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MNK1 Expression Increases During Cellular Senescence and Modulates the Subcellular Localization of hnRNP A1

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Abstract

Heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) is an RNA-binding protein that modulates splice site usage, polyadenylation, and cleavage efficiency. This protein has also been implicated in mRNA stability and transport from the nucleus. We have previously demonstrated that hnRNP A1 had diminished protein levels and showed cytoplasmic accumulation in senescent human diploid fibroblasts. Furthermore, we have shown that inhibition of p38 MAPK, a key regulator of cellular senescence, elevated hnRNP A1 protein levels and inhibited hnRNP A1 cytoplasmic localization. In this study, we have explored the possible involvement of MNK1, one of the downstream effector of p38 MAPK, in the regulation of hnRNP A1. We have demonstrated that pharmacological inhibition of MNK1 by CGP 57380 decreased the phosphorylation levels of hnRNP A1 in young and senescent fibroblast cells and blocked the cytoplasmic accumulation of hnRNP A1 in senescent cells. In addition, MNK1 formed a complex with hnRNP A1 *in vivo*. The expression levels of MNK1, phospho-MNK1, and phospho-eIF4E proteins were found to be elevated in senescent cells. These data suggest that MNK1 regulates the phosphorylation and the subcellular distribution of hnRNP A1 and that MNK1 may play a role in the induction of senescence.

Keywords

Senescence; fibroblasts; hnRNP A1; p38 MAPK; MNK1; CGP57380

Introduction

Cellular or replicative senescence is characterized by an irreversible arrest of the cell division cycle occurring in primary somatic cell culture [1]. Senescent cells lose responsiveness to growth factors, although they remain metabolically active for long periods of time [2]. Cellular senescence contains two senescent states, replicative and premature senescence. Replicative senescence is a quiescent state that can be identified following a

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limited number of cell divisions associated with telomere shortening [3]. Premature senescence is a telomere-independent state induced by stress stimuli and is thus far indistinguishable from replicative senescence [4]. Senescent cells show flat and enlarged morphology, increased acidic β -galactosidase activity, and changes in gene expression pattern [5].

p38 MAPK is one of the members of mitogen-activated protein kinase (MAPK) family and is implicated in a variety of cellular processes, namely stress response differentiation, cell cycle progression, inflammation, proliferation, and cellular senescence [6]. In mammalian cells, four isoforms of the p38 MAPKs have been identified, p38 α , β , δ , and γ . The p38 MAPKs are predominantly activated by two upstream MAPK kinases, MKK3 and MKK6 [7]. It has been demonstrated that p38 MAPK plays a causative role in induction of cellular senescence. p38 MAPK activity is elevated in senescent fibroblast cells [8]. Enforced activation of p38 MAPK by a constitutive active form of MKK3/6 induces premature senescence [9]. Pharmacological inhibition of p38 MAPK activation genetically delays the onset of senescence [10].

Heterogeneous nuclear ribonucleoprotein (hnRNP) family members are the most abundant components of messenger ribonucleoprotein complexes (mRNPs) and play regulatory roles in a variety of biogenesis of mRNA [11,12]. In humans, the hnRNP family consists of at least 24 different polypeptides [11,13]. Among them, hnRNP A1 is a very abundant nuclear protein that is involved in alternative splicing regulation both *in vitro* and *in vivo* [14–16]. Although it is nuclear at steady state, hnRNP A1 shuttles continuously between the nucleus and the cytoplasm, a property common to a subset of hnRNPs [17]. The shuttling activity of hnRNP A1 has been proposed to play a role in cell proliferation, survival, and differentiation of normal and transformed cells [18]. hnRNP A1 is also implicated in postslicing activities, such as mRNA export and cap-dependent and internal ribosome entry site-mediated translation [19–21].

We have previously shown that there is diminished expression and cytoplasmic accumulation of hnRNP A1 protein in senescent human diploid fibroblast cells [22,23]. We have recently demonstrated that hnRNP A1 and p38 MAPK interact *in vivo* and that the p38 MAPK pathway negatively regulates hnRNP A1 expression levels [24]. We have shown that p38 MAPK pathway is required for cytoplasmic accumulation of hnRNP A1 in senescent cells [24]. However, the molecular mechanisms responsible for the regulation of hnRNP A1 downstream of p38 MAPK in human diploid fibroblasts have yet to be identified.

