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Anti-tumor activity of ESX1 on cancer cells harboring oncogenic *K-ras* mutation

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ABSTRACT

Human ESX1 is a 65-kilodalton (kDa) paired-like homeoprotein that is proteolytically processed into N-terminal 45-kDa and C-terminal 20-kDa fragments. The N-terminal ESX1 fragment, which contains the homeodomain, localizes to the nucleus and represses mRNA transcription from the *K-ras* gene. When we inoculated human colorectal carcinoma HCT116 constitutive expressing N-terminal region of ESX1 (N-ESX1) into nude mice, transfectant cells uniformly showed decreased tumor-forming activity compared with that of the parental cells. Furthermore, pretreatment of HCT116 carcinoma cells with a fusion protein consisting of N-ESX1 and the protein-transduction domain derived from the human immunodeficiency virus type-1 TAT protein gave rise to a dramatic reduction in the tumorigenicity of HCT116 cells in nude mice. Our results provide first *in vivo* evidence for the molecular targeting therapeutic application of the *K-ras* repressor ESX1, especially TAT-mediated transduction of N-ESX1, in the treatment of human cancers having oncogenic *K-ras* mutations.

Human ESX1 (extraembryonic, spermatogenesis, homeobox 1 homolog; synonymous with ESXR1 or ESX1L) is a paired-like homeodomain-containing protein, which is encoded by the ESX1 gene located on chromosome Xq22.1 [1,2]. In cells, the full-length 65-kilodalton (kDa) ESX1 protein is proteolytically cleaved into two peptide fragments, a C-terminal 20-kDa fragment consisting of the proline-rich repeat region and an N-terminal 45-kDa fragment that contains the paired-like homeodomain. The C-terminal 20-kDa fragment localizes to the cytoplasm and inhibits the degradation of mitotic cyclins, thereby inducing M phase cell-cycle arrest [2]. On the other hand, the N-terminal 45-kDa fragment is localized to the nucleus, where it specifically binds to the P3 consensus sequence for the paired-like homeodomain, TAATNNNATTA (where N is any nucleotide) and acts as a sequence-specific transcriptional repressor [3]. Among genes whose expression is down-regulated by ESX1 is *K-ras*. Indeed, ESX1 specifically interacts with the TAATGTTATTA sequence that is present within the first intron of the *K-ras* gene termed REK, and represses *K-ras* transcription [3].

K-Ras is a member of the Ras small GTP-binding protein family and functions as a molecular switch that conveys mitogenic signals generated by growth factor–receptor interaction to the downstream signaling pathway in mammalian cells. Missense mutation in codon 12, 13, or 61 of the *K-ras* gene is one of the most frequent genetic changes found in human malignancies. *K-ras* mutation is particularly prevalent in refractory cancers such as pancreatic (70–90%), colorectal (50%), and lung (20–30%) carcinomas [4]. These cancer-associated mutations of *K-ras* are gain-of-function mutations, which encode constitutively active K-Ras mutants. Importantly, the mutant K-Ras activity is required not only for malignant transformation of cells but also for maintenance of the transformed phenotype of the established tumor cells [5–7]. This in turn indicates that oncogenic K-Ras is a critical molecular target in cancer therapy and suggests a potential application of ESX1 for the treatment of human cancers harboring *K-ras* mutations [8].

In a previous study, we demonstrated that ectopic expression of the N-terminal ESX1 fragment, which functions as the *K-ras* repressor, in human cancer cells carrying oncogenic *K-ras* resulted in the inhibition of cell proliferation *in vitro*, whereas it had little effect on the proliferation of cancer cells without *K-ras* mutation [3]. In this work, we show that stable expression of the N-terminal ESX1 region (N-ESX1) significantly reduces *in vivo* tumorigenicity of HCT116 human colorectal carcinoma cells carrying an oncogenic *K-ras* mutation. Furthermore, pretreatment of HCT116 carcinoma cells with a fusion protein consisting of N-ESX1 and the protein-transduction domain derived from the human immunodeficiency virus type-1 (HIV-1) TAT protein, which confers the direct intracellular delivery of the fusion protein [9] strongly inhibits *in vivo* tumorigenicity. These results provide *in vivo* evidence that supports clinical application of the *K-ras* repressor ESX1 to the treatment of human cancers carrying oncogenic *K-ras* mutations.

