Research article

A novel role of Fas in delaying cellular senescence

Chaitrali Saha [a], Jingyu Li [a,b], Xuerong Sun [a], Xinguang Liu [a], Gonghua Huang [a,*]

[a] Guangdong Provincial Key Laboratory of Medical Molecular Diagnostics, The First Dongguan Affiliated Hospital, Guangdong Medical University, Dongguan, 523808, China
[b] School of Medical Technology, Guangdong Medical University, Dongguan, 523808, China

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ABSTRACT

Fas-mediated apoptosis is a major player of many physiological and pathological cellular processes. Fas-regulated immune regulation exhibits either the beneficial or the harmful effects which is associated with the onset or development of immune disorders. Alterations in apoptosis may contribute to age-associated changes. However, the role of apoptosis in the ageing process remains ambiguous. Here we demonstrated Fas signaling-mediated premature senescence in young mouse embryonic fibroblast (MEF) cells. Activated Fas signaling by agonist Jo-2 resulted in declined senescence in young and aged MEFs. Premature senescence induced the early activation of senescence markers, including the increase in the percentage of SA-β-galactosidase (SA-β-gal) cells, the induction of p53 phosphorylation, and the enhanced expression of p16 and p21 protein and elevated IL-6 pro-inflammatory cytokine in the absence of Fas. The elevated production of reactive oxygen species (ROS) in Fas-deficient MEFs was associated with dysfunctional mitochondria. Further, we determined that the known ROS scavenger NAC (N-acetyl-ʟ-cysteine) could reverse the process of premature senescence in absence of Fas. Therefore, this study signifies a novel role of Fas in the control of cellular senescence.

1. Introduction

Cellular senescence and apoptosis (programmed cell death) are two metabolically related and apparently reciprocal processes involved in tissue maintenance, cellular homeostasis, anti-tumor immunity, and age-related pathologies. They are the cellular responses to a variety of intrinsic and extrinsic signals. Senescence stores growth arrested and phenotypically altered ageing cells albeit alive, but apoptosis clears the unwanted cells from the system. Irreversibly growth arrested senescent cells is a selected phenotype, confirming incapacitated cell division of oncogenic damage hence neoplastic growth [1]. Cellular senescence and apoptosis both play an important role in suppressing tumorigenesis, and they potentially contribute to ageing phenotypes and age-related diseases. This suggests the intriguing possibility of these two cellular processes being the examples of antagonistic pleiotropy, which means although in young organisms apoptosis and cellular senescence are beneficial, they may have detrimental effects later in life, contributing to the ageing of old organisms [2,3].

Senescent cells are often associated with survival and with permanent structural and functional alterations, they are non-proliferative but at viable state, distinct from G0 quiescence and terminal differentiation [4]. Cellular senescence occurs in response to numerous stressors, including exposure to genotoxic agents, hypoxia, nutrient deprivation, mitochondrial dysfunction, and oncogene activation [5]. Senescent cells with the feature of an essential irreversible cell-cycle arrest, can be an indicative response

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* Corresponding author.
  E-mail address: gonghua.huang@gdmu.edu.cn (G. Huang).

initiated by detrimental stimuli or abnormal proliferation. These cells show irreversible cell cycle arrest in combination with metabolic and epigenomic changes including senescence-associated SA-β-gal positivity, the senescence-associated secretory phenotype (SASP) [6] and senescence-associated heterochromatin formation (SAHF). The two important cellular pathways orchestrate the onset of senescence mediated stress responses are p53–p21CIP1 and p16INK4a–retinoblastoma (RB) [5,7]. Accumulation of the CDK2 inhibitor p21WAF1/Cip1 (CDKN1A) and CDK4/6 inhibitor p16INK4A (CDKN2A) in senescent cells results in persistent activation of RB family proteins, inhibition of E2F transactivation, and consequent cell-cycle arrest, which, in time, cannot be reversed by subsequent inactivation of RB family proteins or p53. There is evidence supporting that inactivation of either p53 or pRb can significantly delay the onset of senescence, supporting a linear p53-pRb pathway [8]. Whereas in many other cases, both p53 and pRb need to be inhibited to prevent replicative senescence, suggesting co-existence of two independent pathways [9] in senescence. In parallel to p21, p16INK4a (p16) is another CDK inhibitor that leads to pRb hypophosphorylation [10]. In senescent murine cells, at p16INK4a gene locus, ARF—an alternate reading frame protein which activates p53, plays an important role in regulating cell-cycle arrest [11]. However, the fundamentals of p16 and p21 elevation in senescent cells are distinct. In replicative senescent cells a quick increase in p21 expression has been observed [12], whereas p16 enhancement occurs after senescence has been already established [13]. Senescence is often associated with high levels of reactive oxygen species (ROS), which further can be related to dysfunctional mitochondria. Overabundance of ROS is detrimental and leads to lipotoxicity, DNA lesion, and protein damage, which is well connected to cellular senescence [14].

Apoptosis can be triggered by both intrinsic and extrinsic signaling pathways. Fas ligand-induced apoptosis is a well-studied example of the extrinsic pathway [15]. Fas, also known as CD95 or APO-1, is a member of the tumor necrosis factor/nerve growth factor superfamily. Fas-ligand binding to its receptor results in trimerization. Once the Fas receptor interacts with the Fas-associated death domain (FADD), it recruits Fas-associated protein with death domain. Procaspase-8 molecules are then recruited together by FADDs, causing proximity-mediated activation of caspase-8. Next active caspase-8 can activate caspase-3 and caspase-7, which are the executioner caspases because their substrates incorporate several cellular proteins [16]. Following the discovery that mutations in the Fas and Fas ligand (FasL/CD95L) cause the recessive autoimmune lpr syndrome in mice and the autosomal dominant autoimmune lymphoproliferative syndrome (ALPS) in humans, which were the underlying cause of the massive lymphadenopathy and autoimmune lymphoproliferative syndrome, the prime focus was to understand the role of Fas in apoptosis. However, recent studies suggest, that Fas leads to cell proliferation, migration, and inflammation by activating the three main mitogen-activated protein kinase (MAPK) pathways, p38, JNK1/2, and ERK1/2, as well as NF-κB [17]. Thus, Fas stimulation promotes apoptotic signals, such as caspase-8 activation, as well as "non-apoptotic" cellular responses, remarkably NF-κB activation [18]. However, the underlying molecular mechanisms of this physiological outcome of Fas signaling, are not fully understood.

Cellular senescence is a particular state of cell involved in numerous physiological processes and a wide spectrum of age-related diseases. At present targeting senescence therapeutically is of great interest to improve healthy aging and age-related disease. Existence of cellular senescence and apoptosis in ageing tissues are the different sides of the same coin, however the exact concept of their mutual relationships in the ageing study is yet to be elucidated. There is evidence supporting that excessive apoptosis can be found in aged tissues in vivo, as found in aged T-lymphocytes, age-related neurodegenerative processes, normal neuron ageing, and autoimmune diseases [19] in contrast its reported that some senescent cells can resist apoptosis in cultures [20]. Although cellular senescence might be connected to ageing, how apoptosis and senescence can be exclusive in some conditions, and related in others, remains an open question. To address this concern, we have studied the role of pro-apoptotic gene Fas in cellular senescence. We used MEFs, a commonly used model to study cellular senescence. S Kumar team established in 2010 that MEFs can proliferate until passage 5–6, after which their population doubling time increases dramatically and after passage 6, the survived cells recover, and the doubling time decreases again. This is likely due to a population of cells escaping senescence and proliferating again because of spontaneous immortalization, a characteristic of MEFs in culture [21]. In our study we considered MEFs at early passage (P2) the young cells and late passage (P10) the aged cells. To confirm that these cells were indeed entering senescence, a β-galactosidase assay was performed to stain cells that express SA-β-gal activity. To investigate the role of Fas in cellular senescence using MEFs from Fas knock out (KO) mice, we found a surprising appearance of premature senescence in young MEFs in Fas-deficient cells. Further dissecting the molecular mechanism, interestingly we found that this premature ageing leads to induction in ROS and dysfunctional mitochondria. Altogether, our study reveals a novel role of Fas in senescence.

