Senescence-associated Long Non-coding RNA (SALNR) Delays Oncogene-induced Senescence through NF90 Regulation*§

Received for publication, May 5, 2015, and in revised form, October 12, 2015. Published, JBC Papers in Press, October 21, 2015, DOI 10.1074/jbc.M115.661785

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Background: The role of long non-coding RNAs (lncRNAs) in senescence is little known.

Results: Senescence-associated IncRNA (SALNR) expression is reduced during senescence. SALNR delays oncogene-induced senescence through suppressing NF90 nucleolus translocation.

Conclusion: IncRNA SALNR plays an important role in senescence.

Significance: We profiled lncRNAs in senescence and provided novel insights into the role of lncRNAs in senescence.

Long non-coding RNAs (lncRNAs) have recently emerged as key players in many physiologic and pathologic processes. Although many lncRNAs have been identified, few lncRNAs have been characterized functionally in aging. In this study, we used human fibroblast cells to investigate genome-wide lncRNA expression during cellular senescence. We identified 968 downregulated lncRNAs and 899 up-regulated lncRNAs in senescent cells compared with young cells. Among these lncRNAs, we characterized a senescence-associated IncRNA (SALNR), whose expression was reduced during cellular senescence and in premalignant colon adenomas. Overexpression of SALNR delayed cellular senescence in fibroblast cells. Furthermore, we found that SALNR interacts with NF90 (nuclear factor of activated T-cells, 90 kDa), an RNA-binding protein suppressing miRNA biogenesis. We demonstrated that NF90 is a SALNR downstream target, whose inhibition led to premature senescence and enhanced expressions of senescence-associated miRNAs. Moreover, our data showed that Ras-induced stress promotes NF90 nucleolus translocation and suppresses its ability to suppress senescence-associated miRNA biogenesis, which could be rescued by SALNR overexpression. These data suggest that lncRNA SALNR modulates cellular senescence at least partly through changing NF90 activity.

Human fibroblasts have a limited ability to divide in culture, after which they remain viable for many weeks but fail to proliferate despite being given space, nutrients, or growth factors, which is known as senescence (1). Cellular senescence could be triggered through two closely related processes: telomere erosion (replicative senescence) and exposure to damaging conditions (premature senescence) (2). Senescent cells often have an enlarged morphology, elevated activity of lysosomal β-galactosidase, enhanced autophagy, and senescence-associated heterochromatin foci (SAHF). Senescent cells also display the characteristic senescence-associated secretory phenotype (2). Evidence has shown that cellular senescence not only acts as an important anti-cancer mechanism but also influences other disease, such as neurodegeneration, cardiovascular disease, and declining immune function (3).

A number of factors have been implicated in regulating senescence, including transcription factors of the p53 and Ets families (4), the post-transcriptional regulators AUf1 (AU-binding factor 1) (5) and HuR (human antigen R) (6), and small non-coding RNAs (7). A recent report (8) showed that long non-coding RNAs (lncRNAs) exhibit a special expression pattern during senescence and probably act as an important regulator of cellular senescence.

lncRNAs comprise a highly heterogeneous group of transcripts with a wide range of sizes, structures, and subcellular locations. lncRNAs can regulate gene expression by modulating the structure of chromatin remodeling factors and transcription factors and by forming DNA-RNA triple helices (9). lncRNAs also have scaffolding or decoy function affecting gene transcription (9). In addition, lncRNAs post-transcriptionally regulate gene expression via modulating precursor mRNA splicing, mRNA stability, or translation (10–12). The lncRNA-mediated gene expression is involved in cell proliferation, differentiation, chromosomal imprinting, and embryogenesis, whereas lncRNA function is associated with cancer, neurodegeneration, cardiovascular disease, and metabolic disorder (13–15).

Although a growing number of lncRNAs have been characterized in many physiologic and pathologic processes, the abbreviations used are: SAHF, senescence-associated heterochromatic foci; IncRNA, long non-coding RNA; SALNR, senescence-associated IncRNA; NF90, nuclear factor of activated T-cells, 90 kDa; qRT-PCR, quantitative reverse transcriptase PCR; SA-β-gal, senescence-associated β-galactosidase; RIP, RNA immunoprecipitation; RACE, rapid amplification of cDNA ends; miRNA, microRNA; pri-miRNA, microRNA primary transcript; IF, immunofluorescence.
their implications in senescence are largely unknown. In this study, we aimed to identify senescence-associated IncRNAs and intended to characterize their functions in premature senescence.

**Experimental Procedures**

**Cell Cultures and Human Samples**—Human embryonic lung diploid fibroblast 2BS cells (National Institute of Biological Products, Beijing, China) were isolated from female fetal lung fibroblast tissue, and they have been fully characterized previously (16). The cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). WI-38 normal human embryo lung fibroblasts (American Type Culture Collection, Manassas, VA) were cultured in the same manner as described for 2BS cells. Colon adenoma (n = 15) and adjacent normal tissue specimens (n = 15) were collected at Peking University First Hospital.

