipotent stem cells that develop into more specific lineages.[48] Adult stem cells differentiate into progenitor cells that have similar properties as adult stem cells but can replicate only a limited number of times.[49,50] These adult stem cells and progenitor cells have been used in stem cell therapy for regeneration of tissues.[51–54] Especially, mesenchymal stem cells (MSCs) are relatively easy to isolate from specific tissues of a patient and do not result in an immune-rejection response. Thus, these cells provide an accessible source for the usage in stem cell therapies.[55,56]

In order to apply adult stem cells and progenitor cells for cell therapeutics, it is critical to increase their cell numbers by proliferation after extracting them from the body, while maintaining their cellular characteristics. We showed that He gas-based CAP activated the proliferation of diverse mesenchymal adult stem cells, such as adipose tissue-derived stem cells (ASCs), bone marrow-derived mesenchymal stem cells (BM-MSCs), and hematopoietic stem cells (HSCs).[24–26] When ASCs, BM-MSCs, and HSCs were exposed to CAP, increased proliferation was observed in ASCs by 1.6-, BM-MSCs by 1.8-, and HSCs by 2-fold, compared with that of each of the unexposed cells. In the CAP-exposed ASCs, the expression of CD44 and CD105 as the stemness positive markers were maintained, whereas the negative marker CD45 was not at all expressed. In BM-MSCs, the relative expression levels of CD44 and CD105 were increased to 4.2- and 7-fold, respectively, by CAP treatment. Preservation of stemness characteristics by CAP treatment was further tested by examining the expression of Oct4, Sox2, and Nanog, which play major functions in maintaining the pluripotency of stem cells to promote proliferation.[57–59] CAP exposure induced the expression of Oct4, Sox2, and Nanog by 15-, 13.8-, and 14.8-fold, respectively, in ASCs and 3-, 4-, and 3.3-fold, respectively, in BM-MSCs, compared with that of each untreated control. These observations strongly suggest that CAP does not change the stem cell characteristics of mesenchymal stem cells while activating their proliferation.

We also tested whether CAP exposure alters the capability of ASCs to differentiate, and verified that the CAP-exposed ASCs underwent differentiation into adipocytes as efficiently as the unexposed ASCs. One of the major drawbacks of culturing adult stem cells *in vitro* would be cellular senescence.[60] Generally, most stem cells are known to undergo cellular senescence by genotoxic stresses *in vitro*.[61] Thus, we also monitored whether CAP exposure leads to senescence in ASCs using senescence-associated β-galactosidase staining and demonstrated that CAP did not induce cellular senescence in ASCs. Altogether, CAP activates the proliferation of mesenchymal stem cells without affecting stem cell properties or inducing senescence *in vitro*.[24–26]

In addition to the human mesenchymal stem cells, CAP stimulated the proliferation of a progenitor cell *in vitro*. Choi et al.[22] reported that the proliferation of human skeletal muscle myoblasts, which are precursors of muscle cells, increased about 1.2-fold at 72 h after exposure to N_2-based CAP, compared with that of the unexposed cells. They also showed that CAP stimulated the differentiation of myoblasts into muscle cells, as verified by the expression of myosin heavy chain (MHC) and myogenin. CAP exposure also induced the formation of new myofibers in the damaged site of muscle tissue by injury *in vivo*. These results demonstrated that CAP promoted the proliferation and differentiation of human muscle progenitors *in vitro* and the restoration of injured muscle tissue *in vivo*.

These reports strongly support the notion that CAP activates the proliferation of adult stem cells of mesenchymal origin and progenitor cells without disturbing their characteristics. Thus, CAP could be safely applied as an efficient tool to culture various adult stem cells and progenitors extracted from patients *in vitro* to amplify these stem cells for personalized stem cell therapies.

We have to emphasize that the results of our studies solely depend on the device and condition used in the experiments. If the CAP exposure time is increased or if the configuration of the condition is changed, the consequences induced by CAP exposure might cause other effects that we do not expect. Also, although the same condition of CAP is applied, the cellular responses by CAP exposure can be different according to cell types. For example, in the previous study, we showed that cell proliferation was increased in ASCs but cellular apoptosis was induced in various cancer cells, although ASCs and various cancer cells were exposed to the CAP with the same time and condition.[24] Thus, depending on the experimental conditions such as the CAP exposure time, CAP doses, gas used to generate CAP, and cell types, CAP exposure can induce different outcomes.