2. Materials and methods

2.1. Mice

B6.MRL-Tnfrsf6lpr mice were from The Jackson Laboratory. C57BL/6 mice were from Shanghai SLAC Laboratory Animal Center (Shanghai, China). All mice were kept in a specific pathogen-free (SPF) barrier facility maintained by Guangdong Medical University. All the experimental mice were used at 6–10 weeks. Animal protocols were approved by Institutional Animal Care and Use Committee of Guangdong Medical University, China.

2.2. Isolation and culture of primary mouse embryo fibroblasts (MEFs), NIH/3T3 and human umbilical vein endothelial cells (HUVECs)

MEFs have been particularly useful in ageing research, as they have facilitated the identification of the genetic changes that allow cells to overcome senescence and proliferate indefinitely in culture. Harvest embryos from female mice 13–14 days after the appearance of the copulation plug. Tore out the red tissue (heart and liver) from embryo and washed with 1–2 ml of PBS. Tissue was

minced and 10% trypsin (Gibco 15305-014) was added followed by incubating at 37 °C for 10 min. Single-cell suspension was pre-pared and added into 10 cm dishes (designated as passage; P0). Cells need to be split every 3–4 days during the early passages (P1–P6). After that their growth slowed down and they can be split or fed every week. NIH/3T3 is an immortalized mouse embryonic fibroblast. MEFs, and NIH/3T3 were all cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco, USA). Serial passages were performed when the cells reached 80%–90% confluence. Human umbilical vein endothelial cell (HUVEC) line was obtained from iCell Bioscience Inc. (Shanghai, China) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA) in a 37 °C humidified incubator. Cells were passaged every 3–4 days.

2.3. Cell transfection

WT and Fas KO MEFs were seeded in 12-well plates at a density of 4.5×10^4 cells/well at P2 and 5.5×10^4 cells/well at P10 passages and incubated overnight at 37 °C. Post 24 h, Jo-2 (1 μg/ml) was added. Cells were harvested next day, and functional assays were performed.

The transfection protocol of MEFs with siRNA was little different than the transfection of 3T3 cells. In case of MEFs P2 and P10 passages were used for the experiments. At around 80%–90% confluency cells were trypsinized with Trypsin and then cells were seeded in a 12-well plate to achieve 60% confluency for siRNA transfection. After 24 h cells were pre-treated with p38 inhibitor SB203580 (20 μM) for 30 min and then Jo-2 (1 μg/ml) was added. Post 5 h cells were washed twice with 1X PBS and supplemented with fresh DMEM medium. Cells were harvested 72 h post treatment and analyzed for either Western blot or SA-β-gal staining. In case of 3T3 cells, the cells were trypsinized at 80%–90% confluency and seeded to a 12-well plate and at the same time co-transfected with siRNAs. After 24 h cells were pre-treated with 500 μM H_2O_2 to induce senescence. Then after 2 h cells were washed twice with PBS and fresh medium was added followed by pre-treatment with p38 inhibitor (20 μM) for 30 min and then Jo-2 (1 μg/ml) was added. After 5 h cells were washed twice with 1X PBS and supplemented with fresh DMEM medium. Cells were harvested 72 h of treatment and analyzed for either Western blot or SA-β-gal staining. To analyze pp38 level in MEFs treated with Jo-2 and p38 inhibitor we performed an experiment where we pre-treated 60% confluent cells for 30 min with the p38 inhibitor followed by adding Jo-2 and then we harvested the cells at different time points respectively at 0 h, 30 min, 1 h and 2 h and performed Western blot to analyze pp30 protein expression. To observe the possible effect of FAS on HUVEC senescence, HUVECs were treated with CDDP (cisplatin) for 2 days to induce preliminary senescence. Then FAS siRNA transfection was performed and lasted for 3 days. On day 7 after transfection, HUVECs were harvested for analysis.

The small interference RNAs (siRNA) targeting Fas mRNA (designated as Fas-Mus-934) was purchased from GenePharma (Shanghai, China). The sequence of Fas-siRNA is (5′-3′ CCGAAUGUCGCAGAACCUUTT, AAGCUUCUGCGACAUUCGGTT). Transfection of RNA oligoribonucleotide(s) was performed using Lipofectamine RNAiMAX (Life Technologies, USA) according to the manufacturer's instructions. For MEF transfection, MEFs were plated in growth medium at a density of ~60%–70% confluence.

2.4. Pharmacological inhibition of p38 with SB203580 and ROS with NAC and treatment with Jo-2

Cells were pre-treated with p38 inhibitor SB203580 (Merck Calbiochem) at a dose of (20 μM) and purified NA/LE Hamster Anti-Mouse CD95-Jo-2 (Catalogue No. 554254, BD Pharminogen, USA) at a dose of (1 μg/ml). MEFs were treated with ROS inhibitor NAC (Sigma A9165) at the concentrations of 2.5 mM and 5 mM for 5 h.

2.5. Counting the cells

MEFs were counted using a Neubauer hemocytometer. First, the hemocytometer was cleaned with 70% ethanol. Then, 10 μL of cells were diluted with 990 μL of trypan blue. Next, 10 μL of the sample was pipetted onto the hemocytometer and the cells were counted from the four quadrants of hemocytometer.

2.6. Western blot

Initially, cell protein lysates were separated on 12% or 15% SDS polyacrylamide gels, electrophoretically transferred to PVDF membranes (0.22 μm pore size; Millipore, USA). The transferred PVDF membrane was then incubated with rabbit anti-p16 polyclonal antibody (BS1265, Bioworld Technology, USA), rabbit anti-p21 Waf1/Cip1 monoclonal antibody (Catalogue No. 64016, Cell Signaling Technology, USA), rabbit anti-p53 (D2H90) monoclonal antibody (Catalogue No. 32532, Cell Signaling Technology, USA), rabbit pRB monoclonal antibody (Ser807/811) (D20B12) (Catalogue No. 8516, Cell Signaling Technology, USA), rabbit phospho-p38 MAPK (Thr180/Tyr182) (D3F9) (Catalogue No. 4511, Cell Signaling Technology, USA), FAS/CD95 Mouse Monoclonal antibody (Catalogue No. 60196-1-Ig, proteintech, China). Anti-rabbit secondary antibody (Catalogue No. P0025, Beyotime Biotechnology, China) and anti-mouse secondary antibody (Catalogue No. SA00001-1, proteintech, China) conjugated with horseradish peroxidase (HRP) were used and subjected to a commercial enhanced chemiluminescence (ECL) kit (Pierce, USA). Protein loading was estimated using Rabbit α-Tubulin Polyclonal antibody (Catalogue No. 11224-1-AP, proteintech, China).

2.7. RNA isolation and quantitative PCR (qPCR)

For mRNA, 1 μg of total RNA was reverse transcribed using Prime-Script RT Reagent Kit with gDNA Eraser (Takara, Japan)

according to the manufacturer's recommendations. q-PCR was performed in a LightCycler 96 (Roche, Switzerland) with SYBR Select Master Mix (Life Technologies, USA). Total RNA was isolated using Trizol regent and used for reverse transcription. qPCR was carried out. mRNA expression of genes was normalized to Actin.