**IncRNA and miRNA Expression Profiling**—The Human Long Non-coding RNA Array and microarray chip have been described previously (17). The microarray analysis was performed by KangChen Bio-tech (Shanghai, China). We selected candidates with a minimum of 2-fold changes and p < 0.05. Differentially expressed IncRNAs and their chromosomal locations were mapped by Prism version 6.0 (GraphPad Software, La Jolla, CA). The expression profile of miRNAs was determined using the stem-loop RT-PCR-based TaqMan human microRNA array (Applied Biosystems, Foster City, CA) as described previously (18). Relative miRNA levels were determined by ΔΔCt using endogenous controls. The scatter plot is a visualization method used for assessing expression variations between pairs. The values of the x and y axes in the scatter plot are the averaged normalized signal values of the group (log2-scaled). The green lines are -fold change (the default -fold change given is 2.0). The IncRNAs or miRNAs above the top green line and below the bottom green line indicate >2-fold change between pairs.

**Rapid Amplification of cDNA Ends (RACE) Mapping of SALNR**—RACE was performed using a SMARTer™ RACE cDNA amplification kit (Clontech, Palo Alto, CA). The primers (5’Race_GSP1, 5’Race_GSP-1N, 3’Race_GSP1, and 3’Race_GSP-1N) used are shown in Table 1.

**TestCode**—TestCode was developed to identify potential protein-coding regions in nucleic acid sequences by plotting a visualization method used for assessing expression variations between pairs. The values of the x and y axes in the scatter plot are the averaged normalized signal values of the group (log2-scaled). The green lines are -fold change (the default -fold change given is 2.0). The IncRNAs or miRNAs above the top green line and below the bottom green line indicate >2-fold change between pairs.

**Vector Constructs**—The following viral vectors were used in this study: pWZL-Hygro-Ras (H-RasV12) and pZsG-Puro were generously provided by Drs. Liangx Xue and Xiaowei Zhang (Peking University Health Science Center), respectively. SALNR-pZsG was prepared as follows. The full-length SALNR sequence was amplified by PCR on the cDNA of 2BS cells with primers SALNR_F and SALNR_R (Table 1). The PCR fragment was cloned into the NotI/BamHI sites of pZsG-Puro vector. The full-length SALNR was also cloned into the NotI/BamHI sites of the mammalian expression vector pcDNA3.1(+) (Invitrogen) to generate sense SALNR-pcDNA3.1 vector. Antisense SALNR-pcDNA3.1 was constructed by subcloning the antisense of SALNR into BamHI/NotI sites of the pcDNA3.1(−) vector (Invitrogen).

**RNA Interference**—RNA interference was used to knock down SALNR and NF90 in young 2BS cells. The vector used for generating lentivirus was remolded pLL3.7, whose sequence expressing GFP was replaced by sequence expressing G418. The sense strands of shRNAs were as follows: shSALNR, 5’-GGCTTGAGTCTTATGAGTGC-3’; shControl, 5’-TTCTCCGGAACGTCG-3’; shNF90#2, 5’-CAGACTGCTAGCAGCTATCA-3’; shControl, 5’-TTCTCCGGAACGTCG-3’.

**Transfection and Virus Packaging**—All transfections were carried out with Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. To produce infectious viruses, the 293T packaging cell line was co-transfected with the 2BS cells. Colon adenoma (n = 15) were collected at Peking University First Hospital. Colon adenoma (n = 15) and adjacent normal tissue specimens (n = 15) were collected at Peking University First Hospital.

**Table 1**

| Oligonucleotides | Sequences |
|------------------|-----------|
| 5’Race_GSP1      | 5’-GGCTTGAGTCTTATGAGTGC-3’ |
| 5’Race_GSP-1N    | 5’-TTCTCCGGAACGTCG-3’    |
| 3’Race_GSP1      | 5’-CAGACTGCTAGCAGCTATCA-3’ |
| 3’Race_GSP-1N    | 5’-TTCTCCGGAACGTCG-3’    |
| SALNR_F          | 5’-GGCTTGAGTCTTATGAGTGC-3’ |
| SALNR_R          | 5’-TTCTCCGGAACGTCG-3’    |
| IncRNAU1_F       | 5’-GGCTTGAGTCTTATGAGTGC-3’ |
| IncRNAU1_R       | 5’-TTCTCCGGAACGTCG-3’    |
| IncRNAU2_F       | 5’-GGCTTGAGTCTTATGAGTGC-3’ |
| IncRNAU2_R       | 5’-TTCTCCGGAACGTCG-3’    |
| IncRNAU3_F       | 5’-GGCTTGAGTCTTATGAGTGC-3’ |
| IncRNAU3_R       | 5’-TTCTCCGGAACGTCG-3’    |
| IncRNAU4_F       | 5’-GGCTTGAGTCTTATGAGTGC-3’ |
| IncRNAU4_R       | 5’-TTCTCCGGAACGTCG-3’    |
| IncRNAU5_F       | 5’-GGCTTGAGTCTTATGAGTGC-3’ |
| IncRNAU5_R       | 5’-TTCTCCGGAACGTCG-3’    |
| lncRNAD1_F       | 5’-GGCTTGAGTCTTATGAGTGC-3’ |
| lncRNAD1_R       | 5’-TTCTCCGGAACGTCG-3’    |
| lncRNAD2_F       | 5’-GGCTTGAGTCTTATGAGTGC-3’ |
| lncRNAD2_R       | 5’-TTCTCCGGAACGTCG-3’    |
| lncRNAD3_F       | 5’-GGCTTGAGTCTTATGAGTGC-3’ |
| lncRNAD3_R       | 5’-TTCTCCGGAACGTCG-3’    |
| lncRNAD4_F       | 5’-GGCTTGAGTCTTATGAGTGC-3’ |
| lncRNAD4_R       | 5’-TTCTCCGGAACGTCG-3’    |
| lncRNAD5_F       | 5’-GGCTTGAGTCTTATGAGTGC-3’ |
| lncRNAD5_R       | 5’-TTCTCCGGAACGTCG-3’    |
| GAPDH_F          | 5’-GGCTTGAGTCTTATGAGTGC-3’ |
| GAPDH_R          | 5’-TTCTCCGGAACGTCG-3’    |
| SALNRFISH_F       | 5’-GGCTTGAGTCTTATGAGTGC-3’ |
| SALNRFISH_R       | 5’-TTCTCCGGAACGTCG-3’    |
| NF90F_F          | 5’-GGCTTGAGTCTTATGAGTGC-3’ |
| NF90R_F          | 5’-TTCTCCGGAACGTCG-3’    |
| Kras_F           | 5’-GGCTTGAGTCTTATGAGTGC-3’ |
| Kras_R           | 5’-TTCTCCGGAACGTCG-3’    |
| Braf_F           | 5’-GGCTTGAGTCTTATGAGTGC-3’ |
| Braf_R           | 5’-TTCTCCGGAACGTCG-3’    |
| pri-miR-181a_F   | 5’-GGCTTGAGTCTTATGAGTGC-3’ |
| pri-miR-181a_R   | 5’-TTCTCCGGAACGTCG-3’    |
| pri-miR-22_F     | 5’-GGCTTGAGTCTTATGAGTGC-3’ |
| pri-miR-22_R     | 5’-TTCTCCGGAACGTCG-3’    |
| pri-miR-1233_F   | 5’-GGCTTGAGTCTTATGAGTGC-3’ |
| pri-miR-1233_R   | 5’-TTCTCCGGAACGTCG-3’    |