In this study, we demonstrate that hnRNP A1 forms a complex with mitogen-activated protein kinase-interacting kinase 1 (MNK1), a substrate for p38 MAPK, and that pharmacological inhibition of MNK1 activity suppressed the phosphorylation and cytoplasmic accumulation of hnRNP A1 protein. Furthermore, we show that MNK1 displayed similar subcellular distribution to hnRNP A1 both in young and senescent cells and that endogenous MNK1 and phospho-MNK1 levels as well as the phosphorylation level of eIF4E were elevated in aging cells. These results suggest a role for MNK1 in the regulation of hnRNP A1 in senescent cells. To our knowledge, this study reports the first link between MNK1 and cellular senescence.

Materials and Methods

Cell Culture

IMR-90 lung fibroblast cells were cultured at 37°C with 5% CO₂ in DMEM-HAM media (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) and 1%

penicillin-streptomycin (Mediatech, Gibco-Life technologies). Cultures were serially passaged until terminal passage was reached as previously described [25].

Cell lysis and protein quantification

Each culture was rinsed in ice cold 1X phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $\text{Na}_2\text{HPO}_4 \times 7\text{H}_2\text{O}$, 1.4 mM KH_2PO_4), pH 7.4, and 0.75 mL of cold 1% Empigen BB lysis buffer in 1X PBS containing 1mM EDTA, 0.1mM DTT, 10 mM NaF, and 0.1mL of Phosphatase Inhibitor Cocktail I (Sigma, St. Louis, MO) was added to each 100 mm culture dish. Cells were scraped and lysed on ice by sonication three times for 5 sec each. Lysates were cleared by centrifugation at 10,000 rpm at 4°C for 5 min. Supernatants were then transferred into fresh Eppendorf tubes. Protein concentration was quantified in triplicate using the RC DC Protein Assay (Bio-Rad, Hercules, CA).

Western blot analysis

Western blot analysis was performed using standard western procedures [26]. Briefly, all extracts were separated by 10% SDS-PAGE, and electrophoresed at 160 volts until the dye front was at the end of the gel. Proteins were transferred onto PVDF membranes in transfer buffer (10X Tris-Glycine and methanol). The membranes were incubated in cold 1X PBST (1X PBS, 0.1% Tween-20) on a rocker at room temperature for 1 h. Membranes were incubated in the appropriate dilution of primary antibodies in 1X PBST overnight at 4°C. Blots were washed three times for 5–10 min per wash in 1X PBST followed by incubation in appropriately diluted horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (GE Healthcare, Piscataway, NJ). The blots were washed three times in 1X PBST for 5–10 min. The SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL) kit was used for antigen detection.

The primary antibodies used: 4B10 for hnRNP A1, generously provided by Dr. Serafin Pinol-Roma; anti-phospho-serine antibody (Abcam, Cambridge, UK); anti- β -actin antibody (Millipore, Billerica, MA); anti-GAPDH antibody (CHEMICON, Temecula, CA); anti-phospho-eIF4E; anti-eIF4E, anti-phospho-MNK1, anti-MNK1, anti-phospho-p38 MAPK, and anti-p38MAPK antibodies (Cell SignalingTM, Danvers, MA).

Inhibition of MNK1 kinase activity

Young and senescent IMR-90 cells were treated with varying concentrations of CGP 57380 or an equal volume of DMSO for three days at 37°C. Lysates were collected in Empigen buffer. 40 μM CGP 57380 was used for the immunocytochemistry studies.

Immunocytochemistry

IMR-90 cells were fixed with 4% paraformaldehyde in 1X PBS. Cells were washed 3 times for 5 min at room temperature and permeabilized with 0.2% TritonX-100 for 10 min at room temperature. Slides were washed three times with 1X PBS for 5 min and blocked using 10% FBS/PBS at room for 40 min. Slides were treated with monoclonal antibodies diluted in 10% FBS/PBS (blocking buffer) and incubated overnight at 4°C: for hnRNP A1, 4B10 (1:1000), p-p38 MAPK (3:1000; Cell Signaling, Danvers, MA), and MNK1 (1:200; Cell Signaling). Slides were washed 3 times in 1X PBS for 5 min on a shaker at room temperature. Slides were then incubated for 1 h at room temperature with either Alexa Fluor 594 or Alexa Fluor 488 secondary antibodies (Invitrogen, Carlsbad, CA). Slides were washed three times with 1X PBS for 5 min at room temperature and mounted in ProLong Gold Antifade Reagent with DAPI (Invitrogen). Images were captured using a Zeiss Axioplan fluorescence microscope.

