Molecules and Cells

Long-Term Priming by Three Small Molecules Is a Promising Strategy for Enhancing Late Endothelial Progenitor Cell Bioactivities

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Endothelial progenitor cells (EPCs) and outgrowth endothelial cells (OECs) play a pivotal role in vascular regeneration in ischemic tissues; however, their therapeutic application in clinical settings is limited due to the low quality and quantity of patient-derived circulating EPCs. To solve this problem, we evaluated whether three priming small molecules (tauroursodeoxycholic acid, fucoidan, and oleuropein) could enhance the angiogenic potential of EPCs. Such enhancement would promote the cellular bioactivities and help to develop functionally improved EPC therapeutics for ischemic diseases by accelerating the priming effect of the defined physiological molecules. We found that preconditioning of each of the three small molecules significantly induced the differentiation potential of $CD34^+$ stem cells into EPC lineage cells. Notably, long-term priming of OECs with the three chemical cocktail (OEC-3C) increased the proliferation potential of EPCs via ERK activation. The migration, invasion, and tube-forming capacities were also significantly enhanced in OEC-3Cs compared with unprimed OECs. Further, the cell survival ratio was dramatically increased in OEC-3Cs against H_2O_2-induced oxidative stress via the augmented expression of Bcl-2, a pro-survival protein. In conclusion, we identified three small molecules for enhancing the bioactivities of *ex vivo*-expanded OECs for vascular repair. Long-term 3C priming might be a promising methodology for EPC-based therapy against ischemic diseases.

Keywords: cell priming, endothelial progenitor cells, ischemic diseases, vascular repair

INTRODUCTION

Since resident stem/progenitor cells can repair the vascular system in response to ischemic disease-related signals, which induce angiogenic progenitor cell recruitment, the incorporated stem/progenitor cells dynamically contribute to vascular repair for recovering the normal status of the blood system. Accordingly, in the absence or insufficiency of the self-repair capabilities of resident stem cells, there is pathologically abnormal or insufficient vessel formation and lack of an intact blood vessel system, which can lead to the development of various ischemic vascular diseases (Annex, 2013; Carmeliet, 2004) that represent a major cause of death worldwide. Thus, many research groups have focused on the development of *ex vivo*-expanded stem/progenitor cell-

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based therapeutics, leading to the discovery of angiogenic function-modulating factors to achieve that might achieve the sufficient tissue repair of damaged vessels as a next-generation therapy for patients with ischemic diseases (Schaun et al., 2016; Silvestre and Levy, 2002).

Accumulating evidence clearly demonstrates that postnatal neovessel formation is dependent on the sprouting of pre-existing endothelial cells through the process of angiogenesis (Beck and D'Amore, 1997). However, in 1997, Asahara and colleagues (Asahara et al., 1997) reported that purified $CD34^+$ cells isolated from adult peripheral blood were incorporated into neovessels at the sites of ischemia, which were named "endothelial progenitor cells" (EPCs). The discovery of EPCs has therefore greatly changed our understanding of postnatal neovascularization, leading to the general concept of EPC-based cell therapy. This emerging therapeutic strategy involves the transplantation of EPCs in ischemic tissues, which might play critical roles in triggering neo-vessel formation to enhance vascular and tissue repair against pathological ischemic diseases via producing a tremendous amount of angiogenic cytokines, as well as directly differentiating into endothelial lineage cells (Asahara et al., 2011; Fadini et al., 2012; Sukmawati and Tanaka, 2015).

Although EPC-based therapy appears to be a promising strategy for vascular regeneration in ischemic vascular diseases, for this strategy to be most effective, EPC bioactivities should be improved through niche-modulating factors or angiogenic function-related factors. Accordingly, many studies have focused on emerging therapeutic strategies with the short-term priming or preconditioning of EPCs before transplantation with various compounds that could significantly enhance the angiogenic functionalities of EPCs for vascular regeneration (Guo et al., 2017; Vyas et al., 2015; Yamaguchi et al., 2003). For example, tauroursodeoxycholic acid (TUDCA), an endogenous bile acid, promotes blood vessel repair by modulating the dissociation and mobilization of bone marrow-derived stem/progenitor cells, as well as their differentiation into EPCs (Cho et al., 2015). In addition, we recently reported that oleuropein, an olive oil extract, could attenuate oxidative stress and EPC depletion by regulating the ERK1/2-Prdx and Akt-eNOS signaling pathways (Choi et al., 2015). We also reported that fucoidan, a marine sulfated polysaccharide, contributes to the reversal of cellular replicative senescence via the integrin-FAK-Akt signaling axis (Lee et al., 2015a).

