hNRAGE, a human neurotrophin receptor interacting MAGE homologue, regulates p53 transcriptional activity and inhibits cell proliferation

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Abstract hNRAGE, a neurotrophin receptor p75 interacting MAGE homologue, is cloned from a human placenta cDNA library. hNRAGE can inhibit the colony formation of and arrest cell proliferation at the G1/S and G2/M stages in hNRAGE overexpressing cells. Interestingly, hNRAGE also increases the p53 protein level as well as its phosphorylation (Ser392). Further studies demonstrated that hNRAGE does not affect the proliferation of mouse p53−/− embryonic fibroblasts, suggesting that p53 function is required for hNRAGE induced cell cycle arrest. Moreover, the cell cycle inhibiting protein $p21^{WAF}$ is induced by hNRAGE in a p53 dependent manner. The data provide original evidence that hNRAGE arrests cell growth through a p53 dependent pathway.
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Key words: Human neurotrophin receptor p75 interacting MAGE homologue; p53; $p21^{CIP1/WAF1}$; Cell proliferation

1. Introduction

Rat neurotrophin receptor p75 interacting MAGE homologue (NRAGE) encodes a melanoma associated antigen gene (MAGE) family gene that interacts with the intracellular domain of the p75 neurotrophin receptor (p75NTR) [1]. The normal physiological function of this family of genes remains mostly unknown though more than 25 members have been identified in humans [2]. NRAGE has been shown to be involved in cell cycle arrest and apoptotic response upon nerve growth factor (NGF) binding to p75NTR in neural cells [1]. Recently, NRAGE has also been found to interact with XIAP, a member of the inhibitor of apoptosis proteins (IAPs), and to suppress the anti-apoptotic effect of XIAPs, thus augmenting cell death even in Bcl-2 overexpressing 32D cells [3].

Necdin is another MAGE member that is expressed in the neural system [4]. Ectopic expression of necdin in 3T3 cells can significantly arrest cell growth without affecting cell viability [5]. Recent studies reveal that necdin can bind to viral oncoproteins such as large T antigen, E1A and cellular transcription factor E2F [6]. Necdin can also bind p53 protein and suppress its transcriptional activity [7].

We have cloned a group of tumor cell proliferation regulating genes by introducing an expressible cDNA library into human hepatocellular carcinoma cells (so-called functional screening). One of these genes turned out to be the human homologue of rat NRAGE that can inhibit hepatocellular carcinoma cell proliferation. In this paper, we demonstrate that the tumor suppressor p53, which is reported to be essential for p75NTR mediated neuron death [8], played a crucial role for the function of hNRAGE.

2. Materials and methods

2.1. hNRAGE cloning

A mammalian expression cDNA library was constructed from human placenta. After first and second cDNA synthesis, cDNA fragments exceeding 2.0 kb were inserted into the mammalian expression vector pCMV-Script (Stratagene). A total of 0.5 μg plasmid DNA was transfected into HepG2 cells, a hepatocellular carcinoma cell line cultured in 96-well plates. After 10 days G418 selection (800 μg/ml), the genes that could significantly arrest cell growth were selected as candidate cell growth suppressing genes. hNRAGE is one of the candidates and contains a full open reading frame.

2.2. Adenovirus generation and plasmid construction

The adenovirus carrying the hNRAGE gene was generated and produced according to the simplified system introduced by He et al. [9], in which hNRAGE with a myc tag was inserted between *Bgl*II and *Hin*dIII of pShuttle-H1 and recombined with pAdEasy-1 in BJ5183 bacteria, then the virus was generated in 293A cells. hNRAGE was also subcloned into pEFneo and pEGFPc3 vectors (Clontech). The small RNA interference plasmid targeting to hNRAGE was constructed by inserting a 19-nt fragment into the pSUPPER-EGFP vector under the control of an H1 promoter. The sequence of the inserted fragment is GATGAAAGTGCTGAGATTC. Wild-type and mutant p53 (T81A), $p21^{CIP1/WAF1}$ luciferase reporter and β-galactosidase reporter plasmid were kindly gifted by Dr. Z.M. Yin.

