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ERK1/2 mediates unbalanced growth leading to senescence induced by excess thymidine in human cells

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ABSTRACT

Excess thymidine induces unbalanced growth by delaying DNA replication and subsequently induces senescence in every human cell type. Our previous studies with use of inhibitors suggested that ERK1/2 has a major role in these processes. Here we directly assessed the roles of ERK1 and ERK2 in unbalanced growth induced by excess thymidine. Knockdown of ERK2 and ERK1 by vector-based RNA interference prevented loss of colony forming ability and appearance of senescence markers induced by excess thymidine in HeLa and TIG-7 cells, respectively. Such cells continued growing in the presence of excess thymidine. Double knockdown of ERK1 and ERK2 did not improve the effects of single knockdowns of ERK1 and ERK2 in either cell types. These results demonstrate that ERK1 or ERK2 has a major role in manifestation of unbalanced growth in human cells.

1. Introduction

Normal types of somatic cells in culture undergo a terminal growth arrest after a limited number of cell divisions, a phenomenon called replicative senescence or Hayflick limit [1]. Such cells exhibit irregularly enlarged and flat cell shape, and upregulate particular genes called the senescence-associated genes [2]. Normal cells also show a similar phenomenon, termed premature senescence, when they meet various sorts of impediment, namely, oxidative stress, DNA damage, cell cycle perturbations, chromatin destabilization, imbalance of cell signaling, and mutations of particular genes [3–5]. Occasionally, tumor-derived immortal cell lines show similar phenomena by treatment with particular types of stress [6,7]. Due to diversity in the means to induce senescence, senescence is thought to occur through multiple pathways.

As cellular and nuclear enlargement and retardation in DNA replication are common features in senescent cells [7,8], we presented the "unbalanced growth model of senescence", in which delayed progression of S-phase of the cell cycle with normal synthesis of RNA and protein induces cell swelling and subsequently leads to senescence [7]. This model is well agreement with the facts that lifespan of normal cells depends on cell types and culture conditions and that any types of immortal cells can enter a senescence-like state with an appropriate inducer such as 5-bromodeoxyuridine [3] and excess thymidine [7].

Signaling pathways involving the mitogen-activated protein (MAP) kinases such as ERK1/2, p38 and SAPK/JNK play a central regulatory role in the signaling hierarchy to regulate cell growth and cell death. We have characterized these roles in unbalanced growth, and have suggested that the MEK–ERK pathway together with those of p38 or SAPK/JNK mediates the unbalanced growth induced by excess thymidine in HeLa cells and TIG-7 normal human fibroblasts [7,8]. The MEK–ERK pathway was shown to be most important because U0126, a specific inhibitor of MEK1/2 [9] suppressed the unbalanced growth in HeLa cells [7]. Similarly, U0126 substantially suppressed the unbalanced growth in TIG-7 cells, but simultaneous addition of an inhibitor of JNK was necessary to fully suppress it [8]. The MEK–ERK pathway is also shown to mediate proliferation, apoptosis, differentiation [10–12], and responses to hypertrophic and multiple DNA damage stimuli [13–15].

In this study, we characterized the role of ERK1/2 in manifestation of unbalanced growth leading to senescence induced by excess thymidine in HeLa and TIG-7 cells by depleting ERK1 and/or ERK2 by vector-based RNA interference (RNAi). This knockdown experiments can distinguish specific roles of ERK1 and ERK2, and avoid side effects due to use of inhibitors. Excess thymidine is shown to induce unbalanced growth and senescence or senescence–like phenomena in virtually any cell types [7]. It retards DNA replication by inducing pool imbalance of DNA precursors through allosteric regulation of ribonucleotide reductase [16].