Based on these previous findings, we attempted to develop functionally improved EPCs that could be used as therapeutics for ischemic diseases by accelerating the priming effect of the defined physiological molecules. Toward this end, we evaluated the optimal combination of these factors for improving the angiogenic potential of outgrowth endothelial cells (OECs), a subset of EPCs (Lin et al., 2000) by enhancing functionalities such as proliferation, differentiation, and mobilization. Therefore, we identified three small molecules (3-chemical cocktail, 3C), including fucoidan, TUDCA, and oleuropein, and defined their ideal functional combination for enhancing the bioactivities of long-term and *ex vivo*-expanded OECs towards improving their therapeutic potential for neovascularization. Our findings could provide a novel methodology for an effective EPC-based therapeutic strategy against ischemic diseases.

MATERIALS AND METHODS

Isolation and culture of OECs

To isolate human OECs, which are late EPCs, human umbilical cord blood (HUCB) was supplied by the Pusan National University Yangsan Hospital (PNUYH, IRB No. 05-2017-053). Total mononuclear cells (MNCs) were isolated from the HUCB by density-gradient centrifugation using Ficoll (GE Healthcare, Buckinghamshire, UK). The $CD34^+$ cells were obtained from the MNCs using a magnetic activated cell sorting system (CD34 Microbead Kit; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The freshly isolated MNCs were then plated on cell culture dishes with 1% gelatin (Sigma-Aldrich, USA) and cultured in the EGM-2 BulletKit system (Lonza, USA). After 5 days, non-adherent cells were removed, and fresh culture medium was added. Cultures were maintained for 10-14 days until the development of cobblestone-shaped colonies. The medium was changed daily and colonies were re-plated and cultured further. For long-term priming with three small molecules, MNCs isolated from HUCB were consistently *ex vivo*-cultured using EGM-2 medium supplemented with 3 chemical cocktail (Fucoidan 0.1 μg/ml, TUDCA 25 μM, Oleuropein 0.5 μM), designated as OEC-3Cs. In this study, we used OECs and OEC-3Cs (passages 6-10) cultured for the same period from the same donor in all experiments.

EPC colony forming assay

$CD34^+$ cells from the HUCB were expansion-cultured as previously reported (Masuda et al., 2011). For the EPC colony forming assay, expanded $CD34^+$ cells (1000 cells per 35-mm dish) were cultured for 14-21 days in methylcellulose-containing medium (MethoCult SF H4236, StemCell Technologies, Canada) with 20 ng/ml recombinant human stem cell factor (Peprotech, USA), 50 ng/ml vascular endothelial growth factor (VEGF; Peprotech), 20 ng/ml interleukin-3 (Peprotech), 50 ng/ml basic fibroblast growth factor (Peprotech), 50 ng/ml epidermal growth factor (Peprotech), 50 ng/ml insulin-like growth factor-1 (Peprotech), 2 U/ml heparin (Sigma-Aldrich), 1% penicillin-streptomycin (Welgene, Korea), and 30% fetal bovine serum (Thermo Fisher Scientific, USA). The colony forming ability was measured by counting the number of small and large EPC colony forming units (CFU) respectively.

Flow cytometry analysis

Unprimed OECs and those primed with fucoidan, TUDCA, and oleuropein (OEC-3Cs) were compared by flow cytometry analysis using immunofluorescent antibodies specific for the following surface antigens: CD34 (BD Pharmingen, USA), c-Kit (Miltenyi Biotec), CXCR4 (BD Pharmingen), Tie2 (BD Pharmingen), VEGFR2 (BD Pharmingen), CD31 (BD Pharmingen). Samples were analyzed using a fluorescence-activated cell sorter (BD accuri C6).

Cell proliferation assay
Cell proliferation was evaluated by a BrdU incorporation assay kit (Cell Signaling Technology, USA) according to the manufacturer's instructions. In brief, OECs and OEC-3Cs (5×10^3 cells/well) were seeded in 96-well plates in complete EGM2 medium. The cells were then incorporated with BrdU solution for 24 h, and the relative cell proliferation level was determined by measuring the absorbance at 450 nm.

Tube formation assay
Matrigel (BD Biosciences) was added to 96-well plates and incubated at 37℃. The cells (1×10^4 cells) were seeded onto Matrigel-coated wells and then further incubated at 37℃ for 6 h. After incubation, the tube-forming ability was measured by counting the number of branches visualized in one microscopic field per well (40× magnification). Each experiment was repeated three times.

Scratching wound-healing assay
For the scratch migration assays, the cells were seeded in 6-well plates and grown until confluence in complete EGM-2 medium. A linear gap was created by scratching the cells using a SPLScar Scratcher (SPL Life Science, Korea). The cells were washed to remove any detached cells and incubated at 37℃ for 6 h. Cell migration activity was expressed as the relative healing area according to the following formula: [(original scratch area - new scratch area)/original scratch area] × 100%.