2.8. Antibodies and flow cytometry

MitoTracker™ Deep Red FM - Special Packageing (Invitrogen™:M22426), Image-iT™ TMRM Reagent (mitochondrial membrane potential indicator) (Invitrogen™: I34361) CM-H2DCFDA (General Oxidative Stress Indicator) (ROS-FITC) (Invitrogen™:C6827), anti-Ki-67 staining (eBioscience: 12-5698-82) were obtained. Staining was performed according to the manufacturer's instructions. The samples were acquired on a FACSCantoII (BD) or LSRFortessa™ X-20 (BD) and analyzed with FlowJo software (Treestar).

2.9. Detection of ROS

Shortly before performing the experiments, ROS indicator was reconstituted to make a concentrated stock solution and kept tightly sealed until ready to use. Growth media were removed from cells via pipetting and cells were washed with prewarmed PBS. Cells were trypsinized and washed again with prewarmed PBS. Then cells were stained with 10 μM ROS-FITC (1:200) in RPMI/10% FBS and incubated at 37 °C for 30 min. Post 30 min cells were washed with 10% FBS in PBS and resuspend in PBS/FBS and samples were acquired by flow cytometry.

2.10. Analyzing mitochondrial mass and function

Cells were harvested and stained with 50 nM MitoTracker (1:200) and TMRM (1:1000) in PBS/2% FBS at 37 °C for 30 min. Cells were washed and resuspend with PBS/FBS and acquired.

2.11. Senescence-associated β-galactosidase staining

Senescence-associated β-galactosidase staining (SA-β-gal) of cells transfected with indicated RNA oligoribonucleotides is performed utilizing the β-Galactosidase Staining Kit (Catalogue No. CS0030, Sigma, USA). Briefly, the cells are washed and fixed. The fixed cells are then incubated with fresh β-galactosidase staining solution at pH 5.8 and incubated. SA-β-galactosidase-positive cells are detected by inverted bright field microscopy (Nikon, Japan) at 100X magnification.
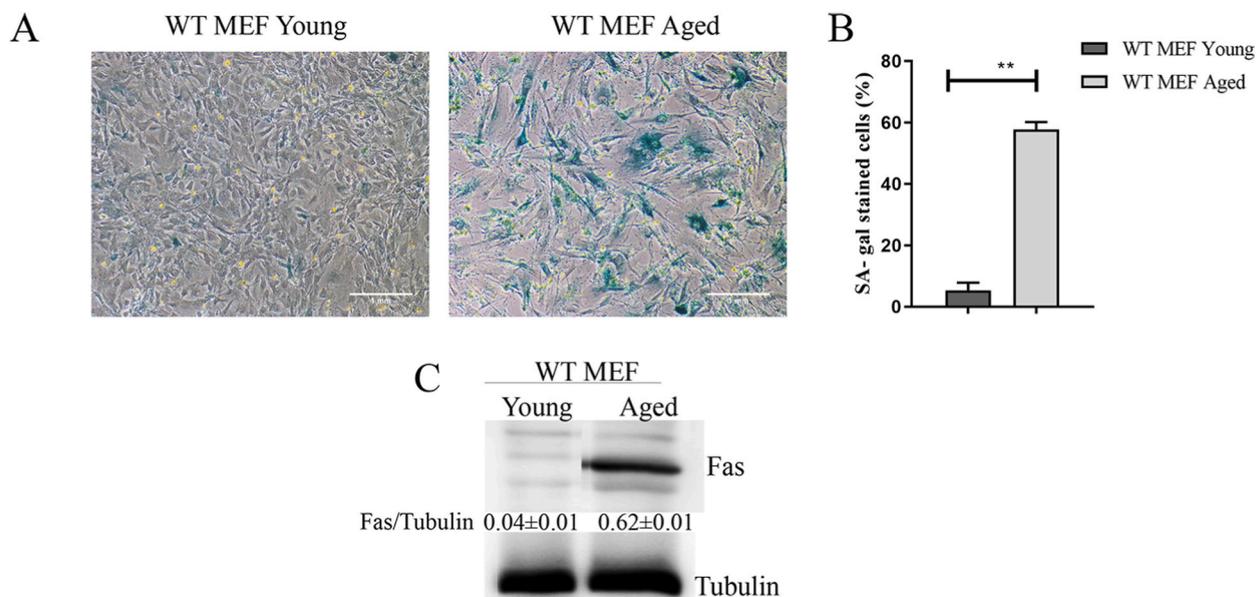
Fig. 1. Elevated Fas in aged WT MEFs. WT MEFs were cultured and maintained from passage 0 till passage 10 for SA-β-gal-staining to determine the status of cellular senescence. Expression of Fas protein was compared between young vs aged WT MEFs. (A) Representative microscopic images of SA-β gal-stained cells in young v/s aged WT MEFs. (B) The graph presents the percentage of SA-β-gal-stained cells. (C) Western blot analysis of Fas protein expression in young v/s aged WT MEFs. The complete Western blot images are presented in Supplementary Fig. S7A. Percentage of SA-β gal-stained cells is the average representation of minimum 10 fields/condition. Data shown are representative of minimum three independent experiments, **p < 0.01.
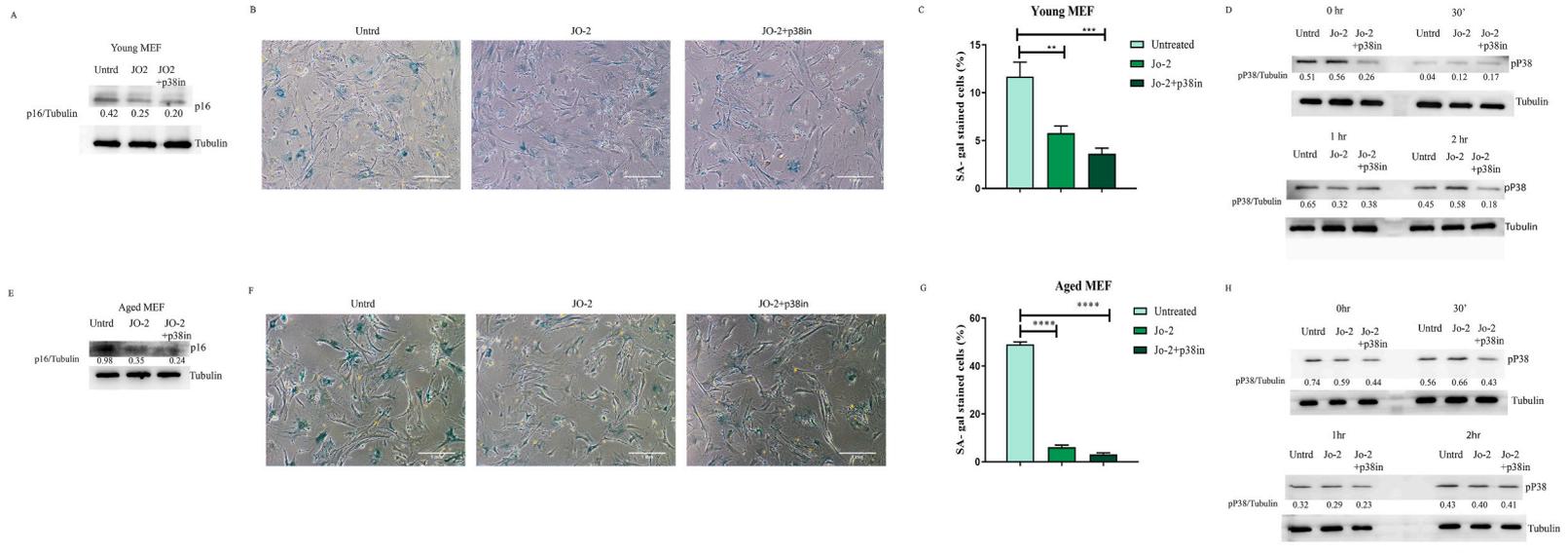
Fig. 2. Jo-2-mediated Fas signaling inhibits MEF cellular senescence and p38 signaling being the barrier. MEFs were stimulated with Fas agonistic antibody Jo-2 which can induce Fas signaling followed by analysing the senescence-associated marker p16 expression by Western blot and stained cells with SA-β-gal. To explore whether p38 signaling pathway contributes in Fas-mediated cellular senescence process, we treated Jo-2-stimulated MEFs with p38 specific inhibitor SB203580 (A) for Western blot analysis of p16 expression of young MEFs treated either with Jo-2 (1 μg/ml) or in combination with Jo-2 and p38 inhibitor SB203580 (20 μM). α-Tubulin was used as an internal control. The complete Western blot images can be found in supplementary Fig. S8A (B). Representative photographs of SA-β-gal staining of young MEFs treated either with Jo-2 or in combination with Jo-2 and p38 inhibitor SB203580. (C) The bar graph represents percentage of SA-β-gal-stained senescence young MEFs. (E) Immunoblot analysis of p16 expression of aged MEFs treated either with Jo-2 (1 μg/ml) or in combination with Jo-2 and p38 inhibitor SB203580. The unaltered images are presented in Fig. S8C (F) Microscopic photographs of SA-β-gal staining of aged MEFs treated either with Jo-2 or in combination with Jo-2 and p38 inhibitor SB203580. (G) The graph presents the percentage of SA-β-gal-stained aged MEFs. Immunoblot analysis of pP38 at 0 h, ½ hr, 1 h and 2 h time points of young (D) and aged MEF (H) treated either with Jo-2 or in combination with Jo-2 and p38 inhibitor SB203580. α-Tubulin was used as internal control for equal loading. The unprocessed blots can be found in supplementary Fig. S8B and Fig. S8D. Data shown are representative of minimum three independent experiments, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

