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Loss of HuR leads to senescence-like cytokine induction in rodent fibroblasts by activating NF-κB

Michihiro Hashimoto [1], Takayuki Tsugawa [1], Hiroyuki Kawagishi, Azusa Asai, Masataka Sugimoto *

Research Institute, National Center for Geriatrics and Gerontology, 35 Gengo, Obu, Aichi 474-8511, Japan

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ABSTRACT

Background: HuR (human antigen R) is a ubiquitously expressed member of the Hu/ELAV family of proteins that is involved in diverse biological processes. HuR has also been shown to play an important role in cell cycle arrest during replicative senescence in both human and mouse cells. Senescent cells not only halt their proliferation, but also activate the secretion of proinflammatory cytokines. A persistent DNA damage response is essential for the senescence-associated secretory phenotype (SASP), and increasing evidence has suggested that the SASP is associated with malignancy.

Methods: Senescence-associated phenotypes were analyzed in MEFs and other cell line in which HuR expression is inhibited by sh-RNA-mediated knockdown.

Results: RNAi-mediated HuR inhibition resulted in an increase in SASP-related cytokines. The induction of SASP factors did not depend on ARF–p53 pathway-mediated cell cycle arrest, but required NF-κB activity. In the absence of HuR, cells were defective in the DNA-damage response, and single strand DNA breaks accumulated, which may have caused the activation of NF-κB and subsequent cytokine induction.

Conclusions: In the absence of HuR, cells exhibit multiple senescence-associated phenotypes. Our findings suggest that HuR regulates not only the replicative lifespan, but also the expression of SASP-related cytokines in mouse fibroblasts.

General significance: RNA-binding protein HuR protects cells from undergoing senescence. Senescence-associated phenotypes are accelerated in HuR-deficient cells.

1. Introduction

Cellular senescence halts the proliferation of cells at risk of malignant transformation and acts as a potent tumor-suppressive mechanism in mammals [1,2]. Senescence occurs following a period of cell proliferation, or is acutely induced in response to various cellular insults such as oxidative stress, oncogene activation, or DNA damaging agents [3]. Cellular senescence depends on the activation of two major tumor suppressor pathways, the $p19^{ARF}$ ($p14^{ARF}$ in human)–p53 and $p16^{Ink4a}$–pRB pathways, which play critical roles in inducing and maintaining cell cycle arrest during senescence [4,5]. The inactivation of these pathways bypasses senescence, allowing damaged cells to proliferate.

Besides cell cycle arrest, senescent cells exhibit several characteristics including a flattened morphology, the formation of senescence-associated heterochromatin foci, and an increase in senescence-associated β-galactosidase activity [6]. Furthermore, recent studies have confirmed that senescent cells secrete numerous inflammatory cytokines, which is called the senescence-associated secretory phenotype (SASP) [7]. Interleukin 6 (IL-6) is the most prominent cytokine among the SASP cytokines, and is known to associate with DNA damage or oncogene-induced senescence in several types of mouse and human cells [8]. The consequence of the SASP varies depending on the biological context. For example, some SASP factors can reinforce cell growth arrest and activate immune systems to clear senescent cells from tissues [9–12]. On the other hand, the SASP also has deleterious effects such as the promotion of malignant phenotypes, angiogenesis, and epithelial–mesenchymal transition [8,13–15]. The SASP may also contribute to age-related diseases because senescent cells accumulate in tissues with age [16]. The mechanism of SASP induction is not fully understood. However, NF-κB, which is activated by a persistent DNA damage response (DDR) or p38 signaling pathway, as well as C/EBPβ appear to play pivotal roles in the induction of SASP-related cytokines [17].

HuR is a ubiquitously expressed member of the ELAV/Hu family and is involved in diverse biological processes [18,19]. HuR is an RNA-

Abbreviations: SASP, senescence-associated secretory phenotype; HuR, human antigen R; IL-6, interleukin 6; MEF, mouse embryonic fibroblast; sh-RNA, short hairpin RNA; DDR, DNA damage response; IR, ionizing radiation; UV, ultraviolet

* Corresponding author at: Research Institute, National Center for Geriatrics and Gerontology, 35 Gengo, Moriokacho, Obu, Aichi 474-8511, Japan. Tel.: +81 562 44 5651; fax: +81 562 46 8463.
E-mail address: msugimot@ncgg.go.jp (M. Sugimoto).

[1] These authors contributed equally to this work.

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binding protein that controls the stability, translation, splicing, and intracellular trafficking of its target mRNA [20,21]. HuR was previously shown to be down-regulated during the senescence of human diploid fibroblasts and human tissues with age [22]. $p16^{Ink4a}$ plays pivotal roles in the induction of cellular senescence in human cells, and HuR destabilizes *Ink4a* mRNA in human cells [23]. In contrast, $p16^{Ink4a}$ is dispensable while the ARF–p53 pathway is essential for senescence in rodent cells [24]. We recently demonstrated that HuR suppressed $p19^{ARF}$ expression in mouse embryonic fibroblasts (MEFs), thereby maintaining the replicative lifespan of these cells [25]. The loss of HuR results in premature cellular senescence with concomitant activation of the ARF–p53 pathway, and deletion of either the *ARF* or *p53* gene enables MEFs to grow in the absence of HuR expression. Thus, HuR plays essential roles in the regulation of cellular senescence in both humans and mice.

We herein showed that HuR regulates not only the replicative lifespan, but also the expression of SASP-related cytokines in MEFs. RNAi-mediated silencing of HuR resulted in the induction of several SASP factors including IL-6. Unlike senescence-associated cell cycle arrest, the *ARF* and *p53* genes were dispensable for the induction of cytokines. NF-κB was activated in HuR knockdown cells, and pharmacological inhibition of NF-κB abrogated the cytokine induction in these cells. Under these conditions, we observed the accumulation of single strand DNA breaks, which may have caused the activation of NF-κB activation and subsequent cytokine expression. Thus, this study confirmed the novel role of HuR in the regulation of senescence-associated phenotypes.

2. Materials and methods

2.1. Cells and culture conditions

NIH-3T3 and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100 U/ml penicillin–streptomycin. Mouse embryonic fibroblasts (MEFs) were cultured in medium supplemented with 0.1 mM nonessential amino acids, 55 μM 2-mercaptoethanol, and 10 μg/ml gentamicin instead of penicillin and streptomycin. To analyze mRNA stability, cells were treated with 5 μg/ml actinomycin D. To perform the clonogenic survival assay, 10^4 cells were seeded on 10-cm-diameter culture dishes and subjected to irradiation (IR or UV). After 12 days, cultured cells were stained with crystal violet, and the number of colonies per dish was counted. To inhibit NF-κB activity, cells were treated with 5 μM BAY 11-7082 (Wako Pure Chemicals Insustries, Osaka, Japan) for 24 h. To inhibit p38 activity, cells were treated with 5 μM SB203580 (Merck Millipore, Billerica, MA) for 24 h.

