**ORIGINAL ARTICLE**

# Lithium inhibits oxidative stress-induced neuronal senescence through miR-34a

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## Abstract

Neuronal senescence, triggered by telomere shortening, oncogene activation, DNA damage, or oxidative stress, has been associated with neurodegenerative diseases' pathogenesis. Therefore, preventing neuronal senescence could be a novel treatment strategy for neurodegenerative diseases. Lithium (Li), the first-line treatment against bipolar disorder, has been shown to have neuroprotective effects in clinical, pre-clinical, and in vitro studies. Li can protect cells from senescence, and its effect on neuronal senescence was investigated in our study. Furthermore, we also investigated the effects of Li on the senescence-associated miR-34a/Sirt1/p53 pathway. In this study, hydrogen peroxide was used as an inducer for the "stress-induced premature senescence" model. In the senescence model, we have assessed Li's effects on senescence by analyzing β-galactosidase activity, Sudan Black B, and senescence-associated heterochromatin foci (SAHF) stainings, and on cell cycle arrest by BrdU staining. Furthermore, expression levels of senescence and cell cycle arrest-related proteins (p53, p21, p16INK4a, and SIRT1) by western blotting. Finally, the effects of Li on senescence-associated miR-34a levels were measured by quantitative PCR. We show via Sudan Black B staining, β-Gal activity assay, and by detecting SAHF, Li protects against senescence in neuronal cells. Then, Li's effect on signaling has also been determined on pathways involved in senescence and cell cycle arrest. Moreover, we have observed that Li has a modulatory effect on miR-34a expression. Therefore, we posit that Li suppresses senescence in neuronal cells and that this effect is mediated through miR-34a/Sirt1/p53 axis.

**Keywords** Oxidative stress · Senescence · Lithium · Neurons

## Introduction

Cellular senescence is a state of permanent cell cycle arrest that results in tissue dysfunction and is associated with aging and age-related diseases [1]. The types of senescence by cause are generally addressed in two: replicative senescence, where cells stop dividing due to a DNA damage response (DDR) induced by critical telomere shortening; and premature senescence, where any of several stressors, including mutations in oncogenes or tumor-suppressor genes, DNA damage, endoplasmic reticulum (ER) stress, and oxidative stress causes the cell cycle arrest [2]. Since DNA damage, which is a defining attribute of replicative senescence, can also appear in premature senescence, definitively identifying the type of senescence a cell has undergone can, in some cases, be challenging [3]. Besides the standard features of arrested cell cycle and function loss, it has been reported that some senescent cells acquire an inflammatory phenotype termed as senescent-associated secretory phenotype (SASP), where they engage in paracrine inflammatory signaling, driving chronic inflammatory diseases [4]. Since one function of senescence is oncogenesis prevention, tumor-suppressor proteins' expression or activity tends to be elevated in senescent cells [2]. Tumor-suppressor proteins most commonly used in identifying senescent cells are p53, p21, and p16/INK4A [5]. The causes of senescence are multifactorial, as stated above, and often overlap. The two nearly universal senescence factors are DDR, whether

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caused by telomere attrition, mitochondrial dysfunction/reactive oxygen species (ROS), ionizing radiation, and elevated activity of the tumor-suppressor proteins p53 and p16/INK4a [6, 7]. Since its discovery, multiple markers of senescence have been identified. A typical senescent cell marker is a change in morphology, where cells become enlarged and flattened [8]. Though depending on the type of cells or senescence trigger, the morphological change can manifest differently; for example, a morphological alteration that appears with ER stress is increased vacuolization in the cytoplasm [2]. Historically, the first biochemical marker of senescence that is still commonly used is increased activity of the β-galactosidase enzyme, usually termed "senescence-associated β-galactosidase (or SA-β-gal)" [9]. While this was initially shown only in cultured cells' replicative senescence, later in vivo studies have reported β-gal activity in senescent-like cells in animals [10, 11].

The senescence of neurons and other central nervous system cells contributes to neurodegenerative diseases [12, 13]. Although senescence in postmitotic cells like neurons may sound counterintuitive, several in vivo and in vitro studies have reported senescent-like states identified by other markers of senescence in neurons and glia [11–13]. Premature senescence, especially SASP, has been implicated in various age-related diseases, including diabetes, osteoarthritis, atherosclerosis, cancer, Alzheimer's disease, and Parkinson's disease [1, 12]. As poor health brought about by aging creates a substantial economic burden [14], it is advantageous to reduce the rate of degenerative aging and increase global health.