2. Materials and methods

2.1. Materials

Plasmids pSU–ERK1 and pSU–ERK1 encoding short hairpin RNA for ERK1 (5′-GCCATGAGAGATGTCTACA-3′) and ERK2

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(5′-GAGGATTGAAGTAGAACAG-3′), respectively, under the control of H1 promoter were kind gifts of Dr. R.C. Bargou of Humboldt University, Germany [17]. Mouse monoclonal antibodies against ERK1/2 and pERK1/2 were purchased from cell signaling, USA. Other monoclonal antibodies were purchased from Santa Cruz Biotech, USA. Reagents used were of reagent grade.

2.2. Cell culture

TIG-7 normal human fibroblast strain originated from a fetal lung and HeLa cervical tumor line were obtained from the Japanese Cancer Research Resources Cell Bank. Cells were cultured in plastic Petri dishes (Thermo Scientific, Nunc) containing ES medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2 and 95% humidity as described previously [6]. We used TIG-7 cells at approximately 40 population doubling levels (PDLs) for proliferating cells and approximately 70 PDLs for senescent cells.

2.3. Colony forming and growth assays

For colony forming assay, RNAi plasmids were introduced to cells together with pPGK-puro encoding puromycin *N*-acetyltransferase mediated by lipofectin (Invitrogen, Japan) for 15 h. The cells were then cultured with 0.5 μg/ml (TIG-7) or 2 μg/ml (HeLa) puromycin for 2 days to select transfected cells. The resulting puromycin-resistant cells were cultured in the presence and absence of 1.5 mM thymidine, and replated to form colonies in normal medium. Colonies formed were stained with Coomassie Brilliant Blue R-250.

For growth assay, the above puromycin-resistant cells were cultured continuously in the presence and absence of 1.5 mM thymidine, and growth was monitored by counting cells at intervals using a hematocytometer.

To observe cellular morphology, RNAi plasmids were introduced to cells by electroporation with a high-efficiency electroporator (type NEPA21, Nepa Gene Co. Ltd., Chiba, Japan). The cells were cultured in normal medium for 2 days and then in medium containing or not containing 1.5 mM thymidine for 7–10 days. Cells were stained with the senescence-associated β-galactosidase as described previously [7].

2.4. Western blot analysis

Cells were suspended in lysis buffer (30 mM Tris–HCl (pH 8.0), 0.1% SDS, 100 pM vanadate, and 15 nM okadaic acid), and disrupted by sonication for 1 min on ice. The resulting cell lysate was centrifuged for 15 min at 15,000 rpm to yield a cell extract. Cell extracts (10 μg) were subjected to 10% SDS polyacrylamide gel electrophoresis, blotted onto a PDVF-membrane and probed with a mouse monoclonal antibody. The mouse antibody was bound with a horseradish peroxidase-conjugated goat antibody against mouse IgG, and detected with a chemiluminescence detection kit (Amersham Bioscience) according to the supplier's instructions. Protein content was determined with a Bradford assay kit (Bio-Rad).

2.5. Indirect immunostaining of pERK1/2

Cells were fixed in 100% methanol at room temperature for 15 min, washed twice with PBS/bovine serum albumin and incubated at 4 °C overnight with anti-pERK1/2 antibody. On the next day, the cells were washed twice with PBS and incubated with alexa 568 (Molecular Probes) as a secondary antibody for 3 h. Finally, the cells were washed twice with PBS, incubated with DAPI (4′,6-diamidino-2-phenylindole) for 30 min, and mounted on a microscopy slide (Olympus, Tokyo).

3. Results

3.1. Activation of ERK1/2 by excess thymidine

We examined activation of ERK1 and ERK2 in HeLa and TIG-7 cells under the same conditions. When HeLa cells were cultured with 1.5 mM thymidine, ERK1 and ERK2 were markedly activated, while JNK and p38 remained unchanged as described previously [7] (Fig. 1A). In contrast, ERK1/2, p38 and JNK were all activated in TIG-7 cells under the same conditions (Fig. 1A). Since ERK1/2 was commonly activated in both cell types, ERK1/2 was suggested to have a key role in responding to excess thymidine.

