Introduction

Malignant melanoma is one of the most aggressive malignancies due to its strong capacity to grow, invade and metastasize, and therefore, it is of high priority to identify novel therapeutic targets and treatment options for this cancer.

Periostin (POSTN), first described in 1993 in mouse osteoblasts as osteoblast-specific factor 2 (OSF-2), is a secreted matrix N-glycoprotein of 93 kDa (Takeshita et al., 1993). The N-terminal region contains four fasciclin-like domains as well as several glycosylation sites. The protein originally was identified in MC3T3-E1 osteoblast-like cells, where it promotes integrin-dependent cell adhesion and motility. It shares homology with the insect cell adhesion molecule fasciclin I, with human β IgH3, and is induced by transforming growth factor-β (TGF-β) (Horiuchi et al., 1999), bone morphogenic protein-2 (Inai et al., 2008), IL-4, IL-13 (Takayama et al., 2006), and PDGF-bb (Li et al., 2006). As a ligand to alpha(V)beta(3) and alpha(V)beta(5) integrins, POSTN appears to activate the Akt/PKB (protein kinase B) pathway, which is known to facilitate cell survival and tumorigenesis (Bao et al., 2004; Gillan et al., 2002; Yan and Shao, 2006).

POSTN promotes the epithelial–mesenchymal transition (EMT), cancer cell growth, angiogenesis, invasiveness, and metastasis in several cancers (Bao et al., 2004; Baril et al., 2007; Erkan et al., 2007; Gillan et al., 2002; Kudo et al., 2006; Li et al., 2002; Puppin et al., 2008; Riener et al., 2010; Sasaki et al., 2001, 2002, 2003; Shao et al., 2004; Soltermann et al., 2008a,b; Tilman et al., 2007; Tischler et al., 2010). Although it was also reported that melanomas expressed POSTN (Tilman et al., 2007), the precise roles and the source of POSTN in malignant melanoma are still unclear.

We investigated the functional role of POSTN during melanoma tumor progression in vitro *and* in vivo. Moreover, we herein demonstrate that stromal cells, normal human dermal fibroblasts (NHDFs), were important sources of POSTN in cutaneous malignant melanoma and that NHDFs promote tumor growth and progression and modulate the tumor microenvironment by secreting POSTN in cutaneous malignant melanoma.

Results

Protein expression profiles in melanoma and normal skin

To identify the proteins associated with the progression of melanoma, we performed comparative protein expression profiling between in situ melanoma tissues and matched normal skin tissue, or between invasive melanoma tissue and matched normal skin tissues. We identified a total of 1062 proteins, and 1036 proteins were quantitatively analyzed by the iTRAQ 4-plex technology using a nano LC-MS/MS analysis. The complete list of all proteins identified is shown in Table S1. Among the identified proteins present at different levels in the invasive melanoma lesions compared with matched normal skin, 30 proteins were found to have increased more than 15-fold, while 67 proteins decreased to <0.25-fold (Table S1). As expected, S100, a protein previously known to be overexpressed in melanoma, was identified as one of the overexpressed proteins. Interestingly, POSTN was found to have a 25.703-fold higher expression in invasive tumor tissue compared with matched normal skin and showed a 4.434-fold higher expression in in situ tissue compared with matched normal skin (Table S1).

Expression of POSTN in melanomas

To confirm the altered expression of POSTN in invasive melanoma, we performed a Western blot analysis using proteins extracted from the same samples. As shown in Figure 1, POSTN was highly expressed in invasive melanoma tissue and slightly expressed in in situ tissue, although POSTN was faint in normal skin tissue (Figure 1A).

We thereafter preformed an immunohistochemical analysis of 20 invasive melanoma tissues and five metastatic lymph nodes. The expression of POSTN was observed in all invasive melanoma tissue samples and metastatic lymph nodes (Figure 1B). POSTN was localized in the stroma of the invasive melanoma, with a mesh-like structure (Figure 1C). Together, these data demonstrate that POSTN was overexpressed in invasive melanoma at the protein level; this was consistent with the results of our iTRAQ analysis.

POSTN is produced by NHDF instead of melanoma cells

We also analyzed the expression of POSTN in the cell lysates from three melanoma cell lines (MeWo, G-361, and VMRC-MELG) and melanocytes by Western blot analysis; however, the expression of POSTN was not observed in these cells (Figure 2A). Because POSTN was expressed in melanoma tissue samples, but not in melanoma cell lines, we hypothesized that an interaction between melanoma cells and NHDFs was required for the optimum expression of POSTN. First, we cocultured NHDFs with the MeWo, G-361, and VMRC-MELG cell lines and performed RT-PCR and a Western blot analysis. An overexpression of POSTN mRNA was only observed in the cocultured cell lysates (Figure 2B), and POSTN protein was detected in the cocultured supernatant in a time-dependent manner (Figure 2C).

To identify the source of POSTN, we cocultured NHDFs with CFSE-labeled MeWo cells for 48 h, and sorted these cells into NHDF and MeWo populations. The expression of POSTN mRNA measured by RT-PCR showed the source of the POSTN to be the NHDFs, not the MeWo cells (Figure 2D).

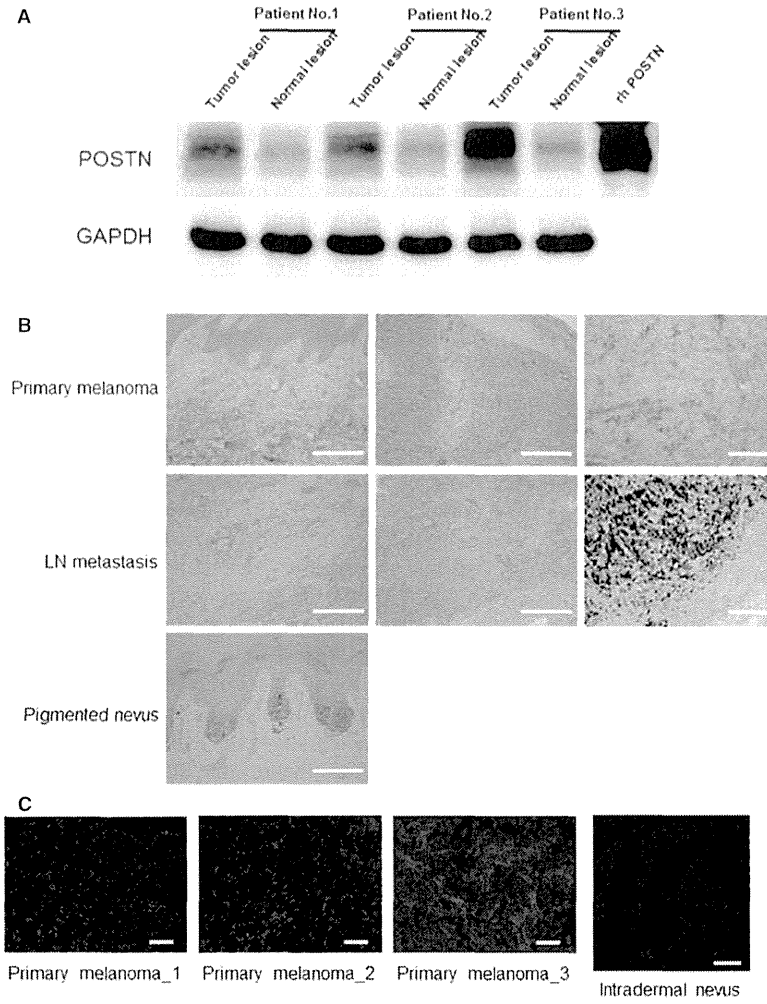

Figure 1. POSTN expression is much higher in melanoma tumor tissues compared with normal lesions. POSTN is predominantly expressed in the stroma of the melanoma tumors not melanoma cells. POSTN was slightly expressed in the in situ melanoma tissues (patient No. 1, No. 2) and highly expressed in the invasive melanoma tissues (patient No. 3) in a Western blot analysis (A). POSTN staining of invasive melanoma samples (upper three panels) and metastatic lymph nodes (*middle three panels*), *but no expression of* POSTN was detected in the pigmented nevus (lower left panel). Positive cells are stained red with ALP colorization. Bar indicates 100 μm (B). The confocal microscopic analysis showed that POSTN was strongly expressed in the stromal tissue with a lattice pattern in the 3 melanoma tissues (left 3 panels) not in the intradermal nevus (right panel). Bar indicates 20 μm for melanoma and 10 μm for intradermal nevus, respectively (C).

TGF-β1 and TGF-β3 mRNA expression in NHDFs after the coculture with melanoma cells

While the coculture of NHDF and melanoma cells was effective for the induction of POSTN expression, it was unclear how POSTN was induced during the coculture. To investigate the effect of soluble factors secreted by melanoma cells, we cultured NHDFs in the conditioned medium from MeWo or G-361 cells, and measured the expression of POSTN. However, the overexpression of POSTN was not observed at the protein level during these experiments (Figure 2E).

Next, to detect the soluble factors inducing POSTN in NHDFs, we examined the expression of POSTN-inducing cytokines, such as TGF-β1, 3, IL-4, IL-13, BMP2, and PDGF-bb, which are known to be soluble inducers of POSTN. During the RT-PCR analysis, IL-4, IL-13, BMP2, and PDGF-bb mRNA were not affected after the cocultured of melanoma cells and NHDFs (Figure 2F). However, TGF-β1 and TGF-β3 mRNA were both significantly upregulated in the cocultured NHDFs (Figure 2F). In addition, neutralization of TGF-β in the coculture markedly blocked the increase in POSTN expression (Figure 2G).

These findings indicate that cell–cell contact between NHDFs and melanoma cells is important for the expression of TGF-βs and POSTN from NHDFs, but the secretion of proteins from melanoma cells is not important for this effect.

Expression of integrin αvβ3 and αvβ5 in melanoma cells

Because integrin αvβ3, αvβ5, and α6β4 are well-known receptors for POSTN, we investigated the expression of these molecules by a Western blot analysis. We observed the expression of integrin αvβ3 and αvβ5 in the MeWo and G-361 cell lines (Figure 3A). On the other hand, integrin α6β4 was not expressed in the melanoma cells (data not shown).