**La Jolla, CA). The expression profile of miRNAs was determined using the stem-loop RT-PCR-based TaqMan human microRNA array (Applied Biosystems, Foster City, CA) as described previously (18). Relative miRNA levels were determined by ΔΔCt using endogenous controls. The scatter plot is a visualization method used for assessing expression variations between pairs. The values of the x and y axes in the scatter plot are the averaged normalized signal values of the group (log2-scaled). The green lines are -fold change (the default -fold change given is 2.0). The IncRNAs or miRNAs above the top green line and below the bottom green line indicate >2-fold change between pairs.**
pZsG-Puro) or 500 μg/ml G418 (for pLL3.7-G418) was added to select stably infected cell populations. After 5 days of selection, stable cell pools were established, and expression was verified by quantitative RT-PCR (qRT-PCR) or Western blot analysis.

RNA Extract and qRT-PCR—Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. qRT-PCR was performed using Superscript™ III transcriptase (Invitrogen) and THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. The expression of lncRNAU1 to -U5, lncRNAD1 to -D5, and microRNA primary transcript (pri-miRNA) was analyzed by qRT-PCR with the use of the primers shown in Table 1. GAPDH was used as an internal control. The mirVana™ qRT-PCR miRNA detection kit and primer sets were purchased from Ambion (Foster City, CA). The cDNA was synthesized from total RNA using gene-specific primers according to the TaqMan microRNA assay protocol (Ambion). qRT-PCR was performed on cDNA generated from total miRNA using the mirVana qRT-PCR miRNA detection kit protocol (Ambion). Amplification and detection of specific products were performed using the ABI Prism 7300 sequence detection system (Applied Biosystems) with a cycle profile according to the instructions of the mirVana qRT-PCR miRNA detection kit. As an internal control, U6 snRNA was used for RNA template normalization.

Cell Extracts and Western Blotting—The isolation of nucleoli fractionation was described previously (20). The primary anti-β-actin, anti-lamin B, and anti-fibrillarin antibodies were from

| UP-REGULATED | DOWN-REGULATED |
|---------------|----------------|
| SEQNAME       | DESIGNED NAME  | CHANGE FOLD | SEQNAME       | DESIGNED NAME  | CHANGE FOLD |
| uc010bkn      | lncRNAU1       | 287.36      | AF085871      | lncRNAD1       | 36.37       |
| AF176921      | lncRNAU2       | 33.69       | AF339813      | lncRNAD2       | 17.93       |
| uc003hhl      | lncRNAU3       | 21.03       | AK091544      | lncRNAD3       | 11.37       |
| uc001hbp      | lncRNAU4       | 18.03       | NR_027180     | lncRNAD4       | 10.81       |
| DB091758      | lncRNAU5       | 12.80       | Z34283        | lncRNAD5       | 10.71       |
Cell Signaling Technology (Beverly, MA). Anti-NF90 antibody was from BD Biosciences. Anti-NF45 antibody was from Abcam (Cambridge, UK). Anti-p16 and anti-p53 antibodies were from Santa Cruz Biotechnology, Inc. Anti-FLAG antibody was from Sigma.

**Senescent Assay and Cell Proliferation Assay**—Detection of senescence-associated β-galactosidase (SA-β-gal) activity and SAHF staining were performed as described previously (21). BrdU was detected using a BrdU incorporation assay kit according to the manufacturer’s instructions (Roche Applied Science). For the cell proliferation assay, 4 × 10^5 cells were plated in 35-mm tissue dishes. Cells were cultured for up to the indicated days and then fixed and stained with crystal violet. Stain was extracted by treatment with 95% ethanol. Subsequently, A_{570} values were measured.