Studies so far have reported both anti and pro-senescence effects of Lithium (Li), which is the first-line treatment against bipolar disorder, has been shown to have neuroprotective effects in clinical, pre-clinical, and in vitro studies [15]. Mechanisms of Li that might help exert its protective role include reducing pro-apoptotic/pro-senescence p53 and increasing anti-apoptotic Bcl-2 and brain-derived neurotrophic factor (BDNF) [16–18]. The effect of Li in senescence induction seems to depend on cell type, the senescence trigger, and the strength of pro-apoptotic versus pro-survival signals [19]. The primary anti-senescence action of Li is the inhibition of glycogen synthase kinase 3 (GSK3) [20], a direct regulator of p53 [21]. Li also reverses the rise in p53 and p21 protein expression and β-gal activity in late passage fibroblasts [22]. It was also demonstrated that Li also restores telomere length in the hippocampus regions of a rat model of depression [23]. In endothelial cells, Li has a pro-senescence effect that is independent of its GSK3 inhibition, where it upregulates the expression of matrix metalloproteinase 1 (MMP1) [24], an enzyme often secreted by SASP cells [1]. Similar results have also been observed in rat nucleus pulposus cells, where Li again induced senescence and a rise in matrix metalloproteinases [25]. A recent study on human iPSC-derived astrocytes (in a model without a senescence trigger) shows that low-dose Li can prevent senescence and SASP markers, including β-gal activity and mRNA expression of p16, p21, and IL-1β [26].

MicroRNAs (miRNAs) are 22-nt members of short non-coding RNA species that regulate expression by binding to 3′-UTR of their target genes. Naturally, miRNAs that target pathways suppressing senescence tend to be upregulated in senescent cells [27, 28]. A well-studied senescence-associated miRNA is miR-34a [29], which suppresses telomerase activity and expression of SIRT1, a histone deacetylase that promotes cell survival [30]. Another biomarker of senescence is the condensation of repressive epigenetic marks in chromatin into multiple focal points, termed senescence-associated heterochromatic foci (SAHF), which can be visually detected by DAPI staining or antibodies for methylations common in senescence such as H3K9me3 [31]. As none of the senescence markers have been specific enough to identify the senescent state exclusively, most studies use multiple markers in combination [7].

In this study, to gain insight into how Li can prevent the occurrence or ameliorate effects of premature senescence in neurons, we have used an $H_2O_2$-induced (i.e., ROS-mediated) premature senescence model, together with Li treatment on the human neuroblastoma cell line SH-SY5Y. We show via Sudan Black B staining, β-Gal activity assay, and by detecting SAHFs, that Li protects against senescence in SH-SY5Y cells. Moreover, we have observed that Li has a modulatory effect on miR-34a-5p, a miRNA that is associated with aging [32, 33] and targets SIRT1 mRNA [34], a longevity-associated protein that boosts mitochondrial function [35]. Therefore, this study aims to examine Li's effects on oxidative stress-induced neuronal senescence and unravel the related signaling mechanisms.

## Materials and methods

### Cell culture and treatments

In this study, SH-SY5Y human neuron-like cells were used. The SH-SY5Y cell line was purchased from ATCC (Catalog number: CRL-2266). Cells were cultured in DMEM: F/12 culture medium supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in an incubator containing 5% $CO_2$ (Thermo Scientific, USA).

SH-SY5Y cells between 5 and 15 passages were used in the experiments. To induce senescence in SH-SY5Y cells, we have optimized $H_2O_2$ concentration by utilizing 25 and 50 µM $H_2O_2$ and determined 25 µM $H_2O_2$ was sufficient to induce senescence. Therefore, cells were incubated with 25 µM $H_2O_2$ for 1 h, then rested for 72 h in DMEM:F12

medium containing 10% FBS. At the end of the incubation periods, senescence markers in the cells were analyzed. To test Li's effects on senescence, cells were incubated with Li (Lithium chloride, Applichem, A6286) at a dose of 2 mM for 24 h before $H_2O_2$ treatment.

## Senescent cell area measurement

To determine the area of senescent cells after treatments, they were incubated in $H_2O_2$ with or without Li and recovered in DMEM:F12 medium for 72 h. At the end of incubation, cells were visualized by phase-contrast microscopy. Cell area after treatments were measured in ImageJ [36].

## β-Galactosidase (β-Gal) activity assay

According to the manufacturer's recommendations, the β-Gal activity was measured using Cellular Senescence Activity Assay (Enzo Life Sciences, USA). Briefly, at the end of treatments, cells were washed once with PBS. Then the cells that were lysed for 5 min at 4 °C with lysis solution, which is supplied with the kit, were centrifuged, and 50 μl of each sample was taken into an empty 96-well plate for analysis, and the incubation buffer was added and kept at 37 °C for 2 h. Finally, fluorescence reading was carried out at 355 nm (excitation) and 460 nm (emission). The increase in cell aging activity is given as a "relative fluorescence unit" (RFU) [37].