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Materials and methods

Cells. U2-OS human osteosarcoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). HCT116 and SW480 human colon carcinoma cells were cultured in DMEM/F12 medium supplemented with 10% FBS.

Plasmids. pcDNA3/N-ESX1 was made by introducing a universal translation terminator sequence into the BamHI site located immediately downstream of the 3′ end of the *ESXR1-ΔC* cDNA in pcDNA3/ESXR1-ΔC. The cDNA encoding *N-ESX1* was then excised from pcDNA3/N-ESX1 and was cloned into pOPTET-BSD [10]. pTet-On vector was purchased from BD Biosciences Clontech (Palo Alto, CA). A bacterial expression vector for TAT/N-ESX1 fusion protein was generated by introducing DNA fragments encoding the protein-transduction domain (PTD) of HIV-1 TAT protein and N-ESX1 into pET-16b plasmid. pcDNA3/TAT/N-ESX1 was made by introducing *TAT/N-ESX1* DNA fragment derived from pET-16b-TAT/N-ESX1.

Stable transfectants. HCT-tet cells were made from HCT116 cells by stably transfecting the pTet-On vector that directs the tetracycline-regulated transactivator. HCT-tet cells were transfected with pOPTET-BSD/N-ESX1 and were selected in DMEM/F12 containing 10% FBS in the presence of 1000 μg/ml of G418 and 20 μg/ml of blasticidin S (BSD). Stable transfectants that express N-ESX1 were single-cell cloned by limiting dilution.

Immunoblotting. Immunoblotting was performed as described previously [3]. Anti-myc (9E10), anti-actin (sc-1615), anti-K-Ras (sc-30), and anti-SHP-2 (sc-280) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and were used for immunoblotting.

Colony formation suppression assay. Twenty micrograms of the expression plasmid was transfected into HCT116 cells together with 1 μg of the puromycin-resistance gene (pBabe-puro) by using Lipofectamine reagent. Transfected cells were selected in DMEM/F12 medium containing 10% fetal bovine serum in the presence of 1 μg/ml puromycin for 2 weeks.

Luciferase reporter assay. Luciferase reporter assay was performed as described previously [3].

Purification of the recombinant TAT/N-ESX1 fusion protein. Recombinant TAT/N-ESX1 fusion proteins were expressed in the *Escherichia coli* BL21 (DE3) strain and were purified as previously described [11]. Briefly, the bacteria were grown in LB medium containing 0.1% glucose and induced with 0.4 mM Isopropyl-β-D-thiogalactopyranoside (IPTG). The sedimented bacteria were re-suspended in Tris buffer (50 mM Tris, 150 mM NaCl, pH 7.4) containing 5 mM imidazole and protease inhibitors and then sonicated and centrifuged. The supernatants were applied to pre-equilibrated Ni–NTA agarose beads (QIAGEN K.K., Tokyo, Japan) and were eluted with Tris buffer containing 500 mM imidazole and protease inhibitors. The eluted fusion proteins were desalted with PD-10 desalting columns (Amersham Pharmacia, Uppsala, Sweden), equilibrated, and dissolved in culture medium.

Nude mouse xenograft assay. Female BALB/c AJcl nu/nu mice (6 weeks old) were purchased from CLEA Japan Inc. Parental HCT-tet cells or transfectant cells were suspended in PBS and were injected subcutaneously in bilateral flanks of mice (1×10^6 cells in 100 μl PBS). For experiments using the recombinant TAT-fusion proteins, HCT116 cells were cultured in F12/DMEM supplemented with 10% FCS in the presence of 0.17 μM TAT/N-ESX1 or ΔTAT/N-ESX1 for 48 h and then subcutaneously inoculated into bilateral flanks of mice.