Immunoprecipitation

Whole cell lysates from young or senescent IMR-90 cells treated in the presence or absence of varying concentrations of CGP 57380 were immunoprecipitated for hnRNP A1 or MNK1 using 4B10 monoclonal and MNK1 antibodies, respectively, via the Dynabeads® Protein A kit (Invitrogen). Dynabeads were resuspended by pipetting. 50 μ L of Dynabeads was transferred to 1.5 mL tubes, placed on a magnet, and the supernatant was removed. The Dynabeads were resuspended in 200 μ L of antibody binding and wash buffer and pre-incubated with 5 μ L of 4B10 antibody or 10 μ L of MNK1 antibody for 30 min at 4°C. Complexes were then incubated with 500 μ g of lysates for 3 h at 4°C. Immunoprecipitates were centrifuged at 14,000 $\times$ g for 5 min, washed 5 times with 200 μ L of wash buffer per wash, then resuspended in 1X sample buffer (62.5 mM Tris-HCl, pH 6.8 at 25°C, 2% w/v sodium dodecyl sulfate (SDS), 10% glycerol, 50 μ M dithiothreitol, 0.01% bromophenol blue), and immediately subjected to 12 % SDS-PAGE.

Results

Change in MNK1 subcellular distribution during aging

We sought to identify kinases downstream of p38 MAPK as possible candidates for the regulation of hnRNP A1 during senescence. One of the direct targets for p38 MAPK, MNK1, seemed to have the potential. MNK1 has been reported to directly phosphorylate hnRNP A1 *in vitro* and *in vivo* [27, 31]. To assess the possibility, we first determined the expression patterns of MNK1 protein in young and senescent fibroblast cells. We performed immunocytochemistry in young and senescent IMR90 cells with antibodies for hnRNP A1, phospho-p38, and MNK1. Young cells displayed a predominant nuclear localization of hnRNP A1 protein (Figure 1A–b). Senescent cells showed predominantly cytoplasmic accumulation (Figure 1A–f). These results are consistent to what we previously reported [24]. Phospho-p38 MAPK was uniformly expressed in young cells (Figure 1A–c) and expressed mainly in the cytoplasm in senescent cells (Figure 1A–g) as well as p38 MAPK as previously reported by us [24]. MNK1 was predominantly localized in the nucleus in young cells (Figure 1B–c). Whereas it showed predominant cytoplasmic accumulation in senescent cells (Figure 1B–g). The localization patterns for both proteins were similar in young and senescent cells (Figure 1B–b and f), suggesting that a physical interaction between MNK1 and hnRNP A1 may potentially take place regardless of the growth state.

Endogenous protein levels of p-eIF4E, eIF4E, p-MNK1, and MNK1

We next determined the endogenous expression levels of MNK1 in different growth states. We prepared total cell lysates from young, pre-senescent, and senescent IMR-90 cells and performed western blot analysis with antibodies for phospho-MNK1, MNK1, phospho-eIF4E, and eIF4E. Eukaryotic initiation factor-4E (eIF4E) is a downstream target of MNK1 and functions as a translation initiation factor that binds to the 5' cap structure of eukaryotic cytosolic mRNAs [28]. Phospho-MNK1, MNK1, and phospho-eIF4E protein levels were found to be up-regulated in pre-senescent and senescent cells (Figure 2). In order to determine the possibility of activation of both proteins during senescence, we measured the relative ratios of phospho-MNK1 and phospho-eIF4E to their respective total protein levels using densitometry. These ratios are shown below Figure 2. Relative to young fibroblasts, the ratio of phospho-MNK1 to total MNK1 protein levels was 1.67 times higher in pre-senescent fibroblasts and 1.75 times higher in senescent fibroblasts. Pre-senescent cells are high passage cells that are about to enter replicative senescence within a few passages. For eIF4E, the ratio of phospho-eIF4E protein levels to total protein was 1.56 times higher in pre-senescent cells and 1.72 times higher in senescent cells compared to young cells. These results indicate that both the protein level and activity of MNK1 increases during senescence. Ours is the first report that shows MNK1 increases during senescence. We and