2.3. Cell culture and transfection

The kidney epithelial cell line HEK293 and human hepatocellular carcinoma cell line HepG2 cells were cultured in Dulbecco's modified Eagle's medium, U2 OS, a human osteosarcoma cell line carrying the wild-type p53 gene, was cultured in RPMI 1640, supplemented with 10% (v/v) fetal bovine serum at 37°C in humidified air with 5% CO_2. Transfection was performed by a modified calcium phosphate precipitation method or lipofectamine. Transient transfection efficiency was

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Abbreviations: hNRAGE, human neurotrophin receptor p75 interacting MAGE homologue; MAGE, melanoma associated antigen gene; p75NTR, p75 neurotrophin receptor

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monitored by cotransfection of green fluorescent protein (GFP) vector (>80%).

2.4. Colony formation assay

U2 OS, HEK293 and HepG2 cells were transfected with pEFneo/hNRAGE or pEFneo vector and selected with G418 (200 μg/ml). After 14 days selection, cells were stained with 0.5% crystal violet in 20% ethanol and photographed. The size and the number of colonies were counted under a stereomicroscope.

2.5. Cell cycle assay

HEK293 cells transfected with pEGFP-hNRAGE, U2 OS cells and mouse p53−/− fibroblasts infected with adenovirus were collected and fixed. After incubated in RNase A (1 μg/ml) for 30 min at 37°C, the cells were stained with propidium iodide (50 μg/ml). Flow cytometric analysis was performed with FACScan (Becton Dickinson, Mountain view, CA, USA) with the CellQuest program.

2.6. $p21^{CIP1/WAF1}$ luciferase assay

pEFneo/hNRAGE (2 μg/well) and $p21^{CIP1/WAF}$ promoter (0.2 μg/well) were co-transfected into HEK293 cells grown to 50% confluence in six-well plates with the calcium phosphate precipitation method. The vehicle vector pEFneo was used as a blank control. β-Galactosidase reporter plasmid (0.1 μg/well) was included in all transfections as an internal control to normalize transfection efficiency. Luciferase activities were determined 48 h after transfection with a luminometer (Lumat LB 9507) using a detection kit from Promega.

2.7. Immunoblot and antibodies

To detect phosphorylated and total p53 levels, HEK293 cells infected with adenovirus were lysed directly in sodium dodecyl sulfate sample buffer. About 50 μg of total protein was loaded in each lane and equal loading was further confirmed by blotting against β-actin. The procedure of immunoblotting followed the method in Molecular Cloning. Anti-p53 monoclonal (DO-1), polyclonal (FL-393) and anti-p-p53 (Ser392) antibodies were purchased from Santa Cruz. Anti-β-tubulin and anti-$p21^{CIP1/WAF}$ antibody were purchased from Sigma.

2.8. Northern blot analysis of NRAGE

The procedure of Northern blotting followed the method in Molecular Cloning. Total RNA was isolated with Trizol reagent (Clontech) and size fractionated by electrophoresis through a 1% agarose-formaldehyde gel. After prehybridization, the blots were hybridized for 16–24 h at 42°C in buffer containing appropriate ^{32}P-labeled hNRAGE probes randomly primed with the Megaprime DNA labeling system (Amersham). The blots were then exposed to X-ray film after thorough washing.

2.9. Statistical analysis

Each treatment was repeated at least three times. The significance of the differences between the treatments and the corresponding controls was analyzed by the pooled *t*-test. A probability value of $P < 0.05$ was taken as statistically significant.

3. Results

3.1. hNRAGE cloning

We have cloned a human cell growth repressing gene by introducing an expressible cDNA library into human hepatocellular carcinoma cells in the early part of 2000 and deposited this gene in EMBL/GenBank data library under accession number AF258554. Later, Barker et al. cloned a NRAGE through yeast two-hybrid screening from rat [1]. By sequence comparison, we found AF258554 is a true homologue of rat NRAGE. Like it does in neuronal cells, we found AF258554 also inhibited colony formation in a hepatocellular carcinoma cell line. The name hNRAGE (AF258554) was used for all the studies in this paper.