## Sudan black B staining

The lipophilic dye Sudan Black B (SBB) detects a biochemical marker of senescence called lipofuscin, an aggregate of oxidized substances in aged tissue or cells [38]. Following the treatment of cells with $H_2O_2$ with or without Li, the medium was replaced with 70% ethanol and then incubated with a saturated SBB solution (in 70% Ethanol) for 8 min [39]. Following SBB incubation, slides were rinsed 50% ethanol and placed in distilled water. Afterward, a 0.1% Nuclear Fast Red stain solution was applied and incubated for 2 min. Finally, sections were washed and mounted in 50% glycerol solution in distilled water. Stained cells were visualized by a light microscope (IX71, Olympus, Japan). From every well, five different fields were quantified by ImageJ software by integrated density measurement [40].

## BrdU incorporation assay

The effect of cell aging on the cell cycle was examined with the commercially purchased BrdU (5-Bromo-2-deoxyuridine) detection kit to determine proliferation (Cell Signaling, USA) according to the manufacturer's instructions. Briefly, at the end of $H_2O_2$ and Li treatments, the cells were

fixed and permeabilized by adding 100 μl 1X fixation solution at room temperature. Then, the fixation solution was removed, and the detection antibody solution was added to each well and incubated for 1 h at room temperature. At the end of the incubation, 100 μl/well of 1X HRP-conjugated secondary antibody solution was applied at room temperature for 30 min. Following final washing steps after secondary antibody 1:1 mixture of Luminol / Enhancer Solution and Stable Peroxide solution was applied to each well, and luminometric measurement was performed using Centro LB 960 microplate luminometer (Berthold Technologies, Switzerland).

## Quantitative PCR

RNA was isolated by the miRNeasy kit protocol (Qiagen, Germany) using TRIzol reagents. cDNA synthesis was done with the miScript II RT Kit (Qiagen, Germany). For real-time PCR, primary, precursor, and mature forms of miR-34a were assessed with specific primers and control RNA primers (U6 and SNORD_95). Relative expression changes were analyzed according to the ΔΔCt method [41].

## Western blotting

Cells were collected by centrifugation at the end of the incubation period for protein extraction. The RIPA lysis solution containing protease inhibitors was added to the pellet for 15 min on ice and occasionally vortexed to facilitate lysis. Afterward, the protein was obtained by 15 min centrifugation at $15,000 \times g$. Protein concentration was determined by BCA protein assay. Protein samples were separated with 12% SDS-PAGE gel and then transferred to polyvinylidene difluoride (PVDF) membrane and blocked in 5% milk solution. After this procedure, samples were incubated overnight at 4 °C with p53, acetylated-p53 (ac-p53, Lys 120), phosphorylated-p53 (p-p53, Ser 15), p21, p16INK4a, Sirt1, and β-actin antibodies. Then the blots were incubated with HRP conjugated secondary antibody, were visualized with the ECL kit based on the membrane chemiluminescence method, and the images were obtained using the Vilber Lourmat chemiluminescence documentation system. Band densities were evaluated densitometrically with Image J software [42].

## SAHF staining

After treatments, cells were fixed with 4% paraformaldehyde for 10 min. The cells were then washed with PBS and incubated with 0.2% Triton X-100 / PBS for 10 min to permeabilize. Subsequently, staining was performed with the H3K9me3 antibody (Abcam, USA) and Alexa Fluor 594 conjugated secondary antibody (Jackson Immunoresearch,

USA). Finally, DNA was stained with DAPI (Sigma Aldrich, Germany) for 1 min. At the end of staining, cells were washed with PBS and imaged with a confocal microscope (Zeiss LSM 880, Germany) to examine heterochromatin formation [43]. Heterochromatins were quantified by integrated intensity measurements of each nuclei using ImageJ software [42].

## Statistical analysis

Statistical data analyses were performed GraphPad Prism 9.0.1 (GraphPad Software Inc., CA, USA). To test the normal distribution of the data, the Shapiro–Wilk test was used. For the cases of three or more independent groups, if the normal distribution was observed, data were analyzed by one-way ANOVA with Sidak's multiple comparisons test. In the cases of non-normal distribution, data were analyzed by Kruskal–Wallis test with Dunn's multiple comparison test. Moreover, for the cases of two independent groups, Mann–Whitney U test was performed. $p < 0.05$ was considered statistically significant. All in vitro experiments were performed at least three times in pentaplicate unless otherwise stated.

## Results

### Effects of Li and oxidative stress on cell proliferation

To determine whether $H_2O_2$ or Li affected cell proliferation, we pre-treated SH-SY5Y cells with 2 mM Li for 24 h, then 25 μM $H_2O_2$ for 1 h, after which the cells were incubated for 72 h in the standard growth medium. At the end of the incubation period, we performed a BrdU incorporation assay to assess the proliferation rate. We observed that Li promoted cell proliferation 23.5% ($\pm 4.16$) when given alone, and $H_2O_2$ treatment caused a 13.52% ($\pm 4.56$) decrease in proliferation in SH-SY5Y cells. Li treatment restored (20.77% $\pm 3.19$) the decreased proliferation rate of the $H_2O_2$ administered cells (Fig. 1).

