Minoxidil Regulates Aging-Like Phenotypes in Rat Cortical Astrocytes In Vitro

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Abstract

Mainly due to the slanted focus on the mechanism and regulation of neuronal aging, research on astrocyte aging and its modulation during brain aging is scarce. In this study, we established aged astrocyte culture model by long-term culturing. Cellular senescence was confirmed through SA-β-gal staining as well as through the examination of morphological, molecular, and functional markers. RNA sequencing and functional analysis of astrocytes were performed to further investigate the detailed characteristics of the aged astrocyte model. Along with aged phenotypes, decreased astrocitic proliferation, migration, mitochondrial energetic function and support for neuronal survival and differentiation has been observed in aged astrocytes. In addition, increased expression of cytokines and chemokine-related factors including plasminogen activator inhibitor -1 (PAI-1) was observed in aged astrocytes. Using the RNA sequencing results, we searched potential drugs that can normalize the dysregulated gene expression pattern observed in long-term cultured aged astrocytes. Among several candidates, minoxidil, a pyrimidine-derived anti-hypertensive and anti-pattern hair loss drug, normalized the increased number of SA-β-gal positive cells and nuclear size in aged astrocytes. In addition, minoxidil restored up-regulated activity of PAI-1 and increased mitochondrial superoxide production in aged astrocytes. We concluded that long term culture of astrocytes can be used as a reliable model for the study of astrocyte senescence and minoxidil can be a plausible candidate for the regulation of brain aging.

Key Words: Astrocytes, Brain aging, Mitochondria, Transcriptomic profiling, Minoxidil

INTRODUCTION

Aging is one of the biggest problems in human society at the moments with the rapidly growing elderly population and the research on aging as well as geriatric diseases has uppermost importance. Therefore, delineating the mechanisms of aging-related changes in cellular functions would be the first step to understand aging and to devise the methods to regulate aging and aging-related diseases. It is obvious that each cell types in the brain including neurons and astrocytes would undergo cell type-specific aging process. Therefore, it is both necessary and important to study the aging process and the mechanism underlying it at the level of each cell types. However, most of the aging studies involving central nervous system (CNS) have been focused on neurons or microglia. This may pose a problem in the interpretation of aging process in the brain considering the magnitude of the cell population and proliferation potency of the other important cell types, i.e. astrocytes. For example, a decrease in brain volume is observed as one of the characteristics of brain aging, but Freeman et al. (2008) reported that number of neurons was not changed in elderly subjects nor Alzheimer’s disease patients. The brain atrophy with aging may be mediated by the dysregulation of glial cells, especially astrocytes. Astrocytes are the largest cell population in the brain with proliferation potential and are well known to exert various functions ranging from structural maintenance of the brain to functional regulation of neurotransmission (Chung et al., 2015; Burda et al., 2016; Siracusa et al., 2019). In addition, astrocytes have a direct effect on neuronal survival and development by secreting neurotrophic factors, assisting cell growth and survival, and contributing to neuronal signal transmission by regulating the concentration of neu-
rotransmitters at synapses (Fiacco et al., 2009). In particular, astrocytes regulate the immune activity in the brain along with microglia, and it is relatively well known that astrocytes and microglia exhibit a mild inflammatory phenotypes with aging (Abbott et al., 2006).

With the multitude of the function served by the astrocytes, functional dysregulation of astrocytes with aging may also affect various other brain cells resulting in the dysregulation of the maintenance of the optimal brain environment (Dossi et al., 2018).

In this study, we developed an in vitro astrocyte aging model by culturing the cells for a long time more than 12 weeks for in-depth study on astrocyte aging and screening of drug candidates with potential to control astrocyte and brain aging processes, which may ultimately be used against neurodegenerative disorders such as Alzheimer’s disease and Parkinson’s disease. We analyzed functional changes of senescent astrocytes in detail and searched for putative senescence regulators through transcriptomic profiling and connectivity map (CMap) analysis (Lamb et al., 2006). Here, we report that long-term culture of astrocytes shows cellular senescent phenotypes along with functional deficits, which even affect deficits in neural support by astrocytes. The potential use of minoxidil as a modulator of astrocyte senescence will be demonstrated.