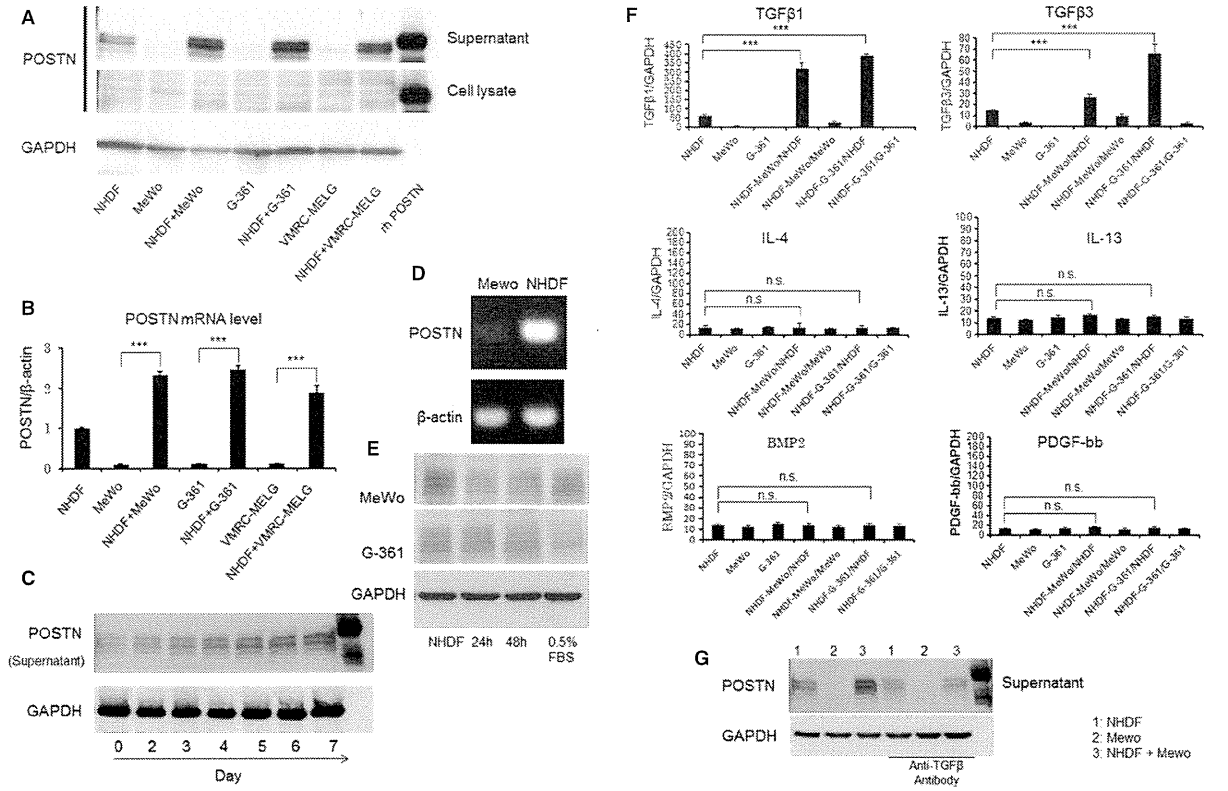

Figure 2. NHDFs secret POSTN during the coculture with human melanoma cells. The POSTN protein was upregulated in the supernatant after the coculture of NHDFs with human melanoma cells as determined by a Western blot analysis (A). POSTN mRNA was upregulated in the cell lysates after the coculture of NHDFs with human melanoma cells (B). POSTN expression was upregulated in the coculture media in a time-dependent manner (C). NHDFs, but not MeWo cells, induced POSTN expression under the coculture conditions (D). POSTN was not upregulated in the treatment with the conditioned media (E). The levels of TGFβ1, TGFβ3, IL-4, IL-13, BMP2, and PDGF-bb from NHDFs were increased after the coculture of NHDFs with melanoma cells (F). POSTN expressions were evaluated in cultured cells with or without anti-TGF-β neutralizing antibody (10 μg/ml, #MAB1835, R&D system, Minneapolis, MN). Neutralization of TGF-β blocked the increase in POSTN periostin in cocultured NHDF (G). *** indicate P-value <0.001.

Recombinant POSTN protein accelerates the proliferation of melanoma cells

To investigate the role of POSTN in the proliferation of human melanoma, we performed the MTT proliferation assay using recombinant human POSTN. The melanoma cells proliferated significantly more than control cells following the treatment with recombinant POSTN (Figure 3B). The proliferation in response to the treatment with recombinant POSTN was suppressed by anti-integrin $\alpha v\beta 3$ and $\alpha v\beta 5$ antibodies, which can neutralize the stimulation by POSTN (Figure 3C).

The phosphorylation of Akt and p44/42MAPK was observed in the cells treated with 100 ng/ml of recombinant POSTN (Figure 3D). However, the proliferation in response to the treatment with recombinant POSTN was abrogated by treatment with a MAPK inhibitor (PD98095), but not with an Akt inhibitor (LY294002) (Figure 3E). These results indicate that POSTN promotes melanoma proliferation *via* the integrin/p44/42MAPK pathway.

NHDF-derived POSTN gene transfection promotes the proliferation of melanoma cells

To investigate the role of NHDF-derived POSTN in melanoma, we transfected the NHDF-derived POSTN gene into MeWo cells (POSTN-low: lower POSTN expressing MeWo cells, POST-high: higher POSTN expressing MeWo cells, Figure 4A) and performed the MTT proliferation assay. The proliferation of POSTN–MeWo cells was significantly upregulated compared with control–MeWo (CTL-MeWo) cells in a time-dependent manner and much higher in POSTN-high cells (Figure 4B).

Significant suppression of human melanoma tumor growth in *POSTN* gene-deficient mice

We established immunodeficient Rag2 knockout mice (*Rag2* KO mice) and POSTN and Rag2 double knockout mice (*POSTN/Rag2* KO mice). We transplanted the MeWo human melanoma cell line subcutaneously onto the back of each of 17 mice and measured the tumor size for 70 days. The resulting tumors were smaller in the

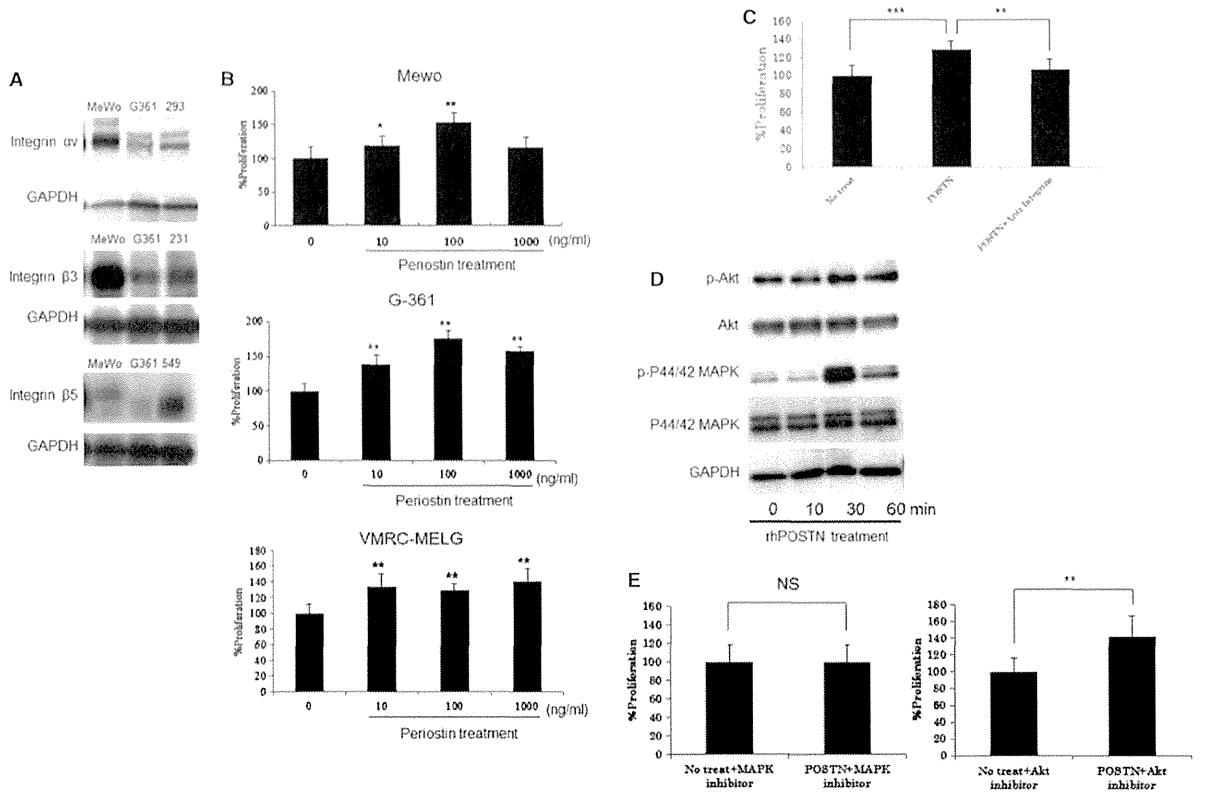

Figure 3. Recombinant POSTN protein accelerates melanoma cell proliferation via the integrin/P44/42MAPK pathway. Integrins αv, $\beta 3$, and $\beta 5$ were expressed in human melanoma cell lines (A). Recombinant POSTN increased the proliferation of human melanoma cells (MeWo, G-361, VMRC-MELG) (B). Neutralization of integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ inhibited MeWo proliferation after the treatment with recombinant POSTN (C). The phosphorylation of Akt and P44/42MAPK in MeWo cells after the indicated treatment (D). Significant inhibition of MeWo cell proliferation after the treatment with recombinant POSTN by MAPK or Akt inhibitor (E). *, **, and *** indicate P-value <0.05, <0.01, <0.001, respectively.

double KO mice on day 56 after transplantation compared with the single KO mice (Figure 5A). The number of Ki-67-positive cells was significantly lower in the double KO mice compared with *Rag2* KO mice which indicated decrease in cell proliferation (Figure 5B, C). The levels of the αSMA protein, known as a marker of myofibroblasts and collagen tissue, which is colored red with E-V (Elastica van Gieson) staining, were decreased (Figure 5B). The growth of implanted melanoma tumors was also significantly suppressed in the double KO mice (Figure 5D).