2.12. Statistical analysis

The data are expressed as the means ± standard deviation and statistical analysis was performed with GraphPad Prism 5. For the statistical analysis of data, one-way analysis of variance (ANOVA) with Tukey's posttest and two-way ANOVA with Bonferroni posttest were used. Two-tailed Student's *t*-test was used when two conditions were compared. p values were indicated and $p < 0.05$ was considered significant. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns, not significant. "n" indicates biological replicates for *in vitro* experiments and number of mice for in vivo studies.

3. Results

3.1. Elevated Fas expression in aged WT MEFs

To confirm that MEFs were indeed entering senescence, the β-galactosidase assay was performed to stain cells that express SA-β-gal activity (Fig. 1A and B). Aged MEFs at P10 showed significantly enhanced SA-β-gal expression compared to young MEFs at P2. We next explored the expression level of Fas in young vs aged MEFs. Western blot data revealed significantly higher expression of Fas in aged MEFs compared to that in young cells (Fig. 1C). In an H_2O_2-induced cellular senescence model, we also found elevated expression of Fas in the H_2O_2-treated 3T3 cells compared to that in the untreated 3T3 (Fig. S2). These data suggest enhanced Fas expression along with the progression of age.
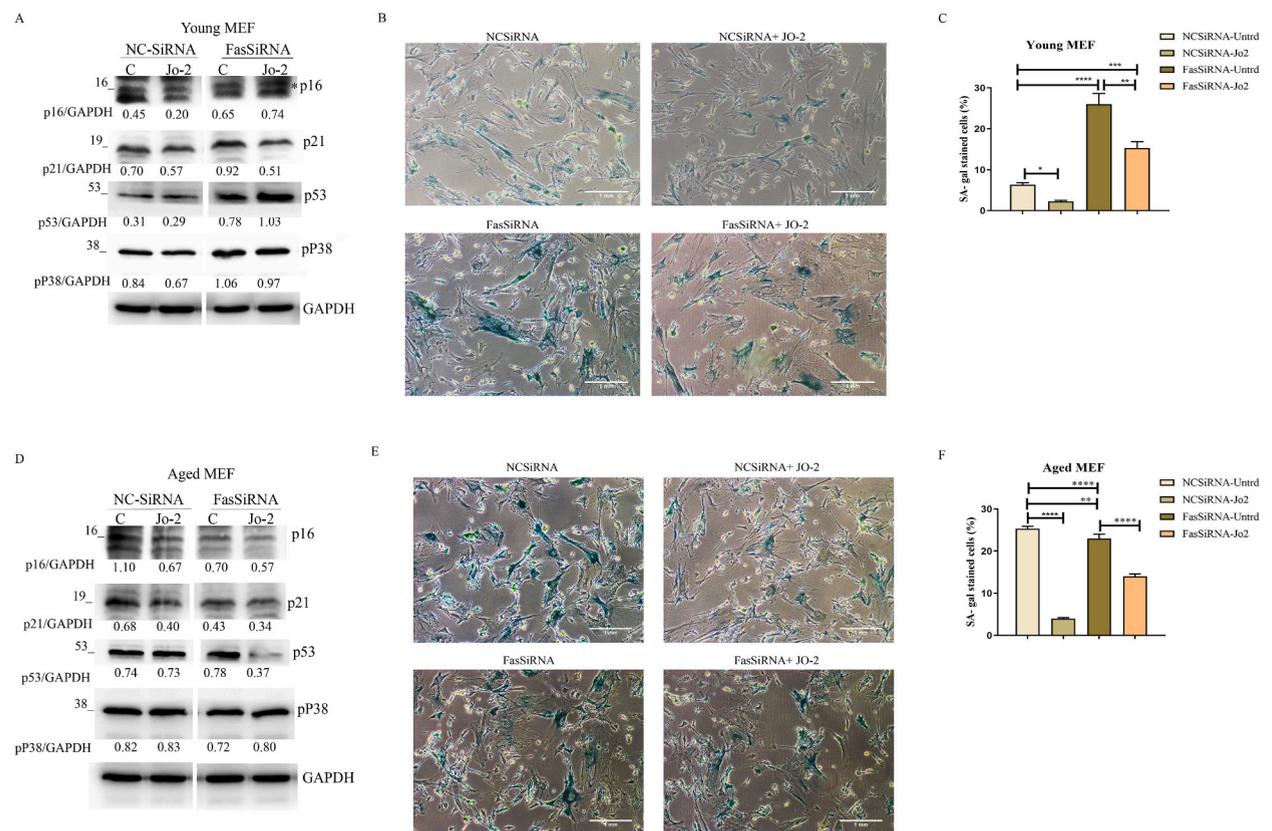
Fig. 3. *In-vitro* knocking down of Fas leads to premature ageing in MEFs. Fas signaling was silenced using siRNA transfection approach *in-vitro*. Young and aged MEFs were transfected with NCsiRNA and FassiRNA and then were treated with or without Jo-2. (A) Representation of Western blot data of p16, pRB, p21, p53 and pp38 in FassiRNA transfected young MEFs treated with Jo-2 (1 μg/ml). GAPDH was used as an internal control for equal loading in Western blot analysis. The blot is contiguous and Lanes 3 and 6 are not presented which had unrelated samples thus those two lanes are omitted. The unprocessed blot images are presented in Fig. S9A. (B) Representative photographs of SA-β-gal stained young MEFs. (C) The bar graph represents the percentage of SA-β-gal stained young MEFs. (D) Western blot data of p16, p21, p53 and pp38 in FassiRNA transfected aged MEFs treated with Jo-2. Lanes 3 and 6 are not displayed which had unrelated samples and the blot is contagious. The complete blots can be found in Supplementary Fig. S9B. (E) Microscope pictures of SA-β-gal stained aged MEFs. (F) The graph shows the percentage of SA-β-gal-stained aged MEFs. The treatment groups include NCsiRNA (Untreated-Control), NCsiRNA + Jo-2, FassiRNA (Untreated) and FassiRNA + Jo-2. Data shown are the mean ± SD from at least three independent experiments, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3.2. Jo-2-mediated Fas signaling inhibits MEF cellular senescence and p38 signaling is the barrier