**Cell Cycle Analysis**—Cells were washed with PBS, detached with 0.25% trypsin, and fixed with 75% ethanol overnight. After treatment with 1 mg/ml RNase A (Sigma), cells were resuspended in PBS and stained with propidium iodide in the dark. Fluorescence was measured with a FACScan flow cytometry system (BD Biosciences).

**Immunofluorescence (IF)**—Cells were fixed with 4% formaldehyde for 15 min and then incubated with PBS containing 0.3% Triton X-100 and 5% normal goat serum for 1 h at room temperature, followed by incubation with antibodies specific for NF90 (BD Biosciences), fibrillarin (Cell Signaling Technology), or NF45 (Abcam) overnight at 4 °C. Cells were then washed with PBS plus 0.3% Triton X-100 and incubated with a fluorochrome-conjugated secondary antibody. Nuclei were stained with DAPI. Cells were imaged on a Zeiss LSM 410 confocal laser-scanning microscope.

The human colon adenoma and control non-adenoma samples were fixed in 10% formalin, routinely embedded in paraffin, and sectioned at 5 μm. Serial sections were deparaffinized in xylene and hydrated through graded concentrations of ethanol. Epitopes were retrieved using microwave in boiling Tris-EDTA buffer, pH 9.0, for 20 min. Blocking, incubations with primary antibodies, and incubations with secondary antibodies were carried out in the same manner as that of culture cells.

**RNA-FISH/IF**—To label the RNA probes, partial cDNA fragments of SALNR were amplified by PCR using the sequence-

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**FIGURE 2. Expression of lncRNAs in senescent fibroblasts.** The activity of SA-β-gal (A), SAHF formation (B), percentage of cells incorporating BrdU (C), and expression of p16 and p53 protein (D) were detected in young, quiescent, replicative senescent, H₂O₂-induced senescent, and Ras-induced senescent 2BS cells. Quiescent cells were incubated in medium containing 0.2% serum for 48 h. H₂O₂-induced senescent cells were treated with a sublethal dose (200 μM H₂O₂) for 2 h. E, qRT-PCR analysis of IncRNA U2, U3, and U5 expression in young (Y), quiescent (Q), replicative senescent (S), H₂O₂-induced senescent, and Ras-induced senescent 2BS cells. F, qRT-PCR analysis of IncRNA A1, D3, and D5 expression in young (Y), quiescent (Q), replicative senescent (S), H₂O₂-induced senescent, and Ras-induced senescent 2BS cells. G, qRT-PCR analysis of SALNR expression in young (Y), quiescent (Q), replicative senescent (S), H₂O₂-induced senescent, and Ras-induced senescent WI-38 cells. Levels are normalized to GAPDH expression and represented as means ± S.D. (error bars) unless otherwise indicated.
specific primers SALNR FISH_F and SALNR FISH_R (Table 1). The PCR product was inserted into pcDNA3.1(+) or pcDNA3.1(−) vector. The plasmid DNA was then linearized and used for transcription with T7 RNA polymerases to generate sense and antisense probes with the FISH tag RNA multicolor kit (Invitrogen) in accordance with the manufa-

FIGURE 3. Molecular cloning of SALNR and analysis of the ORF and phylogenetic conservation of SALNR. **A**, molecular cloning of SALNR. 5’- and 3’-ends of SALNR were extended from 2BS cells using the RACE technique (bottom). A schematic diagram of the full-length SALNR genomic structure is shown at the top. **B**, the ORF of SALNR was analyzed with the NCBI ORF Finder program. Blue boxes, predicted ORFs. **C**, phylogenetic conservation of the SALNR transcript was analyzed using VISTA. The graph is a plot of nucleotide identity for a 100-bp sliding window centered at a given position.
Detection of RNA-FISH/IF was performed as described previously (22).

RNA Pull-down Assay—Sense SALNR-pcDNA3.1(+) and antisense SALNR-pcDNA3.1(−) were linearized with the corresponding restriction enzymes used to clone the vector at the 3′-end to obtain the template DNAs for in vitro transcription. An RNA pull-down assay was performed as described previously (23). The retrieved protein was detected by silver staining or Western blotting. Protein bands differently visualized in sense SALNR compared with antisense were excised and analyzed by mass spectrometry.

RNA Immunoprecipitation (RIP) Assay—An RIP assay was carried out using the Magna RIP™ RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA). The antibody used for RIP was anti-NF90 (BD Biosciences). The co-precipitated RNAs were purified with phenol/chloroform/isoamyl alcohol and detected by qRT-PCR.

Detection of RNA Half-life—Cells were treated with 5 μg/ml actinomycin D. After the indicated incubation period, total RNA was isolated for qRT-PCR as described before. The ratio of the RNA level for SALNR relative to that for GAPDH before actinomycin D treatment was defined as 100%, and the half-life
of IncRNA SALNR was calculated from the time period required for the SALNR RNA to undergo a reduction to one-half of its initial abundance.