2.2. Virus production and infection

293T cells were transfected with sh-SCR or sh-HuR retroviral expression plasmids together with a helper plasmid [26,27]. Viruses were harvested 24 to 60 h after transfection, pooled, and stored on ice. Exponentially growing cells in 10-cm-diameter culture dishes were infected with 3 ml of fresh virus-containing supernatant in complete medium containing 8 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO). Infected cells were selected in the presence of 5 μg/ml puromycin (Sigma-Aldrich).

2.3. Annexin V staining

Annexin V staining was performed using Tali® Apoptosis Kit (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instruction. 50,000 cells were stained for each sample.

2.4. Realtime PCR analysis

Total RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. cDNA was generated using a PrimeScript RT reagent kit with gDNA Eraser (TAKARA BIO, Otsu, Japan), and subjected to PCR using the following primers: for 18S, 5′-AGTCCCTGCCCTTTGTACACA-3′ (sense) and 5′-GATCCGAGGGCCTCACTAAAC-3′ (antisense); for *IL-6*, 5′-CAAGAAAGAC AAAGCCAGAGTC-3′ (sense) and 5′-GAAATTGGGGTAGGAAGGAC-3′ (antisense); for *IL-1beta*, 5′-GAAATGCCACCTTTTGACAGTG-3′ (sense) and 5′-CTGGATGCTCTCATCAGGACA-3′ (antisense); for *Ccl-2*, 5′-TAAA AACCTGGATCGGAACCAAA-3′ (sense) and 5′-GCATTAGCTTCAGATTTA CGGGT-3′ (antisense); for *VEGF*, 5′-CCGAAACCATGAACTTTCTG-3′ (sense) and 5′-AGATGTACTCTATCTCGTCG-3′ (antisense); for *Cxcl1*, 5′-ACTGCACCCAAACCGAAGTC-3′ (sense) and 5′-TGGGGACACCTTTTAG CATCTT-3′ (antisense); for *Cyclin D1*, 5′-GCGTACCCTGACACCAATCTC-3′ (sense) and 5′-CTCCTCTTCGCACTTCTGCTC-3′ (antisense); for *GAPDH*, 5′-AATGGTGAAGGTCGGTGTG-3′ (sense) and 5′-GAAGATGG TGATGGGCTTCC-3′ (antisense). Real-time PCR analysis was performed on a Chromo4 realtime PCR system (Bio-Rad, Hercules, CA). Values were normalized to 18S rRNA or *GAPDH* in each sample.

2.5. Immunoblotting

Cell lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer (10 mM Na-phosphate pH 7.2, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Na-deoxycholate, and 1% NP-40) containing protease inhibitors (Roche, Indianapolis, IN). Nuclear and cytoplasmic lysates were prepared as previously described [26]. Lysates were separated on denaturing polyacrylamide gels containing 0.1% SDS and transferred to PVDF membranes (Millipore, Billerica, MA). Proteins were detected using antibodies to HuR (3A2; Santa Cruz Biotechnology, Santa Cruz, CA), Lamin A/C (H-110; Santa Cruz Biotechnology), α-Tubulin (B-5-1-2; Sigma-Aldrich), phospho-H2AX (γH2AX) (Ser139; Millipore), p38 (Cell Signaling Technology, Danvers, MA), and phospho-p38 (Thr180/182; Cell Signaling Technology). Antibody binding sites were detected using HRP-conjugated antibodies to mouse or rabbit IgG (Jackson ImmunoResearch, West Grove, PA).

2.6. Analysis of mRNA and ribosome association

In vivo ribosome association was analyzed as previously described [25]. Briefly, cytoplasmic extracts were prepared from MEFs expressing GFP or GFP-L10, and immunoprecipitated using GFP antibody-coated magnetic beads (Medical & Biological Laboratories, Nagoya, Japan). RNA was extracted from immune complex, and subjected to realtime PCR analysis.

2.7. Immunofluorescence

Cells seeded on coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, permeabilized in 0.5% Triton X-100/PBS for 15 min at room temperature, and stained with antibodies to NF-κB (C-20; Santa Cruz Biotechnology) or γH2AX. Washed coverslips were incubated with Alexa488-conjugated antibodies to rabbit IgG (Jackson ImmunoResearch) and mounted using Vectashield and 4′, 6′-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA).

2.8. Enzyme-linked immunosorbent assay (ELISA)

Cells were grown to confluent in a 24-well dish and cultured in 1 ml/well fresh cell culture medium for 4 days. The amount of the IL-6 protein in each conditioned medium was determined using the mouse IL-6 Quantikine ELISA kit (R&D Systems, Minneapolis, MN) in accordance with the manufacturer's instructions.

2.9. Luciferase assay

The murine *IL-6* promoter was obtained by PCR from mouse genomic DNA using sense (5′-AAGATCTACATTGTGCAATCTTAATAAG GTTTCC AATCAGCCCCACCCACTCTGGCCCCACCCCCAC-3′) and antisense (5′-AAAAGCTTTCCAGAGCAGAATGAGCTAC-3′) primers. PCR products digested with BglII and HindIII were cloned into a luciferase reporter vector plasmid (pGL3 basic; Promega, Madison, WI). To introduce a mutation in the NF-κB-binding site [28], two PCRs were performed using sense and mutant antisense (5′-TTTGAGACTCAG TCTAAAATCCCACATTTGATAAAAATC-3′) primers, and mutant sense (5′-GGGATTTTAGACTGAGTCTCAAAATTAGAGAG-3′) and antisense primers. PCR products were mixed and subjected to the second PCR using sense and antisense primers, and cloned into the reporter vector. Cells (5×10^4 cells per 3.5-cm diameter dish) were transfected with 380 ng of the luciferase reporter plasmids together with 20 ng of *Renilla* luciferase expression plasmids (Promega) using X-treme Gene 9 (Roche) according to the manufacturer's instructions. Luciferase activities were measured 2 days later using a dual luciferase reporter assay system (Promega). Luciferase activity (firefly) in each sample was normalized by *Renilla* luciferase.

2.10. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using anti-NF-kB (C-20; Santa Cruz Biotechnology) and SimpleChIP Plus Enzymatic Chromatin IP Kit (Cell Signaling Technology). *IL-6* promoter association was quantified by realtime PCR using the following primers: for *IL-6*, 5′-CCCCACCCTCCA ACAAAGAT-3′ (sense) and 5′-ACAGACATCCCCAGTCTCAT-3′ (antisense); for *Ccl-2*, 5′-TCACCATTGCAAAGTGAATTGGC-3′ (sense) and 5′-TCAGATTCTCCGGCCCATGAGAGA-3′ (antisense); for *VEGF*, 5′-CTGAGC CCAGTTTGAAGGGG-3′ (sense) and 5′-CCACTACCGCGAAATGGAAAG-3′ (antisense).