The increased Fas expression in aged MEFs prompted us to examine the role of Fas signaling in mediating cellular senescence. Thus, we stimulated MEFs with Fas agonistic antibody Jo-2 which recognizes mouse Fas and can induce Fas signaling and then analyzed the age-associated p16 expression by Western blot and stained cells with SA-β-gal. We found that both Jo-2-stimulated young and aged MEFs showed reduced p16 protein expression (Fig. 2A and E) and reduced SA-β-gal-stained cells (Fig. 2B, C, F and G). p38 MAPK has been known to be actively involved in ageing process [22]. To explore whether p38 signaling pathway contributes in Fas-mediated cellular senescence process, we treated Jo-2-stimulated MEFs with p38 inhibitor SB203580 and found that inhibiting p38 further abrogated cellular senescence process, suggesting that p38 is an obstacle in the process of declining senescence by Jo-2-mediated Fas signaling. We next performed a time dependent p38 inhibition study on young (Fig. 2D) and aged MEFs (Fig. 2H) and analyzed pp38 level in these cells at different time intervals Western blot. Results showed no difference in pp38 expression level between untreated and Jo-2-treated MEFs at 0 h, ½ hr, 1 h and 2 h time points whereas as expected adding p38 inhibitor showed a marked reduction in p38 expression, suggesting an unaltered pp38 expression in Jo-2 treated young and aged MEFs. Further experiment with aged NIH3T3 treated with Jo-2 and p38 inhibitor, showed significant abrogation in p16 protein expression by immunoblot analysis (Fig. S3A) and weaker SA-β-gal signal (Fig. S3B and S3C) compared to the untreated WT MEFs. These data together suggested a significant role of Jo-2-mediated Fas signaling in inhibiting cellular senescence.

3.3. In vitro knocking down of Fas leads to premature senescence in MEF cell

As Jo-2-treated WT MEFs reduced cellular senescence, we next wanted to elucidate the role of Fas in MEF cellular senescence by *in vitro* knocking down Fas signaling using siRNA transfection. The efficiency of Fas silencing by siRNA was determined by Western blot (Fig. S1A) and qQPCR (Fig. S1B). We first transfected MEFs with NCsiRNA and FassiRNA and then these transfected cells were treated
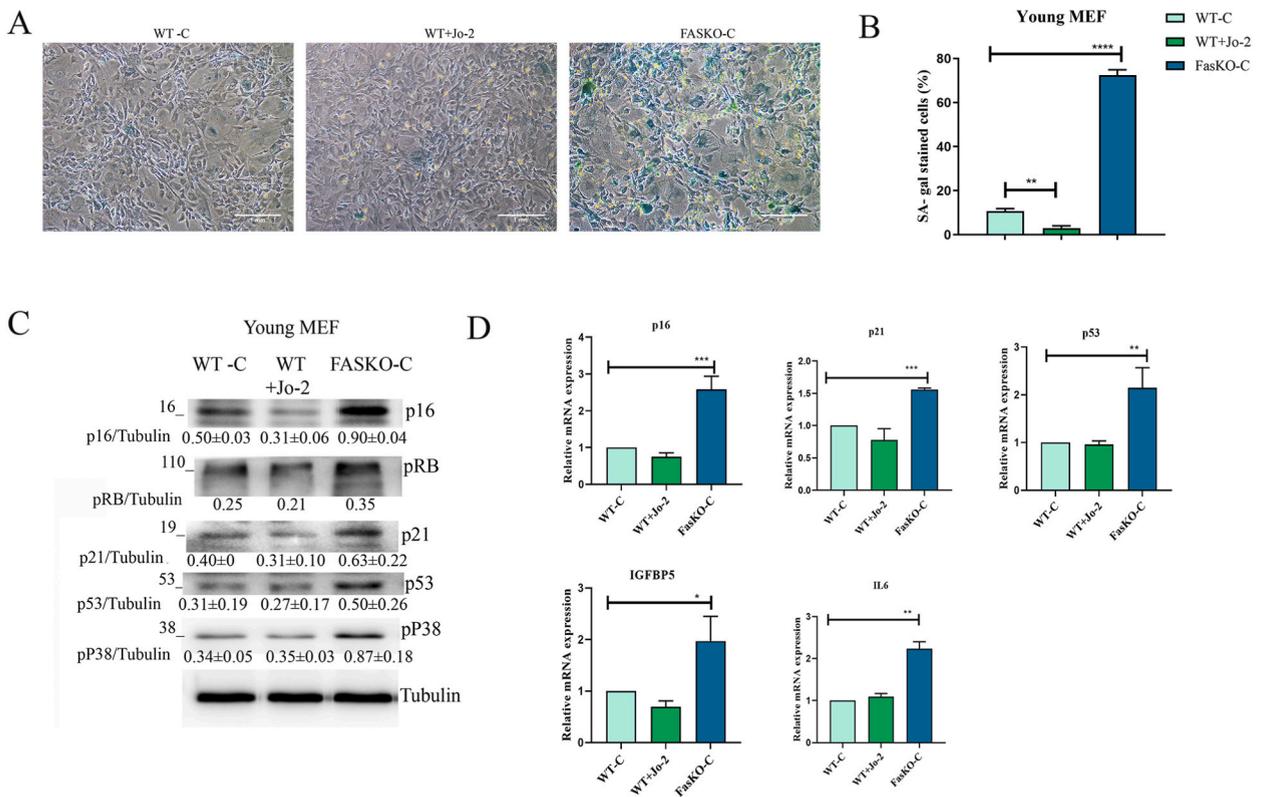
Fig. 4. Fas KO young MEF leads to premature cellular senescence. WT or Fas KO female embryos of 13.5 days old were used to prepare young (P2) and aged (P10) WT or Fas-KO MEFs. These cells were harvested and analyzed for senescence-associated and inflammatory genes expression by Western blot (p16, p21, pRB, p53, pp38), SA-β-gal staining and Real time PCR (p16, p21, p53, IGFBP-5 and IL-6). (A) Photographic representation of SA-β-gal-stained young WT, WT treated with Jo-2 and Fas KO MEFs respectively. (B) The bar graph represents the percentage of SA-β-gal-stained senescence young MEFs. Images are representative of n = 10 microscopic field per experimental group from three experiments. (C) The protein level of senescence-associated genes such as p16, pRB, p21, p53, pp38 and α-Tubulin (loading control) was examined by Western blotting and quantified by densitometry (n = three for each group). The unadjusted Western blot images can be followed in Supplementary Fig. S10A. (D) Relative mRNA expression of senescence markers p16, pRB, p21, p53, IGFBP5 and inflammatory molecule *Il6* was examined by q-PCR (n = three for each group). Data shown are the mean ± SD from at least three independent experiments, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

with or without Jo-2. Comparing the NCsiRNA-untreated with NCsiRNA-Jo-2 treated young MEFs, the latter group showed reduced expression of senescence-associated genes such as p16 and p21 by Western blot (Fig. 3A) and SA-β-gal staining (Fig. 3B and C), confirming that Jo-2 stimulated Fas can reverse the senescence process. Further FassiRNA group showed elevated p16 and p21, p53 and pp38 protein expression compared to the NCsiRNA, suggesting that defect in Fas can induce senescence at young age and Jo-2 stimulation in the absence of Fas cannot correct this effect. Immunoblot (Fig. 3D) and SA-β-gal staining (Fig. 3E and F) analyses of aged MEFs again confirmed that Jo-2-treated NCsiRNA MEFs reduced senescence. However, to our surprise the FassiRNA-treated aged MEFs showed no additive effect on senescence compared to NCsiRNA cells. Overall, the data suggest a role of Fas in delaying cellular senescence. To observe the possible effect of FAS on HUVEC senescence, HUVECs were treated with CDDP (cisplatin) for 2 days to induce preliminary senescence. Then FAS siRNA transfection was performed and lasted for 3 days. On day 7 after transfection, HUVECs were harvested for analysis. The results showed that CDDP weakly induced senescence by increasing p53 and SA-β-gal. FAS knockdown further accelerated the senescence of HUVECs induced by CDDP (Fig. S4).