**NF90-FLAG Vector Construct**—FLAG-NF90 was prepared as follows. The full-length NF90 sequence was amplified by PCR on the cDNA of 2BS cells with primers NF90F_F and NF90F_R (Table 1). The PCR fragment was then cloned into the BamHI/EcoRI sites of pCMV-Tag2B vector.

**Mutational Analysis**—Genomic DNA was extracted from colon adenoma and non-adenoma samples using the QIAamp DNA microkit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. **KRAS** mutation analysis at codons 12 and 13 was performed using direct sequencing of a fragment containing codons 12 and 13 of the **KRAS** gene using primers KRAS_F and KRAS_R (Table 1). These cover the length of the **KRAS** gene and derive a DNA fragment product of 116 base pairs. **BRAF** mutation analysis at codon 600 was performed using direct sequencing of a fragment containing codon 600 of the **BRAF** gene using primers BRAF_F and BRAF_R (Table 1). These cover the length of **BRAF** gene and derive a DNA fragment product of 251 base pairs.

**Statistical Analysis**—Results are expressed as means ± S.D., and the error bars represent S.D. values from three independent experiments unless otherwise indicated. The statistical significance of differences in the experimental data were analyzed using the two-sample Student’s t test (p < 0.05 (*) was regarded as significant).

**Results**

**Genome-wide Screen for Senescence-associated IncRNAs in Human Fibroblasts**—To search for IncRNAs that are associated with senescence, we used the IncRNA microarray to profile IncRNA expression in replicative senescent (divided more than 60 times) and young human 2BS fibroblast. We identified 1,867 IncRNAs (with a ≥2.0-fold change and p < 0.05) in replicative senescent cells compared with young cells, among which 899 were up-regulated and 968 were down-regulated in senescent cells (Fig. 1, A and B). We then picked the five most up-regu-
lated (U1–U5) or down-regulated (D1–D5) lncRNAs for validation (Fig. 1C). As shown in Fig. 1, D and E, the results from real-time PCR were consistent with microarray data. Because of their low abundance, lncRNAU1, lncRNAU4, lncRNAD2, and lncRNAD4 were not studied further. To ensure that the expression change of lncRNAs was senescence-specific, we analyzed the expression of lncRNAs in quiescent and premature senescent cells. The senescence in quiescent and premature senescent cells was quantified and validated by measurement of SA-/H9252-gal activity, SAHF formation, cells incorporating BrdU, and the expression of p16 and p53 (Fig. 2, A–D). For the six selected lncRNAs, similar expression changes were observed in Ras- or H2O2-induced premature senescent cells (Fig. 2, E and F). Interestingly, serum-deprived quiescent cells exhibited a similar change of lncRNAU2, -U3, -D1, and -D5 expression, suggesting that expression change in these four lncRNAs is not senescence-specific (Fig. 2, E and F). Both lncRNAU5 and -D3 expressions did not change in serum-deprived quiescent cells compared with young cells, suggesting that these two lncRNA expression changes were associated with senescence rather than growth arrest (Fig. 2, E and F). In this study, we focused on lncRNAD3 and designated it as SALNR (senescence-associated long non-coding RNA). Decreased expression of SALNR also was observed in senescent WI-38 cells (Fig. 2G), suggesting that SALNR down-regulation is not unique to 2BS cells. To obtain the complete structure of the SALNR gene, we performed 5’-RACE and 3’-RACE using gene-specific primers (Fig. 3A). The 3,788-bp SALNR cDNA contains a putative TATA box (TCTTTTAAT) at –29 bp upstream of the putative transcription start site, without any introns. The full-length cDNA is mapped to 10q23.33 positive strand (NCBI36/hg18). By using the NCBI ORF finder program, we predicted multiple stop codons and several short open reading frames (ORFs) (<201 nucleotides) in SALNR full-length transcript (Fig. 3B). The TestCode value of SALNR is 0.386, which suggests an ncRNA. Furthermore, phylogenetic conservation analysis showed that SALNR was not conservative among humans, dogs, horses, mice, rats, and chickens (Fig. 3C), which appears to be a common feature for most lncRNAs.

SALNR Overexpression Delays Cellular Senescence—To test the significance of SALNR alterations in cellular senescence, we overexpressed SALNR in young cells, followed by RasV12 infection to induce senescence (Fig. 4A). SALNR overexpression resulted in a decrease of SAHF formation (Fig. 4B), a decreased activity of SA-/H9252-gal (Fig. 4C), decreased expression of p16 and p53 protein (Fig. 4D), and an increase in the percentage of cells incorporating BrdU (Fig. 4E). The cell proliferation assay showed that SALNR overexpression prevented cells from entering Ras-induced senescence, and cells maintained proliferation (Fig. 4F). In addition, SALNR overexpression resulted in an increase of S compartment and a decrease in G0/G1 compartment compared with control (Fig. 4G). Furthermore, we found that SALNR overexpression extended the life span of replicative senescent cells (Fig. 5A), accompanied by decreased SAHF formation (Fig. 5B), decreased SA-/H9252-gal activity (Fig. 5C), decreased expression of p16 and p53 protein (Fig. 5D), and an

FIGURE 6. SALNR interacts with NF90. A, subcellular location of SALNR. The antisense of SALNR was used for a control. Fibrillarin is the nucleolar marker, and DAPI staining was used to indicate the cell nucleus. B, identification of SALNR-associated target proteins in young 2BS cells by RNA pull-down assay. The bands specific to sense SALNR were detected with mass spectrometry. C, Western blot analysis of the interaction of SALNR with NF90. Proteins pulled down were detected by NF90 antibody. D, RIP analysis of NF90 binding to SALNR in vivo. RNA was immunoprecipitated using anti-NF90 antibody in young 2BS cells, with IgG as a negative control, and analyzed by qRT-PCR. Error bars, S.D.
increase in the percentage of cells incorporating BrdU (Fig. 5E). These findings suggest that SALNR plays an important role in cellular senescence.