MATERIALS AND METHODS

Materials

The materials used in this study are as follows: Dulbecco’s modified Eagle medium (DMEM)/F12, Penicillin-Streptomycin (P/S), 0.25% trypsin-EDTA, and 10% Fetal Bovine Serum (FBS) were from Gibco BRL (Grand Island, NY, USA); Tween® 20 and ECLTM Western blotting detection reagent were from Amersham Life Science (Arlington Heights, IL, USA); anti-β Actin was obtained from Sigma (St. Louis, MO, USA); anti-INOS and senescence detection kit were the product of Abcam (Cambridge, UK); minoxidil was obtained from Sigma; Agilent Seahorse XF Cell Mito Test Kit was from Agilent Technologies (CA, USA); Alexa Fluor® 594 conjugated Escherichia coli BioParticles® and Tetramethylrhodamine Methyl Ester (TMRM) were purchased from Thermo Fisher Scientific (MA, USA); MitoSOX™ was from Invitrogen (MA, USA).

Astrocyte culture

Animal maintenance and experimental processes were performed following the rules and conditions approved by the Institutional Animal Care and Use Committee (IACUC) of Konkuk University (KU19017). Sprague-Dawley (SD) rats were purchased from Samtako, Inc. (Osan, Korea). Astrocytes were cultured from brain cortex of postnatal day 2 (P2) SD rats as described previously (Bang et al., 2019b). Concisely, brain cortices were dissected and mechanically triturated. The triturated cells were plated on the poly-D-lysine-coated plate (20 μg/mL) and incubated in DMEM/F12 with 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% heat-inactivated FBS in a 95% CO2 incubator at 37°C. The culture medium was changed every 3 or 4 days. After 2 or 12 weeks, the incubated astrocytes were sub-cultured in the well plates. The purity of sub-cultured astrocyte was more than 95% as determined by counting the glial fibrillary acidic protein (GFAP) positive cells.

Neuron culture

Pregnant Sprague-Dawley (SD) rats were purchased from ORIENT (Seongnam, Korea). The primary neurons were dissected from the cortex of embryonic day 18 (E18) SD rats. The isolated cortical neurons were mechanically triturated and seeded on the poly-D-lysine-coated plate (50 μg/mL). The seeded cells were maintained in NBM with B27 supplement and L-glutamine in a 95% CO2 incubator at 37°C. The media were half-changed every 3 days.

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

SA-β-gal positive cells were detected using Senescence Detection Kit (Abcam) in vitro. The experiment was proceeded following the manufacturer’s instructions (Dimri et al., 1995). Density of astrocytes was 2.5×10⁵ cells in 12 well-plate.

Nuclear staining

Cells were incubated on a poly-D-lysine-coated coverslip and were fixed by 4% paraformaldehyde (PFA) for 10 min at 37°C. After then, the cells were permeabilized by 0.1% Triton X-100 for 20 min at room temperature. The fixed cells were stained by DAPI (4’,6-diamidino-2-phenylindole) for 10 min at room temperature. The samples were mounted with GEL/MOUNT (Biomeda Corp., CA, USA) and visualized by a digital microscope (CELENA, Logos Biosystems, Anyang, Korea).

Reverse transcription-polymerase chain reaction (RT-PCR)

The RNA isolation was conducted with TRIzol reagent (Invitrogen). The concentration of extracted RNA was measured using a spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The RNA was used for the synthesis of cDNA with RT reaction mixture containing RevertAid Reverse transcriptase reaction buffer (Thermo Fisher Scientific) and dNTP (Promega, WI, USA). cDNA was used as template for PCR amplification with following protocol: [94°C, 30 s; 60°C, 1 min; 72°C, 30 s]×30 cycles, then 72°C for 10 min; or [94°C, 30 s; 60°C, 1 min; 72°C, 30 s]×23 cycles, then 72°C for 10 min. The sequences of primers used in this study are as follows: IL-1β (sense: 5’-AAA ATG CCT CGT GCT GTG TG-3’/antisense: 5’-CTA TGT CCC GAC CAT TGC TG-3’), IL-6 (sense: 5’-TTG TGC AAT GGG AAT TCT GA-3’/antisense: 5’-TTG AAG ATT GGG TAG GAA GGA-3’), iNOS (sense: 5’-CTG GCT GCC TTG TTC AGC TA-3’/antisense: 5’-AGT GTA GCG TTT CGG GAT CT-3’), p16 (sense: 5’-ATC GCC AAT TCT GA-3’/antisense: 5’-TCT GTG CCT CCC TCC TCT TGG TAA C-3’), GAPDH (sense: 5’-TGG AAG GTG TTG ATG ATT TAT CCT GAC-3’/antisense: 5’-CAT CTG CTG CTG ACC AGC-3’). The PCR products were normalized by GAPDH or 18s RNA mRNA.