IV. CAP STIMULATES THE PROLIFERATION OF TERMINALLY DIFFERENTIATED OSTEOBLASTS, KERATINOCYTES, FIBROBLASTS, AND EPITHELIAL CELLS FOR PROMOTING THE WOUND HEALING PROCESS *IN VITRO* AND *IN VIVO*

CAP exposure stimulates the proliferation not only of adult stem cells and progenitor cells but also of terminally differentiated human cells in various tissues. Eggers et al.[62] reported enhanced proliferation of human osteoblast-like cells (MG63) when exposed to CAP generated by a mixture of nitrogen, oxygen, and argon. Osteoblasts are specialized and terminally differentiated cells from mesenchymal stem cells. CAP-exposed MG63 cells exhibit increased expression of markers of cell proliferation, Ki67 and PCNA, as well as pro-inflammatory markers, such as interleukin-1β (IL-1β), IL-6, IL-8, tumor necrosis factor-α (TNF-α), cyclooxygenase 2 (COX2), and collagen (COL) 1α involved in wound healing. Moreover, CAP-exposed cells displayed faster wound closure in wound healing assay *in vitro*.

In the wound healing process, especially during the inflammatory phase and re-epithelialization phase, keratinocytes, fibroblasts, and epithelial cells, and their interactions play key roles.[63] Fibroblasts secrete several paracrine factors such as basic fibroblast growth factor (bFGF), keratinocyte growth factor (KGF), vascular endothelial growth factor-A (VEGF-A), and insulin-like growth factor-1 (IGF-1), which stimulate keratinocytes to trigger migration and proliferation,[64] and attract epithelial cells to the wounded area for re-epithelialization by proliferation.[65,66] Keratinocytes induce endothelial cell migration and angiogenesis by releasing angiogenic growth factors such as vascular endothelial growth factor (VEGF), and promote the proliferation of fibroblasts by secreting IL-1 and platelet-derived growth factor (PDGF) via double paracrine signaling loops in the wounded area.[64] Therefore, the activation of proliferation in keratinocytes, fibroblasts, and epithelial cells is critical for wound healing.

Some studies have reported that CAP stimulates wound healing by directly increasing the proliferation of keratinocytes, fibroblasts, and epithelial cells and by enhancing cell migration for wound closure. References[36,38] demonstrated that CAP directly affects the proliferation of fibroblasts for wound healing. They exposed Ar-based CAP to murine fibroblast cell line, L929, and observed that the proliferation significantly increased.

Furthermore, they reported increased secretion of epidermal growth factor (EGF) and transforming growth factors-β1 (TGF-β1) in CAP-exposed L929 cells.

Additionally, Brun et al.[42] reported that He-generated CAP exposure to human fibroblast-like primary cells isolated from nonpathological fragments of liver tissue increased the daughter cells by 49.8%, while the unexposed had only 15.9% daughter cells. On exposing the same CAP to the intestinal subepithelial myofibroblasts (ISEMFs) isolated from nonpathological colonic biopsies, the migration of ISEMFs increased by 3.67-fold, compared to that of the unexposed cells. In these cells with increased proliferation and migration, the secretion of IL-6 was increased by 28% in the hepatic stellate cells and by 46% in ISEMFs. These results indicated that CAP exposure activates the proliferation and migration of human fibroblast-like primary cells *in vitro*.

Choi et al.[35] also demonstrated increased proliferation of human keratinocyte HaCaT by Ar-based CAP exposure. CAP exposure induced the expression of cyclin D1 by activating β-catenin, the major regulator of the proliferation of keratinocytes *in vitro*. In a scratch wound healing assay, the wound closure was faster in the CAP-exposed HaCaT cells than in the unexposed cells. Moreover, CAP exposure to wounded mouse skin accelerated the shrinkage of the wounded area by increasing the proliferation of keratinocytes, suggesting that CAP induces the proliferation of keratinocytes, thereby leading to wound closure *in vitro* and *in vivo*.

Using a N_2-based CAP exposure, Won et al.[23] reported increased the proliferation of human bronchial epithelial cell line, BEAS-2B, by 1.5-fold, compared with that of the unexposed cells. In a scratch wound healing assay, BEAS-2B cells incubated with CAP-treated solution displayed decreased scratch area, leading to increased migration. In the CAP-treated BEAS-2B cells of augmented proliferation, the expression of epidermal growth factor receptor (EGFR), a key factor for the wound healing of nasal mucosa, was increased in comparison to the untreated cells. The epithelial-to-mesenchymal transition (EMT) is an essential process of wound healing in which epithelial cells transform into mesenchymal cells by losing the epithelial function.[67,68] The CAP-treated solution activated EMT signaling by activating p-focal adhesion kinase (FAK) and p-proto-oncogene tyrosine protein kinase SRC, subsequently promoting the migration of BEAS-2B cells. In the BEAS-2B cells whose EMT was activated by CAP-treated media, the expression of a mesenchymal marker, slug, increased, while a cell adhesion marker, E-cadherin, decreased. In the wounded mouse model treated with plasma-activated media (PAM), epithelial cells increased in number, suggesting that the PAM activated the proliferation and migration of epithelial cells *in vitro* and *in vivo* to expedite the wound healing process. Collectively, these reports demonstrated that CAP exposure promotes wound healing by activating the proliferation and migration of keratinocytes, fibroblasts, and epithelial cells.