**SALNR Interacts with NF90 and Regulates NF90 Nuclear Localization**—To explore the molecular mechanism underlying SALNR regulation in cellular senescence, we sought to identify SALNR-associated target proteins in young cells by an RNA pull-down assay. First, we found that the normal subcellular localization of SLANR is in the nucleus and excluded from nucleoli (Fig. 6A). Then an RNA pull-down experiment revealed a direct binding of SALNR and a 90-kDa nuclear protein, which was identified as NF90 (nuclear factor of activated T-cells, 90 kDa) by mass spectrometry (Fig. 6B). The interaction between SALNR and NF90 was further confirmed by Western blotting (Fig. 6C). Afterward, we performed RIP to determine whether SALNR binds NF90 in vivo. As expected, NF90 antibody could effectively enrich SALNR RNA compared with nonspecific IgG (Fig. 6D).

NF90 is a double-stranded RNA binding protein and plays an important role in RNA metabolism, including degradation (24, 25). Thus, we proposed two possibilities for the significance of SALNR-NF90 interaction: 1) NF90 regulates SALNR turnover, which probably leads to decreased expression of SALNR during senescence, or 2) SALNR regulates senescence by modulating NF90 function. On one hand, NF90 knockdown in young cells did not affect the half-life of SALNR (Fig. 7A), suggesting that NF90 does not affect SALNR turnover. On the other hand, SALNR did not seem to affect NF90 expression because the
NF90 level was unchanged during replicative or Ras-induced senescence despite the decrease of SALNR expression (Fig. 7B). In addition, SALNR overexpression or knockdown in young cells did not affect NF90 protein level (Fig. 7C). This led us to investigate whether SALNR affects NF90 subcellular distribution. In young 2BS cells, NF90 diffusely distributed throughout the nucleus (Fig. 7D, first row). NF90 was enriched in the nucleolus in Ras-induced senescent (Fig. 7D, second row) but not replicative senescent cells (Fig. 7E, first and second rows), suggesting that NF90 cellular distribution changes upon Ras stress. Overexpression or knockdown of SALNR alone in young cells did not affect NF90 location (Fig. 7E, third to sixth rows), suggesting that NF90 cellular distribution changes upon Ras stress.

NF90 Functions as a Repressor of Senescence—NF90 has been shown to coordinately repress the senescence-associated secretory phenotype (26). Infection of NF90 shRNA in young cells decreased NF90 levels and resulted in an increase of p16 and p53 expression levels (Fig. 8A), SAHF formation (Fig. 8B), and activity of SA-β-gal (Fig. 8C) and a decrease in the percentage of cells incorporating BrdU (Fig. 8D). Similar results were achieved with two different shRNAs, suggesting that NF90 might contribute to repress or delay cellular senescence, consistent with another report with WI-38 and IDH4 fibroblasts (26). However, NF90 overexpression had little, if any, inhibitory effect on Ras-induced senescence (data not shown). This phenomenon may be due to the fact that NF90 is extinguished in nucleoli upon Ras-induced stress (Fig. 8E).

In the replicative senescence model, NF90 may repress the translation of the senescence-associated secretory phenotype factors via binding to their mRNA 3'-UTR; thus, it is presumed to suppress cellular senescence (26). However, this does not seem to be the case in the Ras-induced senescent cells. A recent report has shown that NF90 acts as a negative regulator of miRNA biogenesis, binding pri-miRNA and blocking miRNA processing (25). We surmised that NF90 might regulate cellular senescence through miRNA pathways. We first used TaqMan human microRNA arrays to screen NF90-regulated miRNAs in 2BS fibroblasts. Silencing NF90 by shRNAs resulted in a change (≥2-fold) in the expression of 149 miRNAs (Fig. 9A and supplemental Table S1). A large number of these miRNAs (133 of 149) displayed elevated expression,
consistent with the inhibitory role of NF90 in miRNA biogenesis. These up-regulated miRNAs include several previously characterized miRNAs, such as miR-181a (27) and miR-22 (28), which play a positive role in senescence induction. Using qRT-PCR, we validated our findings of up-regulated miR-181a and miR-22 upon NF90 silencing (Fig. 9B). Interestingly, despite the up-regulation of miRNA levels, their corresponding pri-miRNAs showed a decreased expression in 2BS cells with NF90 knockdown (Fig. 9C), suggesting that these miRNAs are post-transcriptionally inhibited by NF90. To further confirm whether these pri-miRNAs were directly regulated by NF90, we performed RIP analysis using an anti-NF90 antibody. As shown in Fig. 9D, these pri-miRNAs were effectively enriched in NF90 IP, suggesting that NF90 directly regulates the expression of these miRNAs. NF90 did not bind pri-miRNA of control miR-1233 (Fig. 9D), whose expression is unchanged upon NF90 knockdown (Fig. 9B and C), suggesting that NF90 specifically binds to pri-miR-181a and pri-miR-22. Finally, we found that miR-181a and miR-22 promote senescence in young 2BS cells (Fig. 10, A–D), and suppression of miR-181a or miR-22 attenuated shNF90-induced senescence (Fig. 10, E–H), which suggests that shNF90-induced senescence is dependent on miR-181a and miR-22. Taken together, these data indicate that NF90 delays senescence through inhibiting miRNA expression.