Discussion

In this study, we reported the expression and function of POSTN in the ontogeny and progression of human malignant melanoma. We first noted the upregulation of POSTN protein expression in melanoma tissues compared with adjacent normal skin using an iTRAQ analysis, thereafter confirmed that higher expression in invasive melanoma. These results suggested that the upregulation of POSTN expression might be associated with the tumor malignancy. In the confocal microscopic analysis, POSTN was predominantly found to be distinctively localized in the stroma of invasive melanoma tissue, but not in cultured melanoma cells, thus suggesting the possibility that POSTN is derived from NHDFs to affect the melanoma microenvironment. The coculture of human melanoma cells with NHDFs robustly induced POSTN expression. These results indicate that POSTN expression is produced by NHDFs, but not by melanoma cells.

Recent studies have revealed that interactions between tumor cells and the surrounding stroma play an important role in facilitating tumor growth and invasion. In the present study, the induction of TGF-β in NHDF was found by melanoma cell–NHDF cell contact, and the behavior of dermal fibroblasts was altered to promote tumor growth and invasion by the interaction with surrounding melanoma cells. As reported previously, integrin was found to activate autocrine TGF-β signaling (Asano Y et al., 2005). In the present study, integrin was found highly expressed in melanoma cells (Figure 3). It suggested the similar mechanism of activated autocrine TGF-β signaling by integrin might be involved in the interaction of melanoma cell–NHDF.

Recent studies have revealed the importance of the fibrotic microenvironments surrounding cancer cells and the interactions between the host tissue and cancer cells

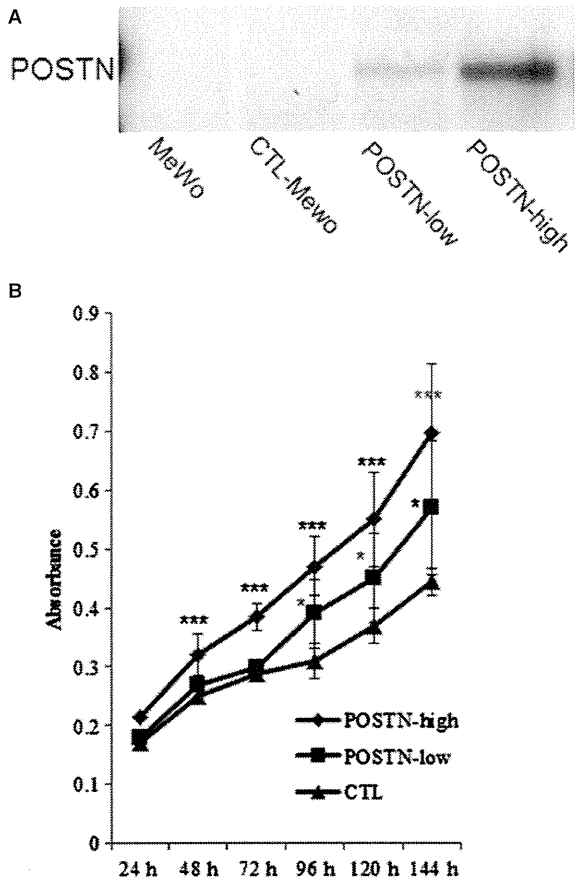

Figure 4. There is a significant increase in the proliferation of *POSTN*-transfected MeWo cells. *POSTN* gene transfection into MeWo cells (A). *POSTN* gene transfection upregulates the proliferation of MeWo cells (B). * and *** indicate P-value <0.05, <0.001, respectively.

for tumor growth and progression, because tumors are dependent on the normal host tissue-derived stromal cells and vasculature for growth and sustenance (Hanahan and Weinberg, 2000; Nyberg et al., 2008; Polyak and Kalluri, 2010; Quaranta, 2002; Yang et al., 2003; Zeisberg et al., 2002). Although there has been no previous report of a role of POSTN as stromal microenvironment in malignant melanoma, our data suggest that such a role exists.

We previously reported that POSTN accelerates dermal fibroblast proliferation, migration (Ontsuka et al., 2012), and myofibroblast differentiation, collagen 1 production (Yang et al., 2012), resulting in dermal fibrosis, and Elliott et al. also revealed the modulation of myofibroblast differentiation by POSTN (Elliott et al., 2012). These data support that NHDF-derived POSTN overexpression in the stroma of melanoma could affect the stromal microenvironment by activating dermal fibroblasts followed by tumor progression. In another report, impaired fibrous capsule formation of the implanted tumor was found in *periostin*-null mice, resulting in accelerating the tumor expansion (Guarino, 2010). Although our present data seem to be opposite of the previous report, MeWo cell established from human melanoma did not histologically form the surrounding fibrous capsule and the tumor immunity was canceled on the basis of Rag2 KO immune-deficient mice. Periostin expressed on intertumor space may be affected to accelerate adjacent melanoma cells in the present study setting.

We also revealed the proliferative effect of POSTN in human melanoma using recombinant and NHDF-derived POSTN. In details, we investigated the phosphorylation of FAK, STAT3, Akt, and p44/42MAPK, which are known to be the downstream pathways of integrin signals (Guarino, 2010). We did not observe any increase in the phosphorylation of FAK or STAT3 (data not shown), but upregulated phosphorylation of Akt and p44/42MAPK was observed after the treatment with recombinant POSTN. We also revealed that the proliferative effect of POSTN in melanoma is mediated by the integrins $\alpha v\beta 3$ and $\alpha v\beta 5$/p44/42MAPK signaling pathway, but not by the Akt pathway which is previously reported pathway (Bao et al., 2004; Ouyang et al., 2009; Yang et al., 2012).

To investigate the agonistic effect of POSTN on melanoma tissue growth in vivo, we transplanted MeWo cells into Rag2 KO mice and POSTN/Rag2 KO mice. The number of cells that were positive for Ki-67 was significantly decreased in the tumors of POSTN KO mice. In addition, the number of αSMA positive cells and the collagen expression which are known to be induced by POSTN in our previous study (Yang et al., 2012) were also decreased in POSTN KO melanomas, thus suggesting that there was suppression of the stromal microenvironment in these melanomas.

It has been reported that the POSTN expression in several cancers plays important roles in cancer progression as a result of increased proliferation, migration (Gillan et al., 2002), EMT (Soltermann et al., 2008a,b), and angiogenesis (Shao et al., 2004). In this study, our findings showed the source of POSTN to be restricted to NHDFs in human melanoma tissues, and that these stromal NHDFs between tumor cells may activate melanoma cell progression and invasion through an enhanced deposition of POSTN in the melanoma microenvironment.

Recent advances in the therapeutic approach for advanced melanoma have led to many clinical trials for patients with melanoma. For example, a BRAF inhibitor was reported to dramatically improve the prognosis of patients with melanoma. However, it was only effective in patients with the *V600E* gene mutation in the tyrosine kinase site (Atefi et al., 2011; Flaherty et al., 2010). Treatment with an anti-CTLA4 antibody can augment the anti-tumor immune response against melanoma tissue by blocking the immune-attenuating molecule, CTLA4, in T cells. However, the administration of the CTLA4 antagonist can induce severe autoimmune reactions, such as colitis and skin rashes (Weber, 2008). Therefore, it is essential to look for other therapeutic modalities with

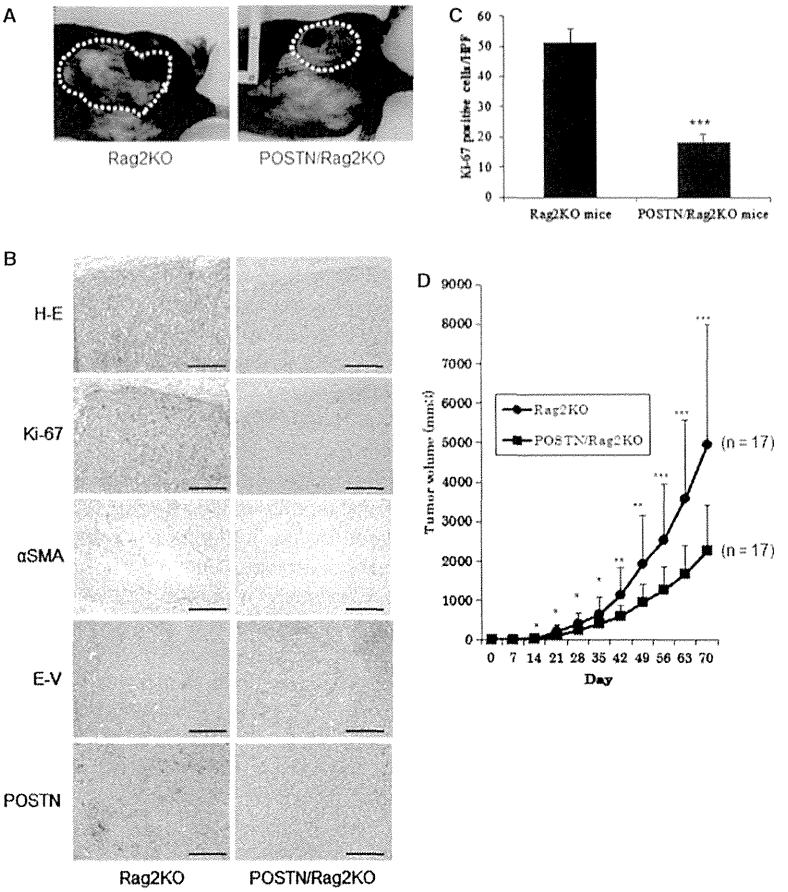

Figure 5. Melanoma tumorigenesis is significantly suppressed in *POSTN* gene knockout mice. Macroscopic feature of the *Rag2* KO and *POSTN/Rag2* KO mice on the day 56 after human melanoma cell implantation (A). There was decreased expression of Ki-67, αSMA, and collagen in the tumors of *POSTN/Rag2* KO mice. Dermal myofibroblast is positive for αSMA staining and collagen tissue becomes red by E-V (Elastica van Gieson) staining. Bar indicates 250 μm (B). There was a significant decrease in the number of Ki-67-positive cells in *POSTN/Rag2* KO mice (C). There was significant suppression of melanoma tumorigenesis in *POSTN* gene knockout mice (D). *, **, and *** indicate P-value <0.05, <0.01, <0.001, respectively.

novel mechanism(s) of action, which are not associated with such life-threatening adverse events. We believe that POSTN is important for the growth of melanoma, because the implanted melanoma cell grew more slowly in *POSTN*-depleted mice compared with matched control mice. A therapeutic approach targeting POSTN and its related signaling may lead to a safer treatment for malignant melanoma, and one that is less likely to be thwarted by resistance of the cancer cells.