3.4. Fas KO young MEFs lead to premature cellular senescence

To further determine the role of Fas in cellular senescence, we used genetic ablation method by using Fas KO mice. WT or Fas KO female embryos of 13.5 days old were used to prepare young (P2) and aged (P10) WT or Fas-KO MEFs. These cells were harvested and analyzed for senescence-associated and inflammatory molecule expression by Western blot (p16, p21, pRB, p53, pp38), SA-β-gal staining and q PCR (p16, p21, p53, IGFBP-5 and IL-6). SA-β-gal analysis showed that young WT MEFs stimulated with Jo-2 had less β-gal-stained cells compared to the untreated WT cells whereas Fas KO MEF showed significantly higher number of β-gal-stained cells (Fig. 4A and B). Western blot analysis revealed induced p16, p21, p53, pp38 protein level in young Fas KO MEFs compared to WT young MEFs (Fig. 4C). Further qPCR analysis detected significantly increased mRNA expression of age-related genes such as p16, p21, p53, IGFBP-5 and elevated expression of IL-6 in young Fas KO MEFs (Fig. 4D). SA-β-gal staining (Fig. S5A and S5B), immunoblot (Fig. S5C) and qPCR (Fig. S5D) of the aged Fas KO MEFs compared to the WT showed almost no significant difference, however qPCR data detected induced *Il-6* in aged Fas KO MEFs. Whereas interestingly Jo-2-stimulated WT MEFs abrogated senescence compared to the WT-untreated at young and as well at aged state consistently. Together the data suggest that Jo-2-mediated Fas can inhibit cellular senescence at young as well as in aged MEFs, whereas Fas alone can only delay the process of senescence.

3.4.1. Oxidative damage to mitochondria leads to mitochondrial dysfunction during stress-induced premature senescence in Fas-deficient young MEFs

Our data so far demonstrated that Fas-deficient MEFs resulted in premature cellular senescence. To provide further insights into this phenomenon, we investigated the underlying mechanism by analyzing whether absence of Fas would affect mitochondria. Mitochondrial function declination and ROS production induction have been hypothesized to be the major contributory factors in senescence hence in ageing [23]. Thus, to underpin the underlying mechanism causing the premature landing of senescence in the absence of Fas, we investigated ROS production using CM-H2DCFDA and evaluated different parameters of mitochondrial biology in living cells using Mitotracker Red and TMRM. CM-H2DCFDA is a chloromethyl derivative of H2DCFDA, an indicator for ROS in cells.
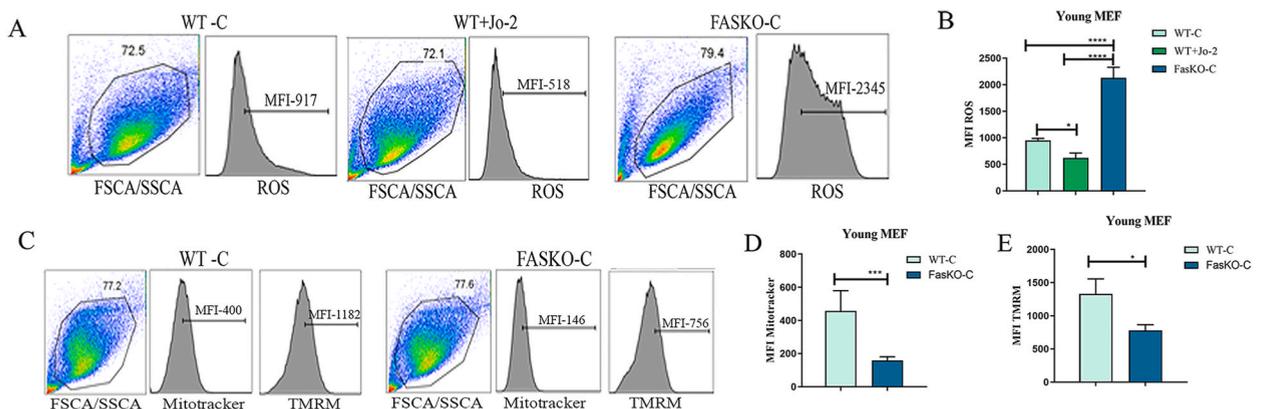
Fig. 5. Oxidative damage to mitochondria leads to mitochondrial dysfunction during stress-induced premature senescence in Fas-deficient young MEFs. ROS production was detected using CM-H2DCFDA and different parameters of mitochondrial biology in living cells was evaluated using Mitotracker Red and TMRM in young WT, Jo-2 stimulated WT and FAS KO MEFs. (A) Young WT and Fas KO MEFs were stimulated in the presence or in absence of Jo-2 followed by detection of ROS production by flow cytometry. (B) The bar graphs represent the MFI of ROS in different treatment groups of young MEFs. Mitochondrial mass was assessed by MitoTracker Red and membrane potential (Δψm) by TMRM incorporation in MEFs. (C) MFI of Mitotracker and TMRM were measured in young WT and Fas-deficient MEFs at different conditions and represented as dot plot. (D) Graphical representation of MFI of Mitotracker in young WT and Fas KO MEFs. (E) The graph represents MFI of TMRM in young WT and Fas KO MEFs. Data are mean ± SEM; data summarized from minimum three independent experiments. P > 0.05 was considered not significant (ns). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

CM-H2DCFDA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols [24]. MitoTracker Red is taken up by negatively charged polarized mitochondria and therefore considered to be dependent on mitochondrial membrane potential ($\Delta\psi$m). It is well established that $\Delta\psi$m changes upon specific stimuli thus, MitoTracker Red can be used to determine gain or loss of mitochondrial functionality [25]. Our flow cytometry data showed significantly induced level of ROS production in young Fas KO MEFs compared to the WT cells and young WT MEFs stimulated with Jo-2 showed reduced ROS activity (Fig. 5A and B). The mean fluorescence intensity (MFI) of Mitotracker and TMRM (Fig. 5C–E) was declined significantly in young Fas KO MEFs. There was no significant difference of ROS production (Fig. S6A and S6B) and mitochondria mass and functionality (Fig. S6C-S6E) in aged WT MEFs comparing to aged Fas KO MEFs. Overall, these results suggest that induced ROS and hampered mitochondria might be the contributing parameters for causing premature cellular senescence in the absence of Fas.

3.5. Blocking of ROS rescues premature cellular senescence in young Fas KO MEFs

To further confirm that accumulation of ROS is the key player resulting in premature cellular senescence in young Fas KO MEFs, we attempted to block ROS accumulation in young MEFs with NAC, a reduced glutathione (GSH) provider and a direct scavenger of ROS [26]. As shown in Fig. 6, cells incubated with 2.5 mM NAC were substantially reduced p16, p21, p53 and pp38 activities of Fas KO young MEFs compared to the WT level (Fig. 6A). The number of young Fas KO MEFs that scored positive for SA-β-gal after 3–4 days was also significantly reduced to the WT level in the presence of NAC (Fig. 6B and C). All these findings indicate that the increased ROS production leads to a premature senescence in young Fas KO MEFs.