Transwell migration and invasion assays
Transwell migration assays were performed using 24-well transwell inserts (8 μm pore size; Costar, USA). Cells (5×10^4) were seeded in the upper inserts, which were placed in 24-well plates containing EGM-2 with 100 ng/ml SDF-1α (R&D Systems, USA) and incubated for 6 h at 37℃. Cell migration activity was quantified by counting the number of cells that migrated in five random microscopic fields (200× magnification). For invasion assays, 5×10^4 cells were placed in the upper inserts coated with Matrigel (BioCoat Matrigel Invasion Chamber; USA). The cells were incubated in EGM-2 containing stromal cell-derived factor 1-alpha (SDF-1α) for 24 h at 37℃, and then the number of invaded cells was quantified in the same way described above for the transwell migration assay.

Annexin V and propidium iodide (PI) staining assay
Apoptosis was measured using a fluorescein isothiocyanate (FITC)-Annexin V apoptosis detection kit (BD Pharmingen) according to the manufacturer's protocol. In brief, the cells were treated with H_2O_2 (800 μM) and incubated for 30 min at 37℃. After staining with Annexin V-FITC and PI reagents, the proportion of viable, apoptotic, and dead cells was analyzed by flow cytometry (BD accuri C6).

Western blot analysis
Whole cell lysates were extracted using PRO-PREP protein extraction solution (Intron Biotechnology, Korea). After determination of the protein concentration using the bicinchoninic acid method (Thermo Scientific, USA), equal amounts of samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, USA). The membrane was blocked with 5% skimmed milk and then incubated with antibodies against p-ERK, ERK, p-Akt, Akt, Bax, Bcl-2 (Cell Signaling Technology), GAPDH, and β-actin (Santa Cruz) overnight at 4℃. After incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, bands were visualized with enhanced chemiluminescence reagents (Millipore).

RNA-Seq analysis
RNA libraries preparation and sequencing were performed by Theragen Bio Institute (Korea) using TruSeq RNA Sample Preparation Kit (Illumina, USA). In brief, purified mRNAs were synthesized as single-stranded cDNAs through random hexamer priming and then double-stranded cDNA synthesis was performed. After the sequential processes of end repair, A-tailing, and adapter ligation, cDNA libraries were amplified with polymerase chain reaction. The quality of the cDNA libraries was evaluated with the Agilent 2100 BioAnalyzer (Agilent, USA). Sequencing progressed as paired-end reads (2 × 100 bp) using HiSeq2500 (Illumina). The RNA-Seq data were analyzed according to a previously described method (Trapnell et al., 2012).

Statistical analysis
Data are presented as mean ± standard error of mean. Comparisons between two groups were performed with Student's *t*-test, and differences with $p < 0.05$ were considered statistically significant.

RESULTS

Identification of three stem-modulating factors for the differentiation of $CD34^+$ hematopoietic stem cells (HSCs) into endothelial lineage progenitors
Based on our previous studies, we investigated the biological effect of natural and physiological factors on $CD34^+$ stem cell differentiation into EPC-lineage cells (Cho et al., 2015). To evaluate the status of the EPC development of $CD34^+$ HSCs with treatment of each factor (TUDCA, fucoidan and oleuropein), we used *ex vivo*-expanded $CD34^+$ cells isolated from HUCB and performed the EPC colony forming assay as described previously (Masuda et al., 2011). Two types of colonies were formed: small EPC-CFUs and large EPC-CFUs, as proliferative populations of small and round-shaped cells, and vasculogenic populations of spindle-shaped cells, respectively (Fig. 1A). We further estimated the EPC colony-forming potential via quantification of the number of small and large EPC-CFUs. In line with the previous report (Cho et al., 2015), we confirmed that TUDCA increased the numbers of both types of EPC-CFUs (Figs. 1B and 1E). In addition, fucoidan and oleuropien also significantly increased the number of large EPC-CFUs (Figs. 1F and 1G), but the number of small EPC-CFUs was not significantly different when compared with that of the control (Figs. 1C and 1D). These results indicated that TUDCA, fucoidan, and oleuropein significantly induce the *in vitro* endothelial lineage differentiation of $CD34^+$ HSCs.