Western blot analysis

Western blot experiment was conducted following a previously described procedure (Bang et al., 2019b). Concisely, cells were harvested with radioimmunoprecipitation assay (RIPA) buffer containing 2 mM EDTA, 0.1% (w/v) SDS, 50 mM Tris-HCl, 150 mM sodium chloride, 1% Triton X-100, and 1% (w/v) sodium deoxycholate. Concentration of the proteins was measured by BCA assay kit (Thermo Fisher Scientific). The protein samples in SDS-PAGE buffer were boiled for 5 min at 100°C. The SDS-PAGE was performed for 120 min at 100 V. The proteins were electrically transferred to nitrocellulose...
BioParticles™ were incubated with cells and the red fluorescence inside the cells was measured by the IncuCyte ZOOM microscope software 2015A (Essen Bioscience, Hertfordshire, UK). Wound width and density were analyzed by the IncuCyte ZOOM microscope software every 30 min for 12 h. The images were analyzed using Image-J software (NIH, USA). The intensity of the bands was normalized using β-Actin as control.

Inverse casein zymography

The plasminogen activator inhibitor-1 (PAI-1) activity was measured through inverse casein zymography following previously described methods (Ko et al., 2015). Briefly, cultured media supernatant of cells was mixed with SDS-PAGE sample buffer lacking β-mercaptoethanol. The samples were conducted SDS-PAGE using gels containing casein and then, gels were rinsed twice in 2.5% Triton X-100 for 30 min. The gels were gently shaken for 5 h at 25°C in reaction buffer (20 mM Tris–HCl, pH 7.6), containing urokinase-type plasminogen activator (uPA; 0.5 IU/mL; American Diagnostica, NY, USA). After then, the inverse-zymography gels were stained with 0.1% Coomassie brilliant blue R-250 (Elpisbio, Daejeon, Korea) and de-stained with de-staining buffer containing 20% methanol and 10% acetic acid. The gels were visualized using the LAS4000 image detection system (Fuji, Tokyo, Japan).

Wound closure assay

Confluent cells were scratched 700 nm-wide using a certified Essen Bioscience automated 96-wound-maker™ (Essen Bioscience, Hertfordshire, UK). Wound width and density were measured using the IncuCyte ZOOM system (Essen Bioscience, MI, USA) and were analyzed using the IncuCyte ZOOM microscope software 2015A (Essen Bioscience).

Phagocytosis

Alexa Fluor™ 594 conjugated Escherichia coli (K-12 strain) BioParticles™ were incubated with cells and the red fluorescence inside the cells was measured by the IncuCyte ZOOM system (Essen Bioscience) every 30 min for 12 h. The images were analyzed by the IncuCyte ZOOM microscope software 2015A (Essen Bioscience).

Determination of mitochondrial membrane potential

The cells were incubated with 100 nM TMRE staining solution in light protection conditions for 30 min at 37°C. After then, the cells were rinsed with phosphate-buffered saline (PBS) and imaged by the IncuCyte ZOOM system (Essen Bioscience). The images were analyzed by the IncuCyte ZOOM microscope software 2015A (Essen Bioscience).