Immunocytochemical analysis. Cells were fixed and treated with an anti-Myc (9E10) antibody at 24 h after transfection. Primary antibody was localized by Alexa Fluor 546-conjugated anti-mouse IgG antibody (Invitrogen, Carlsbad, CA). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Images were acquired by using a confocal fluorescence microscope system.

Results

K-ras repressor activity of the N-terminal ESX1 fragment, N-ESX1

In a previous study, we constructed an expression vector for the N-terminal ESX1 fragment, termed ESXR1-ΔC, by inserting a BamHI fragment of the full-length *ESXR1* cDNA into pcDNA3 [2]. As a result, ESXR1-ΔC consists of the N-terminal 1–230 residues of ESX1 as well as an additional 35-amino acid stretch at the C-terminus that is derived from the pcDNA3 vector sequence. In this work, we first eliminated this C-terminal 35-amino acid sequence from ESXR1-ΔC by introducing a universal translation terminator sequence to the 3′ BamHI site of the *ESXR1-ΔC* cDNA. The resulting pcDNA3/N-ESX1 vector directed to express N-ESX1 consisting of the N-terminal ESX1 fragment (residues 1–229) and the following 4 amino acid residues derived from the universal translational

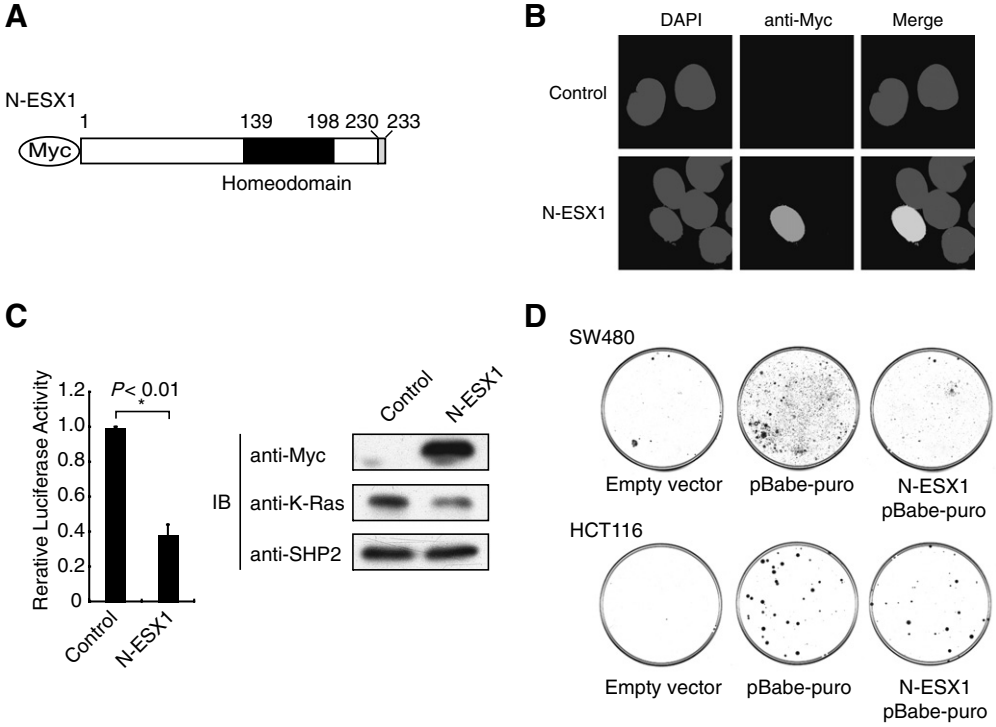

Fig. 1. *K-ras* repressor activity of N-ESX1. (A) Schematic drawing of N-ESX1. (B) Localization of N-ESX1 in HCT116 cells. N-ESX1 or empty control vector was transiently transfected into HCT116 cells. N-ESX1, which was N-terminal Myc-tagged, was detected with the use of an anti-Myc antibody, 9E10. Cell nuclei were stained by DAPI. (C, left) Sequence-specific transcriptional repression by N-ESX1. pcDNA3/N-ESX1 was transfected together with the pGL3-3xREK-Promoter-Luc reporter plasmid into U2-OS cells, and cell lysates were subjected to luciferase assay. Error bars indicate 2 × standard deviation (SD) of data. (C, right) Inhibition of K-Ras expression by N-ESX1. HCT116 cells were transiently transfected with pcDNA3/N-ESX1 or control empty vector. At 24 h after transfection, cells were lysed and the lysates were subjected to immunoblotting with the indicated antibodies as described previously. (D) Colony formation suppression assay. HCT116 and SW480 cells were transfected with the puromycin-resistance gene (pBabe-puro) together with pcDNA3/N-ESX1 or control empty vector. After selection, culture plates were stained and puromycin-resistant colonies were visualized.