In this experimental setting, there was no significant improvement in the overall survival in the POSTN-depleted mice even though the tumor growth was attenuated in KO mice compared with control mice on an immune-deficient background. There is no doubt that the survival is strongly dependent on the metastasis of the inoculated tumor cells, but we did not observe any metastasis in either the KO or wild-type mice, which likely contributed to the lack of a difference in the overall survival between the groups.

In conclusion, in human melanoma tissue, NHDFs interacted with melanoma cells to induce POSTN, which directly promoted melanoma cell proliferation by activating integrin/p44/42MAPK signals and indirectly instituted a fibrotic microenvironment in the tumor, thus resulting in a progression of the melanoma. As a result, the suppression of POSTN represents a novel therapeutic target for cutaneous malignant melanoma.

Methods

Cells and tissue samples

Human melanoma cell lines (MeWo, G-361, and VMRC-MELG) were obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan), and NHDFs were obtained from TaKaRa Bio (Shiga, Japan). 293, 231, and A549 were represented by HEK293, MDA-MB-231, and A549 cancer cell lines loaded as positive controls, respectively (JCRB). All melanoma and normal tissue samples were obtained from patients at Osaka University Hospital (Department of Dermatology, Osaka, Japan). All clinical samples were collected after approval was obtained from the local ethics committee, and informed consent was obtained from each patient for use of the samples. Details of cell culture are described in Data S1.

Mice

Twelve-week-old Rag2-deficient (*Rag2*$^{-/-}$, C57BL/6 background) and periostin-deficient (*Postn*$^{-/-}$, C57BL/6 background) mice were used for the studies (Shimazaki et al., 2010). Experiments were undertaken following the guidelines for the care and use of experimental animals as required by the Japanese Association for Laboratory Animals Science (1987).

Sample preparation and iTRAQ labeling

Proteins were extracted from the frozen tumor and normal skin tissue samples. Details are described in Data S1.

Extracted proteins were purified using a 2D clean-up kit (GE Healthcare, Buckinghamshire, UK). Subsequently, 100 μg of each protein was dissolved, reduced, alkylated, and digested with trypsin, according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). The samples were labeled with iTRAQ reagent: reagent 114 for melanoma in situ, reagent 115 for normal skin lesions of melanoma in situ, reagent 116 for invasive melanoma, and reagent 117 for normal skin lesions of invasive melanoma. The labeled peptide samples were mixed and fractionated as described previously (Serada et al., 2010).

Mass spectrometric analysis and iTRAQ data analysis

NanoLC-MS/MS analyses and iTRAQ data analysis were performed as described in Data S1.

Western blot analysis

Cell lysates and supernatant fluids were used for the Western blot analyses. Details were described in Data S1.

Immunohistochemistry

Patient with paraffin-embedded melanoma tissue sections and in vivo mice melanoma tissue sections were stained with hematoxylin and eosin (H&E). For the immunohistochemical analysis, primary antibodies were used at the following dilutions: the human and mouse anti-POSTN (1:3000; Abcam, Tokyo, Japan), mouse anti-Ki-67 (1:500; Novocastra Laboratories Ltd, Newcastle, UK), and mouse anti-α-smooth muscle actin (α-SMA; 1:3000 dilution; Sigma-Aldrich, St. Louis, MO, USA). Details are described in Data S1.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

To confirm the altered expression of POSTN in melanoma cells and NHDFs, melanoma cells (MeWo, G-361, and VMRC-MELG), NHDFs, and the cocultured cell samples were subjected to RT-PCR. β-actin was used as a housekeeping gene to evaluate and compare the quality of different cDNA samples. The primer sequences and the expected sizes of PCR products were as follows:

periostin, forward, 5′-TTGAGACGCTGGAAGGAAAT-3′

reverse, 5′-AGATCCGTGAAGGTGGTTTG-3′ (199 bp);

β-actin, forward, 5′-AGCCTCGCCTTTGCCGA-3′

reverse, 5′-CTGGTGCCTGGGGCG-3′(174 bp);

Details of total RNA extraction, quantitect reverse transcription, and RT-PCR are described in Data S1.

Quantitative reverse transcription polymerase chain reaction (qT-PCR) analysis

Normal human dermal fibroblasts were cocultured with CFSE-labeled MeWo and G-361 cells for 24 h. Thereafter, we sorted these cells into NHDF, MeWo, and G-361 cells using a FACS system. Next, the total RNA was isolated from the sorted NHDF, MeWo, and G-361 cells, and the products were reverse-transcribed into cDNA. The expression of TGFβ1, TGFβ3, IL-4, IL-13, BMP2, and PDGF-bb was measured using the Power SYBR Green PCR Master Mix (Applied Biosystems, Tokyo, Japan) according to the manufacturer's protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the mRNA, as GAPDH was not affected by the treatment. The primer sequences used were as follows:

TGFβ1, forward, 5′-TCGCCAGAGTGGTTATCTTTTG-3′

reverse, 5′-AGGAGCAGTGGGCGCTAAG-3′;

TGFβ3, forward, 5′-GCCCTTGCCCATACCTCCGC-3′

reverse, 5′-CGCAGCAAGGCGAGGCAGAT-3′;

GAPDH, forward, 5′-GGAGTCAACGGATTTGGTCGTA-3′

reverse, 5′-GCAACAATATCCACTTTACCAGAGTTAA-3′;

IL-4, forward, 5′-ACATTGTCACTGCAAATCGACACC-3′

reverse, 5′-TGTCTGTTACGGTCAACTCGGTGC-3′;

IL-13, forward, 5′-GCAATGGCAGCATGGTATGG-3′

reverse, 5′-AAGGAATTTTACCCCTCCCTAACC-3′;

BMP2, forward, 5′-ACTCGAAATTCCCCGTGACC-3′

reverse, 5′-CCACTTCCACCACGAATCCA-3′;

PDGF-bb, forward, 5′-CAGCGCCCATTTTTCATTCC-3′

reverse, 5′-GTTTTCTCTTTGCAGCGAGGC-3′.

Construction of a NHDF-derived POSTN expression vector

To construct a NHDF-derived POSTN expression vector, the cDNA of human POSTN derived from NHDFs cocultured with melanoma cells was amplified. The amplified cDNA was then inserted into the pcDNA3.1/V5-His-TOPO vector (Invitrogen, Carlsbad, CA, USA) and designated pcDNA3.1-POSTN.

Generation of NHDF-derived POSTN stable transfectant melanoma cells

To generate NHDF-derived POSTN stable transfectant cells (POSTN–MeWo), the MeWo cell line was transfected with pcDNA3.1-POSTN using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, after which the cells were selected with 500 μg/ml of Geneticin (GIBCO; Invitrogen). Stable clones were maintained in 250 μg/ml of Geneticin.

Proliferation assay

The proliferation of MeWo, G-361, VMRC-MELG, and POSTN–MeWo melanoma cells was examined using the Cell Counting Reagent SF (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's recommendations, and then, absorbance was measured with a microplate reader (model 680; Bio-Rad, Tokyo, Japan) at test and reference wavelengths of 450 and 630 nm, respectively.

Kinase inhibition assays

The cells were incubated for 2 h with kinase inhibitors (Cell Signaling Technology, Beverly, MA, USA): LY294002 (10 μM) as an Akt inhibitor and PD98095 (10 μM) as a MAPK inhibitor. Cells were then stimulated with 100 ng/ml of recombinant POSTN in the same media. After stimulation, the MTT proliferation assay was performed.

Statistical analyses

The results are presented as the means + SD. The analyses were carried out using the two-sided, unpaired Student's *t* test or the two-sided Welch test. Multiple comparisons between groups were made by Fisher's or Dunnett's methods. We considered values to be significant when $P < 0.05$.

Acknowledgements

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Disclosure statement

All authors declare no financial support or relationship that may pose conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of all proteins identified by iTraq.

Data S1. Methods.

The translation elongation factor eEF2 is a novel tumor-associated antigen overexpressed in various types of cancers

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Abstract. Recent studies have shown that cancer immunotherapy could be a promising therapeutic approach for the treatment of cancer. In the present study, to identify novel tumor-associated antigens (TAAs), the proteins expressed in a panel of cancer cells were serologically screened by immunoblot analysis and the eukaryotic elongation factor 2 (eEF2) was identified as an antigen that was recognized by IgG autoantibody in sera from a group of patients with head and neck squamous cell carcinoma (HNSCC) or colon cancer. Enzyme-linked immunosorbent assay showed that serum eEF2 IgG Ab levels were significantly higher in colorectal and gastric cancer patients compared to healthy individuals. Immunohistochemistry experiments showed that the eEF2 protein was overexpressed in the majority of lung, esophageal, pancreatic, breast and prostate cancers, HNSCC, glioblastoma multiforme and non-Hodgkin's lymphoma (NHL). Knockdown of eEF2 by short hairpin RNA (shRNA) significantly inhibited the growth in four eEF2-expressing cell lines, PC14 lung cancer, PCI6 pancreatic cancer, HT1080 fibrosarcoma and A172 glioblastoma cells, but not in eEF2-undetectable MCF7 cells. Furthermore, eEF2-derived 9-mer peptides, EF786 (eEF2 786-794 aa) and EF292 (eEF2 292-300 aa), elicited cytotoxic T lymphocyte (CTL) responses in peripheral blood mononuclear cells (PBMCs) from an HLA-A*24:02- and an HLA-A*02:01-positive healthy donor, respectively, in an HLA-A-restricted manner. These results indicated that the *eEF2* gene is overexpressed in the majority of several types of cancers and plays an oncogenic role in cancer cell growth. Moreover, the *eEF2* gene product is immunogenic and a promising target molecule of cancer immunotherapy for several types of cancers.

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Key words: eukaryotic elongation factor 2, tumor associated antigen, cytotoxic T lymphocyte, autoantibody, cancer immunotherapy

Introduction

Cancer immunotherapy consists of therapeutic approaches to elicit effective antitumor immunity through active or passive immunization. Recent studies have shown that cancer immunotherapy have potential to provide anticancer activity as a single agent or in combination with conventional surgery, radiation and chemotherapy as reviewed (1-4). These findings indicate that cancer immunotherapy should be a promising therapeutic option for the cancer treatment.

Strategies of cancer immunotherapy include antitumor monoclonal antibodies, cancer vaccines, adoptive transfer

of *ex vivo* activated T and natural killer cells, and administration of antibodies or recombinant proteins that either costimulate immune cells or block immune inhibitory pathways (5). Among these strategies, cancer vaccines are approaches to specifically activate host T cells against tumor antigens. The target antigens of cancer vaccine should be: i) highly immunogenic; ii) expressed in a significant proportion of cancer patients; iii) not expressed (or expressed in limited populations) in normal tissues; and iv) required for cancer cell growth and/or survival. Although large number of tumor-associated antigens (TAAs) have been identified using recently developed new technologies such as SEREX and protein microarrays (6,7), there are limited number of antigens that fit all of these criteria in current cancer vaccines.

