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NM23H2 inhibits EGF- and Ras-induced proliferation of NIH3T3 cells by blocking the ERK pathway

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ABSTRACT

The NM23 family proteins are involved in a variety of biological processes including tumor metastasis, development, and differentiation; however, their functions in the regulation of cellular proliferation are poorly understood. We have investigated the role of one NM23 family protein, NM23H2, in the regulation of cellular proliferation directed by the extracellular signal regulated kinase (ERK) pathway. The activity of ERKs was enhanced by knockdown of endogenous NM23H2 and blocked by overexpression of NM23H2 in both NIH3T3 and HEK293 cells. Additionally, the epidermal growth factor (EGF)- and oncogenic Ras(G12R)-induced proliferation of both HEK293 and NIH3T3 cells was reduced by NM23H2 overexpression. Furthermore, activation of Raf-1, MEK and the ERKs by either EGF or Ras(G12R) was inhibited by NM23H2 overexpression. Together, our data indicate that NM23H2 is a negative regulator of cellular proliferation stimulated by EGF- and Ras-mediated activation of the ERK pathway.

1. Introduction

The NM23s are a family of proteins involved in a variety of biological functions including cellular proliferation, differentiation, and development [1]. The NM23 family proteins, also known as nucleoside diphosphate kinases (NDPK), are highly conserved from the bacteria to human [2]. Eight genes in the human genome encoding NM23 family proteins have been reported [3]. Two representative members of the NM23 family, NM23H1 and NM23H2, share 88% amino acid similarity, yet they possess some non-overlapping cellular functions. NM23H1 is known to function as a metastasis suppressor [4,5], and it appears to be involved in the inhibition of colonization and motility but not in the regulation of cellular proliferation [6,7]. In contrast, NM23H2, first identified as a purine-binding transcription factor (PuF), functions as a transcriptional regulator of the *c-myc* proto-oncogene [8]. However, several other functions have been reported for NM23H2. For example, NM23H2 regulates endocytosis of G protein-coupled receptors (GPCRs) through direct interactions with the receptor [9]. NM23H2 has been reported to function in β1 integrin-mediated cell adhesion by binding the integrin cytoplasmic domain-associated protein 1α (ICAP-1α) [10]. NM32H2 has been shown to interact with protein phosphatase 2A (PP2A) [11], a known regulator of the ERK pathway component Raf-1 [12,13]. The ERK pathway is a major signaling pathway involving cellular proliferation, and transmits its signal though Ras → Raf → ERK cascade [14]. Roles of NM23H2 in the regulations of the ERK pathway and proliferation are not identified although NM23H1 known to interact with Kinase Suppressor of Ras1 (KSR1), a scaffold protein required for activation of the ERK pathway [15]. Moreover, a relationship between Ras and NM23H2 are not identified.

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Abbreviations: EGF, epidermal growth factor; ERK, extracellular signal regulated kinase; NDPK, nucleoside diphosphate kinase; NM23, non-metastatic clone 23.

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In this study, we have investigated the role of NM23H2 in regulating the ERK signaling pathway. We have used a combination of knockdown and overexpression of NM23H2 to determine its function in both Ras-ERK-regulated cellular proliferation and ERK-mediated activation of the Elk-1 transcription factor. Taken together, the data presented in this study suggest that NM23H2 is a negative regulator of the Ras-ERK pathway, and that it functions to block cellular proliferation.

2. Materials and methods

2.1. Cell lines and culture

NIH3T3 and HEK293 cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). The NIH3T3 cell line containing doxycycline-inducible H-*ras* (G12R) (NIH-Ras) has been described in a previous study [16]. NIH3T3, HEK293, and NIH-Ras G12R cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Life Technologies, Grand Island, NY) in 5% CO_2 at 37 °C. The transfections were performed on cells at 70% confluence with Lipofectamine Plus reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA).