terminator sequence (Fig. 1A). During vector construction, N-ESX1 was also tagged with Myc-epitope at the N-terminus. Biological properties of N-ESX1 were examined by transient transfection of pcDNA3/N-ESX1. When expressed in HCT116 human colorectal carcinoma cells, N-ESX1 was strictly localized in the nucleus (Fig. 1B). Transcriptional activity of N-ESX1 was examined by employing a pGL3-derived luciferase reporter construct, pGL3-3xREK-Promoter-Luc [3]. The reporter and internal control pRK-TK plasmids were transfected into U2-OS human osteosarcoma cells together with pcDNA3 or pcDNA3/N-ESX1. The luciferase activities were normalized with the dual-luciferase system. As shown in Fig. 1C (left), N-ESX1 inhibited the luciferase reporter activity. Consistently, ectopic expression of N-ESX1 downregulated K-Ras levels in HCT116 cells (Fig. 1C, right). In a colony formation suppression assay, N-ESX1 reduced both number and size of the drug-resistant colonies of HCT116 and SW480 human colon carcinoma cells, both of which carry oncogenic *K-ras* (Fig. 1D). Collectively, these results indicate that the newly generated N-terminal ESX1 fragment, N-ESX1, retains the biological activities of ESXR1-ΔC while substantiating the results of the previous work showing that the N-terminal ESX1 fragment transcriptionally represses the *K-ras* gene and thereby inhibits growth of cancer cells carrying oncogenic *K-ras in vitro* [3].

Establishment of HCT116 cells that stably express N-ESX1

To investigate *in vivo* anti-tumor activity of N-ESX1, we sought to establish HCT116 colon carcinoma cells in which N-ESX1 was inducibly expressed through the tetracycline-regulated *tet-on* system. To this end, we first transfected pTet-On into HCT116 cells and established a transfectant clone, HCT-tet, that stably expresses the tetracycline-regulatable (*tet-on*) transcriptional activator. Next, we transfected pOPTET-BSD-N-ESX1 into the HCT116-derived HCT-tet cells and established two transfectant clones that express N-ESX1 in the presence of doxycycline, a water-soluble derivative of tetracycline. Unexpectedly, however, all of the established transfectants expressed ESX1 in an uninduced condition as well. We thus regarded these transfectants as constitutive N-ESX1 expressors. Although the reason why the *tet-on* system is so leaky in HCT116 human carcinoma cells is currently unknown, it seems that the particular cells intrinsically possess promiscuous transcriptional activity that constitutively stimulates the tetracycline-regulated promoter regardless of the presence of tetracycline. Nevertheless, the levels of N-ESX1 constitutively expressed in these transfectants were not high, and consequently suppression of K-Ras expression as well as inhibition of cell proliferation *in vitro* was only marginal (Fig. 2A and B). We consider that the observation is reasonable because constitutive expression of N-ESX1 at high levels should elicit strong downregulation of K-Ras that results in cell-cycle arrest, rendering establishment of such high N-ESX1 expressors practically impossible.

