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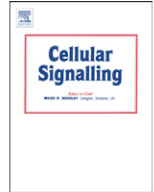

Depletion of ERK2 but not ERK1 abrogates oncogenic *Ras*-induced senescence

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ABSTRACT

In response to oncogenic activation, cells initially undergo proliferation followed by an irreversible growth arrest called oncogene-induced senescence (OIS), an endogenous defense mechanism against tumorigenesis. Oncogenic activation of ERK1/2 is essential for both the initial phase of cellular proliferation as well as subsequent premature senescence, but little is known about the specific contribution of ERK1 versus 2 to OIS. Here we show that depletion of ERK2 but not ERK1 by shRNA knockdown in MEFs leads to continuous proliferation bypassing senescence even in the presence of oncogenic $HRAS^{V12}$. Upon depletion of ERK2, induction of both $p19^{Arf}$ and $p16^{Ink4a}$ was significantly compromised after oncogenic $HRAS^{V12}$ expression, attenuating activation of the key tumor suppressors p53 and pRb. Here we demonstrate that ERK2 but not ERK1 indirectly regulates $p19^{Arf}$ and $p16^{Ink4a}$ both at the transcriptional and translational level. Oncogenic *Ras* expression after ERK2 knockdown downregulates Fra-1 and c-Jun, components of the activator protein-1 (AP-1) heterodimer essential for transactivation of $p19^{Arf}$. Similarly we show a significant decrease in the activation of p38 MAPK and ETS family members which are involved in the induction of $p16^{Ink4a}$. The role of ERK2 in translational regulation is observed by the lack of tuberin (TSC2) and p70 ribosomal S6 kinase 1 (p70S6K1) phosphorylation, components of the mTOR pathway, which enhances $p19^{Arf}$ mRNA translation during oncogenic *Ras*-induced senescence. These observations suggest that ERK2 but not ERK1 contributes to upregulation of $p19^{Arf}$ and $p16^{Ink4a}$ in a transcription- and translation-dependent manner during oncogenic *Ras*-induced senescence. Taken together, our data indicate that ERK2 is the key ERK isoform mediating the senescence signaling pathway downstream of oncogenic *Ras*.

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1. Introduction

The aberrant proliferative signals driven by the activation of oncogenes such as *Ras* or loss of tumor suppressors such as neurofibromin 1 (*NF1*) initially fuel cellular proliferation but subsequently trigger premature cellular senescence, a permanent type of growth arrest. This process has been coined oncogene-induced senescence (OIS) and has been observed in numerous cell types *in vitro* and premalignant neoplastic lesions *in vivo* [1,2]. The widespread occurrence of senescence in benign or premalignant tumors both in mouse models and humans has suggested that senescence functions as a cell-intrinsic tumor suppression mechanism through the removal of cells with neoplastic lesions with the potential to progress to malignancy [3–9].

Previous studies have shown that signaling pathways triggered by oncogenic activation are necessary for the initial burst of cellular proliferation but also required for induction of OIS [10–12]. While multiple mechanisms for OIS have been identified depending on the specific cell type, tissues and oncogenic mutations [1,2], it is clear that OIS requires upregulation of $p19^{Arf}$ and $p16^{Ink4a}$ which in turn leads to stabilization of p53 and dephosphorylation of retinoblastoma tumor suppressor protein (pRb), major downstream effectors of senescence [10–14]. However the molecular basis underlying the of coupling proliferative signals to senescent pathways during OIS is still poorly understood.

Oncogenic *Ras* mutant promotes cell proliferation through activation of both RAF/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/AKT pathways [15]. While activation of the PI3K/AKT pathway suppresses oncogenic *Ras*-induced senescence [16], constitutive hyperactivation of MEK/ERK pathways is crucial for *Ras*-induced senescence, with studies suggesting that the outcome of oncogenic *Ras* activation depends on both the magnitude and duration of MEK/ERK activity [6,11,17,18]. Such observations suggest that MEK/ERK signaling may sense inappropriate proliferative signals derived from

Abbreviations: OIS, oncogene-induced senescence; MEF, mouse embryonic fibroblast; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase; PI3K, phosphatidylinositol 3-kinase; pRb, retinoblastoma tumor suppressor protein; SA-β-Gal, senescence associated β-galactosidase; PML, promyelocytic leukemia; γ-H2AX, phosphorylated histone H2AX; AP-1, activator protein-1; Dmp1, cyclin D-interacting Myb-like protein 1; mTOR, mammalian target of rapamycin; TSC2, tuberous sclerosis 2 or tuberin; p70S6K1, p70 ribosomal S6 kinase 1; EMT, epithelial-to-mesenchymal transformation; shRNA, short hairpin RNA; NS, non-silencing; GFP, green fluorescence protein.