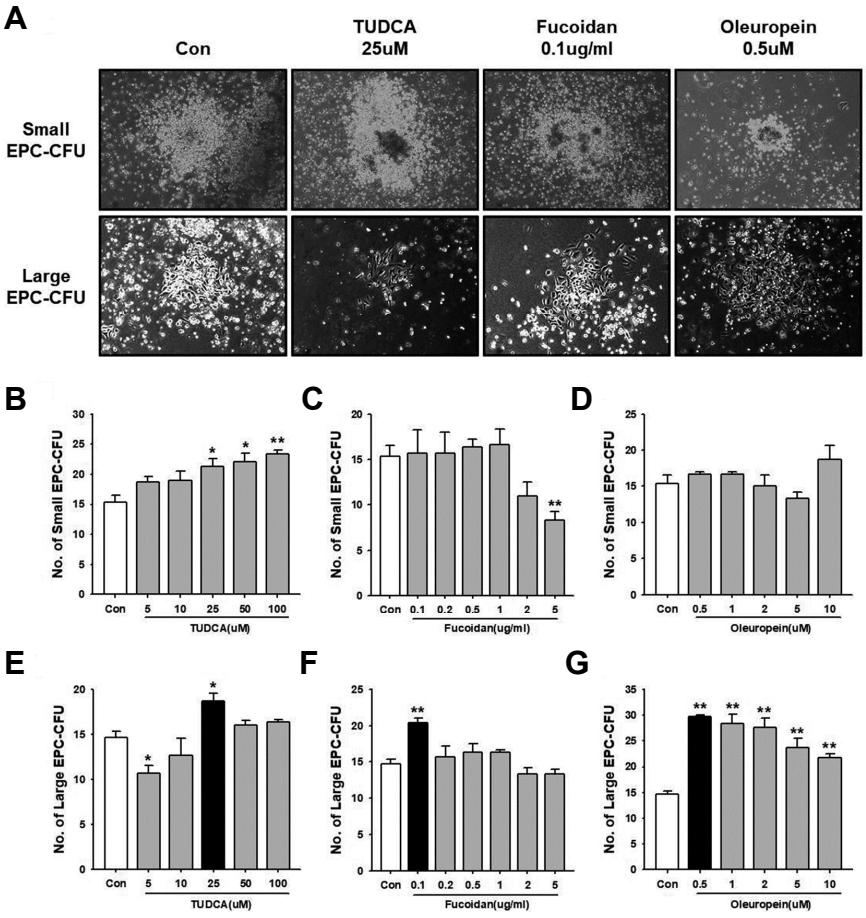

Fig. 1. Effects of each factor on the differentiation of CD34$^+$ HSCs into the EPC lineage. (A) Morphology of small and large EPC-CFUs derived from HUCB CD34$^+$ cells. (B-G) After *ex vivo* expansion of CD34$^+$ HSCs with various concentrations of each factor, the cells were cultured in methylcellulose-containing medium for 14-21 days. Small and large EPC-CFUs were counted. The results are shown as mean ± SEM (∗P < 0.05 and ∗∗P< 0.01 vs. control).

Characterization of OECs and OEC-3Cs

To determine whether the long-term culture of EPCs into late EPCs (i.e., OECs) is functionally enhanced by treatment of the three defined small molecules (3 chemical cocktail: TUDCA, fucoidan, and oleuropein), MNCs isolated from HUCB were *ex vivo*-cultured in EGM-2 medium with or without the 3 chemical cocktail, designated as the OEC and OEC-3C group, respectively. Cell colonies appeared 5 to 7 days after plating. By 10 to 14 days of culture, cells with a cobblestone-like morphology were nearly confluent. As shown in Fig. 2A, both types of OECs exhibited a cobblestone shape, which is the typical endothelial morphology. Next, to identify global transcriptomic signatures associated with the effects of long-term priming by the 3 chemical cocktail, we carried out RNA-seq analysis and compared mRNA expression profiles between OEC-3Cs and OECs. Among the total detected 19,126 genes, only 27 differentially expressed genes with ≥ 2-fold differences were identified in OEC-3Cs when compared with OECs (Fig. 2B, Supplementary Table S1). Furthermore, the biological replicates of OECs and OEC-3Cs were clearly similar to each other, although they definitely differed from adipose tissue-derived mesenchymal stem cells (Fig. 2C). To further confirm the phenotype of OECs and OEC-3Cs, we performed flow cytometry analysis, which demonstrated no significant differences in the expression of EPC surface markers such as CD34, c-Kit, CXCR4, Tie2, VEGFR2, and CD31 between OECs and OEC-3Cs (Figs. 2D-2J). Taken together, these data indicated that the global mRNA expression profiles and typical EPC surface markers of OECs are not affected and are preserved with long-term 3 chemical cocktail preconditioning.

Enhanced cell proliferation via ERK signaling in OEC-3Cs

According to our previous reports, each treatment with TUDCA, fucoidan, and oleuropein activated ERK and Akt (Cho et al., 2015; Choi et al., 2015; Lee et al., 2015a). Additionally, activation of ERK and Akt has been reported to augment EPC function (Wang et al., 2009). We therefore investigated whether the 3 chemical cocktail increases the levels of phosphorylated ERK and Akt (Fig. 3A). As shown in Figs. 3A-3C, the 3 chemical cocktail greatly activated ERK but did not significantly activate Akt in OECs and OEC-3Cs. To investigate the synergistic effect of 3 chemical cocktail, we evaluated the levels of phosphorylated ERK compared to the effects of each factor alone on primed conditions (Supplementary Fig. S1B) and confirmed that the 3 chemical cocktail activated ERK at a similar level to each single factor. The 3 chemical cocktail also increased the proliferation of OECs (Fig. 3D). Treatment with PD98059, a selective inhibitor