Reduced tumorigenicity of HCT116 carcinoma cells expressing N-ESX1

To study anti-tumor effect of N-ESX1, we first investigated the minimal HCT116 carcinoma cell number that is required for tumor formation in a nude mice xenograft assay and found that it was 1×10^6. Given this, we inoculated HCT116-derived HCT-tet cells (1×10^6) that stably express the tet-regulated transactivator into the left flank and each of the N-ESX1 transfectant clones (clone #1, #2) (1×10^6) into the right flank of nude mice. As shown in Fig. 3, tumor formation of the parental HCT-tet cell was uniformly superior to that of the N-ESX1 transfectant. This observation indicates that expression of N-ESX1 significantly impairs *in vivo* tumorigenicity of cancer cells harboring oncogenic *K-ras*. Together with the results shown in Figs. 2 and 3, a slight decrease in the level of oncogenic K-Ras by N-ESX1 appears to elicit potent reduction in the *in vivo* tumorigenicity of cancer cells.

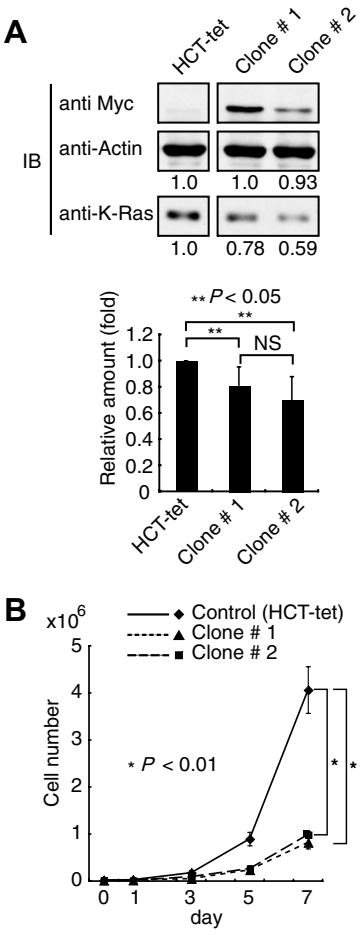

Fig. 2. Establishment of HCT116-derived stable transfectant clones that constitutively express N-ESX1. (A) Inhibition of K-Ras expression in N-ESX1 stable transfectants (top). The levels of K-Ras protein in parental HCT-tet cells and two transfectant clones that constitutively express N-ESX1 were compared by immunoblotting. Numbers below each panel represent relative intensities of K-Ras or actin immunoblotting bands measured by a luminescence image analyzer, which was normalized to a value of 1.0 for HCT-tet cells. Relative K-Ras levels were calculated from the quantitation data using actin as an internal standard (bottom). $P < 0.05$, Student's *t*-test. (B) Growth curves of parental HCT-tet cells and N-ESX1 transfectants, clones #1 and #2. Error bars indicate 2 × SD. $P < 0.01$, Student's *t*-test. NS means not significant.

Generation and biological properties of TAT/N-ESX1 fusion protein

The above-described observations prompted us to investigate potential application of N-ESX1 for the treatment of human cancers carrying *K-ras* mutations. To pursue this possibility, we constructed a bacterial expression vector for TAT/N-ESX1, a fusion protein consisting of N-ESX1 and the protein-transduction domain (YGRKKRRQRRR) that is derived from HIV-1 TAT protein (Fig. 4A). It has been well established that the fusion of this TAT peptide sequence with heterologous proteins is sufficient for the direct intracellular delivery of the desired protein to a variety of cells in a rapid, concentration-dependent manner [12]. As a control, a bacterial expression vector for ΔTAT/N-ESX1, in which the protein-transduction domain was specifically deleted from the TAT/N-ESX1 protein, was also constructed. Recombinant proteins expressed in *E. coli* were purified from the *E. coli* lysates with the use of Ni–NTA beads, which specifically bind to the 10× His-Tag