2.2. Plasmid and siRNA preparation

The pcDNA3.0-NM23H2 and pGEX-NM23H2 plasmids were provided by Dr. S. K. Chae (Paichai University, Dae-Jeon, Korea) [17]. To construct pd2EGFP-NM23H2, a 0.5 kb fragment of the *NM23H2* cDNA was amplified from pcDNA3.0-NM23H2 by polymerase chain reaction (PCR) using the following primers: sense, CTCGAGATGGCC AACCTGGAGCG; anti-sense, GAATTCTTTCATAGACCCAGT-CATGAG (reverse). The PCR was performed with 25 cycles of 30 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C. The PCR products were sub-cloned into T-vector (Real Biotech Corporation, Taipei, Taiwan). Finally, the 0.5 kb *NM23H2* fragment in T-vector was excised with XhoI and EcoRI, and sub-cloned into pd2EGFP-N1 (Clontech, Palo Alto, CA) cut with the same enzymes. The pcDNA3.1-H-ras was described in previous study [16]. The small interfering RNA (siRNA) sequences for mouse *NM23M2* (GenBank Accession No. NM_008705) were designed using a siRNA template design tool (Ambion, Austin, TX). The *NM23M2* mRNA target sequences were: 5′-AAAGAGATCCATCTGTGGTTT-3′ (383–404) and 5′-AACTGATCGACTACAAGTCTT-3′ (414–434). Each siRNA was synthesized using a Silencer™ siRNA construction kit (Ambion).

2.3. Generation and purification of anti-NM23H2 antibody

NM23H2 proteins were expressed in *Escherichia coli* (*E. coli*) BL21 (DE3) pLysS, and bound to glutathione-agarose beads as described previously [18]. The resin-bound GST-NM23H2 was eluted after 5–6 h incubation with GST-cleavage buffer (20 mM Tris–HCl, pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$) containing 50 U thrombin. The eluted NM23H2 proteins attained purities of greater than 95%. The polyclonal antibody for NM23H2 was generated by immunization rabbits as previously described [18]. Total rabbit serum containing anti-NM23H2 antibody was bound to immobilized Protein A beads (Pierce, Rockford, IL), and washed with 5 bead volumes of binding buffer (10 mM NaH_2PO_4, 0.9% NaCl, 10 mM EDTA, pH 7.0). The bead-bound anti-NM23H2 antibody was eluted with elution buffer (0.5 M acetic acid, pH 3.0), and was neutralized by adding 25% (v/v) of a neutralization solution (2 M Tris–HCl, pH 9.0).

2.4. Western blot analysis

Cells grown in a 35 cm dish were transfected with plasmids or siRNAs using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. Cells were harvested 36 h after transfection. For induction of H-Ras (G12R), stable NIH-Ras (G12R) cells were treated with 0.5 μg/ml of doxycycline (Dox) for 12 h before harvesting cells. Attached cells were harvested and lysed in a RIPA buffer (50 mM Tris, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml aprotinin mix, 1 mM Na_3VO_4, 1 mM NaF) for 10 min on ice. Cell debris was then removed by centrifugation at 12,000 rpm for 30 min at 4 °C, and the supernatant was used as total protein lysate. Twenty-five μg of total protein lysate from each sample was separated on a 12% SDS–PAGE gel and then subjected to Western blot analysis [19]. The anti-phospho-ERK (p-ERKs), anti-ERK, and anti-β-actin antibodies were purchased form Santa Cruz Biotechnology (Santa Cruz, CA). The anti-GFP, anti-p-MEKs and anti-α-tubulin antibodies were obtained form Calbiochem (San Diego, CA). Anti-Pan-Ras clone RAS10 and anti-p-Raf-1 (Ser-338) were purchased from Upstate Biotechnology (Lake Placid, NY). The horse radish peroxidase-conjugated secondary antibodies were also purchased form Santa Cruz Biotechnology. The bands were visualized using enhanced chemiluminescence (ECL) and the images were captured by luminescent image analyzer LAS-3000 (Fuji Film, Tokyo, Japan). The quantification of the protein bands were performed using Fuji Multi Gauge v3.0 software.