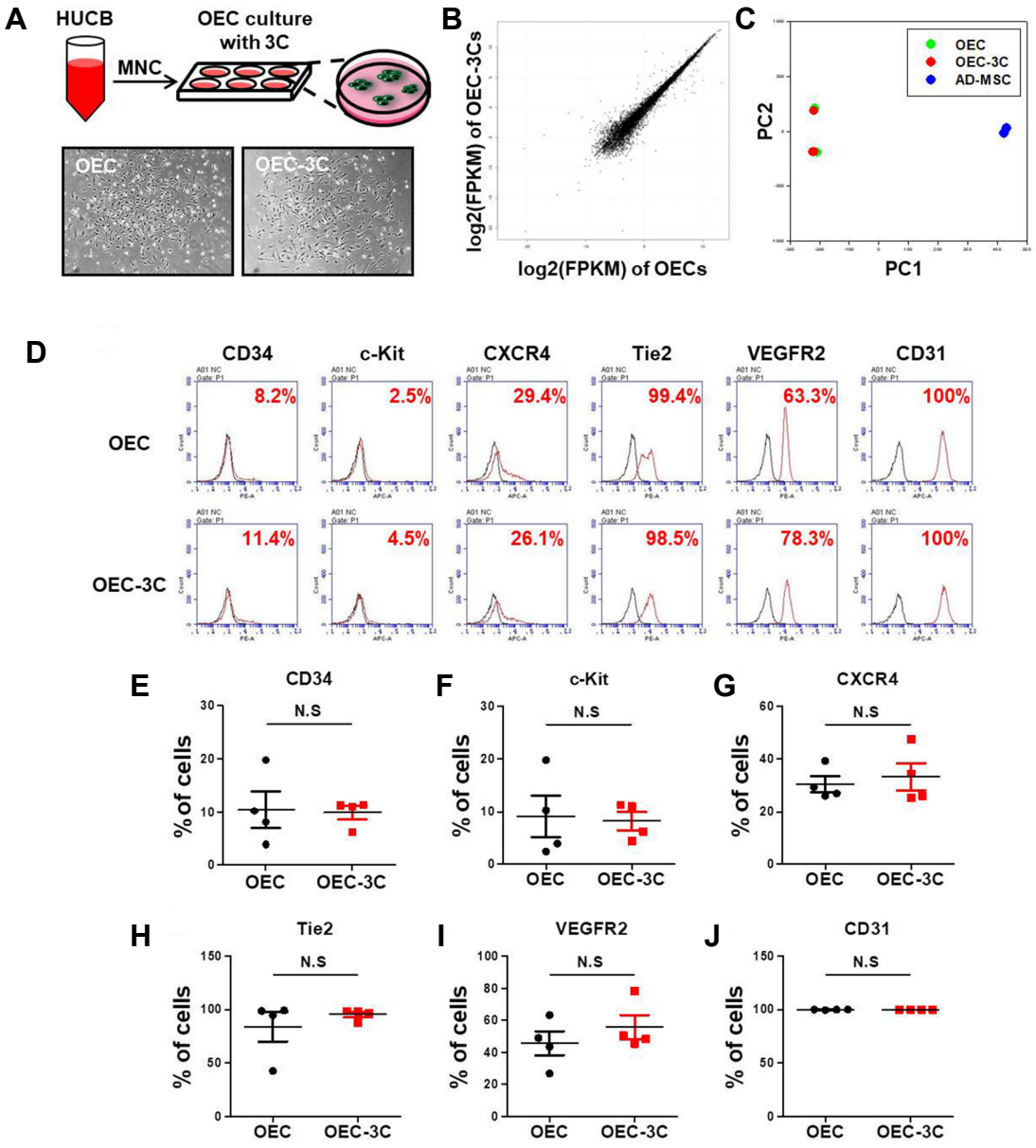

Fig. 2. Characterization of OECs and OEC-3Cs. (A) OEC culture protocol of long-term preconditioning by the 3 chemical cocktail. (B) Scatter plot presenting the log2 (FPKM) values for each gene in OECs (X-axis) versus the OEC-3Cs (Y-axis). (C) Multi-dimensional scale plot representing the biological replicates of OECs, OEC-3Cs, and AD-MSCs. (D) The expression of OEC surface markers (CD34, c-Kit, CXCR4, Tie2, VEGFR2, CD31) was analyzed by flow cytometry. (E-J) Quantification of the flow cytometry results of OEC surface markers. Means ± SEM (n = 4 OEC lines from four separate HUCB samples).

of the MEK/ERK pathway, abolished 3 chemical cocktail-induced proliferation of OECs (Fig. 3E). This finding suggests that ERK signaling, but not Akt, is involved in the proliferative ability of OECs.