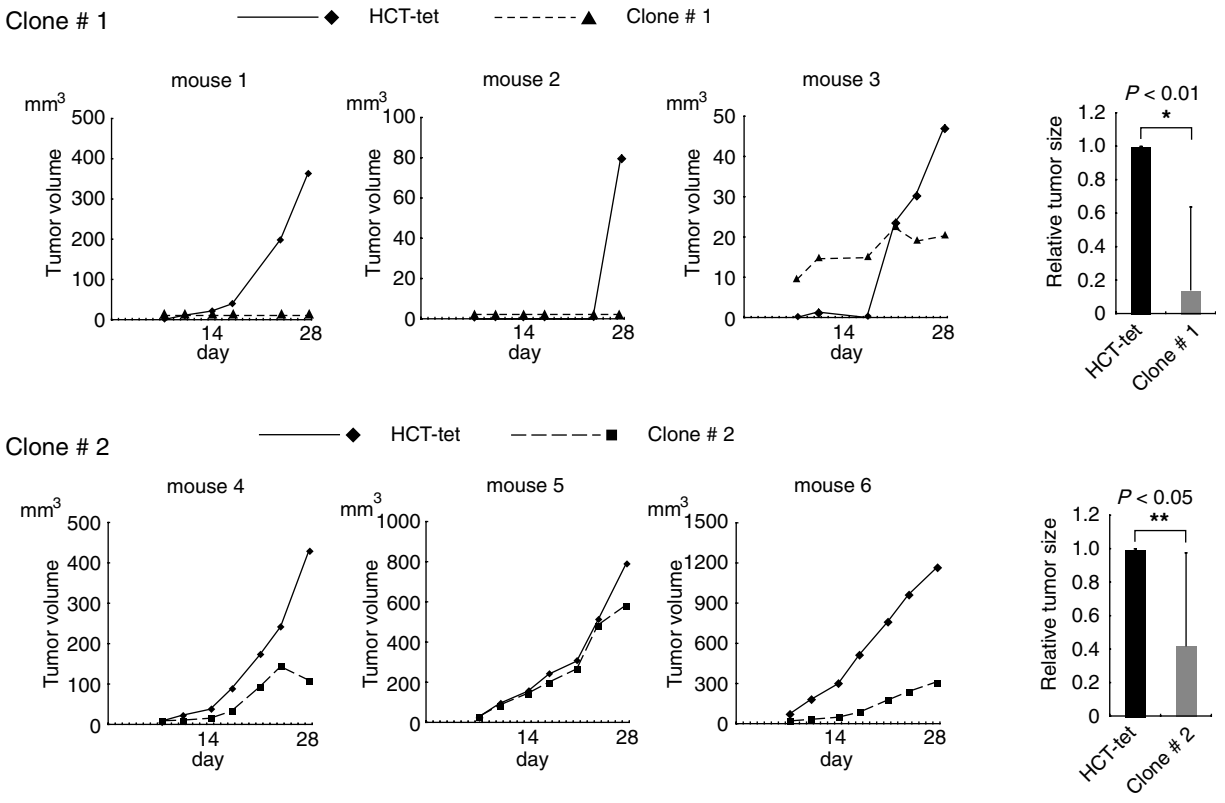

Fig. 3. Effect of N-ESX1 on *in vivo* tumorigenicity of HCT116 cells. *In vivo* tumor formation of parental HCT-tet cells and N-ESX1 transfectants that constitutively express N-ESX1. Parental cells and the transfectant cells (1×10^6 each) were respectively inoculated into the left and right flanks of a single nude mouse (three mice used for each transfectant). The sizes of tumors on day 28 that had developed on the parental cell-inoculated and transfectant-inoculated sides in each mouse are shown by relative tumor volume, with the volume of the tumor developed by parental cells being 1.0 (Right panels). Error bars indicate $2 \times$ SD. $^{*}P < 0.01$, $^{**}P < 0.05$, Student's *t*-test.