3.2. Knockdown of ERK1/2 by RNAi

We employed a widely used method of vector based RNAi to knockdown ERK1/2. Cells were transfected with pSU–ERK1, pSU–ERK2 or both together with pPGK-puro, and protein levels for ERK1 and ERK2 were examined by Western blot analysis.

In HeLa cells, single transfection of pSU–ERK1 and pSU–ERK2 reduced ERK1 (44 kDa) and ERK2 (42 kDa), respectively, to almost

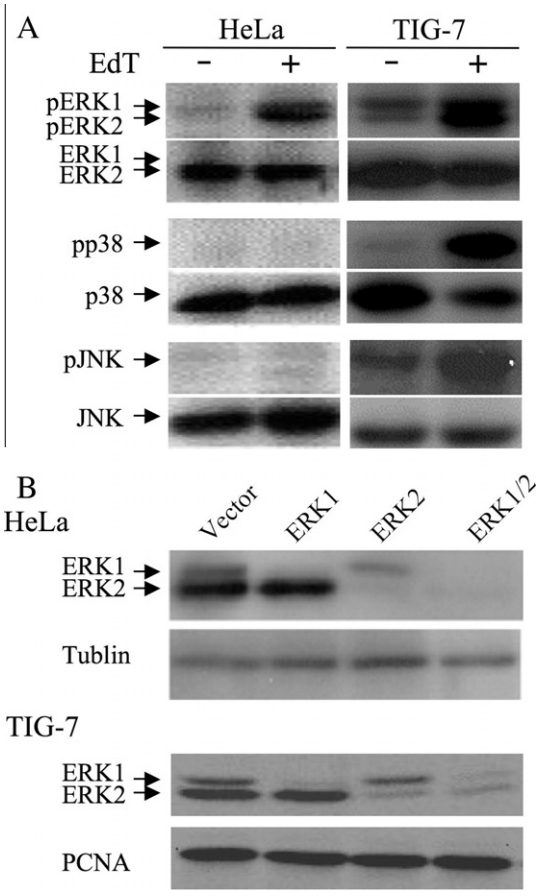

Fig. 1. Western blot analysis of the MAP kinase family. (A) Activation of ERK1/2 in cells cultured with excess thymidine. Cells were cultured with 1.5 mM thymidine (EdT) for 3 days, and cell extracts were prepared from these cells and subjected to Western blot analysis using specific antibodies indicated as described in Section 2. (B) Knockdown of ERK1, ERK2, or both by vector-based RNAi. Cells were transfected with empty vector (vector), pSU–ERK1 (ERK1), pSU–ERK2 (ERK2), or both (ERK1/2) together with pPGK-puro, and cultured with puromycin for 4 days. Cell extracts were prepared from these cells and subjected to Western blot analysis as in (A).

undetectable levels 4 days after culture with puromycin (Fig. 1B). Simultaneous transfection of pSU–ERK1 and pSU–ERK2 reduced both ERK1 and ERK2 to undetectable levels. In TIG-7 cells, single transfection of pSU–ERK1 and pSU–ERK2 decreased ERK1 and ERK2, respectively, to levels less than 20% of control 4 days after culture with puromycin (Fig. 1B). Simultaneous transfection of pSU–ERK1 and pSU–ERK2 reduced both ERK1 and ERK2 similarly to the single transfection experiments.

These results show that our RNAi experiments effectively reduce the protein levels of ERK1 and ERK2 in HeLa and TIG-7 cells as described in other cell types [17].

3.3. Effect of knockdown of ERK1/2 on loss of division potential

We examined the effect of knockdown of ERK1/2 on loss of division potential. This marker is one of the most prominent features in senescent cells. Transfected cells selected by culture with puromycin for 2 days were cultured in medium containing excess thymidine to induce unbalanced growth, and then replated in normal medium to examine their colony forming abilities. The vector-transfected control cells efficiently lost the abilities within 5 days after addition of excess thymidine.