Long-term preconditioning by 3 chemical cocktail promotes the angiogenic function of OECs

We next analyzed the effect of 3 chemical cocktail preconditioning on the migration capacity of OECs. We created a wounded region of confluent monolayers of both types of OECs and measured cell migration to the cell-free area. OEC-3Cs showed significantly increased cell migration compared with OECs (Figs. 4A and 4B). SDF-1α is a key factor in angiogenesis by recruiting EPCs (Yamaguchi et al., 2003; Zheng et al., 2007). Indeed, addition of SDF-1α (100 ng/ml) drastically enhanced the migration and invasion ability of OEC-3Cs (Figs. 4C and 4D). Moreover, the tube-forming capacity was increased in OEC-3Cs compared to OECs cultured in the normal condition (Figs. 4E and 4F). To investigate the additive effect of 3 chemical cocktail on OEC

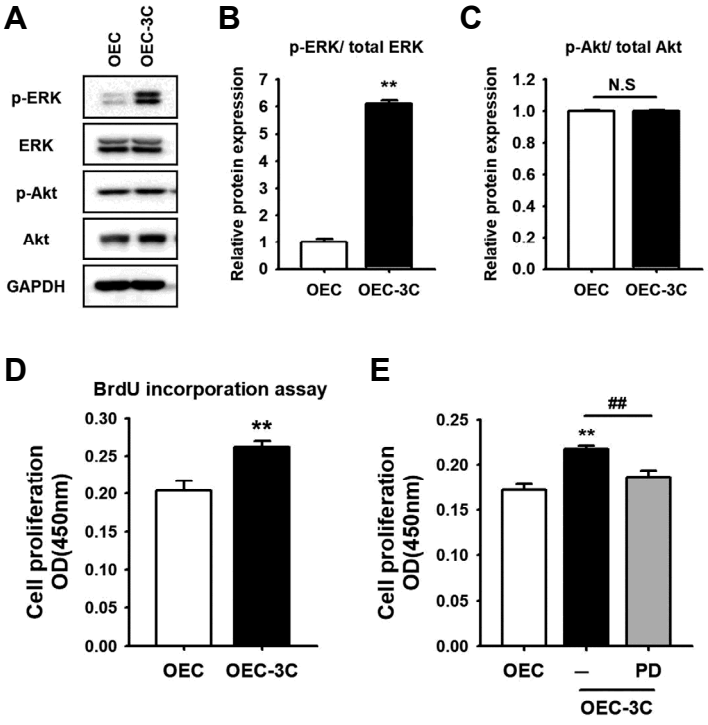

Fig. 3. Enhanced cell proliferation via ERK signaling in OEC-3Cs. (A-C) Activation of ERK and Akt was evaluated by measuring the phosphorylation levels by Western blotting; relative expression levels were determined by densitometry normalized to β-actin expression (D) Cell proliferation was examined using the BrdU incorporation assay. (E) OECs and OEC-3Cs were exposed to PD98059 (10 μM) for 24 h, and cell proliferation was analyzed. The results are shown as mean ± SEM (*$P < 0.05$ and **$P < 0.01$ vs. OEC).