Conversely, some studies have reported that CAP induces wound healing without activating cell proliferation. Arndt et al.[34] demonstrated increased expression of cytokines and growth factors, CD 40 ligand, chemokine ligand 1 (CXCL1), IL-1, IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), and Serpine E1 in primary human dermal fibroblasts exposed to Ar-based CAP. They also observed increased migration of

CAP-exposed human dermal fibroblasts in the scratch wound healing assay *in vitro*; however, the proliferation did not change significantly as compared with the unexposed cells. In a mouse model of scratch wounds, faster wound closure was observed in mice exposed to CAP every day for 5 days, in comparison to that in untreated mice, suggesting that CAP exposure induced the migration of fibroblasts without affecting cell proliferation *in vitro* and *in vivo*. In another study by Duchesne et al.,[44] human microvascular endothelial cells (HMVEC), human primary dermal fibroblasts, and HaCaT keratinocytes were treated with either direct CAP or PAM. In these three cell types used, only direct CAP exposure promoted the migration in scratch assays, and both direct and indirect CAP did not affect the proliferation *in vitro*.

V. MOLECULAR MECHANISMS OF CAP-INDUCED CELL PROLIFERATION *IN VIVO* AND *IN VITRO*

In previous sections, we discussed the evidence that CAP activates the proliferation of diverse human cells including mesenchymal stem cells, progenitor cells, and terminally differentiated cells from different tissues, suggesting the potential for the general application of CAP to activate the proliferation of these cells for regenerative medicine, *in vivo* for wound healing and *in vitro* for cell therapy.

Then, how does CAP exposure activate the proliferation of various human cells? To answer this question in the mesenchymal stem cells, we analyzed the whole genome expression profile of CAP-exposed ASCs using RNA-sequencing (RNA-seq).[26] As shown in Fig. 1, the results confirmed that CAP highly upregulated the expression of various cytokines and growth factors including leukemia inhibitory factor (LIF), heparin-binding epidermal growth factor (HB-EGF), IL-6, IL-11, fibroblast growth factor 16 (FGF16), and IL-1β, and downregulated the expression of cell death-associated genes in ASCs.[26] These results suggested that CAP induces the proliferation of ASCs by upregulating the expression of cytokines and growth factors.

Interestingly, several reports also presented that CAP exposure enhances the expression of cytokines and growth factors, which in turn induce cell proliferation and migration in wounded tissues.[23,34,38,42,62] The genes commonly upregulated by CAP in the ASCs[26] and the wounded tissues are those encoding key cytokines and growth factors including IL-1, IL-6, and IL-8. These observations strongly suggest that the activation of cell proliferation by CAP both in mesenchymal stem cells and in wounded tissues is likely to be regulated by the shared mechanisms and components.

This brings us to the question of which components of CAP are responsible for the increased expression of various cytokines and growth factors and activation of cell proliferation. We found that NO but not ROS generated from the CAP functions primarily to increase the expression of cytokines and growth factors in ASCs.[24,26] As shown in Fig. 1, the expression of major cytokines and growth factors in ASCs considerably increased by CAP exposure, but decreased after treatment with CAP and NO scavenger, carboxy-PTIO. NO, depending on its level, has been reported to regulate the expression of cytokines and growth factors during the wound healing process,[69,70] supporting the important

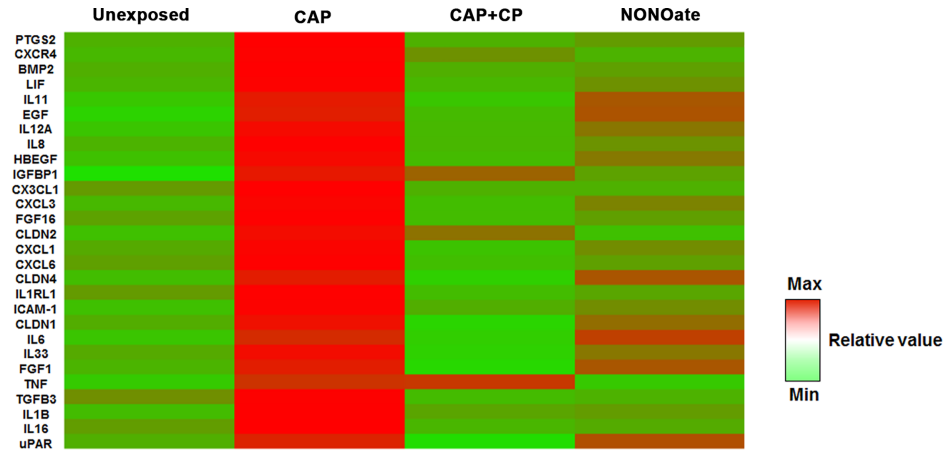

FIG. 1: Nitric oxide (NO) produced by CAP plays a key role in upregulating the expression of cytokines and growth factors in adipose tissue-derived mesenchymal stem cells. Total expressed RNAs were analyzed by RNA-seq in adipose tissue-derived stem cells (ASCs) exposed to CAP for 10 times for 50 sec in every hour (CAP), ASCs exposed to CAP in the presence of carboxy-PTIO (CP) as an NO scavenger (CAP+CP), ASCs treated only with DETA-NONOate as an NO donor (NONOate), and untreated ASCs (Unexposed). The RNA-seq results of these cells were processed for the heatmap analysis by Shinyheatmap software (shinyheatmap.com) and presented[26] (reprinted from Park et al. with permission from Elsevier, copyright 2020).