High level protein biosynthesis is one of the characteristics of cancer cell metabolism (8). Translation is regulated at the initiation and elongation step and deregulated in cancer through a variety of mechanisms (9). Eukaryotic elongation factor 2 (*eEF2*) is a gene that plays an essential role in the polypeptide chain elongation step. Cells control translation levels at elongation step through regulation of eEF2 activity under multiple biological conditions such as cell cycle progression (10) and genotoxic stress (11,12), or in response to endogenous carbon monoxide that exerts antiproliferative effects (13). Previously, we showed that eEF2 was overexpressed in the majority of gastric and colorectal cancers and promoted progression of G_2/M of the cell cycle in association with activation of Akt and a G_2/M regulator, cdc2 proteins, resulting in the enhancement of *in vitro* and *in vivo* cancer cell growth (14). However, the role for eEF2 in the tumorigenesis remains largely unknown and it is undetermined whether eEF2 can be a target molecule of molecule-targeted cancer therapy.

In the present study, we identified eEF2 as an antigen eliciting humoral immune responses in a group of patients with HNSCC or colorectal cancer by immunoblot analysis and showed that eEF2 was overexpressed in the majority of various types of cancers such as lung, esophageal, pancreatic, breast and prostate cancers, HNSCC, glioblastoma multiforme and NHL. Knockdown of eEF2 by shRNA significantly inhibited growth of cancer cells. Furthermore, eEF2-derived 9-mer peptides, EF786 (eEF2 786-794 aa) and EF292 (eEF2 292-300 aa), elicited cytotoxic T lymphocyte (CTL) responses in PBMCs from an HLA-A*24:02- and an HLA-A*02:01-positive healthy donors, respectively, in an HLA-A-restricted manner.

Materials and methods

Cell lines. Lung cancer cell lines PC14 and LU99B, pancreatic cancer cell line PCI6, glioblastoma cell line A172, fibrosarcoma cell line HT1080, gastric cancer cell lines MKN28 and AZ-521, and breast cancer cell line MCF7 were cultured in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum (FBS). Leukemia cell line K562, colon cancer cell line SW480, parent T2 and T2 cells with forced expression of either HLA-A24:02 (T2-2402) (15) or HLA-A02:01 (T2-0201) (16) were cultured in RPMI-1640 medium supplemented with 10% FBS. Leukemia cell line TF-1 was cultured in RPMI-1640 medium supplemented with 10% FBS containing 2 ng/ml human recombinant GM-CSF (Peprotech, Rocky Hill, NJ, USA).

Sera samples. Sera were obtained from 79 colorectal and 80 gastric cancer patients, 10 patients with head and neck squamous cell carcinoma (HNSCC) and 40 healthy individuals with informed consent at Osaka University Hospital and Osaka Rosai Hospital and stored at -80°C until use.

Tissue samples. Tumor tissues were obtained from 31 lung adenocarcinoma, 20 small-cell lung cancer, 15 esophageal squamous cell carcinoma, 21 HNSCC, 28 pancreatic cancer, 8 breast cancer, 16 glioblastoma, 4 prostate cancer and 50 NHL (40 diffuse large B-cell lymphoma and 10 follicular lymphoma) patients. All samples were obtained with informed consent at Osaka University Hospital, Toneyama National Hospital, NHO Osaka Minami Medical Center, and Higashiosaka City General Hospital.

Western blot analysis. Proteins were separated by SDS-PAGE and transferred to Immobilon polyvinylidene difluoride membrane. After blocking of non-specific binding, the membranes were incubated with the first antibodies, followed by incubation with the corresponding secondary antibodies conjugated with alkaline phosphatase, and visualized using BCIP/NBT kit (Nacalai Tesque, Kyoto, Japan). Polyclonal anti-EF2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-GAPDH (Chemicon International, Temecula, CA, USA) were used as the first antibodies.

Density gradient isoelectric focusing. Density gradient isoelectric focusing was performed by the method reported previously (17) with minor modifications. In brief, K562 cells ($5x10^7$ cells) were lysed in 2 ml of 0.1% Triton X-100/PBS. After centrifugation, the supernatant was collected as cytoplasmic fraction. Proteins of the cytoplasmic fraction were precipitated with acetone and the pellet was solved in 1 ml of dH_2O containing 4% CHAPS and 7 M urea. Isoelectric focusing was carried out using an LKB column (NA-1720, Nihon-Eido Co., Tokyo, Japan) according to the manufacturer's instructions. On completion of the run, effluent fractions (3 ml each) were collected and twice dialyzed to 200 volume of de-ionized water for 18 h, and then the proteins were precipitated with acetone and stored at -80°C until use.

MALDI-TOF mass spectrometry. The bands on the silver stained gels were excised with surgical blazor. After dehydration with acetonitrile, the gel slice was dried with Speed Vac. The dried gels were digested with Trypsin (Promega, Madison, WI, USA) at 37°C for 24 h and the tryptic peptides were analyzed. All peptide mass fingerprinting (PMF) spectra were obtained by Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry using an ultraflex spectrometer (Bruker Daltonics, Bremen, Germany). PMF data were then searched with MS-FIT software against NCBInr database.

Immunohistochemistry. Formalin-fixed tissue sections were cut from each paraffin-block. After dewaxing and rehydration, the sections were antigen retrieved using Pascal (Dako Cytometry, Glostrup, Denmark) and reacted with the first antibody at 4°C overnight and then reacted with Dako Envision kit/HRP (Dako Cytometry) at room temperature for 30 min.

After treatment with 3% H_2O_2 solution to reduce endogenous peroxidase activity, immunoreactive eEF2 protein was visualized using diaminobenzidine (DAB). The sections were then counterstained with hematoxylin. The intensity of stain in tumor cells was scored as positive (increased staining in carcinoma cells compared to that in normal cells) or negative (less or negative staining in carcinoma cells) by a pathologist. eEF2-H118 antibody (Santa Cruz Biotechnology) that recognized 741-858aa of eEF2 protein and Sigma-Aldrich #SAB4500695 antibody that recognized the N terminus of eEF2 protein were used as first antibodies. Non-immune rabbit immunoglobulin (Dako Cytometry) was used as negative control for non-specific staining.

Sequencing. The *eEF2* gene overexpressed in tumors was RT-PCR amplified and directly sequenced in both directions by the method previously described (14).

Transient expression of shRNA targeting eEF2. Two different shRNA vectors targeting eEF2 mRNA (shEF-1918 and shEF-2804 targeting 1918-1947 and 2804-2833 nt of eEF2 sequence, respectively) were prepared as described previously (14). shRNA targeting luciferase (shLuc) was used as a control. shRNA vectors were transiently expressed as described previously (14).

Enzyme-linked immunosorbent assay (ELISA). ELISA was established to measure serum eEF2 IgG Ab levels by a method previously reported (18) with modifications. ELISA 96-well plates were coated with recombinant GST-tagged eEF2 fragmented protein (Ref Seq NM_001961, 411-858 aa) (2 μg/well). Plates were blocked with TBS containing 0.05% Tween-20 and 1% gelatin. Sera were diluted at 1:100 in TBS containing 0.05% Tween-20 (0.05% TBST) and pre-absorbed by immobilized GST protein at 4°C overnight. Then, 100 μl of the diluted sera was added to each well for overnight incubation at 4°C. After washing, captured eEF2 IgG Ab was detected using ALP-conjugated goat anti-human IgG Ab (Santa Cruz Biotechnology) and BCIP/NBT kit. Then, absorbance at 550 nm was measured using a microplate reader. All sera were examined in duplicate. The titers of eEF2 IgG Ab were calculated by interpolation from the standard line which was constructed for each assay from the results of simultaneous measurements of serial dilutions of rabbit polyclonal eEF2 H-118 Ab using the corresponding second Ab (data not shown). eEF2 Ab titer that produces the absorbance at 550 nm equal to that produced by 1.0 μg/ml of eEF2 H-118 Ab in the ELISA system was defined as 1.0 EF2-reacting-unit (ERU).

Synthetic peptides. The primary amino acid sequence of human eEF2 was analyzed for consensus motifs for 9-mer peptides capable of binding to HLA-A*24:02 or 02:01 using ProPred-I computer algorithm (Table I). Then, the top 4 candidate peptides for HLA-A*02:01 and 24:02 each were synthesized at immunological grade (Sigma Genosys, Hokkaido, Japan). Synthesized peptide was solved in dH_2O (2 mg/ml) and stored at -20°C until use.

MHC stabilization assay. Binding of the synthetic peptides to HLA-A*24:02 or 02:01 molecules was evaluated by MHC stabilization assay using antigen processing mutant T2-2402 or T2-0201 cells as described previously (19). Expression of HLA-A24 or HLA-A02 molecules was measured with a FACSort flow cytometer (BD Biosciences, San Jose, CA, USA) and the mean fluorescence intensity (MFI) was recorded.

In vitro generation of eEF2 peptide-specific CD8$^+$ T cells. PBMCs were obtained from an HLA-A*24:02-positive and an HLA-A*02:01-positive healthy donors by density gradient centrifugation. CD4$^+$CD25$^+$ Treg cells were depleted from PBMCs by using CD25 MicroBeads (Miltenyi Biotech, Auburn, CA, USA). For generation of autologous dendritic cells (DCs), CD14$^+$ monocytes were isolated from the donor PBMCs using BD IMag CD14 isolation kit (BD Bioscience) and cultured in X-VIVO15 (Bio Whittaker, Walkersville, MD, USA) supplemented with 1% human AB serum (Nabi, Miami, FL, USA) containing IL-4 (1,000 U/ml) and GM-CSF (800 U/ml). After 24 h, IL-1β (10 ng/ml), IL-6 (1,000 U/ml), TNF-α (10 ng/ml), and PGE-2 (1 μg/ml) were added to the culture for DC maturation and the cells were cultured for 48 h. DCs were pulsed with EF2 peptide at the concentration of 10 μg/ml in X-VIVO15 supplemented with 1% human AB serum at 37°C for 2 h, irradiated at 30 Gy, and washed 3 times with RPMI-1640 medium. Then, Treg-depleted PBMCs (2x10^6 cells) were stimulated by co-culture with the EF2 peptide-pulsed DCs at the DC: PBMC ratio of 1:10 in X-VIVO15 supplemented with 5% human AB serum. After 24 h of co-culture, IL-2 (20 U/ml) was added to the culture. The cultured cells were repeatedly stimulated with the EF2 peptide-pulsed, irradiated autologous PBMCs at 10-day intervals. After several times of re-stimulation, the cultured cells were maintained as the established T cell lines in X-VIVO15 supplemented with 5% human AB serum, IL-7 (10 IU/ml) and IL-15 (10 IU/ml) and used for cytotoxic assays.