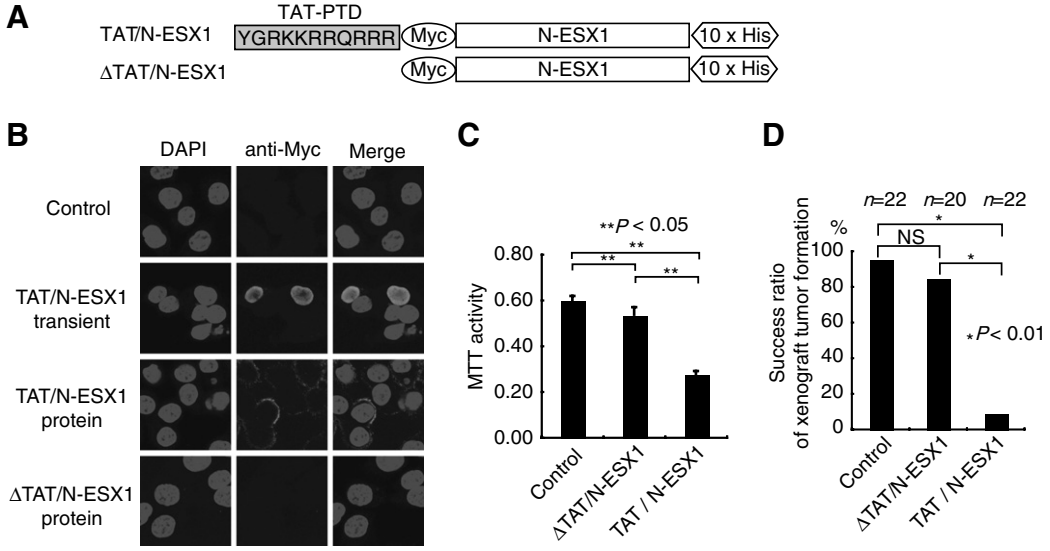

Fig. 4. (A) Schematics of the TAT/N-ESX1 and ΔTAT/N-ESX1 fusion proteins. ΔTAT/N-ESX1 was made from TAT/N-ESX1 by deleting the TAT-derived protein-transduction domain. (B) Immunohistochemical analysis of HCT116 cells treated with TAT/N-EST1 *in vitro*. Cells were treated with 1 μM of recombinant TAT/N-ESX1 or ΔTAT/N-ESXT1 for 1 h, and after fixing, were immunostained with anti-Myc antibody, which recognizes TAT/N-ESX1 or ΔTAT/ESX1. The TAT/N-ESX1 protein transiently expressed in HCT116 cells was used as a control. (C) Effect of TAT/N-ESX1 on *in vitro* proliferation of HCT116 cells. Cells were cultured in a medium containing 10% FBS and recombinant TAT/N-ESX1 or ΔTAT/N-ESXT1 protein at a final concentration of 0.5 μM. At 48 h, cell culture was replaced with a fresh medium containing 0.5 μM of the respective recombinant protein. Cell proliferation was quantitated by MTT assay at day 4. Error bars indicate $2 \times$ SD. $^{**}P < 0.05$, Student's *t*-test. (D) Effect of the TAT/N-ESX1 fusion protein on *in vivo* tumorigenicity of HCT116 carcinoma cells. Nude mice were subcutaneously inoculated with HCT116 cells (control, $n = 22$; n means the number of sites injected), HCT116 cells pretreated with ΔTAT/N-ESX1 ($n = 20$) or HCT116 cells pretreated with TAT/N-ESX1 ($n = 22$). Three weeks after the inoculation, formation of tumor mass was determined. $^{*}P < 0.01$, χ^2 test. NS means not significant.

sequence present at the C-terminus of the fusion protein (Supplemental figure). To investigate whether the purified TAT-fusion protein enters cells, HCT116 cells were treated with the TAT/N-ESX1 or ΔTAT/N-ESX1 protein at a final concentration of 1 μM for 1 h

and were fixed and stained with anti-Myc antibody, which recognizes the Myc tag-epitope of the fusion proteins. As shown in Fig. 4B, TAT/N-ESX1 was detectable inside the HCT116 cells, whereas no specific staining was observed in cells treated with ΔTAT/N-ESX1. Furthermore, treatment with 0.5 μM recombinant TAT/N-ESX1 protein specifically inhibited growth of HCT116 cells *in vitro* (Fig. 4C). These results indicated that the TAT/N-ESX1 fusion protein enters the cancer cells and then inhibits the growth of tumor cells *in vitro*.