role of NO in the CAP-activated expression of cytokines and growth factors in ASCs. However, we expect that CAP may have more complex effects than those mediated solely by NO and other components may also contribute to the increased expression of cytokines and growth factors by CAP, because the observed increase in the expression of cytokines and growth factor in NO donor-treated ASCs was lower than that in CAP-exposed ASCs (Fig. 1).

The following question would be to understand how CAP activates the expression of cytokines and growth factors. In our study, we observed that CAP increased the protein expression of histone deacetyltransferase (HDAC1), while reducing the expression of acetylated histone 3 (Ac-H3). Furthermore, as shown in Fig. 2, CAP-exposed ASCs pre-incubated with inhibitors of histone deacetylation, DNA methylation, and histone methylation (which generally induce epigenetic modifications), had reduced expression of various cytokines and growth factors. These observations strongly suggest that CAP likely induces changes in the global epigenetic process to activate the cytokines and growth factors.

Previously, we mentioned that NO produced by CAP is mainly responsible for up-regulating cytokines and growth factors. Therefore, is it possible for the CAP-generated NO to induce epigenetic modifications to regulate the expression of cytokines and growth factors? Several recent studies reported that NO functions as an epigenetic modifier to control cell fate by regulating gene expression in various cells, including stem cells.[71,72]