3.2. Overexpression of hNRAGE arrested cell growth

To examine the effect of hNRAGE on cell growth, we

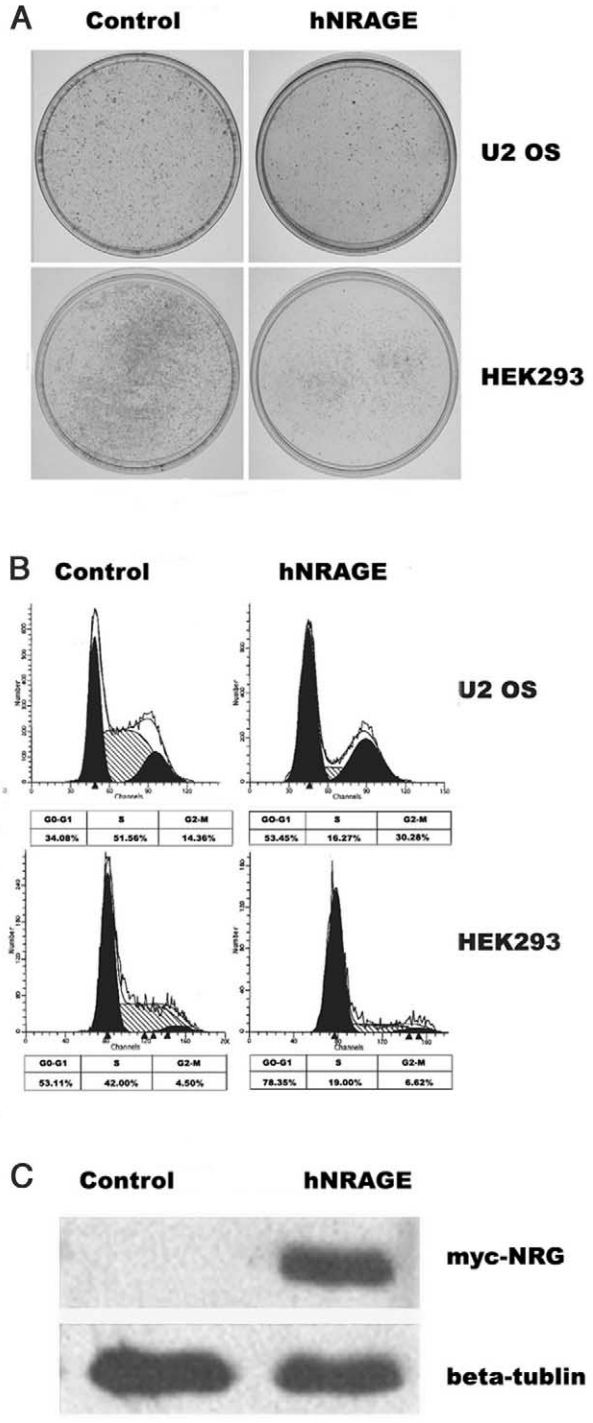


Fig. 1. Cell growth inhibition by NRAGE in HEK293 and U2 OS cells. A: HEK293 and U2 OS cells were transfected with hNRAGE gene and vehicle, then selected with G418 (200 μg/ml). Both the number and size of colonies were significantly decreased by NRAGE. B: Cell cycle analysis by FACS. HEK293 and U2 OS cells were infected with adenovirus carrying human NRAGE or adenovirus vector. Ninety-six hours later, cells were collected and fixed with cold ethanol and then resuspended in 1 ml solution containing 50 mg/ml RNase A and 50 mg/ml propidium iodide. At least 15 000 cells were collected at each treatment by FACScan and analyzed with the CellQuest program (Becton Dickinson). hNRAGE arrested the cell cycle mainly at G1/S and G2/M, whereas no apoptosis was observed. C: Additional expression of hNRAGE was blotted with c-myc antibody.