### Li attenuated oxidative stress-induced effects of cell morphology and cellular senescence

Senescence model has been indued according to a previously published method by Wang et al. [44] by treating 100–200 μM $H_2O_2$. Since 100 μM $H_2O_2$ has cytotoxic effects in SH-SY5Y cells [45, 46], we have tried 25 and 50 μM $H_2O_2$ to induce senescence and our data have shown that 25 μM $H_2O_2$ treatment for 1 h and 72 h recovery in complete medium was adequate to induce senescence significantly ($p = 0.0286$) (Fig. 2A). To confirm $H_2O_2$-induced senescence and look into the effects of Li treatment on $H_2O_2$-induced
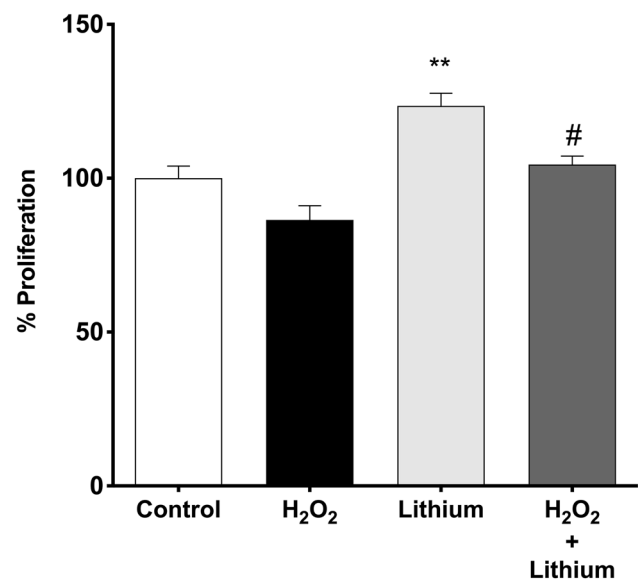


**Fig. 1** Li promotes cell proliferation in SH-SY5Y cells. The chart shows luminometric measurements taken from a BrdU incorporation assay following Li and $H_2O_2$ treatment. Data are mean $\pm$ SEM, n = 5 (**$p < 0.01$ vs. control; #$p < 0.05$ vs. $H_2O_2$). (Color figure online)

senescence, the cells were visualized at the end of their post-treatment incubation with phase-contrast microscopy to observe changes in cellular morphology and cell area. A portion of the $H_2O_2$ administered cells acquired a flat and enlarged shape typical in cellular senescence, while cells with the Li + $H_2O_2$ treatment had a reduced number of flat and enlarged cells (Fig. 2B). When the cell areas were measured, in untreated cells, cell area was 0.047 μm$^2$ ($\pm 0.004$), $H_2O_2$ treatment increased mean cell area to 0.147 μm$^2$ ($\pm 0.021$), and Li treatment reduced $H_2O_2$ caused an increase to 0.066 μm$^2$ ($\pm 0.007$) (Fig. 2C). In another instance of the same experiment, we looked at lipofuscin aggregation as a measure of senescence; and observed a moderate rise in lipofuscin deposition in the $H_2O_2$ group and a reversal of the effect in the Li + $H_2O_2$ group (Fig. 2D). In untreated cells, mean lipofuscin intensity was $3.2 \times 10^6$, $H_2O_2$ treatment increased lipofuscin intensity 2.389-fold, and Li treatment reduced lipofuscin intensity 0.425-fold (Fig. 2E). We also performed a β-galactosidase activity assay as a third measure of senescence and witnessed a 39% rise in β-galactosidase activity in the $H_2O_2$ group, which decreased 32% with Li pretreatment (Fig. 2F).