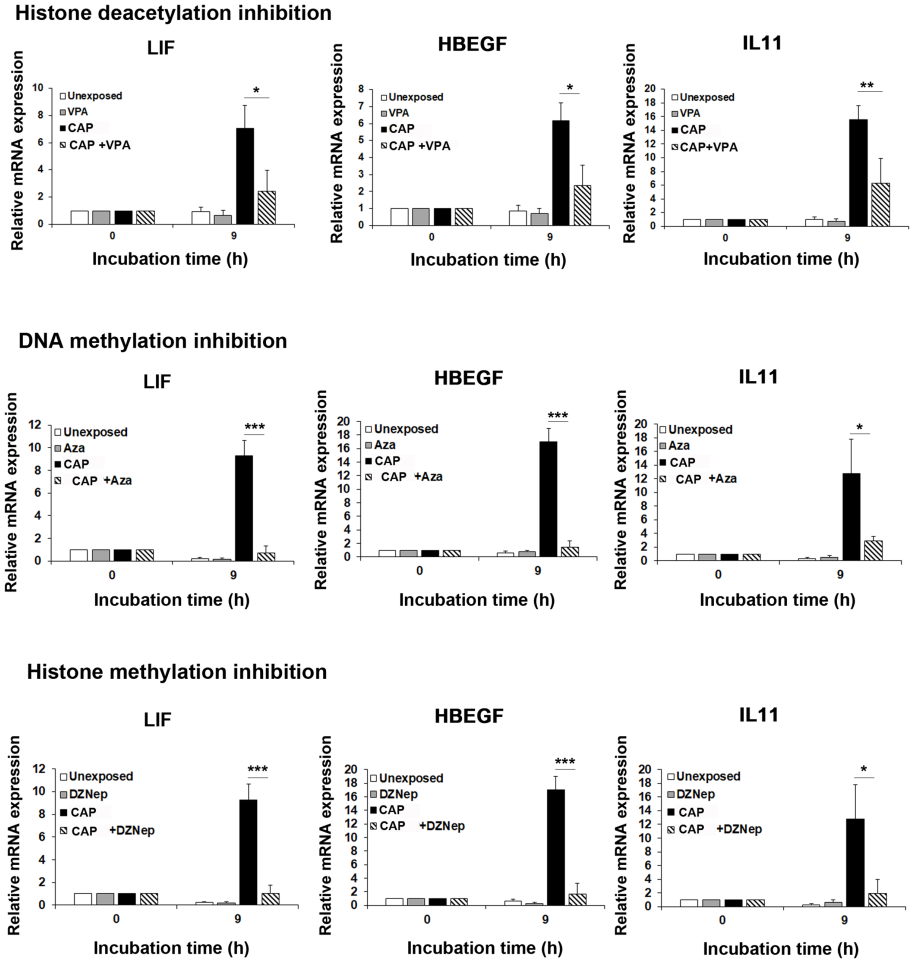

FIG. 2: The global epigenetic modifications are involved in the upregulated expression of cytokines and growth factors in CAP-treated ASCs. ASCs were pretreated with inhibitors for the global epigenetic modifiers, valproic acid (VPA) for histone deacetyltransferase, azacytidine (Aza) for DNA methyltransferase, and 3-deazaneplanocin (DZNep) for histone methyltransferase, for 5 h, and exposed to CAP for 50 s once in an hour for a total of ten times. The mRNA expression of LIF, HB-EGF, and IL-11 was analyzed in VPA, Aza, and DZNep-incubated ASCs with and without CAP exposures by real-time PCR. Their expressions were normalized to the mRNA expression of β-actin in each sample and the relative mRNA expression was presented compared to that in the CAP-unexposed ASCs.[26] The relative mRNA expression of each sample at 9 h over 0 h (before NTAPP-exposure) was plotted as mean ± S.D. from three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (reprinted from Park et al. with permission from Elsevier, copyright 2020).

For example, in mouse embryonic stem cells (mESCs), NO donor reduced the expression of Nanog and Oct4, which are the main factors for maintaining the self-renewal

of stem cells by increasing H4K9me3 and decreasing H3K4me1 and H3 acetylation.[73] Also, an NO donor induced the differentiation of neuronal progenitors by increasing the acetylation of histone 3/4 during neurogenesis.[74] These reports demonstrated that NO acts as the regulator of epigenetic modifications, suggesting that CAP-produced NO mainly functions as an epigenetic regulator to activate cytokines and growth factors.

VI. CONCLUSION

In this review, we overview how CAP has been applied to activate cell proliferation in mesenchymal stem cells, progenitors, and terminally differentiated cells. These accumulated results in various human cells and wounded tissue mouse models strongly support that CAP has a high potential to be developed as an efficient tool to activate cell proliferation *in vitro* for personalized cell therapies and wound healing *in vivo*. Since CAP induced the expression of various cytokines and growth factors in both cultured mesenchymal stem cells and wounded tissue, it can be said that CAP-induced cell proliferation in the culture system *in vitro* and wound healing *in vivo* might share some common mechanisms and components. We showed in the adipose tissue-derived mesenchymal stem cells that NO induces epigenetic changes to increase the expression of cytokines and growth factors and enhances cell proliferation. The functions of NO generated by CAP and its epigenetic modifications should be verified in other cells and in wounded tissue to determine the common mechanism behind enhanced proliferation. To develop CAP as a general tool to activate cell proliferation *in vitro* and *in vivo* for regenerative medicine, further investigations on the mechanism of epigenetic modifications induced by CAP exposure are needed. A detailed understanding of the chromosomal changes in the promoter and enhancer regions of the genes for cytokines and growth factors, which are commonly upregulated in CAP-exposed cells and tissues, may serve as a foundation to apply CAP for regenerative medicine.

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REFERENCES

1. Takeuchi T, Nakamura H. Cell proliferation and development. Dev Growth Differ. 2014;56(5):323.
2. Lander AD. Pattern, growth, and control. Cell. 2011;144(6):955–69.
3. Duronio RJ, Xiong Y. Signaling pathways that control cell proliferation. CSH Perspect Biol. 2013;5(3):a008904.