2.11. Single cell gel electrophoresis assay (comet assay)

A single cell gel electrophoresis assay was performed under neutral or alkaline conditions using the Comet Assay Reagent kit (Trevigen, Gaithersburg, MD) in accordance with the manufacturer's instructions. DNA was visualized by SYBR Green (ThermoFisher Scientific) staining. Fluorescence images were taken using an inverted microscope (Olympus, Tokyo, Japan), and analyzed by Image J with OpenComet.

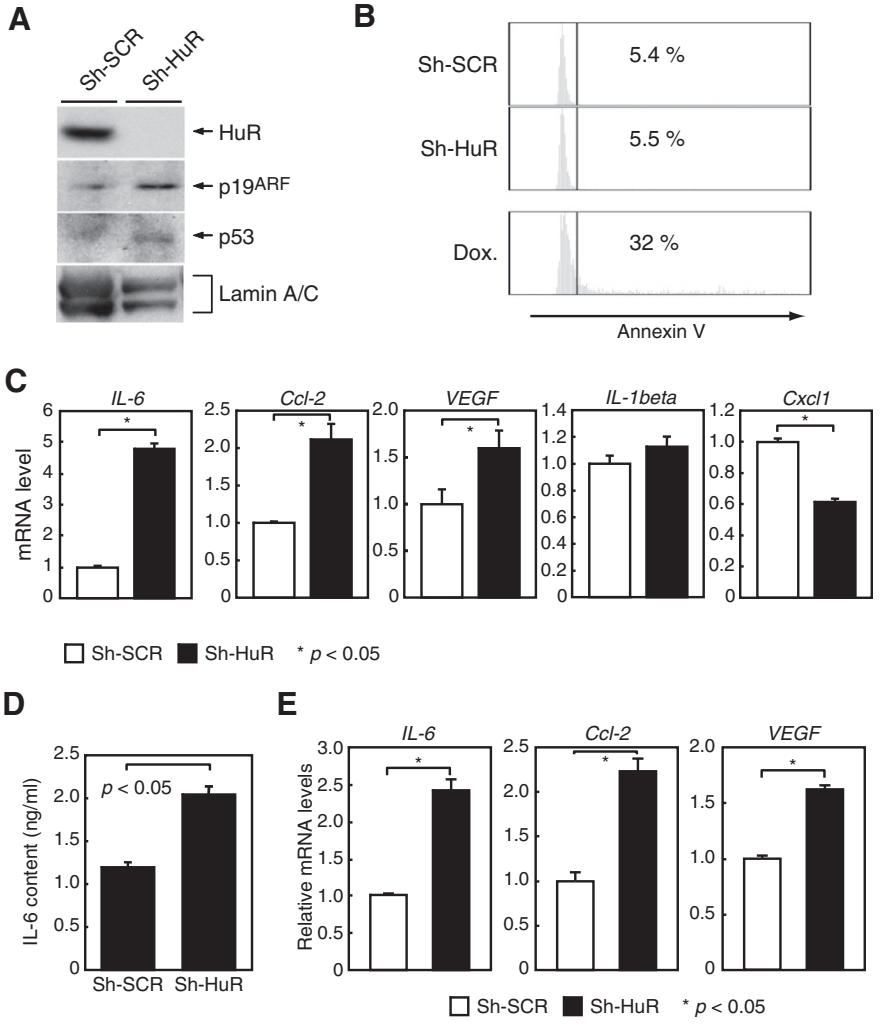

Fig. 1. HuR inhibition enhanced expression of SASP-related cytokines. (A) Wild type MEFs were infected with sh-SCR or sh-HuR retroviruses. Infected cells were selected with puromycin and expression of the indicated proteins was analyzed by immunoblotting. Lamin was used as a loading control. (B) MEFs infected with sh-SCR or sh-HuR were stained with Annexin V. For control, MEFs were treated with 1 μM doxorubicin for 24 h prior to Annexin V staining (Dox.). The numbers represent Annexin V-positive cells. (C) Total RNA was prepared from sh-RNA-infected MEFs and *IL-6*, *Ccl-2*, *VEGF*, *IL-1beta*, and *Cxcl1* mRNA levels were analyzed by realtime PCR. (D) IL-6 contents in conditioned media were measured by ELISA. (E) *IL-6*, *Ccl-2* and *VEGF* mRNA levels in *ARF*/*p53* double knockout MEFs expressing sh-SCR or sh-HuR were analyzed by realtime PCR. Values are means +/− SD of triplicate experiments. Results are representative of at least three independent experiments.

3. Results

3.1. Expression of SASP factors was increased in MEFs upon HuR depletion

To test whether HuR regulates cytokine expression, early-passage (P2) MEFs were infected with retroviruses encoding short hairpin RNA targeting *HuR* (sh-HuR) or scrambled sequence (sh-SCR) [26]. After the selection of infected cells with puromycin, we confirmed that HuR protein levels had decreased to an undetectable level in HuR-knockdown samples (Fig. 1A). As we previously reported [25], HuR silencing resulted in the induction of ARF–p53 pathway. These cells were not undergoing apoptosis as judged by Annexin V staining (Fig. 1B). Thus, together with our previous findings that HuR silencing induces ARF–p53 pathway-dependent cell cycle arrest and senescence-associated β galactosidase [25], senescence, but not apoptotic program was activated upon HuR knockdown. Total RNA was then extracted from these cells, and the expression of SASP-related cytokines (*IL-6*, *Ccl-2*, *IL-1beta*, *Cxcl1*, and *VEGF*) was analyzed by realtime PCR. As shown in Fig. 1C, the knockdown of HuR resulted in increases in *IL-6*, *Ccl-2*, and *VEGF* mRNA levels, while *IL-1beta* and *Cxcl1* levels were unchanged or decreased. Accordingly, we found that the content of IL-6 in the conditioned media also increased in the absence of HuR (Fig. 1D).

Senescence is defined by irreversible cell cycle arrest, and the ARF–p53 pathway plays pivotal roles in the establishment of cell cycle arrest during senescence in MEFs. Our previous study demonstrated that HuR regulated the replicative lifespan of MEFs through the ARF–p53 pathway [25], while p53 was not a major regulator of the SASP in senescent cells [7]. Therefore, we attempted to determine if the increase in cytokine expression in HuR-depleted cells was caused by ARF–p53 pathway-dependent cell cycle arrest. MEFs prepared from *ARF*/*p53*-double null animals were infected with sh-SCR or sh-HuR retroviruses. These cells do not senesce upon HuR depletion [25]. Nevertheless, *IL-6*, *Ccl-2* and *VEGF* mRNA levels increased when HuR expression was inhibited (Fig. 1E). Taken together, these results suggest that HuR regulates not only the replicative lifespan, but also the expression of SASP-related cytokines through distinct mechanisms.