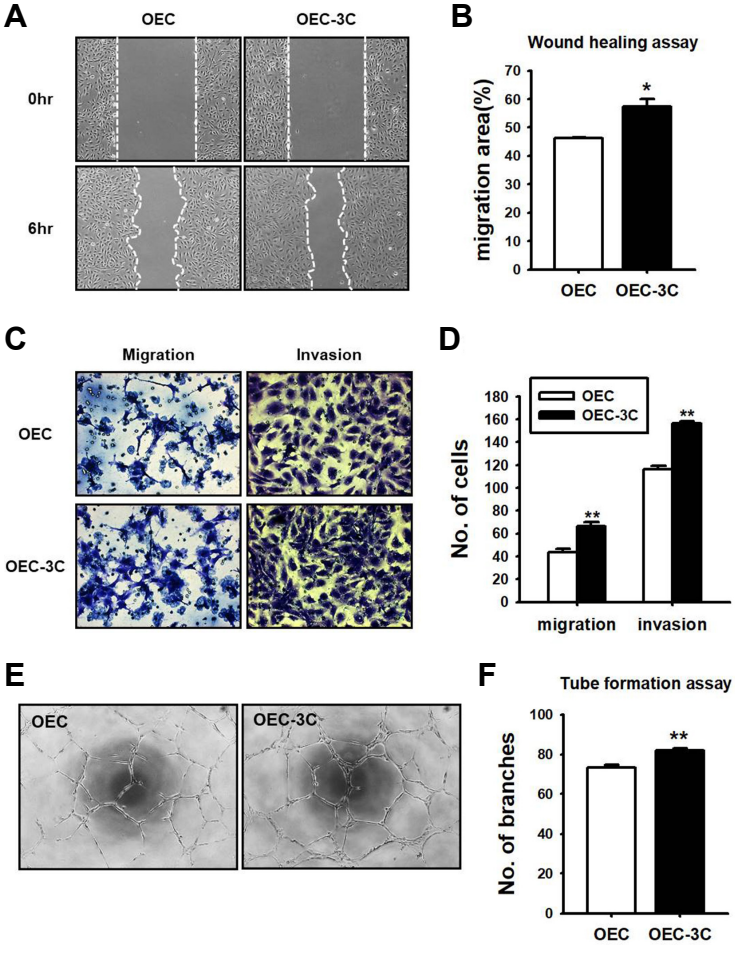

Fig. 4. Enhanced angiogenic function in OEC-3Cs. (A, B) Cell migration was evaluated by scratch wound-healing assays and migration capacity is displayed as the migration area (%). (C, D) Cell migration and invasion were assessed by Transwell migration and invasion assays. The migration and invasion capacity was determined by the numbers of migrating cells in OECs and OEC-3Cs. (E, F) OECs and OEC-3Cs were seeded into Matrigel-coated wells and the angiogenic function of cells was evaluated in a tube formation assay. Representative images of tube formation (magnification 40×) and quantification of the number of tube branches. Data represent the mean ± SEM of three independent experiments (*$P < 0.05$ and **$P < 0.01$ vs. OEC).

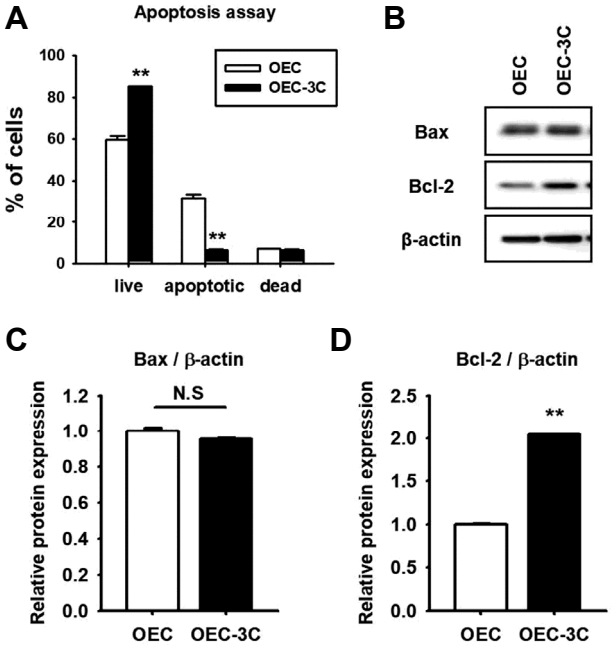

Fig. 5. Cytoprotective effect by the 3 chemical cocktail against H_2O_2-induced oxidative stress. (A) The apoptosis of OECs was analyzed by flow cytometry using AnnexinV/propidium iodide (PI) staining after exposure to H_2O_2 (800 μM, 30 min) as follows: live cells (AnnexinV$^-$/PI$^-$), apoptotic cells (AnnexinV$^+$/PI$^-$), dead cells (AnnexinV$^+$/PI$^+$). (B) Protein expression of Bax (pro-apoptotic) and Bcl-2 (pro-survival) was assessed by western blotting. (C, D) Quantification of expression levels of Bax and Bcl-2 relative to β-actin expression. Data represent the mean ± SEM of three independent experiments (**$P < 0.01$ vs. OEC).

function, we conducted functional assays to compare OEC-3Cs and cells treated with individual factors. Overall, we confirmed that the migration, invasion, tube formation, and survival of OECs were effectively enhanced under 3 chemical cocktail priming conditions compared to the effect of each factor alone (Supplementary Figs. S1C-S1F). Interestingly, 3 chemical cocktail consisting of three small molecules at low concentration, was previously shown to not affect cellular function. These results indicate that treatment with a combination of these factors had a synergistic effect on priming of OECs compared to each factor alone.

Enhanced cell viability in OEC-3Cs against H_2O_2-induced oxidative stress

Increased oxidative stress has been linked to endothelial dysfunction and cellular injury (Cai and Harrison, 2000). Thus, to overcome this reactive oxygen species-mediated cellular damage, various cell defense mechanisms against oxidative stress have been investigated (Franco et al., 2013; Parzonko et al., 2013). Furthermore, we recently reported that oleuropein prevents angiotensin II-meditated oxidative stress and EPC depletion via ERK1/2 signaling (Choi et al., 2015). To test the effects of the 3 chemical cocktail on OEC viability, oxidative stress or cell death was induced by 30-min exposure to H_2O_2 (800 μM) in complete EGM-2 medium. We then measured apoptosis using Annexin V/PI staining and showed that the proportion of apoptotic cells was greatly reduced in OEC-3Cs compared with that in OECs (Fig. 5A). In addition, the live cell population was significantly increased in OEC-3Cs, indicating that viability under oxidative stress was enhanced with 3 chemical cocktail preconditioning. To assess the underlying mechanisms, we examined the protein expression of members of the BCL2 family: Bcl-2 (a pro-survival factor) and Bax (a pro-apoptotic factor). As shown in Fig. 5D, the expression of Bcl-2 was increased by more than 2-fold in OEC-3Cs. However, there was no significant difference in the Bax level between OECs and OEC-3Cs (Fig. 5C). Based on these results, we concluded that Bcl-2 might play a role in the survival of OEC-3Cs.