Mitochondrial oxygen consumption rate (OCR)

The measurement of mitochondrial OCR was proceeded following the Agilent Seahorse XF Cell Mito Test Kit user guide. Briefly, the sensor cartridges were incubated overnight with a calibrator buffer in a non-CO2 incubator at 37°C. Mitochondrial modifiers such as 1.0 μM oligomycin, 1.0 μM FCCP, or 0.5 μM rotenone/0.5 μM antimycin A were put in each port of cartridge as instructed. The cells were incubated with Seahorse XF Base Medium containing 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose for about 1 h. Mitochondrial OCR was measured by Agilent Seahorse XFe96 Analyzer. The results were analyzed by Wave Desktop 2.6 software (Santa Clara, CA, USA).

Measurement of neurite outgrowth

Astrocyte cultured media (ACM) were harvested from astrocyte cultured for 24 h in serum free condition. The rat cortical neurons were incubated for 2 days and added with ACM obtained from young and aged culture. The length of the neurite was measured using the IncuCyte® Live-Cell Analysis system (Essen BioScience, Inc., Ann Arbor, MI, USA).

RNA-sequencing

Astrocytes were incubated for 2 or 12 weeks and sub-cultured. The sub-cultured astrocytes were treated with vehicle (DPBS; Dulbecco’s Phosphate-Buffered Saline) or 10 ng/mL lipopolysaccharide (LPS) and harvested with trizol. The samples were used for RNA-sequencing by a commercial sequencing company (Macrogen, Seoul, Korea).

Measurement of mitochondrial superoxide

The cells were incubated with 5 μM Mitosox™ (Invitrogen) for 10 min at 37°C, protected from light. The samples were washed gently 3 times. The level of mitochondrial superoxide was measured using the IncuCyte® Live-Cell Analysis system.

Statistical analysis

All the experimental data were presented as the mean ± SEM and the statistical analyses were performed using GraphPad Prism version 5 software (GraphPad Software Inc., CA, USA). The group comparisons were conducted using two-way ANOVA followed by Bonferroni’s post-test. Two-sample comparison was analyzed with unpaired t-test. A p-value of <0.05 was considered significant.

RESULTS

Long-term cultured astrocytes displayed aged cell-like phenotypes

Astrocytes were incubated for 2 (short-term cultured astrocytes) or 12 (long-term cultured astrocytes) weeks. Cells were stained with SA-β-gal to confirm whether long-term cultured astrocytes show cellular senescence phenotype. Usually, astrocytes are cultured for 2 weeks and are used for experiments. To establish senescent cells, astrocytes were cultured for 4, 8, 10, 12, 16 and 20 weeks (data not shown). While astrocyte cultures exceeding 16-week period separated from the culture plate, cells cultured for less than 10 weeks did not show a senescent phenotype. As a result, we found that 12-week cultured astrocytes could be maintained for the longest time without being separated from the culture plate. Long-term cultured astrocytes displayed SA-β-gal positive cells 11 times more than short-term cultured astrocytes (Fig. 1A). Senescent cells exhibit nucleus enlargement phenotype (Yoon et al., 2016), therefore, the nuclear size of astrocytes was measured using DAPI staining. After photographing DAPI-stained nuclei, the number of pixels was counted using the Image-J software program. The nuclear size of long-term cultured astrocytes was 17% larger than that of short-term cultured astrocytes.
To determine whether long-term cultured astrocytes show the molecular phenotypes of senescent cells, we first performed RT-PCR to detect mRNA expression, which has been implicated in cellular senescence. Those senescence-related factors include immune- and cell cycle-related factors as well as protease-related factors. In this study, the expression level of *IL-1b* and *IL-6* was significantly increased in long-term cultured astrocytes in basal, non-stimulated condition (Fig. 2A). In addition, the mRNA expression of *p16*, a tumor suppressor and a well-known biomarker of cellular senescence, was increased 1.5-folds in long-term cultured astrocytes (Fig. 2B). To mimic the immune-challenged situation, we stimulated long-term cultured astrocytes with 0 (DPBS), 1, or 10 ng/mL LPS (Fig. 2C, 2D). Although iNOS mRNA and protein expression levels were not detected under basal conditions, immune activation increased the expression of iNOS which is more prominent in long-term cultured astrocytes compared with short-term cultured astrocytes (Fig. 2C). Increased PAI-1 mRNA expression level was also observed in long-term cultured astrocytes (Fig. 2D). Inverse-zymography and Western blot also showed increased activity and protein expression levels of PAI-1, respectively, in long-term cultured astrocytes (Fig. 2E, 2F).