3.2. IL-6 transcriptionally activated by NF-κB in HuR-depleted cells

It is well established that HuR regulates gene expression at post-transcriptional levels including mRNA stability, intracellular trafficking, and translation [20,29]. Hence, it is possible that the induction of SASP-related cytokines in HuR-depleted MEFs is also attributed to such post-transcriptional regulation. HuR has been shown to associate with and regulate the stability of many cytokine mRNAs including *IL-6* [30,31]. In MEFs, however, the knockdown of HuR had only a minor effect on *IL-6*-, *Ccl-2* and *VEGF* mRNA stabilities, while *cyclin D1* mRNA was significantly destabilized as previously reported (Fig. 2A) [32].

To check if HuR knockdown affects the translation of cytokine mRNA, we next analyzed the association of *IL-6*, *Ccl-2* and *VEGF* mRNA and ribosomes in vivo. To this end, MEFs were infected with retroviruses encoding GFP or GFP fused to ribosomal protein L10 together with sh-SCR or sh-HuR retroviruses. Cytoplasmic lysates were immunoprecipitated using GFP antibody to purify ribosome–mRNA complexes [33,34]. RNA was recovered from immune complexes and subjected to realtime PCR analysis. *IL-6*, *Ccl-2* and *VEGF* mRNA were enriched in GFP-L10 immune complex, but we did not observe further increase in the mRNA and ribosome association in HuR-knockdown cells

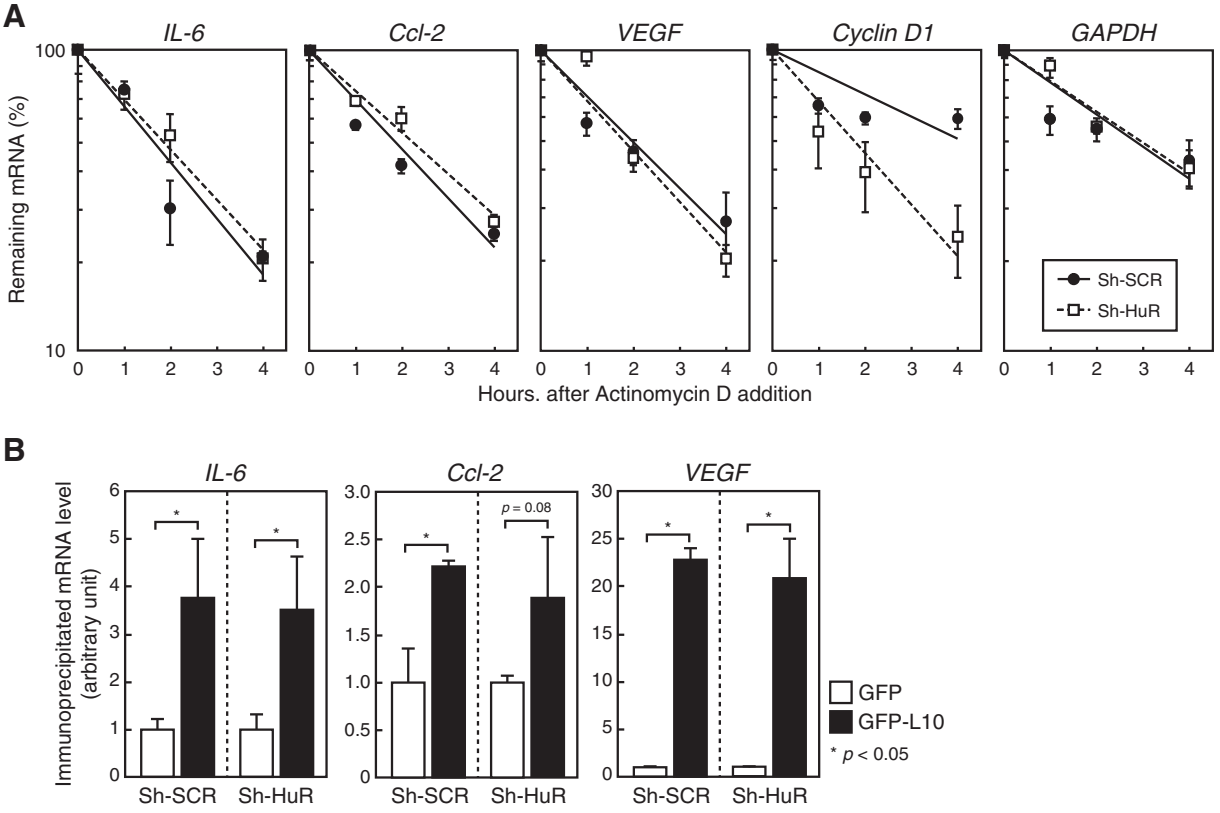

Fig. 2. Effects of HuR knockdown on mRNA stability and translation. (A) MEFs expressing sh-SCR or sh-HuR were treated with 5 μg/ml actinomycin D for the indicated periods, and *IL-6*, *Ccl-2*, *VEGF*, *Cyclin D1* and *GAPDH* mRNA were analyzed by realtime PCR. (B) Sh-SCR and sh-HuR MEFs were infected with GFP or GFP-L10 retroviruses. GFP-tagged ribosomes were immunopurified from cytoplasmic extracts and *IL-6*, *Ccl-2* and *VEGF* mRNA in the ribosome complex was analyzed by realtime PCR. mRNA level was normalized to input signal in IP sample. Data are means +/− SD of triplicate sample and representative of two-independent experiments.

(Fig. 2B). Therefore, loss of HuR expression seems not to modulate the translation efficiency of cytokine mRNA in MEFs.

We next attempted to determine if transcriptional regulation was involved in the activation of SASP-related cytokines in HuR-knockdown cells. Among the transcription factors, C/EBPβ and NF-κB play essential roles in transcriptional induction of the SASP [17]. However, *C/EBPβ* mRNA is a *bona fide* target of HuR, and the loss of HuR results in a reduction in C/EBPβ protein levels [25,35]. Therefore, it is unlikely that C/EBPβ caused cytokine induction in HuR-knockdown cells.

Hence we tested if NF-kB was activated in HuR-knockdown cells. Immunofluorescence analysis revealed that the number of cells exhibiting nuclear NF-κB was increased upon HuR knockdown (Fig. 3A/B), which is indicative of the activation of NF-κB. To further confirm these results, nuclear and cytoplasmic fractions were analyzed by immunoblotting. As shown in Fig. 3C, although HuR status did not affect the total NF-κB amount, we observed a slight increase in the nuclear NF-κB level in HuR-knockdown cells. Consistently, HuR knockdown promoted recruitment of NF-κB to the *IL-6*, *Ccl-2* and *VEGF* promoter regions (Fig. 3D).