3.6. Hampered cell proliferation and lower apoptosis in the absence of Fas in young MEFs

Senescence is characterized *in vitro* by a spontaneous decline in growth rate and culmination of a terminal arrest of cell cycle G1 phase [27]. Therefore, we were interested in understanding the proliferation status of the cells in the absence of Fas. We used Ki-67 to analyze the proliferation state of MEFs because Ki-67 is generally expressed strongly in proliferating cells and poorly in quiescent cells
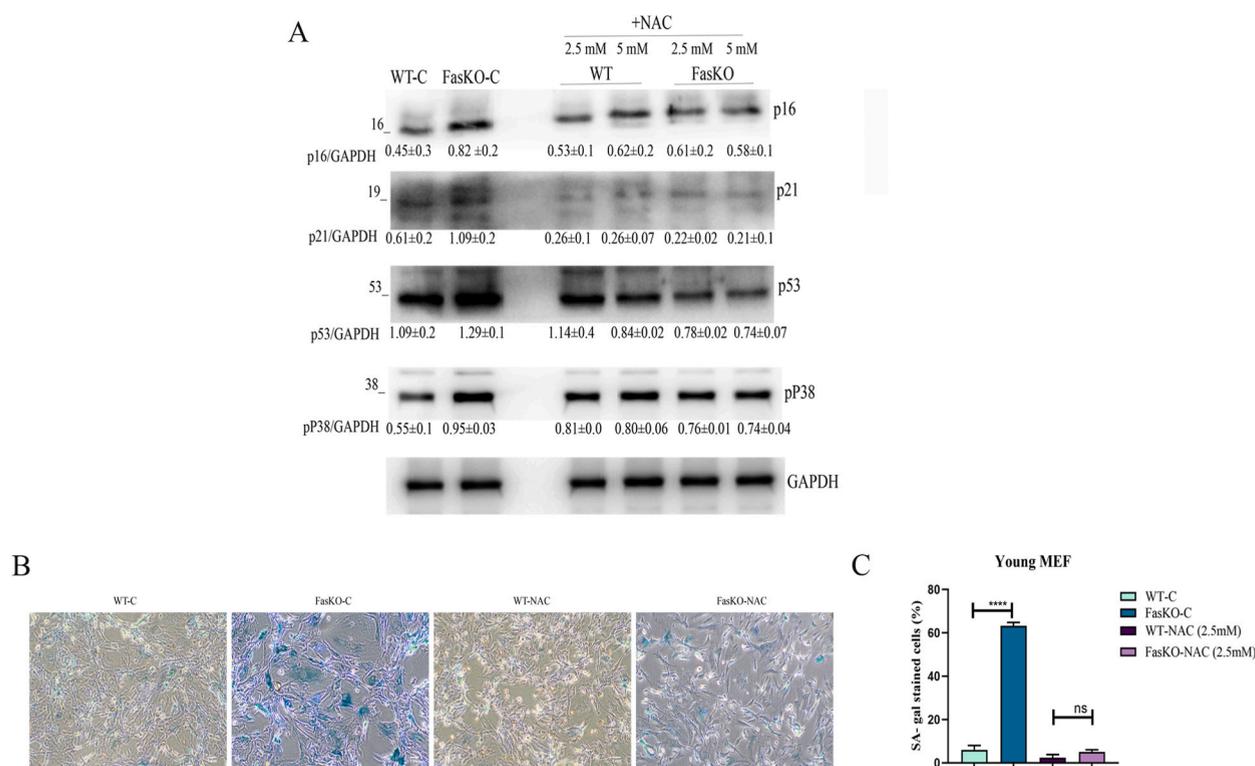
Fig. 6. Blocking of ROS rescues premature cellular senescence in Fas KO young MEFs. ROS accumulation in young MEFs was blocked with NAC, a reduced glutathione (GSH) provider and a direct scavenger of ROS. (A) Young WT and Fas KO MEFs treated in the presence or absence of 2.5 mM and 5 mM NAC and post 5 h cell lysates obtained were subjected to immunoblot analysis with anti-p16, anti-p21, anti-p53, anti-pp38 antibodies. The complete blot images are presented in Supplementary Fig. S11A. (B) Microscopic pictures of SA-β-gal-positive cells of WT and Fas KO young MEFs treated with or without 2.5 mM NAC for 5 h. Images are an average of 10 different fields for each group. (C) Bars correspond percentage of SA-β-gal-stained cells. Results represent mean values of at least three independent experiments, and error bars show the standard deviation, ****p < 0.0001.

[28]. Flow cytometry analysis of the percentage of Ki-67$^+$ cells was significantly reduced in the young Fas KO MEFs (Fig. 7A and B). Annexin V and propidium iodide (PI) were used to analyze apoptotic and necrotic cells. Further flow cytometry analysis of apoptotic cells by Annexin V–PI staining in young Fas KO MEFs revealed significant higher necrotic cells (PI positive) and tendency of lower apoptotic cells (Annexin V positive) (Fig. 7C–E). Together this data suggest that the absence of Fas leads to dampened proliferation of young MEFs and results lower apoptosis and higher necrotic cells.

4. Discussion

ALPS is a non-infectious and non-malignant unchecked proliferation of lymphocytes caused by defects in FAS or FASL associated with defective lymphocyte apoptosis [29,30]. Behaviour of Fas-mediated immune cells is complex and censorious for maintaining immune homeostasis. Study from Yoshio Hayashi group showed that Fas-mediated apoptosis can lead to age-associated dysregulation of CD4$^+$ T cells in primary Sjögren's Syndrome [31]. Jing-He Tan group demonstrated that FasL/Fas signaling facilitates oocyte ageing [32]. Fas-deficient mice are prone to premature death and loss of c-REL greatly prolongs the lifespan of Fas lpr/lpr mice [33]. Morphologically senescent cells are large, flat and have acidic SA-β-galactosidase enzymatic activity. They are associated with profound growth defects [34] as they prevent cell proliferation. On the other hand, senescent cells may be resistant to apoptosis and thus probably allow cells to undergo clonal selection for mutations [35]. Although there are few previous studies which mentioned involvement of Fas in ageing but the role of Fas in the development of autoimmune diseases in Fas KO mice and its relationship with ageing have not been explored in detail. Thus, our group is keen to understand the role of Fas in cellular senescence which would help to underpin the steps to target reversal of senescence process and in this study, and we demonstrate a novel and interesting role of Fas in delaying cellular senescence, hence inhibiting ageing.

Our study shows that Fas-agonist Jo-2 can significantly abrogate cellular senescence in young as well as in aged WT MEFs as shown by reduced SA-β-gal activity and abrogated p16 protein level. This was further diminished with the treatment of p38 inhibitor suggesting that p38 signaling is a stumbling block in this process. In the siRNA transfection experiment, knocking down Fas in young MEFs led to enhanced cellular senescence compared to the control MEFs which was further enhanced with Jo-2 stimulation, suggesting that absence of Fas resulted in the induction of cellular senescence and Jo-2 was unable to rectify this effect. However, the aged MEFs transfected with FassiRNA did not show any modification in cellular senescence compared to the control aged MEFs. Interestingly, in young Fas KO MEFs absence of Fas led to premature cellular senescence detected by strong SA-β-gal activity, significantly enhanced ageing-associated markers such as p16, p21, p53, pp38 by Western blot and elevated p16, p21, p53, IGFBP5 mRNA level by qPCR. However, the aged WT and Fas KO MEF behaved almost similar without having any additive effect of Fas signaling in the cellular senescence.