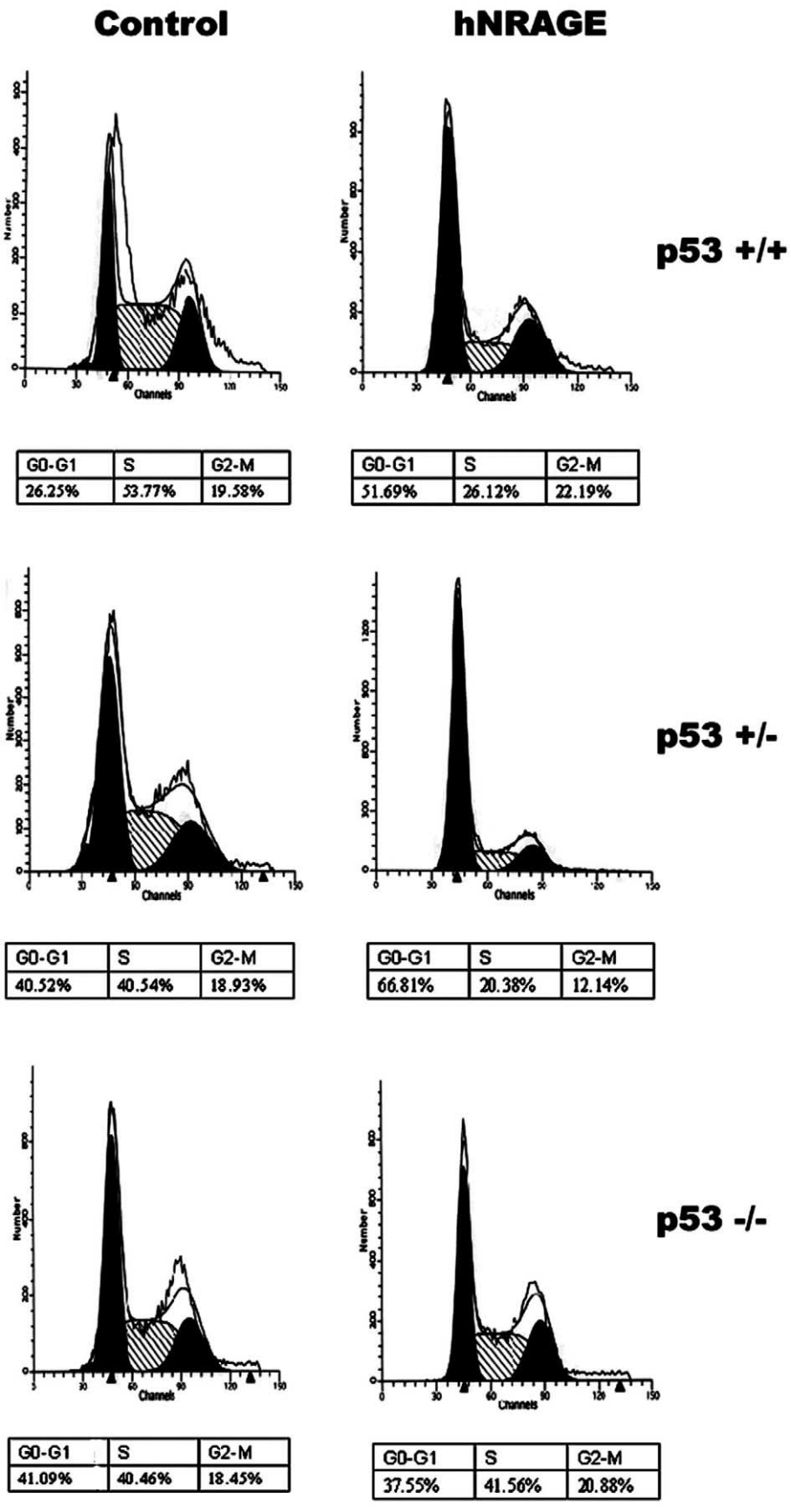


Fig. 2. hNRAGE arrests the cell cycle through a p53 dependent pathway. Adenovirus of NRAGE was used to infect mouse primary fibroblasts derived from p53+/+, +/−, and −/− mice. The cell cycle suppressing function of hNRAGE was totally inhibited in p53−/− fibroblasts. The infection efficiency was monitored with GFP expression.