These results suggest that NF-κB is activated in the absence of HuR, which likely leads to the transcriptional induction of the cytokines. To test whether NF-κB is required for the induction, we performed a luciferase reporter assay using the mouse *IL-6* promoter (Fig. 4A). Reporter plasmids were transfected into MEFs expressing sh-SCR or sh-HuR together with *Renilla* luciferase expression plasmids. *IL-6* promoter activity was significantly increased in MEFs in which HuR expression was suppressed (Fig. 4B). However, HuR knockdown failed to activate the *IL-6* promoter when the NF-κB binding site was mutated (Fig. 4B, *right*) [36]. To further confirm the involvement of NF-κB, we utilized NF-κB inhibitor BAY 11-7082. In the presence of NF-κB inhibitor, *IL-6* promoter activity was suppressed to below the basal level and HuR silencing did not increase the promoter activity (Fig. 4C). Moreover, BAY 11-7082 totally abrogated the cytokine induction in sh-HuR MEFs (Fig. 4D), suggesting that NF-κB is prerequisite for the transcriptional induction of cytokine in HuR-knockdown cells. Thus, cytokine induction in HuR-knockdown cells is mostly attributed to transcriptional activation by NF-κB.

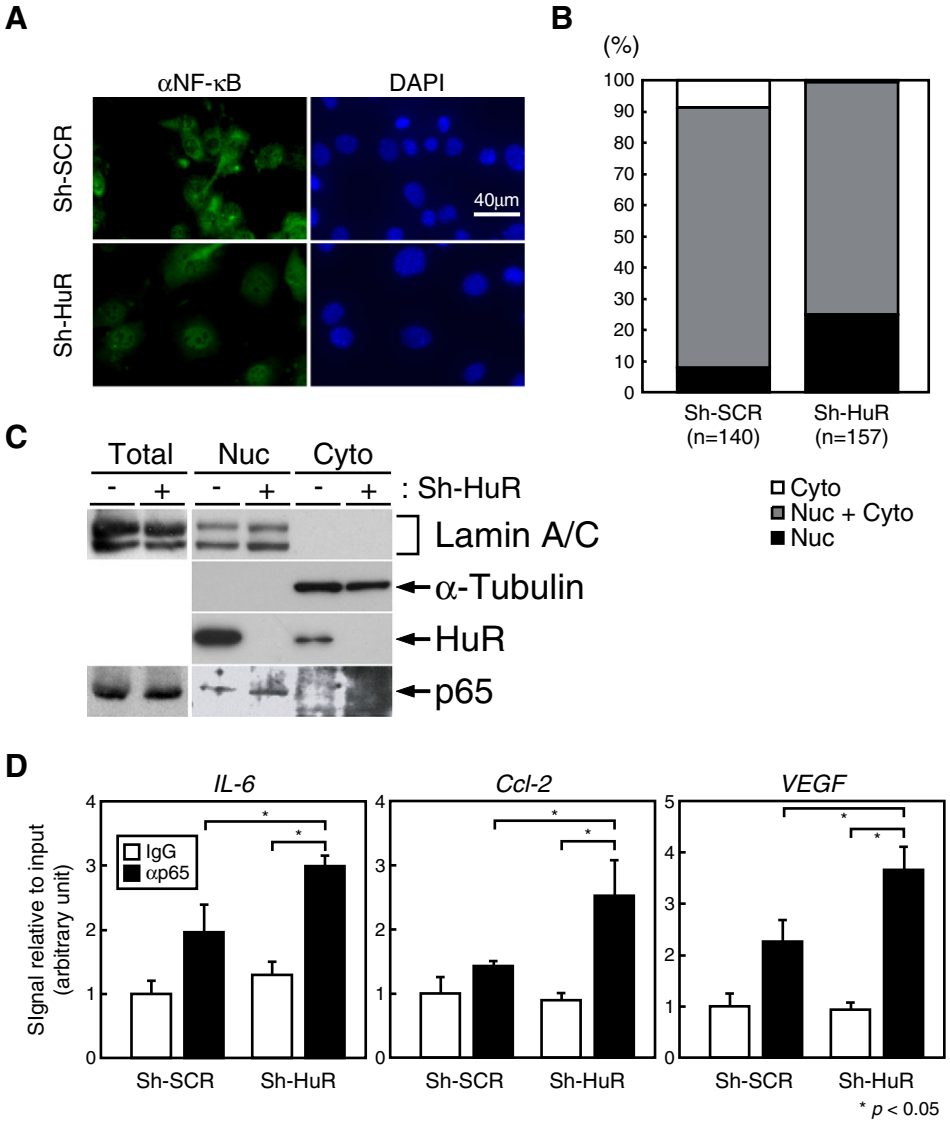

Fig. 3. NF-κB was activated in the absence of HuR. (A) Immunofluorescence analysis was performed using NF-κB antibodies in sh-SCR and sh-HuR-expressing MEFs. Cells were counterstained with DAPI. (B) The percentages of cells exhibiting predominantly cytoplasmic (Cyto), nuclear (Nuc), and both cytoplasmic and nuclear (Nuc + Cyto) NF-κB staining were calculated. (C) Total, nuclear (Nuc) and cytoplasmic (Cyto) fractions were analyzed by immunoblotting. (D) Chromatin immunoprecipitation was performed using control IgG or anti-NF-κB (p65) in MEFs expressing sh-SCR or sh-HuR. *IL-6*, *Ccl-2* and *VEGF* promoters were quantified by realtime PCR. Values were normalized to input signal in each IP. Data are means +/− SD and representative of three independent experiments.