DISCUSSION

The objective of this study was to investigate the long-term priming effects of a 3 chemical cocktail including fucoidan, TUDCA, and oleuropein on the angiogenic cell functionalities of OECs. We previously reported the short-term priming effects of these three physiologically natural factors on enhancing the bioactivities of EPCs, including cell proliferation, differentiation, mobilization, and senescence (Cho et al., 2015; Choi et al., 2015; Lee et al., 2015a). Based on these previous findings, we hypothesized that long-term priming of EPCs with an optimal combination of these three small molecules might be a promising strategy to improve the functionalities of EPCs against ischemic vascular diseases.

In the EPC colony forming assay, we found that the number of large EPC-CFUs, definitive EPCs, was increased by pretreatment of fucoidan, TUDCA, and oleuropein, indicating that each factor has an impact on the differentiation of CD34+ HSCs into endothelial lineage progenitors. Based on these effects, we cultured MNCs from HUCB in EGM-2 medium with or without the 3 chemical cocktail and examined its long-term priming potential on OECs, also known as late EPCs (Hur et al., 2004) or ECFCs (Yoder et al., 2007), under continuous 3C treatment conditions. Our results clearly demonstrated that long-term priming by 3C had no impact on the OEC phenotype, including cell morphology and expression of typical EPC surface markers (CD34, c-Kit, CXCR4, Tie2, VEGFR2 and CD31). Similarly, global transcriptome analysis revealed that biological replicates of OECs and OEC-3Cs had very high Pearson correlation coefficients (0.950-0.969), indicating that the two OEC types are very closely related. Taken together, these data suggest that 3C-primed OECs are indeed a type of late EPC without any significant difference in gene expression profiles, indicating that long-term treatment of 3C in OECs might be a safe methodology for cell therapy.

To clarify the functional differences between OECs and OEC-3Cs, we first evaluated the proliferation capacity of OECs. According to our previous reports, fucoidan and TUDCA promote the proliferation of EPCs via activation of Akt and ERK. In particular, ERK activation plays a key role in the mediation of EPC proliferation by these factors by increasing the expression of cell cycle-associated proteins (Cho

et al., 2015; Lee et al., 2015a). However, our present findings showed that long-term priming by 3C increased the proliferation capacity of OECs via ERK activation but not Akt activation. Moreover, there was no significant difference in the expression of cell cycle-associated proteins (cyclin D1, Cdk4, cyclin E, and Cdk2) between OECs and OEC-3Cs (data not shown). These results indicate that only the ERK signaling cascade is mainly involved in the effects of priming by the 3 chemical cocktail on OEC proliferation; thus, further investigation is required to elucidate the precise downstream mechanism of ERK signaling in OECs. Next, we demonstrated that the migration, invasion, and tube-forming abilities were all drastically improved in OEC-3Cs compared with OECs. In addition, long-term priming by the 3 chemical cocktail could prevent cellular damage against H_2O_2-induced oxidative stress and upregulated the expression of Bcl-2, which is an important marker for apoptosis as a pro-survival protein (Czabotar et al., 2014). It has been reported that advanced glycation end products, which are proteins or lipids glycated as a result of exposure to sugars, could induce EPC apoptosis by downregulating Bcl-2 expression (Shen et al., 2010).

Interestingly, although the two types of OECs showed similar phenotypes and expression patterns of the global transcriptome, various functionalities such as proliferation, migration, and tube formation were greater in OEC-3Cs than in OECs. One possible explanation is the difference in post-translational modifications (PTMs), including methylation, sumoylation, and ubiqitination, between OECs and OEC-3Cs. A PTM is a biochemical modification that occurs on one or more amino acids in a protein during translation to diversify the limited genome of organisms (Prabakaran et al., 2012). Recently, the role of PTMs in the regulation of cellular pluripotency has been reported (Wang et al., 2014). To clarify the roles of the 3 chemical cocktail in intracellular signaling cascades, further mechanistic studies regarding PTM-based gene and protein alterations in 3C-primed OECs are warranted.

In RNA-seq analysis, we found that some genes that may contribute to the angiogenic ability of OECs. Among the 27 DEGs between OECs and OEC-3Cs, STAT3, an important transcription factor that regulates anti-apoptotic pathways (Duan et al., 2013; Lee et al., 2015b), was significantly upregulated in OEC-3Cs. MDGA1, which plays a critical role in controlling neuronal adhesion and migration (Diaz-Lopez et al., 2010), was also increased in OEC-3Cs. Further studies are needed to confirm the contribution of these genes to the enhanced angiogenic ability of OECs.

In conclusion, our results demonstrate that priming of OECs with our optimized 3 chemical cocktail significantly improves the proliferation potential of the cells as well as their differentiation, migration, and tube-forming capacities. Therefore, this work provides strong evidence for the first time that long-term preconditioning of OECs with the 3 chemical cocktail might be an effective strategy for developing functionally improved EPC therapeutics against ischemic vascular diseases.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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