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oncogenic *Ras* and trigger senescence as a fail-safe mechanism to limit the transforming potential of abnormal *Ras* signaling. However mechanisms that monitor the integrity of MEK/ERK signaling and switch from its proliferative to senescent activity remain largely undetermined.

Despite the critical role ERK plays in both cell proliferation and premature senescence as a consequence of oncogenic *Ras activation*, little is known about the individual contribution of its two isoforms, ERK1 and ERK2, to control of those processes. Since these evolutionary conserved kinases exhibit high sequence homology, have similar biochemical properties *in vitro*, share activators and substrates, and are coexpressed in a majority of cell lines and tissues examined, they have been considered to be functionally redundant and thus compatible to each other in directing most biological processes [19–25]. However, accumulating evidence indicates that each ERK isoform executes distinctive biological functions under certain contexts [26–33]. Indeed, a study using immortalized mouse fibroblasts has shown that individual depletion of each ERK isoform differentially affects transforming activity of oncogenic *Ras* [31]. In addition, a recent study has demonstrated specific contribution of ERK2 to *Ras*-induced epithelial-to-mesenchymal transformation (EMT) [33]. Such observations raise the possibility that ERK1 and ERK2 play distinct roles in mediating oncogenic *Ras* signaling.

Here we show that depletion of ERK2 but not ERK1 abrogates premature senescence induced by oncogenic *Ras* in mouse embryonic fibroblasts (MEFs). Silencing of ERK2 attenuates the induction of $p19^{Arf}$ and $p16^{Ink4a}$ through altering transcriptional and translational pathways known to positively regulate their expression. Together, our results demonstrate that ERK2 but not ERK1 is necessary for driving senescence pathways in response to oncogenic *Ras* activation.

2. Materials and methods

2.1. Cell culture

MEFs were isolated from wild-type C57BL/6 embryos at embryonic day 13.5 as previously described [10]. Human embryonic kidney (HEK) 293T/17 cells were obtained from ATCC (Rockville, MD, USA). All cultures were maintained in high-glucose DMEM (WelGENE, Seoul, Korea) supplanted with 10% FBS (Hyclone, Logan, UT, USA), 2 mM L-glutamine (Gibco BRL, Grand island, NY, USA), 50 U/ml penicillin, and 50 μg/ml streptomycin (WelGENE).

2.2. Lentiviral shRNA production and infection

Lentiviral pGIPZ vectors encoding microRNA-adapted short hairpin RNAs ($shRNA^{miR}$s) against either *Erk1* or *Erk2* and non-silencing (NS) $shRNA^{miR}$ as a negative control were purchased from Open Biosystems (Thermo Scientific, Waltham, MA, USA). To generate lentiviral vectors co-expressing oncogenic $HRAS^{V12}$ with shRNAs, *GFP* gene in pGIPZ vector was replaced with $HRAS^{V12}$ gene derived from pBabe-puro-$HRAS^{V12}$ vector [10]. The sequences of shRNA are as follows: *Erk1*, 5′-TAATGATGCAATTAAGGTC-3′; *Erk2*, 5′-TCAACTTCAATCCTCTTGT-3′; NS, 5′-TCTCGCTTGGGCGAGAGTAAG-3′.

For lentiviral production, HEK 293T/17 cells were co-transfected with Δ8.2 lentiviral construct encoding *gag-pol* and *rev*, pVSV-G expressing vesicular stomatitis virus envelope G protein (VSV-G), and pGIPZ vectors using TransIT-293 transfection reagent (Mirus Bio LLC, Madison, WI, USA). The media containing viruses was then harvested at 48 h after transfection and used to infect MEFs as previously described [34]. MEFs at early passage were plated in 10 cm tissue culture plates (Nunc, Rochester, NY, USA), incubated with virus-containing media for overnight, selected for 3 days with 2 μg/ml puromycin (Gibco BRL) and used for experiments.