2.5. Flow cytometric analysis

NIH3T3 cells were plated onto coverslips at a confluency of 40%. The cells were transfected with pd2EGFP-N1 or pd2EGFP-NM23H2 together with the specified siRNAs, and then labeled with BrdU as previously described [16]. For quantification of the BrdU incorporation, the cells were fixed with 3.7% paraformaldehyde for 10 min at room temperature, permeabilized with permeabilization buffer (phosphate buffered saline (PBS) without Mg^{2+} and Ca^{2+}, 1% FBS, 0.1% saponin), and then incubated with anti-BrdU monoclonal antibody (DAKO, Glostrup, Denmark) (1:100) followed by Alexa Fluor® 488-conjugated goat anti-mouse IgG (1:200) (Molecular Probes, Eugene, OR). The cell cycle profile was determined using a Becton Dickinson FACS Caliber and analyzed with Cell Quest Version 3.3 (Becton-Dickinson Immunocytometry Systems, San Jose, CA).

2.6. Immunocytochemistry

NIH-Ras (G12R) cells were plated onto coverslips at a confluency of 40%. The cells were transfected with pd2EGFP-N1 or pd2EGFP-NM23H2 together with the specified siRNAs. For induction of H-ras (G12R), cells were treated with 0.5 μg/ml Dox for 12 h. Cells were then grown for 4 h in DMEM containing 20 μM BrdU before immunocytochemical analyses. The cells were fixed in 70% ethanol at room temperature for 30 min, permeabilized with PBS containing 0.1% Triton X-100 at room temperature for 30 min, and incubated with 2 N HCl for 30 min. The cells were then blocked with PBS containing 10% normal goat serum (NGS), incubated with anti-BrdU monococlonal antibody (1:100) for 16 h at 4 °C, and then incubated for 1 h with Alexa Fluor® 555-conjugated goat anti-mouse IgG (1:250) (Molecular Probes). The cells were then treated with 1 μM 4′, 6′-diamidine-2-phenylindole dihydrochloride (DAPI) in PBS for 10 min, and the cells were washed in distilled water. Samples were mounted for imaging on a Radiance 2100 Laser Scanning System (Bio-Rad, UK).

2.7. Reporter assays

For reporter gene assays, HEK293 cells were seeded into 6-well dishes at 2×10^5 cells per well. Cells were co-transfected with either pcDNA3.1 or pcDNA3.1-NM23H2 and the pFA2-ELK1/pFRLuc vector system [20]. Transfection efficiencies were normalized by co-transfection with 0.5 μg of pCMV β-gal reporter (Clontech, Palo Alto, CA). Twenty-four hours after transfection, cells were treated with 20 ng/ml EGF and then were incubated for an additional 8 h. The cells were then rinsed twice with ice-cold PBS, harvested and resuspended in reporter lysis buffer (Promega, Madison, WI) for luciferase activity assay. Luciferase activities were normalized to β-galactosidase levels as an internal control.

3. Results

3.1. NM23H2 inhibits EGF-induced proliferation of NIH3T3 cells

We first wished to investigate the functions of NM23H2 on cellular proliferation. To determine the effect of NM23H2 overexpression on cellular proliferation, we transfected NIH3T3 cells with a plasmid driving high-level expression of NM23H2, labeled S-phase cells with BrdU, and quantified BrdU incorporation by FACS. The numbers of cells incorporated BrdU, decreased from 40.81% in control cells to 29.18% in cells overexpressing NM23H2 (Fig. 1A). When NIH3T3 cells were transfected with a NM23H2-GFP, we observed a decrease in BrdU-positive cells from 24.6% to 15.5% in GFP-expressing cells (Fig. 1B; upper panel). The percentage of BrdU-positive cells, which increased from 24.6% to 50.9% following EGF stimulation in control cells, was reduced to 36.3% in cells transfected with NM23H2-GFP. In contrast, the fraction of BrdU-positive cells, under both basal and EGF-stimulated conditions, was up-regulated by siRNA-mediated knockdown of endogenous NM23M2 (Fig. 1B; lower panel). Together, these data suggest NM23H2 negatively regulates EGF-stimulated proliferation of NIH3T3 cells.