^{51}Cr release cytotoxicity assay. Effector cells were prepared from the established T cell lines using Human CD8 T Lymphocyte Enrichment Set-DM (BD Bioscience). Target cells (listed in Table III) were labeled with 100 μCi of ^{51}Cr (Perkin-Elmer, Waltham, MA, USA) at 37°C for 1.5 h and the target cells (1x10^4 cells) were added to wells containing varying numbers of effector cells in 96-well plates. After 4 h of incubation at 37°C, 100 μl of supernatants were collected from each well and measured for radioactivity. The percentage of specific lysis was calculated as follows: percentage of specific lysis = (cpm of experimental release - cpm of spontaneous release)x100/(cpm of maximal release - cpm of spontaneous release). Radioactivity of the supernatant of the target cells that were cultured without effector cells and the radioactivity of target cells that were completely lysed by the treatment with 1% Triton X-100 was used for spontaneous and maximal release, respectively. The characteristics of target cells in cytotoxicity assay are listed in Table III.

Statistics. The statistical significance in a difference between arithmetical means of test groups was assessed by unpaired t-test or Kruskal-Wallis test. After Kruskal-Wallis test, Scheffe's F-test was used as a post hoc test.

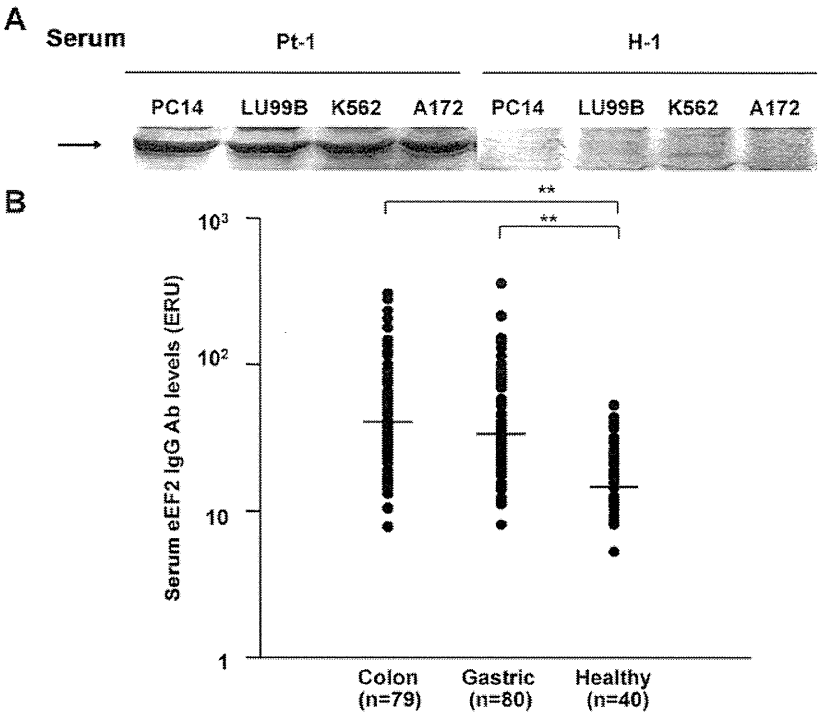

Figure 1. Elevation of serum eEF2 IgG autoantibody levels in cancer patients. (A) Cytoplasmic proteins from PC14, LU99B, K562 and A172 cells were subjected to immunoblot analysis using sera as the first antibodies. Representative results with sera from an HNSCC patient (Pt-1) and a healthy control individual (H-1) are shown. Arrows indicate the protein that is recognized by IgG autoantibody in the sera from the HNSCC patient. (B) Elevation of serum eEF2 IgG autoantibody levels in cancer patients. Assays were performed in duplicate. Colon, colorectal cancer; gastric, gastric cancer; and healthy, healthy individuals. Standard bar represents median value. $^{**}p<0.01$. eEF2 Ab levels that produces the absorbance at 450 nm equal to that produced by 1 μg/ml of anti-eEF2 H-118 Ab in the ELISA system were defined as 1.0 eEF2-reacting-unit (ERU).

Results

Production of IgG autoantibody against eukaryotic elongation factor 2 (eEF2) in cancer patients. To identify novel tumor-associated antigens (TAAs) with high molecular weight (more than 100 kDa), which were difficult to isolate by standard two dimensional electrophoresis methods because they could not be absorbed into a strip gel, proteins from tumor lysates were first separated by SDS-PAGE, transferred to PVDF membrane, and then probed with sera from tumor-bearing patients. As shown in Fig. 1A, an approximately 100 kDa protein was recognized by sera from 4 of 10 HNSCC and 2 of 3 colon cancer patients in cytoplasmic proteins from two lung cell lines (PC14 and LU99B), one leukemic cell line (K562) and one glioblastoma cell line (A172), whereas it was not recognized by the sera from 5 healthy individuals. To identify this protein, cytoplasmic proteins of K562 cells were fractionated by density gradient isoelectric focusing, separated by SDS-PAGE, and subjected to immunoblot analysis using sera from an HNSCC patient as the first antibody. Since immunoblot analysis detected this protein in fractions of pH 6.62 and pH 6.75, the silver-stained band corresponding to this protein was excised from the SDS-PAGE gel and the protein was analyzed by MALDI-TOF Mass Spectrometry. The search for NCBInr database by MS-Fit software identified the protein as human eukaryotic elongation factor 2 (eEF2) that had M.W. of 95.3-kDa and calculated pI of 6.4.

Elevation of serum eEF2 IgG antibody levels in cancer patients. Serum eEF2 IgG Ab levels were examined by ELISA in 79 colorectal and 80 gastric cancer patients and 40 healthy individuals and detected in all the samples examined (Fig. 1B). eEF2 IgG Ab levels ranged from 7.8 to 301.7 (median 41.1), from 8.1 to 353.9 (median 33.6) and from 5.2 to 53.0 (median 20.6) ERU in colorectal and gastric cancer patients and healthy individuals, respectively. eEF2 IgG Ab levels were significantly ($p<0.01$) higher in both colorectal and gastric cancer patients than healthy individuals.

Overexpression of eEF2 in various types of human cancers. eEF2 protein was immunohistochemically examined in 51 lung cancers, 15 esophageal squamous cell carcinomas, 21 HNSCCs, 28 pancreatic cancers, 8 breast cancers, 16 glioblastoma multiformes, 4 prostate cancers and 50 NHLs. Immunohistochemical analysis with two different anti-EF2 antibodies recognizing different regions of eEF2 protein showed similar results. Overexpression of eEF2 protein was detected in 71.0% (22 of 31) of lung adenocarcinoma, 95.0% (19 of 20) of small-cell lung cancer, 73.3% (11 of 15) of esophageal cancer, 60.7% (17 of 28) of pancreatic cancer, 50.0% (4 of 8) of breast cancer, 75.0% (3 of 4) of prostate cancer, 52.4% (11 of 21) of HNSCC, 75.0% (12 of 16) of glioblastoma multiformes, and 94.0% (47 of 50) of NHL. Results are summarized in Table I. Representative results are shown in Fig. 2.

Table I. Overexpression of eEF2 in human cancers.

Cancer	Overexpression of eEF2 (%)
Lung cancer	80.4 (41/51)
Lung adenocarcinoma	71.0 (22/31)
Small cell lung cancer	95.0 (19/20)
Esophageal squamous cell carcinoma	73.3 (11/15)
Head and neck squamous cell carcinoma	52.4 (11/21)
Pancreatic cancer	60.7 (17/28)
Breast cancer	50.0 (4/8)
Glioblastoma	75.0 (12/16)
Prostate cancer	75.0 (3/4)
Non-Hodgkin's lymphoma	94.0 (47/50)
Diffuse large B cell lymphoma	92.5 (37/40)
Follicular lymphoma	100 (10/10)

Expression of eEF2 protein in human cancers was examined by immunohistochemistry. Immunostaining was evaluated as positive when cancer cells were stained brown in >10% of the cells.

Overexpressed eEF2 gene is a non-mutated, wild-type. To examine whether or not the overexpressed *eEF2* gene was non-mutated, wild-type, the 5' (84-1334 nt) and the 3' (1314-2660 nt) sequences of eEF2 mRNA (coding sequence: 84-2660 nt) from five lung adenocarcinomas and five HNSCCs were amplified by RT-PCR and direct sequencing. No mutation was found in the *eEF2* gene in the 10 cancers examined (data not shown).

Knockdown of eEF2 inhibits cancer cell growth. To examine the role of eEF2 in cancer cell growth, either of two different shRNAs targeting eEF2 (shEF-1918 and shEF-2804) or a control shRNA targeting luciferase (shLuc) was transfected into four eEF2-expressing cells, lung cancer PC14, pancreatic cancer PCI6, fibrosarcoma HT-1080, and glioblastoma A172 and eEF2-undetectable breast cancer MCF7 cells. After culture for 72 h, both of the two shRNAs targeting eEF2 (shEF-1918 and shEF-2804) reduced eEF2 protein expression levels (Fig. 3A) and significantly inhibited cell growth in all the four eEF2-expressing cells examined (Fig. 3B). However, neither of the two shRNAs targeting eEF2 inhibited growth of eEF2-undetectable MCF7 cells.

*Identification of eEF2 peptides that bind to HLA-A*24:02 or HLA-A*02:01 molecules.* Epitope candidates of eEF2 that bound to HLA-A*24:02 or HLA-A*02:01 molecules were first analysed using ProPred-I computer algorithm (Table II).