Knockdown of ERK1 had no effect on the loss of division potential in HeLa cells cultured with excess thymidine for 5 days (Fig. 2A). However, knockdown of ERK2 almost completely prevented loss of division potential under the same culture conditions. Double knockdown of ERK1 and ERK2 only partially prevented such loss of division potential. In contrast, knockdown of ERK1 markedly prevented loss of division potential in TIG-7 cells cultured with excess thymidine for 5 days, whereas knockdown of ERK2 and double knockdown of ERK1 and ERK2 did not and only partially prevented it, respectively (Fig. 2B).

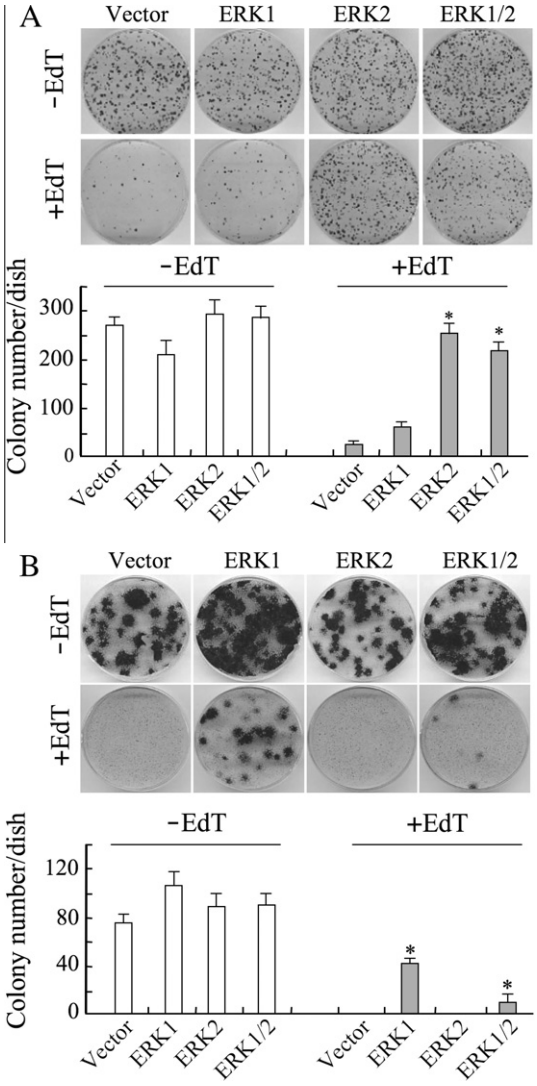

Fig. 2. Effects of knockdown of ERK1, ERK2, or both on division potential of cells. (A) HeLa cells were transfected with the plasmid as in Fig. 1B and selected with puromycin as described in Section 2. The cells were cultured with 1.5 mM thymidine (EdT) for 5 days, replated, and cultured in normal medium for 7 days to form colonies. Upper panel, photographs of colonies; lower panel, histograms of the numbers of colonies ± SE in triplicate assays ($n = 3$); $^{*}P < 0.01$ in comparison with the vector control colonies. At least three independent experiments gave similar results. (B) TIG-7 cells transfected with the plasmids to form colonies as in Fig. 2A except that colonies were stained after culture for 14 days.