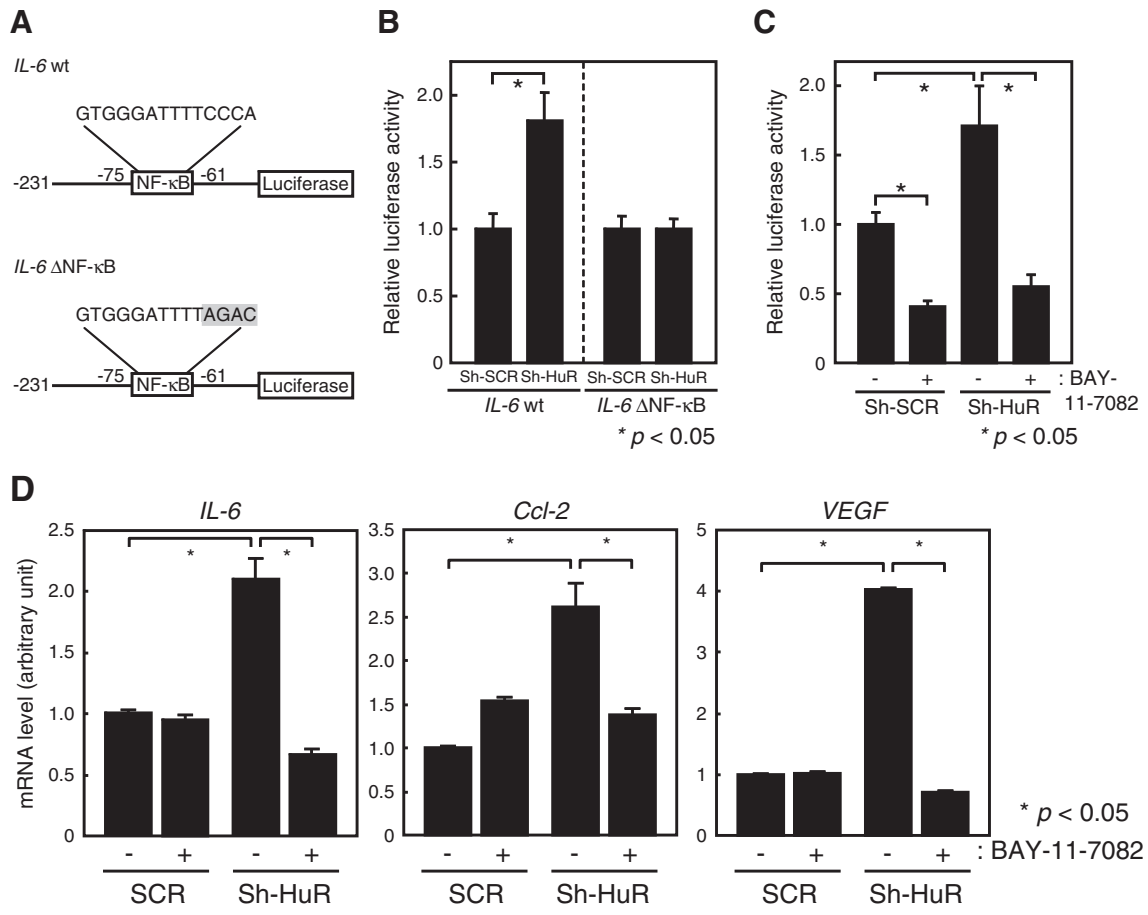

Fig. 4. NF-kB activity was required for *IL-6* induction in HuR-knockdown cells. (A) Schematic representation of the *IL-6* reporter (firefly luciferase) plasmids. (B) MEFs expressing sh-SCR or sh-HuR were transfected with the indicated *IL-6* reporter plasmids together with SV40-driven *Renilla* luciferase plasmids (pRL-SV). Lysates were prepared 2 days later and luciferase activity was measured. Values represent relative luciferase activity (firefly/*Renilla*). (C) MEFs expressing indicated sh-RNA were transfected with wild type *IL-6* reporter plasmids together with pRL-SV. 24 h later, cells were treated with NF-κB inhibitor BAY 11-7082 and cultured for 24 h, and luciferase activity was measured. (D) MEFs were treated with BAY 11-7082 for 24 h and *IL-6*, *Ccl-2* and *VEGF* levels were analyzed by realtime PCR. Values are means +/− SD. Results are representative of three independent experiments.

3.3. DNA damage accumulated in HuR-knockdown cells

The activity of NF-κB is regulated by several signaling pathways and the pathways that are particularly important for SASP induction include p38 and a persistent DDR [17]. We firstly compared the activity of p38 in sh-SCR and sh-HuR cells, but did not detect any increase in phospho-p38 in HuR-knockdown cells (Fig. 5A). Nonetheless, induction of *IL-6*, *Ccl-2* and *VEGF* was abrogated when p38 activity was inhibited by chemical inhibitor SB 203580 (Fig. 5B), suggesting that basal activity of p38 is required for cytokine induction in HuR-knockdown cells.

We then investigated whether the DNA damage signal was activated in HuR-knockdown cells. As shown in Fig. 5C, immunoblot analysis revealed that γH2AX, an indicator of DNA damage, was increased in HuR-knockdown MEFs. Consistent with these results, we were able to detect distinct γH2AX foci in these cells by an immunofluorescence experiment (Fig. 5D), which indicated that DNA strand breaks accumulate in the absence of the HuR protein. Immunoblot analysis using an antibody recognizing phospho-ATM/ATR substrates (SQ/TQ) revealed that DDR signaling pathway is indeed activated in HuR-knockdown cells (data not shown). These results suggest that DNA damage spontaneously accumulates in the absence of HuR and activates the DDR signaling pathway.

3.4. Single strand breaks accumulated in the absence of the HuR protein

To gain further insight into the DNA damage observed in HuR-knockdown cells, we characterized the DNA breaks in these cells. In the following experiments, we utilized NIH-3T3 cells since MEFs are very heterogeneous, and senesce or enter crisis before they form colonies in clonogenic survival assay (Fig. 7). NIH-3T3 cells are derived from mouse embryo tissue, and have lost the *ARF*/*Ink4a* genes during immortalization, yet retain the wild type *p53* allele, therefore are able to proliferate even when their HuR expression is inhibited [25]. We firstly established if cytokine expression is induced in NIH-3T3 cells by inhibiting HuR expression. Total RNA were extracted from control and HuR-knockdown NIH-3T3 cells, and the expression of *IL-6* mRNA was analyzed by realtime PCR. We detected an increase in *IL-6* mRNA in NIH-3T3 cells, although the magnitude was somewhat weaker than that observed in MEFs (Supplementary Fig. 1). As observed in MEFs (Figs. 4 and 5), the *IL-6* promoter was activated upon HuR depletion in NIH-3T3 cells, and NF-κB-binding was prerequisite for activation of the promoter. DNA damage (γH2AX) also accumulated in these cells when HuR was inhibited, which demonstrated that the loss of HuR induced SASP-related cytokines in NIH-3T3 cells through DDR and NF-κB pathways.

DNA damage is largely classified into two categories, namely, double-strand and single-strand breaks. These are thought to activate distinct and overlapping damage signal pathways [37]. To distinguish which type of DNA break accumulated in HuR-knockdown cells, we performed a single cell gel electrophoresis assay (comet assay) under neutral and alkaline conditions. Only double strand breaks are detected under neutral conditions because broken strands still anneal to the other unharmed strands; therefore, DNA remains virtually intact after