others have previously shown that p38 MAPK activity was elevated in senescent fibroblast cells [9,10,24]. Taken together, these results suggest an age- and p38 MAPK-dependent increase in MNK1 levels and activity. Another substrate for p38 MAPK, MAP kinase-activated protein kinase 2 (MAPKAPK2), is known to phosphorylate hnRNP A0, which has structural similarity to other hnRNPs [29]. Western blot analyses of phospho-MAPKAPK2 and MAPKAPK2 levels demonstrated that the levels of these proteins showed no age-dependent change (data not shown). These results lend support to the possibility that MNK1 specifically regulates cellular senescence downstream of p38 MAPK.

Effect of MNK1 inhibition on hnRNP A1 expression

We have previously demonstrated that inhibition of p38 MAPK activity with SB203580 upregulated hnRNP A1 protein levels in young IMR-90 fibroblasts [24]. Therefore, we wanted to determine whether or not inhibition of MNK1 kinase activity affected the expression levels of hnRNP A1 protein. We blocked MNK1 kinase activity using 4-Amino-5-(4-fluoroanilino)-pyrazolo[3,4-d]pyrimidine (CGP 57380) in young and senescent cells and conducted western blot analyses with phospho-eIF4E, eIF4E, and the 4B10 monoclonal hnRNPA1 antibody. This inhibitor is a selective inhibitor of MNK1 ($IC_{50} = 2.2 \mu M$) with no inhibitory activity against p38 MAPK, JNK1, ERK1/2, PKC, or Src-like kinases [30]. At 40 μM , CGP 57380 blocked kinase activity, as indicated by the disappearance of the 25 kDa band in young cells that corresponds to phospho-eIF4E (Figure 3B). Likewise, CGP 57380 at 20 μM and 40 μM blocked MNK1 kinase activity in senescent cells (Figure 3C). Our results suggest that CGP 57380 works as an inhibitor of MNK1 in our system and that MNK1 kinase is active in both young and senescent fibroblasts. We have previously shown that hnRNP A1 protein levels are lower in senescent cells as compared to young cells (Figure 3A, [24]). The levels of endogenous hnRNP A1 in young and senescent cells were unchanged by CGP 57380 compared to DMSO-treated cells (Figures 3B and 3C). These results suggest that MNK1 does not alter hnRNP A1 protein levels.

Inhibition of MNK1 activity modulates the phosphorylation level of hnRNP A1

Because MNK1 phosphorylation of hnRNP A1 has been described by others *in vitro* and *in vivo* [27, 31], we investigated whether this phosphorylation occurred as a function of cellular aging. Young IMR-90 cells were treated in the presence or absence of varying concentrations of CGP 57380 for three days. The cell lysates were then immunoprecipitated with the hnRNP A1 specific monoclonal antibody, 4B10. Subsequently, membranes were probed with a phospho-serine antibody. A 34 kDa band was detected in the complex, representing the presence of phospho-hnRNP A1 (Figure 4A, upper panel). The blots were stripped and then re-probed with 4B10 antibody to confirm the presence of immunoprecipitated levels of hnRNP A1 in the lysates (Figure 4A, lower panel). We found that the phospho-hnRNP A1 band was downregulated with increasing concentrations of the inhibitor (Figure 4A, upper panel). We performed the same experiment with senescent cells. We found a dose-dependent decrease in the phosphorylation levels of hnRNP A1 (Figure 4B, upper panel). These results suggest that hnRNP A1 is a putative substrate of MNK1 in young and senescent cells. This supports the possibility of a correlation between the age-dependent increase in MNK1 and phospho-MNK1 levels (Figure 2) and the age-dependent increase in endogenous phosphorylation levels of hnRNP A1 [24].