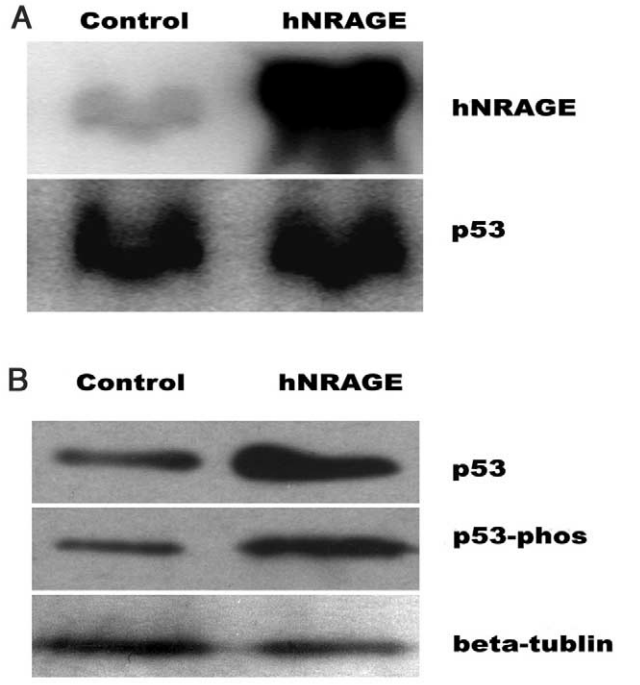


Fig. 3. hNRAGE induces post-translational modification of p53. A: Although the mRNA level of NRAGE was very high after overexpression in HEK293 cells, the mRNA level of p53 remained unchanged. B: Western blotting showed that both the total protein level and phosphorylation level of p53 were increased after overexpression of hNRAGE in HEK293 cells.

tested the colony formation ability of U2 OS, HepG2 and HEK293 cells after transfection with hNRAGE gene. Transfection of hNRAGE resulted in a decrease of the colony number or size in the three cell lines (Fig. 1A, only the data of U2 OS and HEK293 are shown). To further determine whether NRAGE induces apoptosis or arrest of cell cycle, FACS analysis was applied to HEK293 cells transfected with GFP-hNRAGE gene and U2 OS cells infected with hNRAGE adenovirus. Unexpectedly, no apoptotic cells were found in hNRAGE overexpressing cells. The number of S phase cells, however, was decreased significantly in both cell lines. Ninety-six hours after infection, the percentage of S phase U2 OS cells was decreased from 51.56% to 16.27%, whereas the percentage at G1 and G2/M phase was increased from 34.08% to 53.45% and 14.36% to 30.28%, respectively (Fig. 1B). Similar results were observed in HEK293 cell (S phase cells from 42.00% to 19.00%, G1 phase cell from 53.11% to 78.35%). These data clearly demonstrate that overexpression of hNRAGE caused cell cycle arrest at G2/M or G1 phase. Fig. 1C shows that hNRAGE adenovirus infection could highly increase the protein level of hNRAGE in HEK293 cells.

3.3. p53 was required for cell growth arrest induced by NRAGE

p53 is an important tumor suppressor gene regulating both cell apoptosis and proliferation. Is p53 involved in hNRAGE mediated cell cycle arrest? To answer this question, we infected mouse embryonic fibroblasts (MEF) derived from p53 null, heterozygous and wild-type mice. Two days after infection, FACS analysis showed that the percentage of S phase cells was decreased from 53.77% to 26.12%, and G1 phase was increased from 26.25% to 51.69% in p53+/+ MEF. p53+/− cells displayed similar number changes (40.54% to 20.38% for S phase and 40.52% to 66.81% for G1 phase). However, p53−/− MEF showed no significant change in all G1, S phase and G2/M phase cell numbers upon hNRAGE overexpression (Fig. 2), suggesting p53 was required for hNRAGE induced cell cycle arrest.