4. Otto T, Sicinski P. Cell cycle proteins as promising targets in cancer therapy. Nat Rev Cancer. 2017;17(2):93–115.
5. Letai A. Apoptosis and cancer. Annu Rev Canc Biol. 2017;1:275–94.
6. Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. Nature. 2008;453(7193):314–21.
7. Krafts KP. Tissue repair. The hidden drama. Organogenesis. 2010;6(4):225–33.
8. Hladik D, Hofig I, Oestreicher U, Beckers J, Matjanovski M, Bao XW, Scherthan H, Atkinson MJ, Rosemann M. Long-term culture of mesenchymal stem cells impairs ATM-dependent recognition of DNA breaks and increases genetic instability. Stem Cell Res Ther. 2019;10.
9. Franzen J, Zirkel A, Blake J, Rath B, Benes V, Papantonis A, Wagner W. Senescence-associated DNA methylation is stochastically acquired in subpopulations of mesenchymal stem cells. Aging Cell. 2017;16(1):183–91.
10. Xie XY, Hiona A, Lee AS, Cao F, Huang M, Li ZJ, Cherry A, Pei XT, Wu JC. Effects of long-term culture on human embryonic stem cell aging. Stem Cells Dev. 2011;20(1):127–38.
11. Park YB, Kim YY, Oh SK, Chung SG, Ku SY, Kim SH, Choi YM, Moon SY. Alterations of proliferative and differentiation potentials of human embryonic stem cells during long-term culture. Exp Mol Med. 2008;40(1):98–108.
12. Venkatachalam G, Surana U, Clement MV. Replication stress-induced endogenous DNA damage drives cellular senescence induced by a sub-lethal oxidative stress. Nucleic Acids Res. 2017;45(18):10564–82.
13. Lukasova E, Rezacova M, Bacikova A, Sebejova L, Vavrova J, Kozubek S. Distinct cellular responses to replication stress leading to apoptosis or senescence. FEBS Open Bio. 2019;9(5):870–90.
14. Bernardo ME, Zaffaroni N, Novara F, Cometa AM, Avanzini MA, Moretta A, Montagna D, Maccario R, Villa R, Daidone MG, Zuffardi O, Locatelli F. Human bone marrow–derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. Cancer Res. 2007;67(19):9142–9.
15. Ng CP, Sharif ARM, Heath DE, Chow JW, Zhang CBY, Chan-Park MB, Hammond PT, Chan JKY, Griffith LG. Enhanced ex vivo expansion of adult mesenchymal stem cells by fetal mesenchymal stem cell ECM. Biomaterials. 2014;35(13):4046–57.
16. Walasek MA, van Os R, de Haan G. Hematopoietic stem cell expansion: Challenges and opportunities. Ann NY Acad Sci. 2012;1266:138–50.
17. Ahn HJ, Kim KI, Hoan NN, Kim CH, Moon E, Choi KS, Yang SS, Lee JS. Targeting cancer cells with reactive oxygen and nitrogen species generated by atmospheric-pressure air plasma. PLoS One. 2014;9(1):e86173.
18. Ahn HJ, Kim KI, Kim G, Moon E, Yang SS, Lee JS. Atmospheric-pressure plasma jet induces apoptosis involving mitochondria via generation of free radicals. PLoS One. 2011;6(11):e28154.
19. Kim GJ, Kim W, Kim KT, Lee JK. DNA damage and mitochondria dysfunction in cell apoptosis induced by nonthermal air plasma. Appl Phys Lett. 2010;96(2):021502.
20. Ma YH, Ha CS, Hwang SW, Lee HJ, Kim GC, Lee KW, Song K. Non-thermal atmospheric pressure plasma preferentially induces apoptosis in p53-mutated cancer cells by activating ROS stress-response pathways. PLoS One. 2014;9(4):e91947.
21. Panngom K, Baik KY, Nam MK, Han JH, Rhim H, Choi EH. Preferential killing of human lung cancer cell lines with mitochondrial dysfunction by nonthermal dielectric barrier discharge plasma. Cell Death Dis. 2013;44(5):e642.
22. Choi JW, Kang SU, Kim YE, Park JK, Yang SS, Kim YS, Lee YS, Lee Y, Kim CH. Novel therapeutic effects of non-thermal atmospheric pressure plasma for muscle regeneration and differentiation. Sci Rep-UK. 2016;6:28829.
23. Won HR, Kang SU, Kim HJ, Jang JY, Shin YS, Kim CH. Non-thermal plasma treated solution with potential as a novel therapeutic agent for nasal mucosa regeneration. Sci Rep-UK. 2018;8:13754.
24. Park J, Lee H, Lee HJ, Kim GC, Kim DY, Han S, Song K. Non-thermal atmospheric pressure plasma