3.4. Effect of knockdown of ERK1/2 on cell growth

We examined the effect of knockdown of ERK1/2 on growth in the presence of excess thymidine (Fig. 3). Transfected cells selected by culture with puromycin for 2 days were continuously cultured in normal growth medium to monitor growth. Knockdown of ERK1, ERK2, or both reduced the growth rate of HeLa cells in normal medium by approximately 30%, 40%, and 50%, respectively, as compared to the vector-transfected control cells. These knockdowns reduced the growth rate of TIG-7 cells by approximately

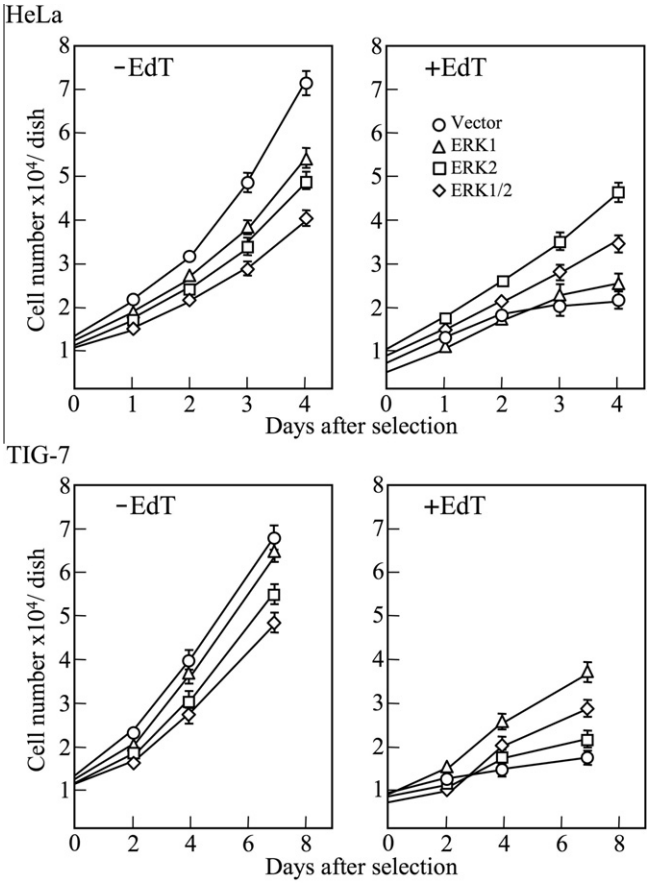

Fig. 3. Effects of knockdowns of ERK1, ERK2, or both on growth of cells. Cells were transfected with the plasmid indicated, cultured with puromycin for 2 days, replated, and cultured with or without 1.5 mM thymidine (EdT) as described in Section 2. At intervals, cell numbers were counted. The values are means ± SE in triplicate assays ($n = 3$). At least three independent experiments gave similar results.

10%, 20%, and 30%, respectively. These results show that full expression of both ERK1 and ERK2 are necessary for normal growth, but the presence of either of ERK1 or ERK2 can support sufficient growth.

HeLa and TIG-7 cells transfected with the empty vector gradually slowed down their growth rates and almost completely stopped growing by 4–5 days after addition of excess thymidine. In HeLa cells, knockdown of ERK1 had no effect, but knockdown of ERK2 enabled the cells to continuously grow in medium containing excess thymidine although their growth rates were significantly slower than in normal medium. Knockdown of ERK1 and ERK2 exhibited intermediate growth. In TIG-7 cells, knockdown of ERK1 enabled the cells to continuously grow in the presence of excess thymidine. Knockdown of ERK2 had no effect and knockdown of both ERK1 and ERK2 exhibited intermediate growth.

3.5. Effect of knockdown of ERK1/2 on cellular morphology

To observe cellular morphology, we introduced the RNAi plasmids into cells using a high-efficiency electroporator, which was established very recently. Transfection efficiencies of a plasmid encoding GFP were more than 95% and 80% in HeLa and TIG-7 cells, respectively, with more than 90% viability in both cell types.

Cells transfected with plasmids were cultured in normal medium for 2 days to allow for decay of ERK1/2, and then replenished with medium containing excess thymidine to observe their cellular morphology (Fig. 4). In HeLa cells, knockdown of ERK1 had no effect. In contrast, knockdown of ERK2 prevented appearance of senescent markers such as irregularly enlarged and flat cell shape and blue images stained with the senescence-associated β-galactosidase. Simultaneous knockdown of ERK1 and ERK2 partially did so. In TIG-7 cells, knockdown of ERK1 significantly prevented the appearance of the senescent markers, whereas knockdown of ERK2 had no effect. Simultaneous knockdown of ERK1 and ERK2 was partially effective.