To identify functional significance of the astrocyte senescent phenotypes, astrocyte migration, phagocytosis, and mitochondrial function were examined. Migration of cells was investigated through wound-scratch assay. After making scratch wound with a width of about 700-800 μm on the confluent astrocyte cultures, the width and density of wound were automatically determined using computer-aided analytical tools (Incucyte, Essen Bioscience). Wound closure of long-term cultured astrocytes was significantly slower than cells cultured for short-term (Fig. 3A). The rate of decrease in wound width was slower in long-term cultured cells and the relative cell density in wound, which represents the cell density in the wound area compared to the cell density outside the wound area that changes over time, was higher in the short-term cultured cells compared to the long-term cultured cells. Next, phagocytosis function of astrocytes was investigated using *E. coli* bio-particles uptake assay. The intensity of *E. coli* bio-particles inside the cells was decreased in long-term cultured astrocytes, suggesting the reduced phagocytic activity of the aged cells (Fig. 3B). To identify mitochondrial function of long-term cultured astrocytes, mitochondrial membrane potential was determined using TMRM. Doxorubicin, a well-known mitochondrial toxin, was used as a positive control. Long-term cultured astrocytes showed significantly reduced TMRM intensity compared to short-term cultured astrocytes (Fig. 3C). In addition, spare respiratory capacity was increased in long-term cultured astrocytes (Fig. 3D) although no differences in basal respiration, proton leak and ATP production were observed between short- and long-term cultured astrocytes.

**Conditioned media from long-term cultured astrocytes show altered capacity to support neurons**

Astrocytes and neurons interact closely, and consequently, we reasoned that dysfunction of astrocytes in long-term culture could adversely affect neuronal function such as neurite outgrowth and mitochondrial OCR (Lee et al., 2020; Zhang et al., 2022). Thus, we tried to examine the effects of long-term cultured astrocytes on neuron through the application of astrocyte culture-conditioned media (ACM) to neuron. After applying ACM to neuron, neurite outgrowth was monitored and analyzed every 3 h. Control ACM treated neurons displayed longer length of neurite than non-treated group. However, long-term cultured ACM treated neurons measured shorter neurite length than short-term cultured ACM treated neurons (Fig. 4A). In mitochondrial OCR experiment of long-term cultured ACM treated neurons, spare respiratory capacity of neuron was increased (Fig. 4B) suggesting that the long-term cultured astrocytes adversely affect their capability to modulate neuronal function.

**Fig. 1.** Morphological changes of long-term cultured astrocytes. (A) Cultured astrocytes were with stained senescence-associated beta-galactosidase (SA-β-gal). The bar graph shows fold changes of SA-β-gal positive cell number. (B) Cultured astrocytes were indicated by DAPI to determine size of nuclei. The bar graph representative nuclei size of cells. ***p<0.001 vs. short-term cultured astrocytes. The data analysis was performed using unpaired t-test and Error bars indicate SD.
Gene expression signature of long-term cultured astrocytes

RNA-seq was performed to examine the gene expression profile of long-term cultured astrocytes and comparative transcriptome analysis was performed according to the workflow in Fig. 5A. The gene ontology of the selected target gene was identified through GENE MENIA and GOrilla. Genes which showed a significant change (38 up-regulated genes, and 64 down-regulated genes (|log2 FC|>1.5, p-value <0.05) in long-term culture, were further analyzed for the prediction of function of gene sets through GENE MENIA analysis and the related cellular components were analyzed using GOrilla. Genes that were significantly increased by long-term culture showed a substantial correlation with the extracellular region, and the significantly decreased gene set exhibited a high correlation with the membrane (Supplementary Fig. 1A, 1B).