hnRNP A1 forms a complex with MNK1

We next investigated whether or not there is a physical interaction between hnRNP A1 and MNK1 using coimmunoprecipitation studies in the presence or absence of CGP 57380. Cell lysates were immunoprecipitated with 4B10 antibody and then the membranes were probed with MNK1 antibody. MNK1 co-immunoprecipitated with hnRNP A1 as indicated by a 52 kDa band in the complex (Figure 5A, DM and 5 μM lanes). We next conducted the

reciprocal analysis to ascertain whether hnRNP A1 was co-immunoprecipitated using a MNK1 specific antibody. A faint 34 kDa band was detected in the complex, representing the presence of hnRNP A1 (Figure 5B). These results indicate that a reciprocal interaction exists between MNK1 and hnRNP A1. The ratio of MNK1 to hnRNP A1 and hnRNP A1 to MNK1 did not change as the concentration of the inhibitor increased. Taken together, the data from Figure 4 suggest that phosphorylation of hnRNP A1 is not required for its interaction with MNK1.

MNK1 is required for the subcellular distribution of hnRNP A1

We have previously demonstrated that activation of p38 MAPK is required for the cytoplasmic accumulation of hnRNP A1 in senescent cells [24]. It has been previously shown that MNK1/2 promoted stress-induced phosphorylation and cytoplasmic accumulation of hnRNP A1 in HeLa cells [31]. These findings raised the possibility that MNK1 might potentially exhibit the cytoplasmic accumulation of hnRNP A1 downstream of p38 MAPK in senescent cells.

To assess this, we treated young and senescent IMR-90 cells with CGP 57380 or an equivalent amount of DMSO, then determined the localization pattern of hnRNP A1 by immunocytochemistry. Young CGP 57380 treated cells did not show any alteration in their nucleo-specific localization pattern (Figure 6f) compared to control cells (Figure 6b). In contrast, CGP 57380 treatment inhibited the cytoplasmic accumulation of hnRNP A1 in senescent cells, as the majority of the protein was localized in the nucleus (Figure 6n). These results indicate that the accumulation of hnRNP A1 in the cytoplasm of senescent cells requires the kinase activity of MNK1. We subsequently determined the localization pattern of MNK1 before and after inhibition with CGP 57380. CGP 57380 inhibition did not induce any change in MNK1 subcellular distribution in young (Figure 6g) and senescent (Figure 6o) cells, indicating that the kinase activity of MNK1 is not required for its subcellular distribution. The fact that we detected MNK1-dependent hnRNP A1 phosphorylation and a reciprocal interaction between these proteins (Figures 4 and 5) suggests that MNK1 promotes the cytoplasmic accumulation of hnRNP A1 via phosphorylation in senescent cells. There may, however, be different mechanisms involved in younger cells.

Discussion

Phosphorylation and cytoplasmic localization of A1 by MNK1

p38 MAPK plays a causative role in cellular senescence [9,10]. We have previously shown that p38 MAPK is required for the subcellular distribution of heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) in senescent cells and regulates its protein levels in young cells [24]. In this study, we investigated molecular mechanisms responsible for the regulation of hnRNP A1 downstream of p38 MAPK during senescence. We found that MNK1, a substrate for p38 MAPK, modulates the phosphorylation and subcellular distribution, but not the expression level of hnRNP A1. These results suggest that p38 MAPK regulates hnRNP A1 phosphorylation and localization in a MNK1-dependent manner and the protein level in a MNK1-independent manner. We suspect that phosphorylation of hnRNP A1 by MNK1 is critical for its subcellular distribution. This hypothesis is supported by the findings of others that MNK1 phosphorylates three serine residues of hnRNP A1 at its C-terminal peptide, F-peptide [25]. Phosphorylation of the F-peptide results in hnRNP A1 cytoplasmic accumulation by reducing its interaction with the nuclear receptor, transportin [32]. We have found that siRNA transfection against MAPKAPK-2, one of the substrates for p38 MAPK, did not alter hnRNP A1 protein levels in young IMR-90 cells (data not shown). Thus, the change in hnRNP A1 protein levels by

p38 MAPK inhibition may be mediated by other kinases and/or transcription factors downstream of p38 MAPK.