3.4. NRAGE affects the phosphorylation and accumulation of p53

Because Northern blotting showed that the mRNA level of the p53 gene was not changed by hNRAGE overexpression (Fig. 3A), hNRAGE might affect p53 activity in a post-tran-

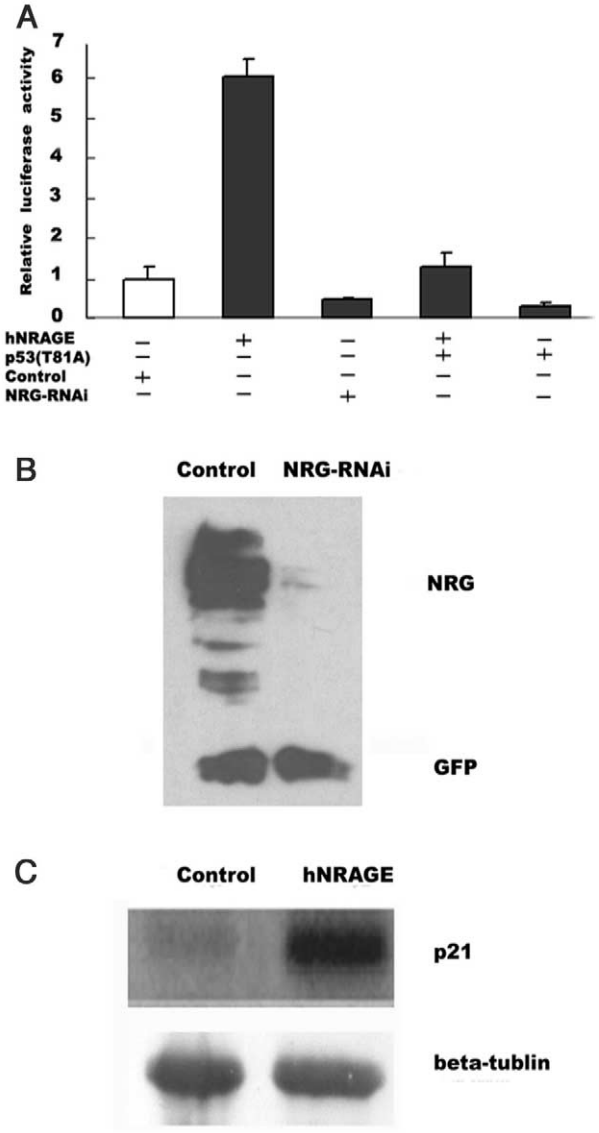


Fig. 4. hNRAGE could induce expression of $p21^{CIP1/WAF}$ through a p53 dependent pathway. A: NRAGE could stimulate the luciferase activity that is under the control of the $p21^{CIP1/WAF}$ promoter, but the p53 mutant (T81A) could abolish the induction of p21 by NRAGE. B: siRNAi of hNRAGE could eliminate the expression of hNRAGE in HEK293 cells. siRNA plasmid was co-transfected with pEGFP-hNRAGE. The expression of GFP-hNRAGE was tested by GFP antibody. C: The induction of p21 by NRAGE was further confirmed by Western blotting. About 50 μg of total protein was loaded in each line.

scriptional manner through changing its phosphorylation and accumulation. Western blotting of p53 showed that both the phosphorylation (Ser392) and the accumulation level of p53 protein were increased significantly after overexpression of hNRAGE (Fig. 3B). It is possible that the increase of p53 phosphorylation might account for the elevation of the p53 protein level. These data indicate that hNRAGE could somehow modify p53 and increase its stability.