efficiently promotes the proliferation of adipose tissue-derived stem cells by activating NO-response pathways. Sci Rep-UK. 2016;6:39298.
25. Park J, Lee H, Lee HJ, Kim GC, Kim SS, Han S, Song K. Non-thermal atmospheric pressure plasma is an excellent tool to activate proliferation in various mesoderm-derived human adult stem cells. Free Radical Biol Med. 2019;134:374–84.
26. Park J, Suh D, Tang T, Lee HJ, Roe JS, Kim GC, Han S, Song K. Non-thermal atmospheric pressure plasma induces epigenetic modifications that activate the expression of various cytokines and growth factors in human mesoderm-derived stem cells. Free Radical Biol Med. 2020;148:108–22.
27. Bekeschus S, Masur K, Kolata J, Wende K, Schmidt A, Bundscherer L, Barton A, Kramer A, Broker B, Weltmann KD. Human mononuclear cell survival and proliferation is modulated by cold atmospheric plasma jet. Plasma Process Polym. 2013;10(8):706–13.
28. Bundscherer L, Wende K, Ottmuller K, Barton A, Schmidt A, Bekeschus S, Hasse S, Weltmann KD, Masur K, Lindequist U. Impact of non-thermal plasma treatment on MAPK signaling pathways of human immune cell lines. Immunobiology. 2013;218(10):1248–55.
29. Haertel B, Volkmann F, von Woedtke T, Lindequist U. Differential sensitivity of lymphocyte subpopulations to non-thermal atmospheric-pressure plasma. Immunobiology. 2012;217(6):628–33.
30. Shi XM, Zhang GJ, Yuan YK, Ma Y, Xu GM, Yang Y. Effects of low-temperature atmospheric air plasmas on the activity and function of human lymphocytes. Plasma Process Polym. 2008;5(5):482–8.
31. Kang SU, Kim HJ, Kim DH, Han CH, Lee YS, Kim CH. Nonthermal plasma treated solution inhibits adipocyte differentiation and lipogenesis in 3T3-L1 preadipocytes via ER stress signal suppression. Sci Rep-UK. 2018;8:2277.
32. Park JK, Kim YS, Kang SU, Lee YS, Won HR, Kim CH. Nonthermal atmospheric plasma enhances myoblast differentiation by eliciting STAT3 phosphorylation. FASEB J. 2019;33(3):4097–106.
33. Tominami K, Kanetaka H, Sasaki S, Mokudai T, Kaneko T, Niwano Y. Cold atmospheric plasma enhances osteoblast differentiation. PLoS One. 2017;12(7):e0180507.
34. Arndt S, Unger P, Wacker E, Shimizu T, Heinlin J, Li YF, Thomas HM, Morfill GE, Zimmermann JL, Bosserhoff AK, Karrer S. Cold atmospheric plasma (CAP) changes gene expression of key molecules of the wound healing machinery and improves wound healing in vitro and in vivo. PLoS One. 2013;8(11):e79325.
35. Choi JH, Song YS, Song K, Lee HJ, Hong JW, Kim GC. Skin renewal activity of non-thermal plasma through the activation of beta-catenin in keratinocytes. Sci Rep-UK. 2017;7:6146.
36. Liu JR, Xu GM, Shi XM, Zhang GJ. Low temperature plasma promoting fibroblast proliferation by activating the NF-kappa B pathway and increasing cyclinD1 expression. Sci Rep-UK. 2017;7:11698.
37. Schmidt A, Dietrich S, Steuer A, Weltmann KD, von Woedtke T, Masur K, Wende K. Non-thermal plasma activates human keratinocytes by stimulation of antioxidant and phase II pathways. J Biol Chem. 2015;290(11):6731–50.
38. Shi XM, Xu GM, Zhang GJ, Liu JR, Wu YM, Gao LG, Yang Y, Chang ZS, Yao CW. Low-temperature plasma promotes fibroblast proliferation in wound healing by ROS-activated NF-kappa B signaling pathway. Curr Med Sci. 2018;38(1):107–14.
39. Laroussi M. Cold plasma in medicine and healthcare: The new frontier in low temperature plasma applications. Front Phys-Lausanne. 2020;8.
40. Lunov O, Zablotskii V, Churpita O, Chanova E, Sykova E, Dejneka A, Kubinova S. Cell death induced by ozone and various non-thermal plasmas: Therapeutic perspectives and limitations. Sci Rep-UK. 2014;4:7129.
41. Duchesne C, Frescaline N, Lataillade J-J, Rousseau A. Comparative study between direct and Indirect treatment with cold atmospheric plasma on in vitro and in vivo models of wound healing. Plasma Med. 2018;8(4):379–401.
42. Brun P, Pathak S, Castagliuolo I, Palu G, Brun P, Zuin M, Cavazzana R, Martines E. Helium generated cold plasma finely regulates activation of human fibroblast-like primary cells. PLoS One. 2014;9(8):e104397.