2.3. Proliferation assay

5×10^4 MEFs infected with lentiviruses were then plated in hexiplicate in 6 well tissue culture plates (Nunc) and the cell growth was monitored by counting cell numbers every 2 days using a Multisizer 4 Coulter counter (Beckman Coulter, Fullerton, CA, USA). Cumulative population doublings were determined using the formula Log N/Log2, where N is the number of cells counted divided by the initial number of cells plated, as previously described [35].

2.4. Senescence associated β-galactosidase (SA-β-Gal) staining

5×10^4 infected MEFs were seeded in triplicate in 6 well tissue culture plates. At day 6, cells were fixed, stained for SA-β-Gal activity using senescence cells histochemical staining kit (Sigma, St. Louis, MO, USA) following the manufacturer's instructions and observed under bright field at 100–200× magnification.

2.5. Immunofluorescence

10^4 MEFs infected with lentiviruses were seeded in triplicate in 4 well chamber slides (Nunc). At day 6, cells were fixed with 4% formaldehyde (Sigma) in PBS for 15 min, permeabilized with 0.2% Triton X-100 (Sigma) for 15 min, blocked with 10% normal goat serum (Millipore, Billerica, MA, USA) in PBS for 30 min at RT, incubated with antibodies against PML (Millipore) or phospho-H2AX (S139) (Cell Signaling Technology, Danvers, MA, USA) diluted in blocking solution for 1 h at RT, further incubated with secondary antibodies conjugated with Alexa Fluor-594 (Invitrogen, Carlsbad, CA, USA) for 1 h at RT, counterstained with Hoechst dye (Sigma) to visualize cell nuclei and examined under a fluorescence microscope (Carl Zeiss, Jena, Germany).

2.6. Immunoblotting

3×10^5 infected MEFs were plated in 10 cm tissue culture plates, harvested, lysed with RIPA buffer containing 10 mM NaF, 1 mM Na_3VO_4, 1 μM DTT, 1 mM PMSF (Sigma) and protease inhibitor cocktail (Roche, Mannheim, Germany), separated in SDS–PAGE, transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA) and probed for phospho-ERK (Thr202/Tyr204), total ERK, p53, phospho-Fra-1 (Ser265), phospho-c-Jun (Ser63/Ser73), phospho-MEK (Ser217/Ser221), MEK, phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK (Cell Signaling), HRAS, $p21^{CIP1}$, $p16^{INK4a}$, Fra-1, c-Jun, ETS1, ETS2, TSC2, p70S6K1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), pRb (BD Pharmingen, San Diego, CA, USA), $p19^{ARF}$ (Novus Biological, Littleton, CO, USA), Dmp-1 (AVIVA System Biology, San Diego, CA, USA), phospho-ETS1 (Thr38) (Assay Biotech, Sunnyvale, CA, USA), phospho-ETS2 (Thr72) (Invitrogen), phospho-TSC2 (Ser664) (Abcam, Cambridge, UK), phospho-p70S6K1 (Thr389) (Millipore) and β-actin (Sigma, St. Louis, MO, USA) as a loading control. To measure relative gel densities, band densities were quantified with densitometric analysis using ImageJ software (NIH) and then normalized to β-actin or control protein bands as previously described [36,37].

2.7. RNA isolation and real-time PCR

Total RNAs were extracted from MEFs infected with lentiviruses using TRIZOL reagent (Invitrogen), purified with RNeasy columns (QIAGEN, Hilden, Germany) and reverse transcribed to cDNAs with oligo (dT) primers using ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA) following the manufacturers' instructions. Quantitative real-time PCR was then performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with Power SYBR Green PCR master mix (Applied Biosystems). The sequences of primers used for real-time PCR are as follows: murine $p19^{ARF}$ forward, 5′-GCTCTGGCTTTCGTGAACATG-3′; murine $p19^{ARF}$

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