### Effects of Li and oxidative stress on epigenetic markers of senescence

To further probe for senescence signs, we stained the SH-SY5Y cells for SAHF on their chromatin, using antibodies against the common methylation marker H3K9me3. The imaging indicated that $H_2O_2$ administration induces
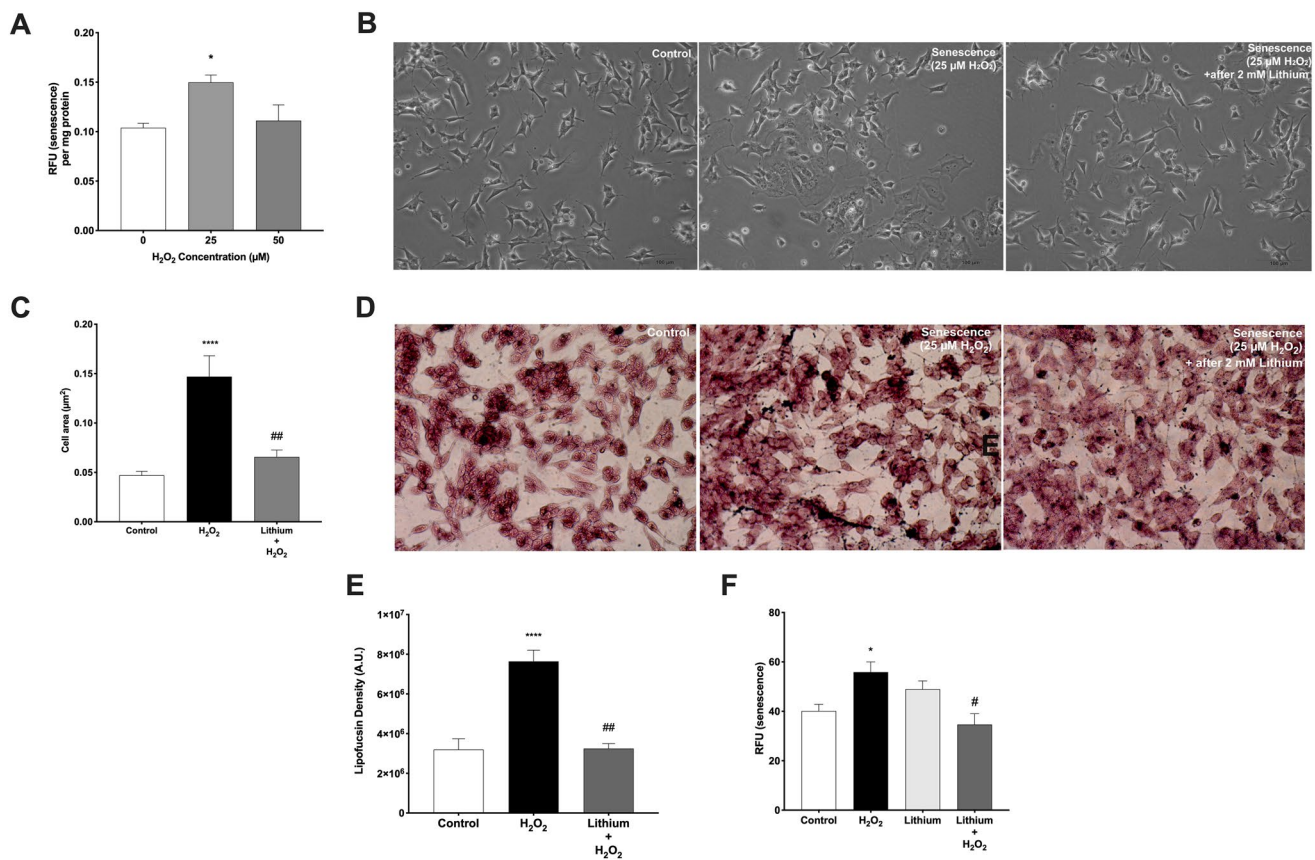
**Fig. 2** SH-SY5Y cells show senescence upon H$_2$O$_2$ administration, which is partially reversed with Li treatment. **A** The morphologies of SH-SY5Y cells without intervention, after H$_2$O$_2$ administration, and after Li+H$_2$O$_2$ administration. **B** Quantification cell area revealed senescence-induced cell flattening was reversed by Li treatment. **C** Sudan Black B staining, showing senescence-associated lipofuscin deposits (dark areas) in the three different groups (coun- terstain: Nuclear Fast Red). **D** Quantification lipofuscin staining revealed senescence-induced lipofuscin formation was reversed by Li treatment. **E** The β-galactosidase activity of the different groups as measured in RFUs. Data in C are mean±SEM, n=5 (*p<0.05, ****p<0.0001 vs. control; #p<0.05, ##p<0.01 vs. H$_2$O$_2$). (Color figure online)

SAHF formation, while Li treatment downregulated this effect (Fig. 3A). In untreated cells, mean SAHF fluorescent intensity was 3238±179.2, H$_2$O$_2$ treatment increased lipofuscin intensity to 4521±280.3, and Li treatment reduced H$_2$O$_2$-induced lipofuscin intensity to 3005±238.4 (Fig. 3B).

## Li treatment reversed oxidative stress-induced expression alterations of cell cycle arrest and longevity-related genes

To detect whether tumor suppressor pathways were also activated in our model, we explored the change in protein levels of senescence-associated tumor suppressors p53, p21, and p16/INK4A. While the expression of total p53 was primarily unchanged, the protein levels of acetylated and phosphorylated p53 (ac-p53 and p-p53, respectively), p21, and p16/INK4A were increased with H$_2$O$_2$

administration and reverted closer to basal levels with Li treatment (Fig. 4A-E). Similarly, levels of the anti-senescence/pro-survival protein SIRT1 fell slightly with H$_2$O$_2$ and rose back to basal levels with Li. (Fig. 4A, F).