The roles of MNK1 in cellular senescence

MNKs have been reported to promote protein synthesis, cell cycle progression, and proliferation in tumor cells through phosphorylation of its substrate, eIF4E [33], which is a central regulator for cap-dependent translation initiation. On the other hand, we found an age-dependent increase in MNK1 and phospho-MNK1 expression and eIF4E phosphorylation in senescent cells (Fig 2) and that MNK1 activity was required for the phosphorylation of eIF4E in the cells (Fig. 3C). Overexpression of eIF4E increases cellular senescence in mice as measured by β -galactosidase staining [34]. Senescence is delayed and lifespan is extended in *Caenorhabditis elegans* mutants that are defective in eIF4E [35]. The MNK1-eIF4E pathway in senescent cells appears to have an opposite regulating role in protein synthesis from that in tumor cells. It has been shown that protein synthesis is elevated in carcinogenesis, whereas the rate of general protein synthesis is diminished during aging [36]. Moreover, negative effects of MNK1/2 activity and phosphorylation of eIF4E on cap-dependent translation have been demonstrated by others [30,37]. Therefore, we suggest a putative role for MNK1 in senescence. Possible mechanisms that can explain the age-dependent effects by MNK1 is that the increase in the phosphorylation of eIF4E by MNK1 somehow decreases cap-dependent translation followed by a decline in *de novo* synthesis of the proteins required for longevity. It would be intriguing to assess whether the rate of protein synthesis during senescence is affected by inhibition of MNK1 activity. One study showed that knockdown of MNK1/2 was essential for the phosphorylation of eIF4E in embryonic fibroblasts and adult tissue, but not for general protein synthesis, cap-dependent translation, and cell growth in development [38]. It seems that MNKs do not play a crucial role in the early development of normal mice. However, the role of MNK has not been studied in senescent mice.

We have previously demonstrated the downregulation of hnRNP A1 and/or A2 by siRNA transfection contributes to a partial senescence-like morphology [24]. hnRNP A1 is known to be involved in a variety of activities involving RNA, including alternative splicing and mRNA transport and stability [15,39]. Our findings suggest that the MNK1 induced changes in localization of hnRNP A1 contribute to the genetic features of senescence through altered regulation of its target mRNAs. In general, genes required for stress and immune/inflammatory responses are up-regulated during senescence [40]. Some may be potentially regulated by the MNK1/hnRNP A1 pathway. To support this idea, it has been shown that MNK1 induces the synthesis of tumor necrosis factor (TNF) α via hnRNP A1 phosphorylation in Jurkat cells [25]. Activation of MNK1 by stress stimuli causes phosphorylation and cytoplasmic accumulation of hnRNP A1 in stress granules [31] whereas stress responsive mRNAs are translated. Further analysis of RNA metabolism regulated by the MNK1-hnRNP A1 pathway could reveal potential molecular mechanisms, that establish the senescent phenotype. To begin to address this issue, we inhibited MNK1 activity with CGP 57380 in young and pre-senescent (late passage) IMR90 fibroblasts to determine a potential role for MNK1 in cell growth. Based on our studies discussed above, we had initial thought that inhibiting MNK1 would delay either the onset of senescence. Instead, we found that CGP 57380 inhibited the growth of both young and late passage cells. These results are shown in Supplemental Figure A and indicate that MNK1 has pleiotropic effects. In Supplemental Figure B, we measured senescence-associated β -galactosidase (a biomarker for replicative senescence) in MNK1 inhibited cells and did not observe an induction of senescence. Thus, studies are warranted to look more closely at eIF4E or some other unknown downstream target of MNK1 and their contributions to senescence.

This is the first report, to our knowledge, that shows a link between MNK1 phosphorylation and hnRNP A1 during cellular senescence. In addition, this is the first report to show increased MNK1 protein levels in senescent cells. Our findings imply that the MNK pathway could provide a novel contributing factor to study age-related disorders, including Alzheimer's disease, heart disease, and diabetes. The continued study of the role of MNK pathways in hnRNP A1 functionality will extend our knowledge of signaling pathways beyond that of carcinogenesis and inflammatory responses. Further studies are warranted to elucidate the precise molecular mechanisms by which MNK1 regulates cellular senescence through hnRNP A1 and eIF4E.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

hnRNP A1	heterogeneous nuclear ribonucleoprotein A1
p38 MAPK	p38 mitogen-activated protein kinase
MNK1	mitogen-activated protein kinase-interacting kinase 1
eIF4E	Eukaryotic initiation factor-4E
MAPKAPK2	MAP kinase-activated protein kinase 2

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Highlights

- MNK1 and not MAPKAPK2 phosphorylates hnRNP A1
- MNK1 has elevated levels in senescent cells, this has not been reported previously
- MNK1 activity induces cytoplasmic accumulation of hnRNP A1 in senescent cells
- Altered cytoplasmic localization of hnRNP A1 may alter gene expression patterns
- Our studies may increase our understanding of RNA metabolism during cellular aging.