3.5. NRAGE could induce $p21^{CIP1/WAF}$ expression in a p53 dependent manner

Because the cell cycle inhibitor protein $p21^{CIP1/WAF}$ is a downstream target gene of p53, we examined the expression of $p21^{CIP1/WAF}$ in hNRAGE overexpressing cells. Results revealed that hNRAGE induced an about five-fold increase in $p21^{CIP1/WAF}$-Luc reporter gene activity (Fig. 4A). The results were further confirmed by a loss of function experiment, in which siRNA of hNRAGE could abolish the stimulation of hNRAGE overexpression. Fig. 4B shows that siRNA of hNRAGE could eliminate the expression of GFP-hNRAGE. The data of the luciferase assay that hNRAGE could induce $p21^{CIP1/WAF}$ transcriptional activity were further confirmed by Western blotting experiments that the protein level of $p21^{CIP1/WAF}$ was indeed increased in hNRAGE expressing cells (Fig. 4C). These results suggest that hNRAGE could inhibit cell proliferation through increasing the expression of the cell cycle inhibitor protein $p21^{CIP1/WAF}$. Moreover, the upregulation of $p21^{CIP1/WAF}$ by hNRAGE depends on the p53 activity because this effect was completely abolished by co-transfection of dominant negative p53 (T81A) (Fig. 4A).

4. Discussion

Over 25 MAGE genes have now been cloned in humans since the identification of the first MAGE gene in 1991. However, the physiological role of most MAGE proteins remains a mystery. Recent studies are now beginning to provide insights into MAGE gene functions. For instance, necdin was found to regulate the cell cycle and be involved in the pathogenesis of Prader–Willi syndrome [10]. First identified as a binding partner for p75NTR, NRAGE also blocks PC12 cell cycle progression and enhances apoptosis in the presence of NGF though little is known about the mechanisms [1].

Our studies found that hNRAGE suppresses cell colony formation in HEK293, U2 OS and HepG2 cells. The cell cycle arrest occurs at the G2/M and G1 stage. By using p53−/− MEF, our results clearly proved that p53 activity is required for the suppression of cell proliferation by hNRAGE. It is worth mentioning that all the other cell lines we used carry wild-type p53 [11,12]. In addition, we also demonstrated that the upregulation of $p21^{WAF}$ expression by hNRAGE also depends on the wild-type p53 activity because dominant negative mutant p53 completely abolishes hNRAGE's function. Moreover, we observed that hNRAGE enhances the phosphorylation and accumulation of p53 protein without changing the mRNA level of p53. Therefore we propose that hNRAGE upregulates p53 activity through post-transcriptional modification of p53. This characteristic of hNRAGE resembles that of the Wilms' tumor suppressor WT-1 that binds to p53 directly and enhances p53 dependent transactivation [13]. It will be extremely interesting to further examine whether and how hNRAGE indirectly or indirectly regulates the components in the p53 regulation pathway.

Previous studies have indicated that p75NTR can activate the p53 pathway. When p53 is diminished in p53−/− mice or ablated using the adenovirus E1B55K protein, p75NTR associated sympathetic neuron death is inhibited [8]. But there is also a report that suggests that p75NTR might also mediate apoptosis in a p53 independent pathway [14]. So the precise signaling event linking p75NTR to apoptosis activation is still unknown. But hNRAGE, based on our data and previous reports [1,3], can still be thought to be a very important mediator of apoptosis and cell proliferation. We speculated that hNRAGE should act as a protein recruiter or mediator of this processes. When signal molecule binding to its receptor, hNRAGE can be activated, for instance bind the intracellular domain of p75NTR in NGF issue, and then recruit and activate other proteins such as p53. Actually we really found that hNRAGE can affect the stability of some intracellular molecules like p53 (Fig. 3) and β-catenin (data not shown). Recently, Salehi et al. have reported that hNRAGE could induce caspase activation and cell death through the c-Jun N-terminal kinase (JNK)/c-Jun pathway [15]. In response to stress, JNK is phosphorylated on both Thr-183 and Thr-185 residues by MKK4/7 [16,17], which then leads to the phosphorylation of JNK substrates, which include p53 [18,19]. It is unknown whether hNRAGE induced p53 activation is linked to the JNK pathway.

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