3.2. NM23H2 inhibits H-Ras(G12R)-induced proliferation of NIH3T3 cells

To further investigate the role of NM23H2 in the regulation of proliferation directed by the Ras-ERK pathway, we measured the effect of NM23H2 on proliferation induced by oncogenic H-Ras(G12R). We used a NIH3T3-derived cell line, NIH-Ras, engineered to expresses H-Ras(G12R) upon doxycycline (Dox) treatment. When NIH-Ras cells were treated with Dox, the percentage of BrdU-positive cells increased from 34.3% (untreated) to 51.6% (Dox-treated) (Fig. 2A; qualitative results are shown in right panel). However, when NIH-Ras cells were first transfected with NM23H2-GFP, the Dox-stimulated increase in BrdU-positive cells was completely abolished (Fig. 2A). In contrast, the fraction of BrdU-positive cells was increased following transfection with a *NM23H2* siRNA. Moreover, the percentage of BrdU-positive cells, which was increased by Dox-mediated H-Ras(G12R) induction, showed a marked further increase following transfection with *NM23H2* siRNA (Fig. 2A). In addition, we found that when NIH-Ras cells were transfected with NM23H2-GFP, no incorporation of BrdU was detected in any of the GFP-positive cells (Fig. 2B). These data indicate that NM23H2 inhibits proliferation induced by oncogenic H-Ras in NIH3T3 cells.

3.3. NM23H2 negatively regulates the ERK pathway downstream of EGF and Ras

Using Western analysis to measure the level of p-ERKs, the active forms of these kinases [21], we found that the level of p-ERKs was dose dependently reduced by transfection with NM23H2 (Fig. 3). Conversely, the level of p-ERKs was increased by siRNA-mediated knockdown of endogenous NM23H2. This suggests that NM23H2-mediated suppression of the ERK pathway. We next examined the effects of NM23H2 on EGF-stimulated activation of MEK and Raf-1, two kinases that function upstream of the ERKs. As expected, EGF stimulation resulted in increased activities of both Raf-1 and MEK, and this was concomitant with ERK activation (Fig. 3B). Interestingly, the activity of each of these ERK pathway kinases was reduced following transfection of NM23H2 into HEK293 cells (Fig. 3B). Similarly, the Ras(L61)-induced activation of Raf-1, MEK, and ERK were also reduced by transfection of NM23H2 into HEK293 cells (Fig. 3C). These data suggest that NM23H2 inhibits activation of the ERK pathway at the level of Raf-1 or farther upstream.

To identify the mechanism of inhibition of EGF- and Ras-mediated proliferation by NM23H2, we examined the status of the ERK pathway in response to changes in the expression level of NM23H2.

Activation of the ERK pathway ultimately results in activation of the ELK-1 transcription factor [20,22]. We found that overexpression of NM23H2 inhibited both EGF- and Ras(G12R)-mediated stimulation of an Elk-1-dependent promoter in HEK293 and NIH-Ras cells (Fig. 4A and B). These data indicate that NM23H2 suppresses activation of the ERK pathway in response to EGF stimulation or expression of oncogenic Ras.

4. Discussion

The NM23 family proteins regulate growth regulatory signals induced by transforming growth factor-β1 (TGF-β1), nerve growth factor (NGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF-1) [23]. NM23 proteins are abundant in highly metastatic cell lines [24,25], and they have been shown to regulate tumor metastasis [8,26–28]. NM23H2 was originally identified as a purine-binding transcription factor, and it has also been suggested to be a suppressor of tumor metastasis [8].

In this study, we have investigated the role of NM23H2 in cellular proliferation directed by EGF- or oncogenic H-Ras-stimulation of the ERK pathway. The ERK signaling pathway regulates mitogen-dependent cellular proliferation, and transient activation of the ERK has been used as a marker for cell proliferation [20,29]. Growth factor signaling to the Raf-1 → MEK → ERK cascade requires activation of the Ras signaling molecule [30].