As candidate epitope peptides that bound to HLA-A*24:02 molecules, EF78, EF786, EF701 and EF412 peptides were selected and analyzed for binding affinity to HLA-A*24:02 molecules by the MHC stabilization assay. These peptides were pulsed to T2-2402 cells and the expression of HLA-A*24:02 molecules on the cell surface was analyzed by flow cytometry. As shown in Table II, all the four peptides increased the expression of HLA-A24:02 molecules on T2-2402 cells as a result of the stabilization of HLA-A24:02 molecules. Among the four peptides, EF786 peptide showed binding affinity higher than CMVpp65$_{328\text{-}336}$, which was an exogenous cytomegalovirus antigen epitope, to the HLA-A*24:02 molecules. As candidate peptides that bound to HLA-A*02:01 molecules, EF292, EF739, EF519 and EF671 peptides were selected and analyzed for binding affinity to HLA-A*02:01 molecules by the MHC stabilization assay. As shown in Table II, all the four peptides increased the expression of HLA-A02:01 molecules on T2-0201 cells and EF292 peptide showed the highest binding affinity to HLA-A*02:01 molecules among the four HLA-A*02:01-binding peptides examined.

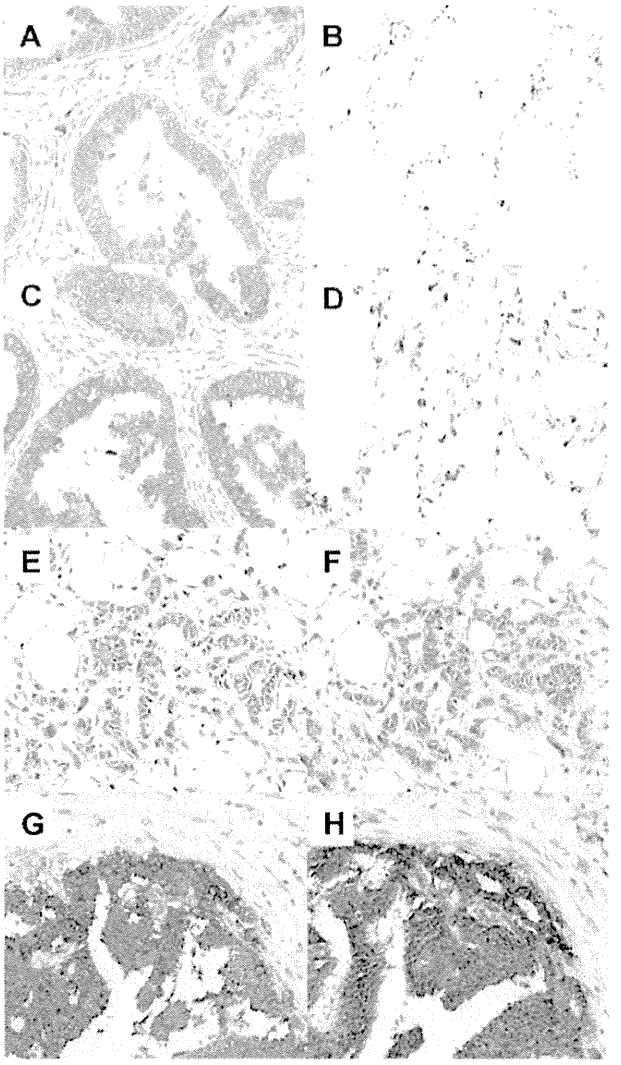

Figure 2. Overexpression of eEF2 in various types of cancers. Representative results of immunohistochemical analysis for eEF2 protein expression in (A and C) lung adenocarcinoma, (B and D) normal lung cells, (E and F) breast cancer, and (G and H) prostate cancer. eEF2 was stained with (A, B, E and G) eEF2-H118 antibody or (C, D, F and H) #SAB4500695 antibody. eEF2 protein was stained brown. Macrophages are non-specifically stained in normal lung tissues.

*Generation of EF2-specific CTLs from HLA-A*24:02- or HLA-A*02:01-positive donors.* Treg-depleted PBMCs from

Table II. Characteristics of EF2-derived peptides and results of the MHC stabilization assay.

Peptide	Position (aa)	Sequence	Score	%MFI increase
HLA-A*24:02-binding peptides				
EF78	78-86	FYELSENDL	360	40.5
EF786	786-794	AYLPVNESF	252	1552.1
EF701	701-709	RFDVHDVTL	40	297.3
EF412	412-420	AFGRVFSGL	33.6	47.9
CMVpp65 328-336		QYDPVAALF		1344.1
HLA-A*02:01-binding peptides				
EF292	292-300	LILDPIFKV	3290	183.3
EF739	739-747	RLMEPIYLV	2426	141.1
EF519	519-527	KLVEGLKRL	705	58.9
EF671	671-679	YLNEIKDSV	642	89.6

The primary amino acid sequences of human eEF2 were analyzed for consensus motifs for 9-mer peptides capable of binding to HLA-A*24:02 or 02:01 molecules using ProPred-I software. Percentage MFI increase in MHC stabilization assay was calculated as follows: percentage MFI increase = (MFI with the given peptide - MFI without peptide)/(MFI without peptide) x 100.

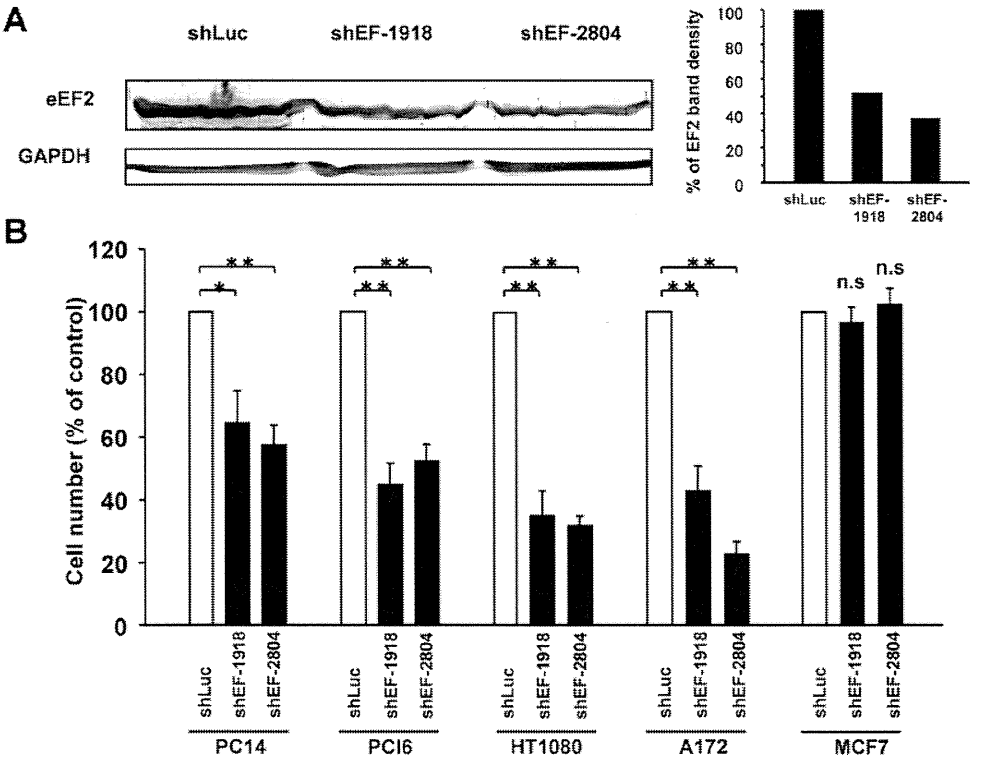

Figure 3. Knockdown of eEF2 inhibits cancer cell growth. Two shRNA vectors targeting different sequences of eEF2 (shEF-1918 and shEF-2804 targeting 1918-1947 and 2804-2833 nt of eEF2 sequence, respectively) or control shRNA targeting luciferase (shLuc) was transfected into PC14, PCI6, HT1080, A172 and MCF7 cells. (A) Reduction in eEF2 protein expression levels in HT1080 cells. Results of western blot analysis are shown. (B) After 72 h of transfection, the cell numbers were examined. *p<0.05; **p<0.01. Experiments were independently performed three times.

HLA-A*24:02- or HLA-A*02:01-positive healthy donors were repeatedly stimulated with EF2 peptides (EF786 and EF292 peptides for HLA-A*24:02- and HLA-A*02:01-positive healthy donors, respectively) and pulsed irradiated autologous DCs and EF2 peptide-specific CTLs were established.

To examine whether EF2 peptides are capable of eliciting CTL responses, CTL activities of established CTLs were examined. As shown in Fig. 4A, EF786-specific, HLA-A*24:02-restricted CTLs lysed EF786 peptide-pulsed T2-2402 cells but not unpulsed ones. The EF786-specific

Table III. Characteristics of target cells in the killing assay.

Target cells	HLA-A*24:02 expression	HLA-A*02:01 expression	eEF2 expression
T2	-	-	Undetectable
T2-2402	+	-	Undetectable
T2-0201	-	+	Undetectable
SW480	+		+
AZ-521	-		+
MKN28	-		+
TF-1		+	+
K562	-	-	+
MCF7		+	Undetectable

Cell surface protein expression of HLA-A molecules was confirmed by flow cytometry. Expression of eEF2 protein was analyzed by western blot analysis.

CTLs lysed HLA-A*24:02-positive, eEF2-expressing SW480 cells, but not HLA-A*24:02-negative, eEF2-expressing AZ-521 and MKN28 cells. As shown in a Fig. 4B, EF292 peptide-specific, HLA-A*02:01-restricted CTLs lysed EF292 peptide-pulsed T2-0201 cells but not unpulsed ones. Moreover, the EF292-specific CTLs lysed HLA-A*02:01-positive, eEF2-expressing TF-1 cells, but not HLA-A*02:01-negative, eEF2-expressing K562 cells and HLA-A*02:01-positive, eEF2-undetectable MCF7 cells (Fig. 4B).