## Li affects the senescence-associated expression of miR-34a

In order to gain insight into how Li might be protecting SH-SY5Y cells from senescence, we analyzed the expression levels of the senescence-associated microRNA miR-34a, which decreases with Li treatment [16]. The qPCR results indicate that mature miR-34a and its pri- and pre- forms are downregulated with Li alone (− 20% for mature miRNA) and upregulated in the senescence model (+ 63% for mature miRNA). In addition to senescence inhibition, Li acts protectively, inhibiting the rise in miR-34a in H$_2$O$_2$
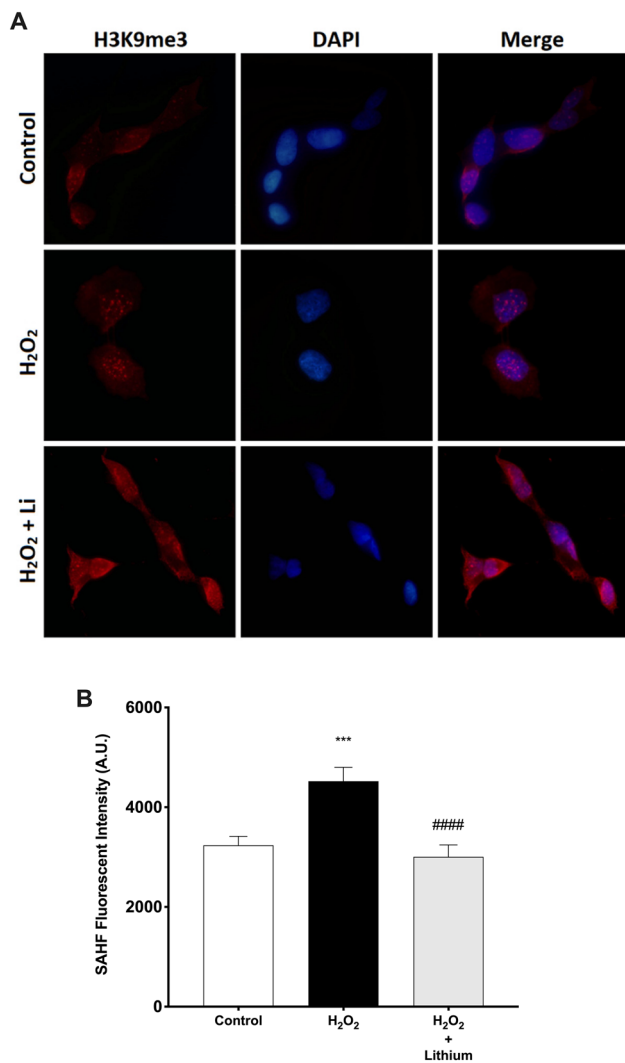
**Fig. 3** $H_2O_2$ induces SAHF formation in SH-SY5Y cells; Li treatment prevents this effect. **A** The images show senescence-associated heterochromatic foci (stained for H3K9me3) in control, $H_2O_2$, and $H_2O_2$+Li groups. Blue (DAPI) shows cell nuclei, red shows H3K9me3 spots on chromatin. **B** Quantification SAHF staining revealed senescence-induced SAHF formation was reversed by Li treatment

administered cells, albeit slightly (− 10.5% for mature miRNA) (Fig. 5).

## Discussion

In our study, we show by multiple techniques that Li protects against $H_2O_2$-induced senescence in SH-SY5Y cells. Moreover, we have observed that Li has a modulatory effect on miR-34a-5p, associated with aging, and targets the mRNA of SIRT1, a longevity-associated protein. Therefore, we suggest that Li suppresses senescence in neuroblastoma cells

and that this effect is mediated at least partially through miR-34a-5p.

Previous research has confirmed markers of neuronal senescence in brain sections of old mice [11, 47] and long-term cultures of rat cortical neurons [48]. These markers include increased β-galactosidase activity, accumulation of lipofuscin deposits, cytoplasmic enlargement, and a rise in heterochromatic foci. Furthermore, in mice, it was shown that for DNA-damaged neurons to senesce, the presence of p21 was essential [11], so a rise in p21 expression is to be expected in senescence. Accordingly, we have probed for these markers in our $H_2O_2$ induced senescence model to confirm that $H_2O_2$ does indeed produce previously established markers of senescence in neurons.

We have used a ROS-mediated senescence model, where administration of $H_2O_2$ leads to an accumulation of ROS-dependent DNA damage [49]. A 2014 study that employs 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) to induce ROS-mediated senescence in SH-SY5Y cells has demonstrated elevated β-galactosidase activity and increased protein levels of p21 and p16/INK4A [50]. Our similar findings indicate that neurons undergoing ROS-mediated senescence are likely to display these markers.