In addition, CMap was performed to find perturbagens showing opposite phenotype to the changes in gene expres-
Minoxidil reverses phenotypic changes in long-term cultured astrocytes

We confirmed whether the functional dysregulation of long-term cultured astrocytes can be modulated by selected perturbagens identified with CMap analysis (Fig. 5B). We preliminarily screened several candidates and minoxidil showed most significant functional changes in long-term cultured astrocytes. During the long-term culture period, minoxidil was administered for the last 2 weeks, and it was examined whether the functional changes of astrocytes by long-term culture can be reversed. The number of SA-β-gal positive cells increased during long-term culture, which was significantly reduced by minoxidil treatment (Fig. 6A). In addition, the size of the nucleus was enlarged by long-term culture that was significantly reduced in the minoxidil-treated group (Fig. 6B). The size of nucleus in min-

![Fig. 3.](image)

**Fig. 3.** Functional changes of long-term cultured astrocytes. (A) Cultured astrocytes were measured for migration ability through wound healing scratch assay. (B) Cells were confirmed phagocytosis using E. coli particles for 12 h. The cells were also checked for mitochondrial function by (C) mitochondrial membrane potential and (D) mitochondrial oxygen consumption rate (OCR). *p<0.05, **p<0.01, and ***p<0.001 vs. short-term cultured astrocytes. The data analysis was performed using two-way ANOVA and Error bars indicate SD.
oxidil treated group was exhibited similar size with short-term cultured astrocytes (Fig. 6B). In the zymography experiments, the increased PAI-1 activity was normalized by minoxidil treatment (Fig. 7). The mitochondrial function was examined using MitoSOX™. The increased production of mitochondrial superoxide by long-term culture was mitigated in the minoxidil-treated

Fig. 4. Functional changes of cultured neurons by astrocytes conditioned media (ACM) treatment. Cultured cortical neurons were treated ACM. (A) The neurons were subjected to live cell imaging to check the neurite length for 42 h. (B) Mitochondrial OCR of neurons were measured using Cell Mito Test Kit (Agilent Technology) and Agilent Seahorse XFe96 Analyzer (Agilent Technology). The data analysis was performed using two-way ANOVA and Error bars indicate SD.

Fig. 5. Big data analysis of long-term cultured astrocytes. RNA-sequencing was performed on cultured astrocytes and analyzed. (A) RNA-sequencing results were analyzed following workflow. (B) Cmap used for finding novel therapeutics.
Fig. 6. Minoxidil reversed morphological changes on long-term cultured astrocytes. Long-term cultured astrocytes were pretreated with vehicle (DMSO) or 10 μM minoxidil before 2 weeks. (A) Cultured astrocytes were inspected for SA-β-gal positive cells. (B) Nuclei size were examined on cultured astrocytes. Scale bar=25 μm. The graph shows nuclei size of cells. *p<0.05 vs. vehicle treated astrocytes. **p<0.001 vs. long-term cultured astrocytes. Error bars indicate SD.

Fig. 7. Minoxidil reversed molecular changes on long-term cultured astrocytes. Long-term cultured astrocytes were pretreated with vehicle (DMSO) or 10 μM minoxidil for 2 weeks. Protein activity was examined through zymography and inverse-zymography. * indicates p<0.05, and ** indicates p<0.01 vs. short-term cultured astrocytes. #p<0.05 vs. long-term cultured astrocytes. Error bars indicate SD.

Fig. 8. Minoxidil reversed functional changes on long-term cultured astrocytes. Long-term cultured astrocytes were pretreated with vehicle (DMSO) or 10 μM minoxidil for 2 weeks. Mitochondrial superoxide was detected by MitoSOX dye. *p<0.05 vs. vehicle treated astrocytes. Error bars indicate SD.

discuss further in the future study.


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cells delays the onset of aging or age-related diseases (Baker et al., 2008, 2011). Whether the increased expression of p16 in aged astrocytes as observed in this study contribute to the regulation of aging process per se and relative importance of stem cell senescence (or inhibition of proliferation) compared with astrocyte senescence on the development of neurodegenerative disorders awaits further investigation.