We found that overexpression of NM23H2 blocked cellular proliferation, whereas proliferation was enhanced by knockdown of NM23H2, indicating that this protein negatively regulates cellular proliferation. NM23H2 inhibited both EGF- and oncogenic H-Ras-stimulated proliferation in HEK293 and NIH3T3 cells, suggesting that it could sup-

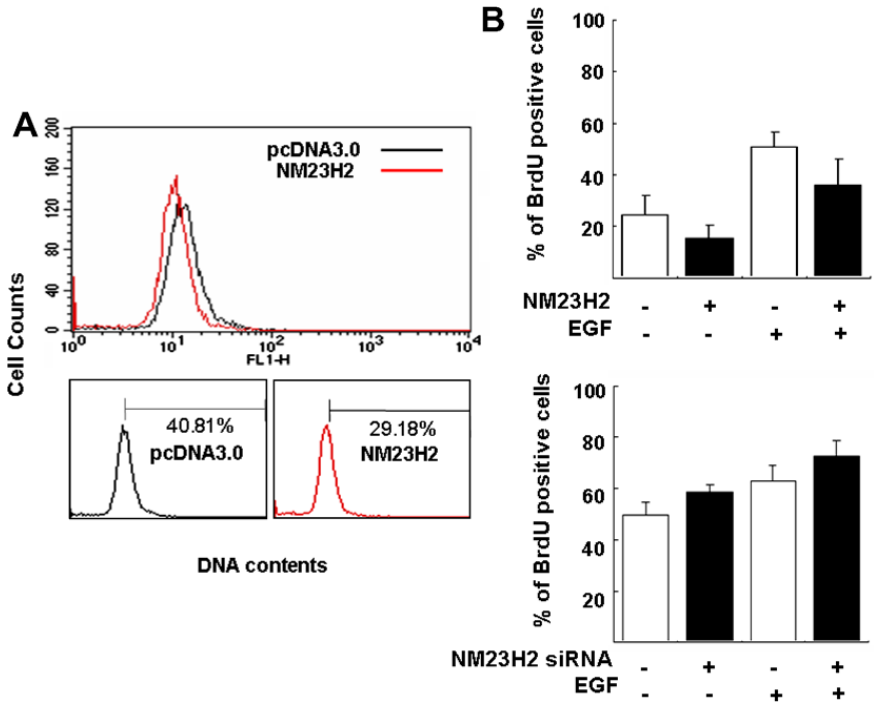

Fig. 1. Effect of NM23H2 on EGF-induced proliferation of NIH3T3 cells. (A) NIH3T3 cells were transfected with 1 μg of either pcDNA3.0 or pcDNA3.0-NM23H2. The cells were labeled with BrdU as previously described [16]. Cells were fixed, permeabilized, and incubated with BrdU antibody followed by Alexa Fluor®488-conjugated goat anti-mouse IgG. The relative intensities of Alexa Fluor®488-conjugated BrdU were measured by FACS. (B) NIH3T3 cells were grown on coverslips and transfected with 0.5 μg of pd2EGFP-N1, NM23H2-GFP, or 1 μg of *NM23M2* siRNA for 36 h. The cells were stimulated with 20 ng/ml EGF 24 h, labeled with BrdU, and harvested for immunocytochemical analyses. The relative percentages of BrdU-positive cells compared to total cells (stained with DAPI) were counted and quantified as described previously [16].