These results show that knockdowns of ERK1 and/or ERK2 prevented manifestation of unbalanced growth regardless of whether cells were selected with puromycin or not.

3.6. Location of phosphorylated ERK1/2

We examined the localization of phosphorylated ERK1/2 (pERK1/2) in HeLa and TIG-7 cells during culture with excess thymidine by indirect immunofluorescence staining (Supplementary Fig. 1). During culture in normal medium, pERK1/2 located predominantly in the nucleoplasm in both cell types. When the cells were added with excess thymidine for one week, pERK1/2 was found homogeneously in the cytoplasm in both cell types. A similar patterns was observed in TIG-7 cells undergoing replicative senescence.

4. Discussion

The RNAi experiments shown here confirmed our previous study [17] in which U0126, a MEK1/2 inhibitor, suppressed loss of colony forming ability induced by excess thymidine in HeLa and TIG-7 cells. Moreover, ERK1 and ERK2 were shown to mediate unbalanced growth in HeLa and TIG-7 cells, respectively. In other

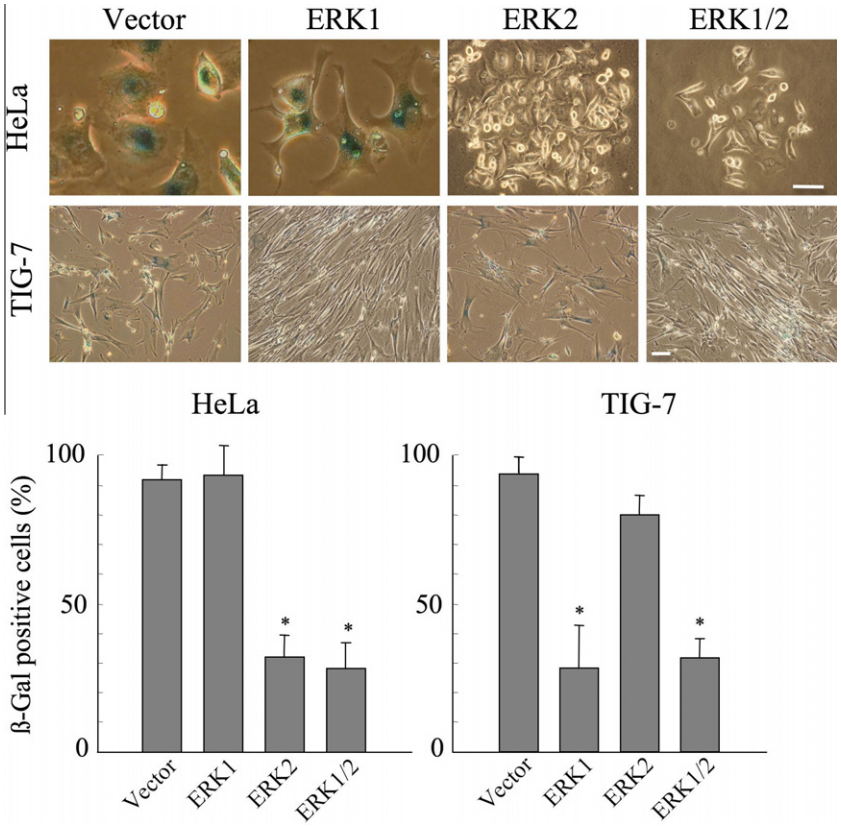

Fig. 4. Effects of knockdown of ERK1, ERK2, or both on cellular morphology. Cells were transfected with the plasmids indicated in Fig. 1, cultured in normal medium for 2 days, and then cultured with or without excess thymidine for 8 and 10 days in TIG-7 and HeLa cells, respectively, as described in Section 2. Upper panel, photographs of cells stained with the senescence-associated β-galactosidase; lower panel, histograms of the numbers of cells positive for the β-galactosidase ± SE ($n = 3$); $^*P < 0.01$ in comparison with vector control cells. Bars, 50 μm. Three independent experiments gave similar results.