Functional examination of aged astrocytes in this study showed prominent defects in cell migration. In this regard, it is noteworthy that tPA and PAI-1 are well known targets and regulators of proliferation and migration pathways (Czekay et al., 2011) as well as aging and immune responses (Valentijn et al., 2018; Zhang et al., 2021). In addition, IPA and PAI-1 plays crucial roles in the regulation of neurite outgrowth (Xin et al., 2010; Lee et al., 2014; Yu et al., 2019). Considering the changes in the IPA and PAI-1 activity in this study, these proteins may underlie the dysregulation of migration of long-term cultured astrocytes, which hampers the regenerative potential of astrocytes after injury as well as the potential to posterior neurite extension from damaged or newly generated neurons. Mitochondrial dysfunction including mtDNA mutations, decreased mitochondrial potential. Actually, the imbalance in mitochondrial dynamics is regarded as one of the indicators of aging. (Sugrue and Tatton, 2001; Seo et al., 2010; Srivastava, 2017). The reduced mitochondrial membrane potential observed in this study, might be related to the increased non dividing cells at their G0 phase at which the mitochondrial membrane potential decreased (Sugrue and Tatton, 2001). In addition, senescent cells are resistant to metabolic challenge by increasing mitochondrial OCR to meet the energy demand (Kim et al., 2018b), which might adversely affect the cells by increased ROS accumulation and increased fusion of mitochondria (Son et al., 2017). Dysregulation of mitochondrial function may adversely affect astrocyte function, which in turn accelerate mitochondrial dysfunction that might perpetuate neural dysfunction in aged brain. In this study, spare respiratory capacity (SRC) is the component of mitochondrial OCR showing most prominent changes in aged astrocytes. It has been argued that SRC is the robust parameters to evaluate mitochondrial reserve and non-proliferative cells might have higher proliferative potential (Marchetti et al., 2020), consist-ent with the reduced proliferative potential of the aged astrocytes observed in this study. Interestingly it is also reported that higher SRC is related to the reduced nuclear reprogramming and hence the reduced iPSC induction capacity (Zhou et al., 2017). Whether the aged astrocytes may have defective roles in stem cell induction and proliferation needs to be explored in the future study.

Transcriptomic profiling using GENE MANIA and GOrilla, revealed major changes in cytokines/chemokines and related functions as well as regulations of extracellular regions. This is consistent with the previous research suggesting the up-regulation of immune-related factors such as SASP with aging (Coppe et al., 2010; Davalos et al., 2010; Michaud et al., 2013; Cuello et al., 2020). Interestingly, among the gene sets showing significantly reduced expression, those related with TNF production are identified. There are several reports that TNF is involved in wound-healing, neuronal survival and strengthening of synapses (Zhang et al., 2011; Ashcroft et al., 2012; Kim et al., 2018a; Heir and Stellwagen, 2020).

Through the CMap analysis and subsequent validation studies, minoxidil has been identified as a potential candidate to reverse or prevent astrocyte aging. Minoxidil is a pyrimidine derivative drug used to treat high blood pressure and hair loss. Interestingly, minoxidil has been implicated in neuroprotection, induction of cell growth factor, and stimulation of cell proliferation (Mesenger and Rundegren, 2004; Chen et al., 2017). In addition, it has been reported that minoxidil modulates signaling pathways known to regulate cellular senescence such as insulin signaling pathways and mtTOR, suggesting that these signaling pathways may contribute to reversing the phenotype of aged astrocytes including inflammation, mitochondrial dysfunction and abnormal proteostasis (Sanders et al., 1996; Campisi et al., 2019; Liu and Sabatini, 2020). Our results demonstrated that minoxidil normalized morphological phenotype and expression of immune-related factor as well as PAI-1 in long-term cultured astrocytes. Moreover, mitochondrial superoxide was reduced by minoxidil treatment in aged astrocytes raising the possibility that minoxidil can be used as a potential candidate to delay or inhibit the astrocyte aging. However, further studies are needed on the mechanism involved in reversing aged astrocytes.

In conclusion, we demonstrated that long-term culture can be used as an appropriate cell model for astrocyte aging research and for screening of anti-aging drugs. Genetic profiling analysis using aged astrocytes may provide us chances to excavate a potential therapeutic candidate against aging and various neurodegenerative diseases that is, in this case, minoxidil.

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