Li administration induced cell proliferation around 23% and restored senescence induced cell cycle arrest in our study. In this case, Li treatment may be inhibiting p53 activity and downstream molecules, such as p16 and p21, which contribute to cell cycle arrest [51]. Next, we observe in our $H_2O_2$ induced senescence model an increase in p21 and p16 levels (and similarly, the activity of p53, the primary inducer of p21, appears to be increased). These proteins are potent cell cycle inhibitors and are cell cycle arrest agents in senescence [52]. Li treatment reverses this trend, rescuing cells from cell cycle arrest. It is reported that the longevity-associated deacetylase SIRT1 inhibits the p53/p21 pathway [53] and expression of p16 [54]. In agreement with that, we also see that SIRT1 decreases in the senescent cell group and returns to normal levels in the Li-treated group. The SIRT1 drop is in line with a previous finding that nuclear SIRT1 declines in senescent-like neurons with lipofuscin deposits in old mice [47]. The $H_2O_2$-induced decrease in SIRT1 is accompanied by a rise in miR-34a, which targets SIRT1 [34], in our study. Seeing how Li reverses the senescence-associated changes in both SIRT1 and miR-34a, we suggest that Li induces a drop in miR-34a expression, which would result in a restoration of SIRT1 levels and inhibition of p53, p21 and p16 (though further experiments would be required to demonstrate a more evident causation link).

It is also possible that the decrease in miR-34a levels is induced by the reduced activity of p53, as the two molecules are reported to create a positive feedback loop [55]. Thus, Li might be suppressing p53 activity and accumulation by
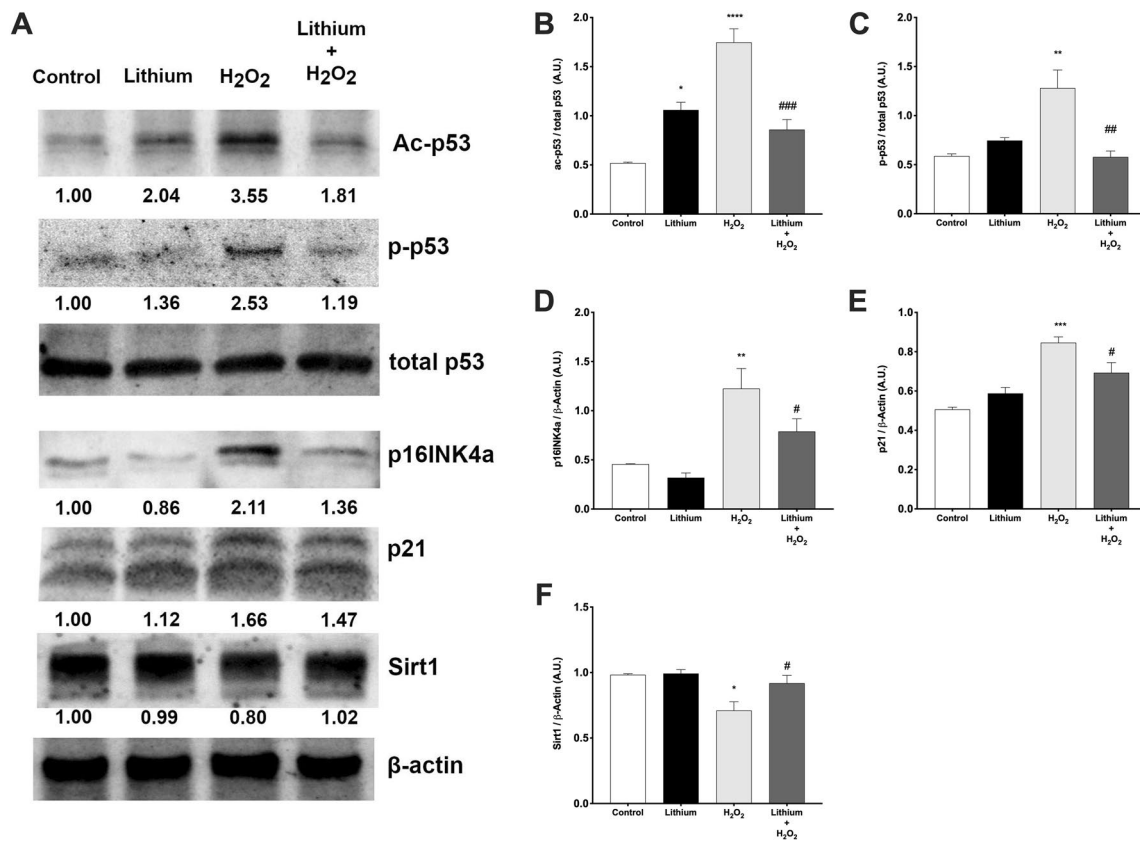
**Fig. 4** Protein levels of senescence-associated tumor-suppressor proteins rise with $H_2O_2$ administration and return to normal with Li treatment in SH-SY5Y cells. **A** The image shows the relevant proteins on a western blot. Numbers below the bands are band intensities as measured by densitometry analyzing software. **B** Ac-p53, **C** p-p53, **D** p16INK4a, **E** p21, and **F** SIRT1 protein levels were determined by western blot analysis. The results are mean $\pm$ SEM of three independent experiments. ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ vs. control; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ vs. $H_2O_2$)
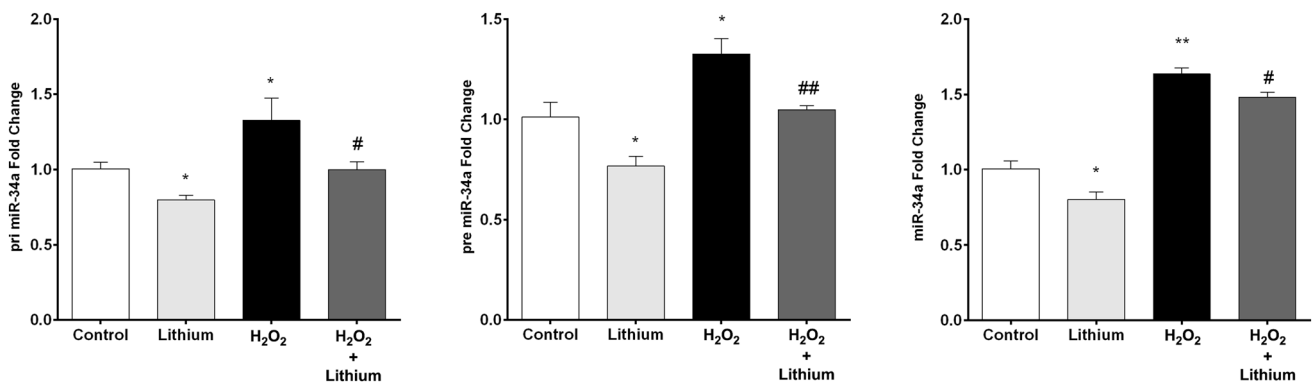