TAT/N-ESX1 fusion protein inhibits in vivo growth of human cancer cells carrying oncogenic K-ras

To evaluate anti-tumor activity of TAT/N-ESX1, HCT116 carcinoma cells were *in vitro* treated with 0.17 μM TAT/N-ESX1 or ΔTAT/N-ESX1 for 48 h and then subcutaneously inoculated into the flanks of nude mice bilaterally (1×10^6 cells/injection). Formation of tumor mass at the site of inoculation was evaluated 3 weeks after the injection of the carcinoma cells. As shown in Fig. 4D, tumorigenicity of HCT116 cells was dramatically reduced when they were pretreated with TAT/N-ESX1 but not ΔTAT/N-ESX1. From these observations, we concluded that treatment of human colon carcinoma cells carrying oncogenic *K-ras* with TAT/N-ESX1 potently impaired *in vivo* tumorigenicity.

Discussion

Given that oncogenic K-Ras, which induces growth factor-independent cell proliferation and cell survival, is required for maintenance of the cancer phenotype [5–7], the *K-ras* gene or the encoded K-Ras protein has been considered to be a critical molecular target in the treatment of cancers harboring oncogenic *K-ras* [8]. In fact, several strategies to inhibit *K-ras* expression using antisense oligonucleotides, siRNA [13,14], or farnesyltransferase inhibitors as anti-cancer agents that target Ras [15] have been developed and extensively studied. However, these approaches have been limited by the ability to safely and effectively deliver nucleic acids to cancer cells or the results of clinical trials are not promising [16]. To date, however, little attention has been focused on the transcriptional regulation of *K-ras* in the molecular targeting therapy of cancer.

We previously demonstrated that ectopic expression of the N-terminal 45-kDa ESX1 fragment downregulates K-Ras expression through transcriptional repression of the *K-ras* gene and thereby causes growth inhibition of cancer cells harboring oncogenic *K-ras in vitro* [3].

The present study extends our previous *in vitro* work by demonstrating anti-tumor activity of the N-terminal ESX1 region (N-ESX1) *in vivo*.

Considering practical application of N-ESX1 for molecular cancer therapy, the most critical issue is the strategy by which N-ESX1 is delivered/expressed in cancer cells with high efficiency and selectivity. Although retrovirus vector-mediated or adenovirus vector-mediated gene therapies have been developed, subsequent clinical trials using these vectors revealed serious side effects, most notably secondary leukemias [17]. Thus, we employed in this work an alternative approach—transmembrane delivery of N-ESX1 into cancer cells. The TAT protein of HIV-1 possesses a small basic peptide consisting of 11 amino acids, YGRKKRRQRRR, termed the "protein-transduction domain". The fusion of this TAT peptide sequence with heterologous proteins confers the direct intracellular delivery of the desired protein to a variety of cells in a rapid, concentration-dependent manner [12]. Although the detailed molecular mechanism of protein transduction remains to be elucidated, TAT-fusion proteins seem to enter cells by fluid-phase macropinocytosis, a specialized form of endocytosis that is independent of caveolae, clathrin or dynamin [9]. Transmembrane delivery of tumor suppressor proteins such as p27 or p16 by TAT-mediated transduction has been achieved *in vitro* [12]. Furthermore, intraperitoneal injection of the TAT-p53 activating peptide caused a significant reduction in tumor growth and an extension in lifespan in an *in vivo* nude mice xenograft model [18]. Immunocytochemical analysis of cells treated *in vitro* with the recombinant TAT/N-ESX1 protein revealed that the fusion proteins directly entered the cells, although the staining indicated that only a small amount of the TAT-fusion proteins was transduced into cells in our experimental setting. Nevertheless, TAT/N-ESX1 strongly reduced *in vivo* tumorigenicity of human cancer cells harboring oncogenic *K-ras*, indicating that TAT/N-ESX1 exerts potent anti-tumor activity through targeting *K-ras*.

In summary, the present work provides the basis and rational for the molecular cancer therapy that targets oncogenic *K-ras*. The work further suggests the potential clinical application of TAT-mediated transduction of N-ESX1 in the treatment of human cancers with *K-ras* mutations.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.03.062.

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