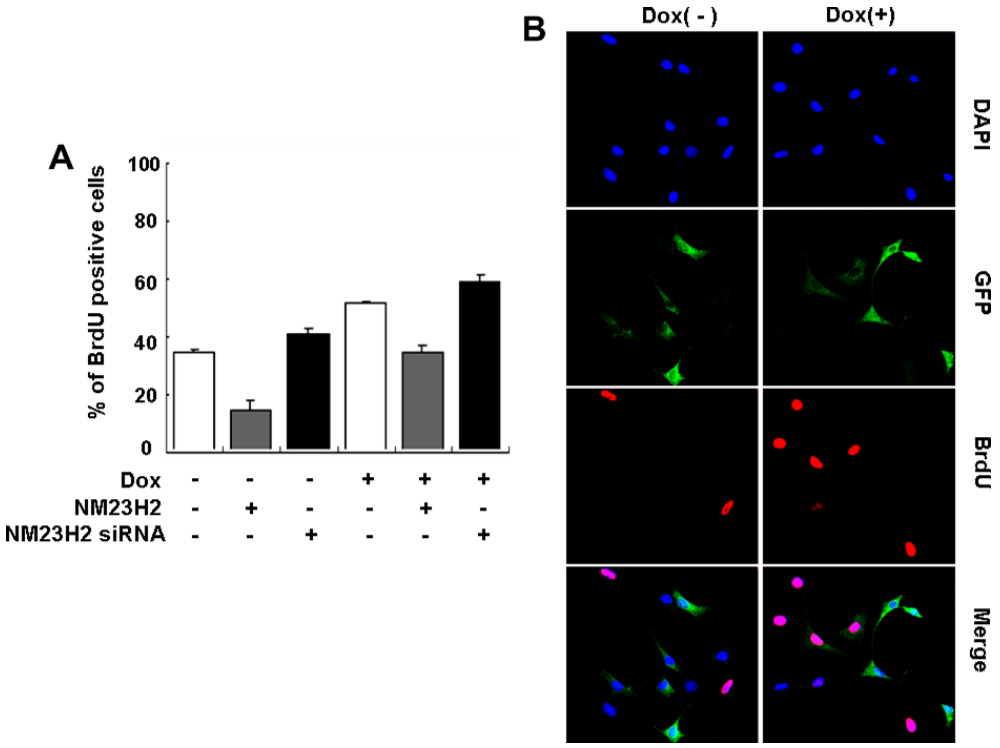

Fig. 2. Effect of NM23H2 on H-Ras-induced proliferation of NIH3T3. (A) NIH-Ras(G12R) cells [16] were transfected with 0.5 μg of pd2EGFP-N1, NM23H2-GFP, or 1 μg of si*NM23M2* for 36 h. The cells were treated with or without 0.5 μg/ml of Dox for 12 h before performing immunocytochemical analysis with anti-GFP and anti-BrdU antibodies [34]. Cells were labeled with 20 μM BrdU for 4 h prior to fixation. Cell nuclei were stained with DAPI. Cells with BrdU signal in the nucleus were scored as BrdU-positive. These experiments were performed at least three times. Error bars indicate the standard deviations of three independent experiments. The right panels are representative images of immunocytochemical analyses.