experiments, knockdown of both ERK1 and ERK2 was found to be necessary to suppress unbalanced growth induced by transfection of TIG-7 cells with plasmid encoding activated Ras (Kobayashi, Y. et al., unpublished data). These results suggest that the roles of ERK1 and ERK2 depend on cell types and inducers. Recently, RNAi experiments have revealed that ERK1 and ERK2 determine hepatocyte survival and proliferation, respectively, and dual inhibition stabilizes a highly differentiated state of hepatocytes [18].

ERK1/2 is activated by multiple DNA damage stimuli, and facilitates DNA damage-induced cell cycle arrest or apoptosis in response to DNA insults [15,19,20]. This DNA damage response is mediated by activation of the ATR network [21,22] to prevent the collapse of replication forks during S phase of the cell cycle. Inhibitors of ERK1/2 such as U0126 and PD98059 were shown to attenuate apoptosis induced by a high dose of etoposide, adriamycin, or UV, and partially release G_2/M cell cycle arrest induced by etoposide [23].

Knockdown of ERK1 or ERK2 is also shown to reduce etoposide-induced CDC25C S216 phosphorylation and significantly compromise etoposide-induced G_2/M arrest in MCF7 cells [24]. Conversely, enforced activation of ERK1/2 by overexpression of activated MEK-1 sensitized cells to DNA damage-induced apoptosis [23]. As CDC2 kinase activity is required for mitosis, ERK1 and ERK2 are thought to play important roles in preventing mitotic entry in response to DNA damage through CDC2 Y15 phosphorylation.

If ERK1/2 responds to multiple DNA damage stimuli, then is excess thymidine recognized as DNA damage? At present, we have no convincing evidence that it works as DNA damage. Senescent cells accumulate at multiple points of the cell cycle, namely, G_1, S, and G_2, depending on inducers. It is well known that single thymidine block arrests cells at middle to late S-phase, and double blocks early S-phase. Recently, we have found that an inhibitor of Chk1, which is activated by ATR, but not of Chk2, which is activated by ATM, destabilizes unbalanced growth and leads to cell death (Nozawa, M. et al., unpublished data). Therefore, a possibility arises that pool imbalance of DNA precursors retard movement of DNA replication forks and the resulting longstanding presence of single-stranded DNA *per se* may active ERK1/2 as false DNA damage.

On the other hand, U0126, an inhibitor of MEK1/2, blocked cell swelling with accumulation of protein unaltered in both HeLa and TIG-7 cells [7]. It is therefore evident that ERK1/2 has a role in cell volume regulation, i.e., regulatory volume increase, in response to hypertrophic stimuli besides DNA damage stimuli. Taken together, ERK1/2 has dual roles in unbalanced growth, one in checking DNA replication during S–G_2 phase and the other in cell swelling.

Historically, unbalanced growth was observed in many cell types and with various inducers. Any agents or genetic conditions that directly or indirectly inhibit DNA replication lead to unbalanced growth. Multiple signaling pathways seem to mediate these processes. Therefore, identification of each role of the pathways is helpful for understanding diverse facets of unbalanced growth leading to senescence. However, senescence can occur without experiencing unbalanced growth in very special cases as exemplified by 5-bormodeoxyuridine. This thymidine analog changes DNA conformation and destabilizes nucleosome positioning, leading to collapse of the nuclear envelope as is seen in swelled cells [25]. Finally, these experiments imply that unbalanced growth and senescence can be prevented suggesting that aging processes can be controlled.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.006.

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