**Fig. 5** Li downregulates miR-34a expression. Charts show miR-34a-5p fold change (in its pri-, pre- and mature forms) with Li treatment, $H_2O_2$-induced senescence and both treatments together. Data are mean $\pm$ SEM, n = 5 ($*p < 0.05$, $**p < 0.01$ vs. control; $\#p < 0.05$, $\#\#p < 0.01$ vs. $H_2O_2$ treatment)

inhibiting GSK3 [21, 22], leading to a drop in miR-34a levels. However, the relationship between GSK3 and p53 is disputed, as another study indicates inhibition of GSK3 results, conversely, in an increase in transcriptionally active p53 [56]. Cell state (the cell's propensity to undergo senescence, apoptosis, or proliferation) may influence how GSK3 inhibition would affect p53 levels and activity.

The ROS-induced senescence model is not the only model of senescence, and the effect of Li in other instances of physiological senescence may vary. The effect might also change when translated from cell culture into in vivo. The Li dose would need to be adjusted for clinical usage as a senostatic (suppressor of senescence). Li's effects could be studied in other cell types, preferably primary cells, to represent the physiological milieu better and shed more light on Li's senescence-related properties. For our study, we have used LiCl for our experiments. Since it is soluble in water, we have dissolved it in cell culture medium. In our previous report, published in 2015, we have shown that LiCl is cytotoxic at 10 mM and above concentrations [16] and in that study we have utilized 2–5 mM LiCl as neuroprotectant. Also another study suggests that 2 mM Li exerts neuroprotection against glutamate excitotoxicity in hippocampal neurons [57]. Moreover, 5 mM LiCl was also shown to be neuroprotective against $H_2O_2$ toxicity in NSC34 neural stem cell line [58]. Furthermore, previous studies have shown that Li is still neuroprotective at 2 mM concentration in amelospheroid-induced toxicity in rat forebrain cholinergic neurons [59]. Thus, based on the previous in vitro and in vivo studies, we have selected 2 mM concentration for LiCl treatments.

There is also the issue that mere suppression of senescence may not necessarily benefit an organism in the long term [60]. Since in a physiological context, senescent cells arise due to cellular stress or DNA damage and display aberrant function. It would be preferable to dispose of them entirely rather than risk their deleterious influence on their surroundings (as in the case of SASP). Thus, while our study shows that Li has a senostatic effect, it remains to be shown that this suppression of senescence also cancels the harmful paracrine actions and restores senescent neurons' functionality.

In conclusion, our data establish that $H_2O_2$ induces senescence efficiently in SH-SY5Y cells. Our neuronal senescence model displays previously confirmed senescence markers that appear with ROS or old age in mammalian neurons. We also provide evidence that Li protects against ROS-mediated senescence in neuronal cell culture. Our finding that this protection is coupled with a decrease in tumor-suppressor proteins p53, p21, p16/INK4a, a decline in miR-34a, and a rise in SIRT1, suggests that Li's modulation of these proteins is the primary cause of its senostatic effect.

**Author contributions** All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by KUT, BA, ET, and TS, KUT wrote the first draft of the manuscript, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## Declarations

**Conflict of interest** The authors have no conflicts of interest to declare that are relevant to the content of this article.

**Ethical approval** This study does not require any ethical statement.

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