NF90 Is Required for SALNR to Delay Cellular Senescence—NF90 nucleoli translocation in Ras-induced senescent cells prompts us to investigate whether its localization altered its activity. Here, we chose NF90 inhibition of miRNA expression as an indicator of NF90 activity. An RIP assay showed that NF90 was not able to bind these pri-miRNAs in senescent cells compared with young cells (Fig. 11A). Consistently, NF90-targeting miRNAs displayed increased expression in Ras-induced senescent cells (Fig. 11B), suggesting loss of the inhibitory effect of NF90 on pri-miRNA processing in senescent cells. Because NF90 binds pri-miRNAs in the form of NF90-NF45 complex, in addition to NF90 itself, NF45 also affects NF90 binding of pri-miRNAs (25). To rule out the possibility that the inability of NF90 to bind pri-miRNAs results from NF45 changes, we analyzed NF45 protein expression level and cellular distribution. Senescent cells displayed protein levels and cellular distribution of NF45 similar to that in young cells (Fig. 11, C and D), suggesting that translocation of NF90 nucleoli is associated with its activity in Ras-induced senescent cells. Indeed, SALNR overexpression blocked NF90 nucleoli translocation and promoted the interaction between NF90 and its targeting pri-miRNAs, leading to decreased expression of targeting miRNAs in senescent cells (Fig. 11, E and F). The inhibitory effect of SALNR on expression of senescence-associated miRNAs is dependent on NF90 because NF90 shRNA antagonized not only NF90 interaction with these pri-miRNAs but also the suppression of two target miRNAs in senescent cells (Fig. 11, E and F). Overexpressing SALNR did not change the NF90 level, which rules out the possibility that SALNR suppression of miRNAs is due to increased expression of NF90 (Fig. 7C, left). Our findings indicate that translocation of NF90 into nucleoli is associated with NF90 activities, exemplified by loss of its suppressive effect on senescence-associated miRNAs, thus weakening its inhibitory role in senescence.

Then we investigated whether NF90 is required for SALNR to delay cellular senescence. 2BS cells with SALNR overexpression...
were infected with NF90 shRNA or control lentivirus, followed by senescence induction with activated RasV12. NF90 knockdown attenuated SALNR-induced decrease of p16 and p53 expression (Fig. 12A), SAHF formation (Fig. 12B), and activity of SA-β-gal (Fig. 12C) and increase of cells incorporating BrdU (Fig. 12D) and cellular proliferation (Fig. 12E). This suggests that NF90 contributes to SALNR delay of cellular senescence.

Finally, we tested whether miR-181a and miR-22 are involved in SALNR delaying oncogene-induced senescence. Young 2BS cells with SALNR overexpression were transfected...
with a control scrambled oligonucleotide or synthetic miR-181a or miR-22, followed by infection with RasV12 to induce senescence. Overexpression of miR-181a or miR-22 attenuated SALNR-induced decrease of p16 and p53 expression (Fig. 13A), SAHF formation (Fig. 13B), and SA/H252-gal activity (Fig. 13C) and increase of cells incorporating BrdU (Fig. 13D).

The Expression of SALNR Decreased in Preneoplastic Lesions—SALNR down-regulation during Ras-induced senescence prompted us to ask whether it might also be decreased in preneoplastic lesions associated with senescent cells. Colon adenomas are preneoplastic lesions associated with telomere shortening-induced replicative senescence (29) and oncogene-induced premature senescence (30). The expression of p16INK4A, an in vivo senescent marker (31), was much more abundant in colon adenomas than in normal colon tissues (Fig. 14A and Table 2), which was consistent with a previous report (32). Compared with adjacent normal tissues, SALNR level was also significantly down-regulated in colon adenomas (Fig. 14B).
Consistently, the expression of miR-181a and miR-22 was elevated in colon adenoma tissues compared with normal colon tissues (Fig. 14, C and D). Moreover, we performed immunofluorescence staining of colon adenomas to identify the location of NF90. As illustrated in Fig. 14E, NF90 diffusely distributed throughout the nucleus of normal colon cells (first row) but was enriched in the nucleolus of colon adenoma cells (second to fourth rows), which is in accordance with the location of NF90 in Ras-induced senescent cells. These findings show that the signature of SALNR expression occurs not only in vitro but also in vivo.

Discussion

IncRNAs play crucial roles in various biological processes, but little is known about the significance of IncRNAs in cellular senescence. A large number of IncRNAs by RNA sequencing differently expressed during replicative senescence (8), suggesting that IncRNAs are involved in cell senescence. In this study, we also found that a set of IncRNAs displayed different expression during Ras-induced premature senescence by IncRNA microarray and identified a 3.8-kb IncRNA, SALNR, regulating cellular senescence. We revealed that SALNR regulation of senescence is achieved by its binding and blocking of NF90 nucleolus translocation. Also, NF90 knockdown may antagonize SALNR delay of cellular senescence.