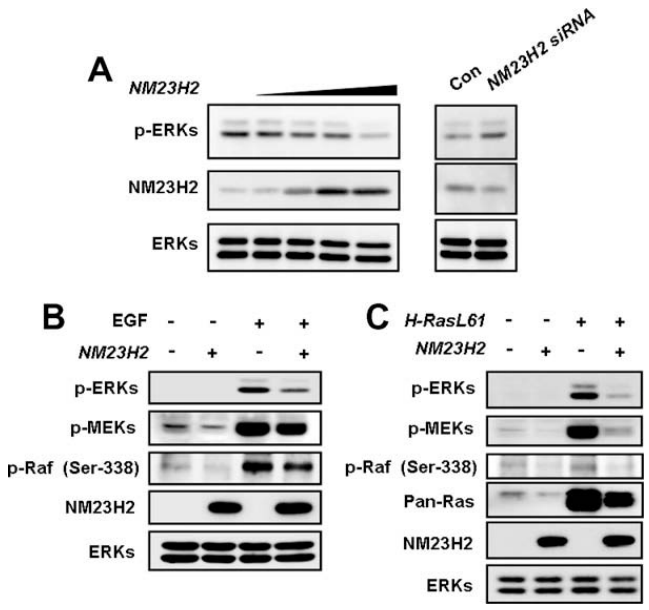

Fig. 3. NM23H2 blocks EGF and Ras(L61)-mediated activation of ERKs and their upstream kinases. (A) Left, NIH3T3 cells were transfected with 1 μg of pcDNA3.0 or with 0.125, 0.25, 0.5, or 1 μg of pcDNA3.0-NM23H2. Right, NIH3T3 cells were transfected 1 μg of control siRNA (Con) or siRNAs for mouse *NM3M2*. (B) HEK293 cells were transfected with 0.5 μg of pcDNA3.1 or pcDNA3.1-NM23H2 for 36 h. Cells were treated with or without 20 ng/ml of EGF for 30 min before harvesting for Western blot analyses. (C) HEK293 cells were transfected with pcDNA3.1 and pcDNA3.1-NM23H2 together with 0.5 μg of pMT3-Ras-L61 for 36 h. Western blots of total cell lysates were performed with anti-p-ERK, anti-p-MEK, anti-p-Raf-1 (Ser-338), anti-Pan Ras, anti-NM23H2, or anti-ERK antibody.

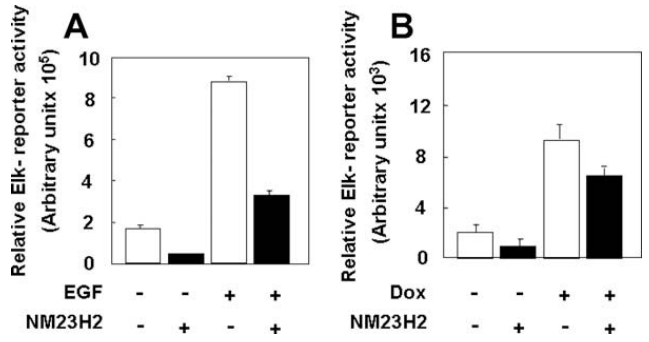

Fig. 4. NM23H2 EGF- in or Ras(G12R)-directed activation of an Elk-1 responsive reporter in HEK293 and NIH3T3-Ras cells. (A) HEK293 cells were transfected with pcDNA3.1 and pcDNA3.1-NM23H2 together with 0.5 μg of pFR-Luc, pCMV β-gal reporter, and 50 ng of pFA2-Elk-1. Cells were stimulated with 20 ng/ml of EGF 8 h before harvesting. (B) NIH3T3-Ras cells were transfected with pcDNA3.1 and pcDNA3.1-NM23H2 together with 0.5 μg of pFR-Luc, pCMV β-gal reporter, and 50 ng of pFA2-Elk-1. Cells were treated with 0.5 μg/ml of Dox for 12 h before harvesting. Cells were harvested 36 h post-transfection and relative reporter activities were measured by luciferase assay followed by corrections with the β-galactosidase activities. Each data point represents the mean of three independent experiments. Error bars indicate the standard deviations of three independent experiments.

press the activation of the ERK pathway. To test this hypothesis, we examined the effect of NM23H2 on EGF- or H-Ras-mediated ERK activation and found that NM23H2 overexpression potently suppressed ERK activation in response to these stimuli. Furthermore, we found that overexpression of NM23H2 blocked the activation of Raf-1 and MEK, as well as the downstream transcription factor ELK-1. The ELK-1 is subjected to regulation by ERK pathway and subsequent proliferation [31,32].

These results indicate that NM23H2 may regulate the ERK pathway and cellular proliferation at the level upstream of Raf-1. This is further supported by the finding that NM23H2 blocks ERK pathway activation by oncogenic Ras. We interestingly found reductions of both endogenous and overexpressed mutated Ras by NM23H2 overexpression. These results indicate that NM23H2 inhibits the ERK pathway via down regulation of Ras level. The Ras proteins are mostly known to be regulated its activity by GDP/GTP exchange [33]. However, recent studies indicated Ras protein also could be regulated at the level of protein stability [16,34].

In addition to the ERK pathway, the PI3K-Akt signaling pathway is also regulated by Ras, and this pathway also impinges upon the regulation of cellular proliferation [35,36]. However, we did not observe any regulation of Akt in cells overexpressing NM23H2 (data not shown), indicating that the effect of NM23H2 is restricted to the Ras-ERK pathway. Taken together, our data show that NM23H2 negatively regulates cellular proliferation, and that this regulation is mediated by blocking the activation of the Ras-ERK signaling pathway.

5. Conflicts of interest Statement

All authors dose not have any financial and personal relationships with other people or organisations that could inappropriately influence (bias) our work.

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