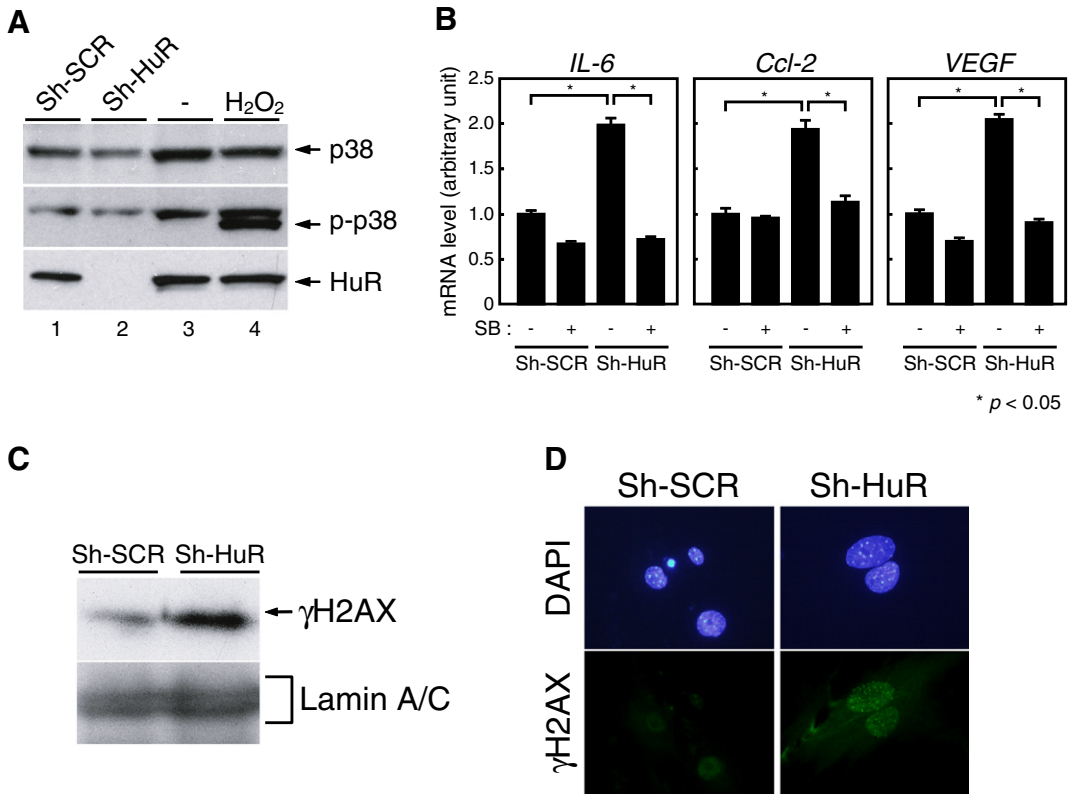

Fig. 5. DNA damage was accumulated in HuR-knockdown MEFs. (A) p38 phosphorylation was analyzed by immunoblotting in MEFs expressing sh-SCR and sh-HuR. p38 (total) was used as a loading control. For positive control, MEFs were treated with 0.1 mM H_2O_2 for 30 min (lane 4). (B) MEFs expressing indicated sh-RNA were treated or untreated with 5 μM SB203580 (SB) for 24 h. Total RNA was analyzed for the expression of indicated mRNA. (C) Lysates prepared from MEFs expressing sh-SCR and sh-HuR were analyzed for γH2AX levels by immunoblotting. Lamin A/C was used as a loading control. (D) Immunofluorescence analysis was performed using γH2AX antibodies. Cells were counterstained with DAPI.

electrophoresis. On the other hand, DNA is denatured under alkaline conditions, thereby enabling the detection of both single and double strand breaks. As shown in Fig. 6, no significant difference was detected in the comet tails of sh-SCR and sh-HuR cells under neutral conditions. However, significant increases in both comet tail intensity and the moment in sh-HuR cells were observed under alkaline conditions (the denatured condition). Pharmacological inhibition of NF-κB did not affect the DNA breaks observed in HuR-knockdown cells (Supplementary Fig. 2), which may be consistent with previous report that DDR acts as upstream of NF-κB pathway [38]. Taken together, these results strongly suggest that the loss of HuR results in the accumulation of single strand-specific DNA damage, which subsequently activates the DNA damage signal and NF-κB, leading to SASP expression.

3.5. HuR was required for the single strand DNA damage response

We next attempted to identify a possible cause for the accumulation of single strand DNA breaks in HuR-knockdown cells. One possible explanation for this phenomenon is that HuR is required for a certain type of DNA damage response; therefore, cells are not able to repair single strand breaks that spontaneously occur during DNA replication. To test this possibility, NIH-3T3 cells expressing sh-SCR or sh-HuR were exposed to ionizing radiation (IR) or ultraviolet C radiation (UV), and cell viability was determined by clonogenic survival. IR decreased cell survival in a dose-dependent manner, and no significant difference was observed in the rates of survival between sh-SCR and sh-HuR cells (Fig. 7, *top*). In contrast, fewer colonies were recovered from sh-HuR cells after UV irradiation (*bottom*). This effect was more evident in the presence of a higher dose of UV; the survival rate of HuR-knockdown cells was less than one-tenth that of control cells after 10 J/m^2 of UV irradiation. Thus, HuR is required for a proper DNA damage response, although its function appears to be limited among the DNA damage signaling pathways.

4. Discussion

In this study, we demonstrated the roles of HuR in cellular senescence. Although the magnitude of SASP induction is much less prominent in rodent cells compared to human cells as previously reported [39], down-regulation of HuR expression leads to increases in several SASP-related cytokines. Because HuR expression declines during cellular senescence [22,25], these phenomena can contribute to the establishment of senescent phenotypes in physiological settings. Although the mechanism to down-regulate the HuR level in senescent cells is still unknown, miRNA may be involved because miR-519, which targets *HuR* mRNA, is increased in senescent cells [40].

The p53 pathway is not absolutely required for SASP expression [7]. This is also the case for HuR-knockdown cells, as the induction of SASP-related factors has been observed in *ARF*/*p53*-double null MEFs and an immortalized cell line (NIH-3T3). Nevertheless, cytokine induction in HuR-knockdown cells may be different from naturally occurring SASP, as not all of the SASP components were induced in these cells. Therefore, other factor(s) that are activated or repressed during cellular senescence may be required for SASP induction in addition to HuR.

HuR has multiple functions in post-transcriptional gene regulation. In many cases, HuR stabilizes its bound mRNA. Numerous studies have demonstrated that HuR stabilizes several cytokine mRNAs [41]. Our above results indicate that in addition to such post-transcriptional control, HuR also contributes to the transcriptional regulation of cytokines through NF-κB. Indeed, our luciferase reporter analysis indicated that transcription of the *IL-6* gene was activated in the absence of HuR. The transcription factors NF-κB and C/EBPβ play pivotal roles in