Cellular senescence is a "chronic inflammatory" condition supported by elevated levels of circulatory proinflammatory cytokines, including IL-6, which is one of the senescence-associated secretory phenotype components accelerating senescence [36]. During cellular senescence the detrimental role of inflammation is further evidenced by clinical data [37]. The increased levels of chronic inflammation in these circumstances are known as inflammageing [38]. In senescence, metabolic activation yields many metabolites, cellular energy, and reactive oxygen species that accelerate senescence with secretion of proinflammatory cytokines such as IL-6 [39]. Senescent cells are stimulated by various types of stresses, including that induced by ROS. Elevated oxidative stress in senescent cells
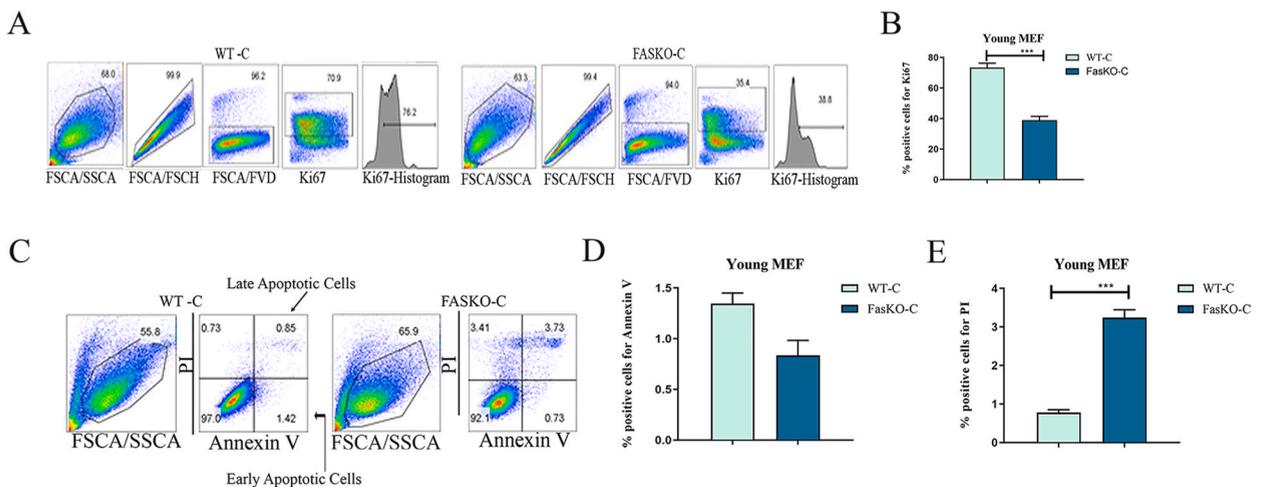
Fig. 7. Hampered cell proliferation and lower apoptosis in the absence of Fas in young MEFs leading to premature senescence. Ki-67 analysis in young Fas KO MEFs to investigate the proliferation state of MEFs. Annexin V and PI were used to analyze apoptotic and necrotic cells in young Fas KO MEFs. (A) Representative flow cytometry plots of Ki-67 staining of young WT and Fas KO MEFs in different treatment groups to study the cell proliferation status. (B) Bar graphs are the representation of the % positive cells for Ki-67. (C) Flow cytometry dot plots represent the percentage of Annexin V and PI-positive cells. (D) Graph represents the % positive cells for Annexin V. (E) Graph represents the % positive cells for PI. Data are mean ± SEM; data summarized from minimum three independent experiments. ***p < 0.001, ****p < 0.0001.

has been linked to the dysfunctional mitochondria. Indeed, senescent cells are characterized by changes in mitochondrial mass, membrane potential and mitochondrial morphology [40]. While exploring the underlying mechanism, our data revealed significantly induced level of IL-6 in Fas KO young as well as aged MEFs, suggesting a favourable proinflammatory environment in absence of Fas. Jo-2 stimulated young WT MEFs resulted in abrogated ROS activity, indicating that Fas-agonist could favour an anti-senescent effect. Interestingly, ROS was significantly increased in young Fas KO MEFs compared to the WT followed by dampened mitochondrial mass and potential. Further inhibiting ROS with NAC could reverse the effect of absence of Fas in favouring senescence in Fas KO MEFs. All of these finding strongly suggest that ROS accumulation is responsible for the senescence phenotype induced by young Fas KO MEFs.

Morphologically, Fas KO MEFs from early to late all passages looked flattened, enlarged, and clustered under microscope indicating an aged phenotype. Moreover, Fas KO MEFs were less proliferative since the young state, and they exhibited significantly induced accumulation of necrotic cells. Cell death can be of both types: necrotic death and programmed death (apoptosis). Oxidative stress is required in many instances for execution of the apoptotic program. Several reports suggested that ROS mediates necrosis primarily not apoptosis. Since apoptosis was not inducible in senescent fibroblasts, it indicated that ROS mainly induces apoptosis in proliferating cells by acting in the Gl phase of the cell cycle and increasing the p53 and p21WAFi/ciPi protein levels [41]. Our results showed less apoptotic cells and more necrotic cells in the absence of Fas. Lower apoptosis meaning unwanted cells were building up in the system which is a characteristic of senescent cells, and more necrosis is the result of more inflammatory environment which might be linked to elevated ROS, altogether this can be contributing to the low proliferative ability of MEFs which are deficient of Fas.

The interesting observation of this study is that the young MEFs lead to premature senescence however the aged MEFs did not show any significant difference either in the presence or in the absence of Fas. But the inflammatory IL-6 level was elevated in both young and aged MEFs. Noteworthy, in aged MEFs which were deficient of Fas, the intensity of SA-β-gal-stained cells was much stronger, even though the percentage of SA-β-gal-stained cells were unaltered. The possible reason could be P2 young Fas KO MEFs acquired senescent phenotype much early, resumed this phenotype until they approached to aged P10, contributing to the intensity of the β-gal-stained cells instead having effect in the number of aged cells compared to WT aged MEFs. We detected induced Fas expression in aged WT MEFs compared to the young WT MEFs. However, the overall observation suggests that the presence of high Fas in aged WT MEFs would not be the major driver of normal ageing process of these cells and it might be the consequence. Whereas Jo-2-mediated Fas exerts reduction in cellular senescence at any age indicating Jo-2-mediated Fas signaling exerts anti-senescent role. Fas KO mice displayed early signs of senescence and substantially accelerated the onset of cellular senescence, resulted in landing premature senescence in young MEFs, indicating the presence of Fas is necessary to delay senescence. Biological process of ageing is very complex and is the result of genetic and, to a greater extent, environmental factors, and time. The alterations associated with ageing can be divided into a few categories such as normal ageing, somatic diseases and multiple chronic conditions, psychological, cognitive and social changes [42,43]. We believe no difference observed between aged Fas KO MEFs vs WT MEF could be because WT cells had undergone the complete canonical process of ageing whereas in absence of Fas the immune compromised Fas KO mice cells landed up premature ageing hampering the normal ageing process in these cells, obtaining substantial senescence at young age which might be a static process. Importantly we observed Fas KO mice having much shorter life span than the WT.

In conclusion, our observations of Fas-mediated delay in cellular senescence and the role of Jo-2-mediated Fas signaling in reducing senescence are interesting. Premature senescence induced the early activation of senescence markers, including the increase in the percentage of SA-β-galactosidase (SA-β-gal) cells, the induction of p53 phosphorylation, and the enhanced p16 and p21 protein and elevated IL-6 pro-inflammatory cytokine in Fas KO MEFs. Induced ROS followed by mitochondrial dysfunction in the absence of Fas could be the possible mechanism in promoting premature senescence. However, the reason behind no significant difference observed between Fas-efficient and Fas-deficient aged MEFs need to be further studied. Nevertheless, our data suggest the importance of the presence of Fas in controlling the cellular senescence process.

Author contribution statement

Chaitrali Saha: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Jingyu Li: Performed the experiments; Analyzed and interpreted the data.

Xuerong Sun: Conceived and designed the experiments; Analyzed and interpreted the data.

Xinguang Liu: Contributed reagents, materials, analysis tools or data.

Gonghua Huang: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Funding statement

Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e13451.

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