43. Lee HW, Park GY, Seo YS, Im YH, Shim SB, Lee HJ. Modelling of atmospheric pressure plasmas for biomedical applications. J Phys D Appl Phys. 2011;44(5).
44. Duchesne C, Frescaline N, Lataillade J, Rousseau A. Comparative study between direct and indirect treatment with cold atmospheric plasma on in vitro and in vivo models of wound healing. Plasma Med. 2018;8(4):379–401.
45. Kaushik NK, Ghimire B, Li Y, Adhikari M, Veerana M, Kaushik N, Jha N, Adhikari B, Lee SJ, Masur K, von Woedtke T, Weltmann KD, Choi EH. Biological and medical applications of plasma-activated media, water and solutions. Biol Chem. 2018;400(1):39–62.
46. Gorbanev Y, Privat-Maldonado A, Bogaerts A. Analysis of short-lived reactive species in plasma-air-water systems: The dos and the do nots. Anal Chem. 2018;90(22):13151–8.
47. Chagastelles PC, Nardi NB. Biology of stem cells: An overview. Kidney Int Suppl. 2011;1(3):63–7.
48. Zakrzewski W, Dobrzynski M, Szymonowicz M, Rybak Z. Stem cells: Past, present, and future. Stem Cell Res Ther. 2019;10.
49. Clarke MF, Fuller M. Stem cells and cancer: Two faces of Eve. Cell. 2006;124(6):1111–5.
50. Weissman IL, Anderson DJ, Gage F. Stem and progenitor cells: Origins, phenotypes, lineage commitments, and transdifferentiations. Annu Rev Cell Dev Biol. 2001;17:387–403.
51. Hou YN, Li CY. Stem/progenitor cells and their therapeutic application in cardiovascular disease. Front Cell Dev Biol. 2018;6.
52. Mirzaei H, Sahebkar A, Sichani LS, Moridikia A, Nazari S, Nahand JS, Salehi H, Stenvang J, Masoudifar A, Mirzaei HR, Jaafari MR. Therapeutic application of multipotent stem cells. J Cell Physiol. 2018;233(4):2815–23.
53. Montagnani S, Rueger MA, Hosoda T, Nurzynska D. Adult stem cells in tissue maintenance and regeneration. Stem Cells Int. 2016;2016.
54. Rohban R, Pieber TR. Mesenchymal stem and progenitor cells in regeneration: Tissue specificity and regenerative potential. Stem Cells Int. 2017;2017.
55. Hombach-Klonisch S, Panigrahi S, Rashedi I, Seifert A, Alberti E, Pocar P, Kurpisz M, Schulze-Osthoff K, Mackiewicz A, Los M. Adult stem cells and their trans-differentiation potential-perspectives and therapeutic applications. J Mol Med. 2008;86(12):1301–14.
56. Rizvanov AA, Persson J, Sahin F, Bellusci S, Oliveira PJ. Hematopoietic and mesenchymal stem cells in biomedical and clinical applications. Stem Cells Int. 2016;2016.
57. Han SM, Han SH, Coh YR, Jang G, Ra JC, Kang SK, Lee HW, Youn HY. Enhanced proliferation and differentiation of Oct4-and Sox2-overexpressing human adipose tissue mesenchymal stem cells. Exp Mol Med. 2014;46.
58. Kim DS, Lee MW, Lee TH, Sung KW, Koo HH, Yoo KH. Cell culture density affects the stemness gene expression of adipose tissue-derived mesenchymal stem cells. Biomed Rep. 2017;6(3):300–6.
59. Sun Z, Han Q, Zhu YS, Li ZY, Chen B, Liao LM, Bian CJ, Li J, Shao CS, Zhao RC. NANOG has a role in mesenchymal stem cells' immunomodulatory effect. Stem Cells Dev. 2011;20(9):1522–9.
60. Tower J. Stress and stem cells. WIREs Dev Biol. 2012;1(6):789–802.
61. Estrada JC, Torres Y, Benguria A, Dopazo A, Roche E, Carrera-Quintanar L, Perez RA, Enriquez JA, Torres R, Ramirez JC, Samper E, Bernad A. Human mesenchymal stem cell-replicative senescence and oxidative stress are closely linked to aneuploidy. Cell Death Dis. 2013;4.
62. Eggers B, Marciniak J, Memmert S, Kramer FJ, Deschner J, Nokhbehsaim M. The beneficial effect of cold atmospheric plasma on parameters of molecules and cell function involved in wound healing in human osteoblast-like cells in vitro. Odontology. 2020;108(4):607–16.
63. Werner S, Krieg T, Smola H. Keratinocyte-fibroblast interactions in wound healing. J Invest Dermatol. 2007;127(5):998–1008.
64. Wojtowicz AM, Oliveira S, Carlson MW, Zawadzka A, Rousseau CF, Baksh D. The importance of both fibroblasts and keratinocytes in a bilayered living cellular construct used in wound healing. Wound Repair Regen. 2014;22(2):246–55.

65. Bartkova J, Gron B, Dabelsteen E, Bartek J. Cell-cycle regulatory proteins in human wound healing. Arch Oral Biol. 2003;48(2):125–32.
66. Brugues A, Anon E, Conte V, Veldhuis JH, Gupta M, Colombelli J, Munoz JJ, Brodland GW, Ladoux B, Trepat X. Forces driving epithelial wound healing. Nat Phys. 2014;10(9):683–90.
67. Stone RC, Pastar I, Ojeh N, Chen V, Liu S, Garzon KI, Tomic-Canic M. Epithelial-mesenchymal transition in tissue repair and fibrosis. Cell Tissue Res. 2016;365(3):495–506.
68. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol. 2014;15(3):178–96.
69. Kobayashi Y. The regulatory role of nitric oxide in proinflammatory cytokine expression during the induction and resolution of inflammation. J Leukocyte Biol. 2010;88(6):1157–62.
70. Schwentker A, Vodovotz Y, Weller R, Billiar TR. Nitric oxide and wound repair: Role of cytokines? Nitric Oxide. 2002;7(1):1–10.
71. Socco S, Bovee RC, Palczewski MB, Hickok JR, Thomas DD. Epigenetics: The third pillar of nitric oxide signaling. Pharmacol Res. 2017;121:5–8.
72. Vasudevan D, Bovee RC, Thomas DD. Nitric oxide, the new architect of epigenetic landscapes. Nitric Oxide. 2016;59:54–62.
73. Mora-Castilla S, Tejedo JR, Hmadcha A, Cahuana GM, Martin F, Soria B, Bedoya FJ. Nitric oxide repression of Nanog promotes mouse embryonic stem cell differentiation. Cell Death Differ. 2010;17(6):102–33.
74. Nott A, Riccio A. Nitric oxide-mediated epigenetic mechanisms in developing neurons. Cell Cycle. 2009;8(5):725–30.