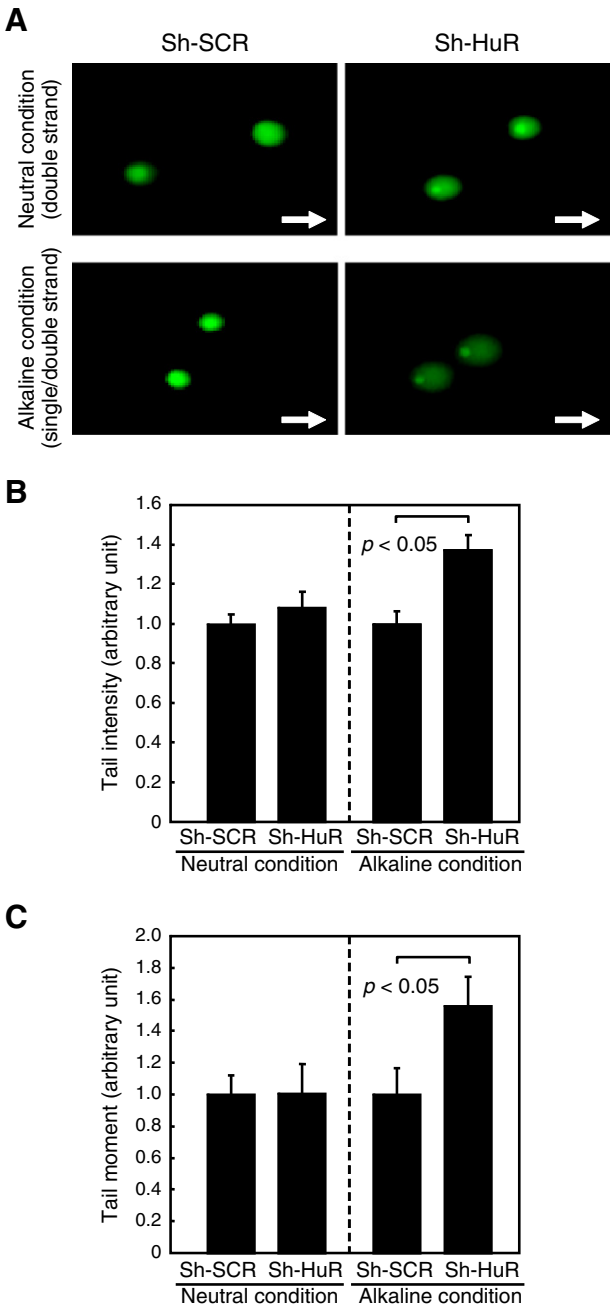

Fig. 6. Single-strand breaks accumulated in HuR-knockdown cells. (A) NIH-3T3 cells expressing sh-SCR and sh-HuR were subjected to the single cell gel electrophoresis assay under neutral or alkaline conditions. Representative images were shown. Arrows indicate the direction of electrophoresis. DNA was visualized by SYBR Green. (B and C) The comet tail intensities (B) and moment (C) were measured. At least 100 cells were counted in each sample.

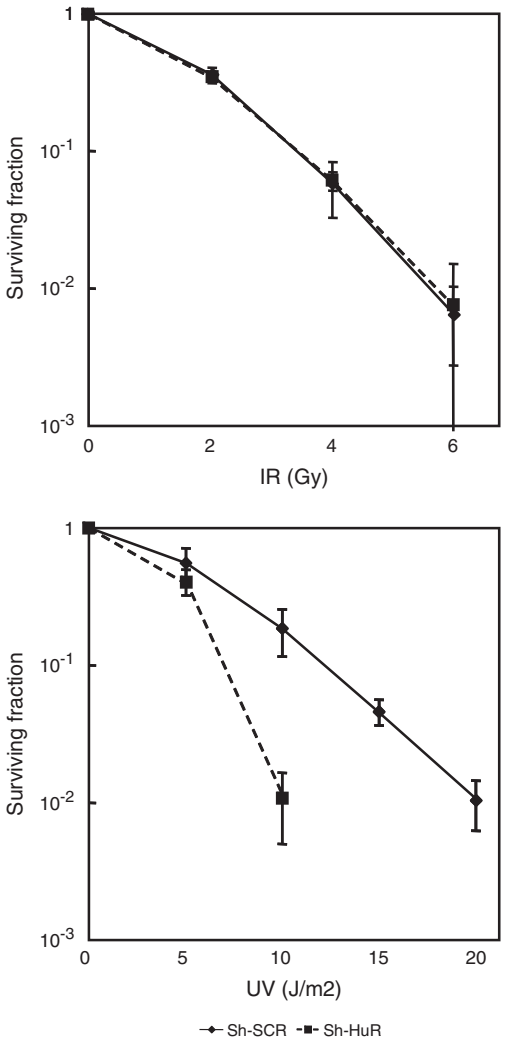

Fig. 7. Effects of HuR knockdown on sensitivity to genotoxic stress. NIH-3T3 cells expressing sh-SCR or sh-HuR were treated with the indicated doses of IR (*upper panel*) or UV (*lower panel*). After 12 days, the numbers of colonies in each plate were enumerated, and their survival was compared to nonirradiated control cultures. Values are means +/− SD, and results were representative of two independents.

SASP induction [17]. Our above results reveal that at least NF-κB is essential for *IL-6*, *Ccl2* and *VEGF* induction. We have not ruled out the possibility that C/EBPβ also contributes to its induction. Nevertheless, this is unlikely because our previous study revealed that C/EBPβ was down-regulated in the absence of HuR in MEFs [25].

NF-κB is activated by p38 and/or DDR signals to induce SASP expression during cellular senescence [17]. A recent report has suggested that the loss of HuR expression may activate p38 through Hsf1 and SIRT1 in human fibroblasts, thereby activating NF-κB and the subsequent expression of the SASP [42]. However, p38 phosphorylation was under detection limit in HuR-knockdown cells. Therefore it is possible that basal activity of p38 is required for activation of NF-κB. The DDR signaling pathway instead appears to be more important for cytokine induction in HuR-knockdown MEFs and NIH-3T3 cells. DNA damage spontaneously accumulated in the absence of the HuR protein, and the signaling pathway was activated in these cells. Single strand breaks rather than double strand breaks preferentially accumulate in the absence of HuR. It remains to be clarified what is causing the accumulation of single strand break. One possibility is that HuR is essential for the DNA damage signal pathway specifically required for the repair of single strand breaks, which can naturally occur during DNA replication. Our findings support this idea because the loss of HuR specifically sensitizes cells more to UV-induced DNA damage than to IR-induced damage. Additionally, HuR has been shown to regulate the expression of several genes that function in the DNA damage response [43].

The loss of HuR induces acute cell cycle arrest [23,25]. This has been attributed to changes in the post-transcriptional regulation of *INK4a* and *ARF* genes. HuR predominantly acts on *ARF* mRNA in mouse cells, while *INK4a* targets human cells. The loss of HuR accelerates the translation of *ARF* mRNA, thereby inducing ARF–p53 pathway-dependent cell cycle arrest. In the absence of HuR, *ARF* mRNA relocalizes to nucleoli, in which it associates with nucleolin. Therefore, certain mRNAs are localized to nucleoli following DNA damage, leading to an increase in its

translation [44]. Therefore, it is possible that DDR also contributes to the translation of *ARF* mRNA in HuR-knockdown cells.

Global changes in gene expression occur during cellular senescence, which is attributed to elevations or reductions in transcription, mRNA turnover, and translation. It has become more apparent that HuR and other RNA-binding proteins play crucial roles in the induction of cellular senescence by regulating several genes involved in cell cycle control. The present study has demonstrated that HuR regulates not only cell proliferation, but also other senescence-associated features such as the SASP. Cellular senescence acts as a potent tumor suppressive mechanism, and has also been suggested to associate with age-related disorders. HuR was previously shown to be deregulated in many types of human cancers that had bypassed senescence [45], and adipose-specific *HuR* knockout accelerated the aging phenotype by inducing cellular senescence [25]. Thus, the regulation of senescence-associated phenotypes by HuR may underlie human pathologies. In conclusion, our findings lend further support to the function of HuR in cellular senescence, and will extend understanding on the mechanisms regulating senescence-associated phenotypes.

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