Here we provide evidence to show that NF90 acts as a repressor of cellular senescence. Although the mechanisms underlying NF90 repression of senescence are unclear, a recent report (26) indicated that NF90 may interact with 3′-UTR of numerous senescence-associated secretory phenotype mRNAs, including two important senescent regulators, IL6 and IL8, and inhibit their expression, thus contributing to suppression of senescence. Here, we showed that NF90 suppresses senescence by inhibiting expression of senescence-associated miRNAs, such as miR-22 and -181a. In the Ras-induced senescent model, NF90 is sequestered into nucleoli, which leads to the loss of its inhibitory activity on miRNA biogenesis. It also contributes to increased expression of target miRNAs and induction of cellular senescence. SALNR antagonized NF90 translocation into nucleoli and rescued its inhibitory activity on senescence-associated miRNA expression. Thus, SALNR delays cellular senescence at least partly via suppressing NF90-mediated miRNA...
expression. This also reveals a cross-talk between lncRNAs and miRNAs.

NF90 is predominantly expressed in the nucleus and can regulate transcription in mammalian cells (33). However, it has been shown that some stimuli are able to cause its translocation to the cytoplasm, where NF90 controls mRNA turnover or translation via binding mRNA 3’UTR (24, 34). In this study, we found that NF90 nucleoli translocation takes place upon exposure of normal fibroblasts to Ras stress. On one hand, nucleoli translocation leads to loss of inhibitory activity of NF90 on miRNA biogenesis or likely NF90 transcription regulation function. On the other hand, as an RNA-binding protein, NF90 translocates in nucleoli binding pre-rRNAs and inhibiting rRNA biogenesis, thus slowing protein synthesis in senescent cells. SALNR regulates cellular senescence, probably by multiple NF90-dependent pathways. Interestingly, SALNR knockdown alone might not be sufficient to induce nucleolar translocation of NF90 (Fig. 7E, fifth and sixth rows), and NF90 was not enriched in the nucleolus in replicative senescent cells (Fig. 7E, first and second rows), suggesting the presence of an additional mechanism in favor of NF90 nucleolar localization in the absence of SALNR, such as NF90 modification or interaction with nucleolar protein.

It appears that ectopic SALNR may substantially extend the replicative life span (Fig. 5A), whereas NF90 is not enriched in nucleoli during replicative senescence (Fig. 7E, first and second rows), which suggests that SALNR extends the replicative life span through an NF90-independent pathway. Replicative senescence was first attributed to telomere attrition caused by the successive DNA replications because overexpression of human telomerase reverse transcriptase corrects telomere erosion and could delay replicative senescence (35). When telomeres shorten below a certain threshold, they are recognized as double strand breaks of DNA and trigger the DNA damage response, which ultimately activates the mechanisms of cellular arrest (36). Intriguingly, oncogenes also induce telomeric lesions and stochastic telomere attrition in primary fibroblasts (37). However, oncogene-induced senescence was initially reported to be independent of telomere length and telomerase activity (38). In human cells, aberrant oncogenic signaling initially causes cells to hyperproliferate, which leads to abnormal high DNA replication rates, leading to frequent DNA replication fork stalling events, including improperly or prematurely terminated replication forks. As a result, single strand DNA breaks and double strand breaks are generated in the vicinity of collapsed replication forks.
and activate a robust DNA damage response and induce cell senescence (39, 40).

Oncogene caused a high degree of non-telomeric and telomeric DNA damage response foci (37), but replicative senescence may be triggered only by telomeric DNA damage response. Therefore, we think that SALNR may participate in telomere maintenance in replicative and oncogene-induced senescence independent of NF90 and participate in non-telomeric replication fork repair in oncogene-induced senescence dependent of NF90. NF90 is also involved in DNA break repair, which supports this hypothesis (41). Moreover, a recent study compared gene expression levels between replicative and oncogene-induced senescence (42). In this study, gene set enrichment analysis showed that the gene ontology sets most enriched in replicative senescence

TABLE 2

Information on 12 colon adenoma samples

| Case number | Histopathological diagnosis | KRAS       | BRAF     |
|-------------|-----------------------------|------------|----------|
| 1           | Tubular adenoma             | MUT 12 (GGT to GAT) | WT       |
| 2           | Villous adenoma             | MUT 12 (GGT to GAT) | WT       |
| 3           | Tubular adenoma             | WT         | WT       |
| 4           | Tubulovillous adenoma       | WT         | WT       |
| 5           | Tubulovillous adenoma       | MUT 12 (GGT to GAT) | WT       |
| 6           | Tubular adenoma             | WT         | WT       |
| 7           | Tubular adenoma             | WT         | WT       |
| 8           | Tubulovillous adenoma       | MUT 13 (GGT to GAC) | WT       |
| 9           | Tubular adenoma             | WT         | WT       |
| 10          | Villous adenoma             | MUT 12 (GGT to GAT) | WT       |
| 11          | Tubulovillous adenoma       | MUT 12 (GGT to GAT) | WT       |
| 12          | Tubulovillous adenoma       | WT         | WT       |
| 13          | Tubulovillous adenoma       | NA         | NA       |
| 14          | Tubular adenoma             | NA         | NA       |
| 15          | Tubular adenoma             | NA         | NA       |

* NA, not applicable.
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