Figure 1. Subcellular distribution of hnRNP A1, P-p38, and MNK1 in young and senescent cells Young and senescent IMR-90 cells were subjected to immunocytochemistry for hnRNP A1, P-p38, and MNK1. DAPI was used to stain nuclei. Shown are representative images of three independent experiments. Identical fields were taken for DAPI, hnRNP A1, and MNK1 fluorescent signals. The corresponding merged images (Merged) are shown on the right (panels d, h, l, and p). White dotted lines indicate cell boundaries. Magnification: 400X in each field. The scale bar represents 20 μ m.

Figure 2. Western analysis of downstream p38 MAPK signaling pathway in IMR-90 young, pre-senescent, and senescent cells

Whole cell lysates were electrophoresed on a 12% SDS-PAGE and probed with the indicated antibodies. The blot was then stripped and reprobed to detect total β-actin levels. The relative ratios of phospho-MNK1 and phospho-eIF4E to their respective total protein levels are shown below and were measured using densitometry. The blots are representative of at least three independent experiments.

Figure 3. Effect of MNK1 inhibition on hnRNP A1 expression

5 μ g of total cell lysates from young and senescent IMR-90 cells were subjected to 12.5% SDS-PAGE and probed with the 4B10 hnRNP A1 antibody (A). Young (B) and senescent (C) IMR-90 cells were treated with various concentrations of CGP 57380 or DMSO. 60 μ g of total cell lysate were loaded onto 12.5% SDS-PAGE gels and immunoblotting analyses were performed with antibodies specific for either p-eIF4E or eIF4E. The blots are representative of at least three independent experiments.

Figure 4. Effect of MNK1 inhibition on phosphorylation level of hnRNP A1

Young (A) and senescent (B) IMR-90 cells were treated in the presence or absence of varying concentrations of CGP 57380. Cell lysates were immunoprecipitated with the 4B10 hnRNP A1 antibody immobilized beads, subjected to 12% SDS-PAGE followed by immunoblotting with phosphoserine antibody to detect phospho-hnRNP A1. Mock IP reactions without lysates were included to establish the positions of the antibody heavy and light chains. Blots were stripped and reprobed with the 4B10 monoclonal antibody to detect total hnRNP A1 (lower panels).

Figure 5. MNK1 forms a complex with hnRNP A1 *in vivo*

Total lysates from young IMR-90 cells treated in the presence or absence of varying concentrations of CGP 57380 were immunoprecipitated using the hnRNP A1 antibody (A) or the MNK1 antibody (B), subjected to 12% SDS-PAGE, and probed with MNK1 (A) or 4B10-hnRNP A1 (B) specific antibodies. Blots were stripped and reprobed with the hnRNP A1 monoclonal antibody (A, lower panel) and the MNK1 antibody (B, lower panel) to detect total hnRNP A1 and MNK1, respectively. (C) Binding specificity of hnRNP A1 and MNK1. 300 µg of total cell lysates from young IMR-90 cells were immunoprecipitated with the 4B10 and MNK1 antibodies and probed with a GAPDH antibody.

Figure 6. Effect of MNK1 inhibition on the subcellular distribution of hnRNP A1 and MNK1
 Young and senescent IMR-90 cells were treated with 40 μ M of CGP 57380 (panels e–h and m–p) or an equal volume of DMSO (panels a–d and i–l) for 3 days and then subjected to immunocytochemistry for hnRNP A1 and MNK1. DAPI was used to stain nuclei. Shown are representative images of three independent experiments. Identical fields were taken for DAPI, hnRNP A1, and MNK1 fluorescent signals. The corresponding merged images (Merged) are shown on the right (panels d, h, l, and p). White dotted lines indicate cell boundaries. Magnification: 400X in each field. The scale bar represents 20 μ m.