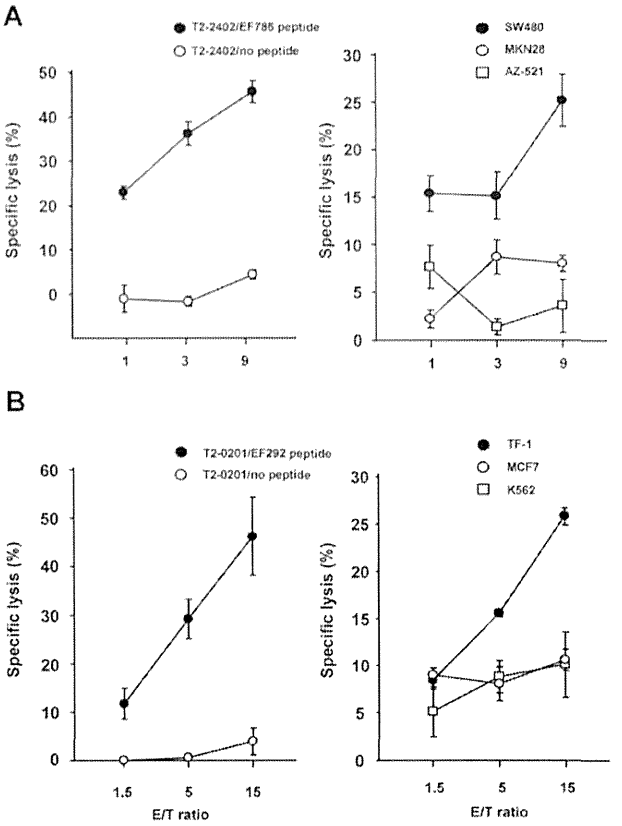

Figure 4. Generation of eEF2-specific CTLs. (A, left panel) Specific lysis of EF786 peptide-pulsed T2-2402 cells by EF786-specific, HLA-A*24:02-restricted CTLs. (A, right panel) Specific lysis of eEF2-expressing, HLA-A*24:02-positive SW480 by EF786-specific, HLA-A*24:02-restricted CTLs. AZ-521 and MKN28 are eEF2-expressing, but HLA-A*24:02-negative. (B, left panel) Specific lysis of EF292 peptide-pulsed T2-0201 cells by EF292-specific, HLA-A*02:01-restricted CTLs. (B, right panel) Specific lysis of eEF2-expressing, HLA-A*02:01-positive TF-1 cells by EF292-specific, HLA-A*02:01-restricted CTLs. K562 is eEF2-expressing and HLA-A*02:01-negative, and MCF7 is eEF2-undetectable and HLA-A*02:01-positive. E/T, effector/target ratio. CTL cytotoxic assays were performed in triplicate.

Discussion

We showed that eEF2 was overexpressed in the majority of various types of tumors such as lung, esophageal, pancreatic, and breast cancer and promoted growth of various types of cancer cells. Moreover, *eEF2* gene product elicited both humoral and cellular eEF2-specific immune responses. The production of eEF2 IgG autoantibody was enhanced in patients with colorectal and gastric cancer and 9-mer eEF2 peptides elicited EF2-specific CTLs from healthy donors. These results indicated that overexpressed eEF2 played an oncogenic role and served as a TAA in these tumors.

It is considered that production of autoantibody indicates the potential of its antigen as a target of cancer immunotherapy (20). In the present study, we showed the elevation of serum EF2 IgG levels in colorectal and gastric cancer patients, indicating that eEF2 overexpressed in cancer cells was recognized by the host immune system and induced eEF2-specific immune responses. Since production of IgG autoantibody needed help from $CD4^+$ helper T cells (Th cells) for class switch from IgM to IgG, elevation of EF2 IgG Ab levels indicated the activation of EF2-specific Th cells. It is well established that Th cells play an important role in the immune responses against cancer (21). $CD4^+$ Th cells are required for activation and maintenance of $CD8^+$ CTLs, but they could also exert cytotoxic function against cancer in the absence of $CD8^+$ CTLs recognizing antigenic peptides presented by MHC class II molecules (22,23). These results indicated that EF2 protein was an immunogenic molecule that is capable of eliciting not only humoral but also cellular immune responses. In fact, eEF2-derived EF786 peptide showed the binding affinity higher than CMVpp65328-336, an exogenous viral antigen epitope, and elicited *in vitro* EF786-specific CTLs from PBMCs of HLA-A*24:02-positive healthy donors. Taken together, eEF2 protein is highly immunogenic and a promising target molecule for cancer immunotherapy.

Expression of target molecules in tumor cells is the first requisite for TAA-targeting cancer immunotherapy. Survivin is a member of the family of the inhibitor of apoptosis proteins and functions as a key regulator of mitosis and programmed cell death (24). Survivin is overexpressed in various types of tumors with the frequency of 34.5% in gastric cancers (25), 50-60% in colorectal cancers (25,26), 64% in malignant gliomas (27), 53-72% in lung cancers (28,29), and 70.7% in breast cancers (30). Cancer vaccines to induce an antigen-specific immune responses against survivin-expressing tumor cells have been developed with

promising results (31,32). Thus, survivin appears to be a promising TAA. However, survivin-targeted immunotherapy may be applicable to a limited population of patients because of its low expression rates in several tumors. In addition, the frequency of survivin-positive tumor cells may vary in individual tumors (25). Thus, the existence of tumor cells lacking survivin could result in tumor evasion from CTL responses against survivin induced by vaccination. NY-ESO-1 is a member of cancer testis antigens and is expressed in a variety of common cancers. Clinical trials that evaluate therapeutic responses against NY-ESO-1 are underway in various cancers (33). However, NY-ESO-1 protein was expressed in only 20 to 30% of lung (34), bladder and ovarian cancers (35) and melanoma and was undetectable in colon and renal cancers (36). Thus, therapeutic strategy against NY-ESO-1 is applicable to a minor population of cancer patients. Compared to these TAAs, eEF2 is more attractive as a target molecule of cancer immunotherapy because of its high frequency of overexpression in various types of cancers. The frequency of eEF2 overexpression exceeded 70% in lung, esophageal, breast and prostate cancers, and 90% in gastric and colorectal cancers and NHL, as shown in the present and previous (14) studies. These results indicated that eEF2-targeted immunotherapy should be a therapeutic strategy that would be applicable to the majority of cancer patients. WT1 is also a promising target molecule of immunotherapy and was ranked as top of TAAs (37). WT1 is overexpressed in the majority of leukemia (38) and various types of tumors such as lung (39), colorectal (40) and pancreatic cancer (41), and glioblastoma multiforme (42). However, WT1 might be less expressed in malignant lymphoma. In diffuse large B-cell lymphoma the most common type of NHL, WT1 protein was detected in only 33% of the cases examined (43). Thus, eEF2-targeted immunotherapy may have a priority for NHL.

One mechanism for escape from immune surveillance is the loss of expression of target molecules in cancer cells (44). Therefore, it is important to know whether or not loss of eEF2 expression affects tumor growth in consideration of the potential of eEF2 as a target molecule for cancer immmunotherapy. As shown in the present study, knockdown of eEF2 by shRNA significantly inhibited cancer cell growth. Also, we have demonstrated that eEF2 was overexpressed in the majority of gastric and colorectal cancers and promoted progression of G_2/M in the cell cycle, resulting in the enhancement of *in vitro* and *in vivo* cancer cell growth (14). Based on these findings showing the involvement of eEF2 in cancer cell growth, it is unlikely that antigenic loss of eEF2 could become a mechanism of tumor escape from eEF2-specific immune responses.

A primary goal of cancer immunotherapy is generation of effective CTL responses through the expansion of robust pre-existing, naturally occurring $CD8^+$ CTL precursors and the establishment of long-lasting memory $CD8^+$ T cells. This critically depends on the activation of pre-existing antigen-specific CTL precursors as the initial step to induce immune responses. In the present study, eEF2-specific CTL clones were established from HLA-A*24:02- or HLA-A*02:01-positive healthy donors. In addition, eEF2 IgG autoantibody is detected at low levels in healthy individuals examined. Since these results indicated the existence of not only eEF2-specific CTL precursors but also eEF2-specific B and Th cells even in healthy donors without cancer, the host immune system of cancer patients should have a potential to make robust immune responses against eEF2-expressing cancers by vaccination with EF2 protein or peptide.

In conclusion, eEF2 that is overexpressed in a wide variety of cancers is a promising cancer antigen that can elicit both humoral and cellular immune responses and shows promise as a target molecule of cancer immunotherapy.

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ORIGINAL ARTICLE

Histamine Contributes to Tissue Remodeling via Periostin Expression

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Histamine is thought to have a critical role in the synthesis of extracellular matrix in skin and may be involved in tissue remodeling of allergic diseases. Recent studies revealed that periostin, a matricelluar protein, contributed to tissue remodeling; however, a link between periostin and histamine remains unproven. We investigated whether periostin was involved in histamine-induced collagen production. Cultured dermal fibroblasts derived from wild-type (WT) or periostin knockout ($PN^{-/-}$) mice were stimulated with histamine, and then collagen and periostin production was evaluated. Histamine induced collagen gene expression in WT fibroblasts in the late phase but not in the early phase, whereas no effect on collagen expression was observed in histamine-stimulated $PN^{-/-}$ fibroblasts. In WT fibroblasts, histamine directly induced periostin expression in a dose-dependent manner, and an H1 receptor antagonist blocked both periostin and collagen expression. Histamine activated extracellular signal-regulated kinase 1/2 (ERK1/2) through the H1 receptor. Periostin induction was inhibited by either H1 antagonist or ERK1/2 inhibitor treatment *in vitro* and was attenuated in $H1R^{-/-}$ mice. Elevated expression of periostin was found in lesional skin from atopic dermatitis patients. These results suggest that histamine mediates periostin induction and collagen production through activation of the H1 receptor–mediated ERK1/2 pathway; furthermore, histamine may accelerate the chronicity of atopic dermatitis.

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INTRODUCTION

Tissue remodeling is both a cause and a consequence of allergic inflammation and is considered a target for therapeutic intervention.

Understanding the underlying mechanisms that cause tissue remodeling is progressing gradually, and certain factors that are correlated with allergic inflammation have been found to be involved in tissue remodeling. Among them, histamine has been found to induce *de novo* synthesis of collagen from fibroblasts in both *in vivo* and *in vitro* experiments (Sandberg, 1962, 1964; Cohen *et al.*, 1972; Murota *et al.*, 2008). Furthermore, it is well known that antihistamine drugs used for the treatment of allergic disorders improve hypertrophic scars (Murakami *et al.*, 1998). These results indicate that histamine may be involved in the mechanism of tissue remodeling in allergic diseases; however, it remains unknown how histamine contributes to tissue remodeling.

Recent studies showed that expression of periostin, a matricellular protein with profibrogenic function, increased in sera and lesional tissue from patients with allergic diseases, such as allergic rhinitis, asthma, and atopic dermatitis (AD), and that this expression was associated with airway or other tissue remodeling (Takayama *et al.*, 2006; Hur *et al.*, 2012; Masuoka *et al.*, 2012). Furthermore, periostin has also emerged as a key regulator in the development of wound healing and scleroderma (Ontsuka *et al.*, 2012; Yang *et al.*, 2012). To the best of our knowledge, the impact of histamine on the expression level of periostin is unknown. Therefore, in this study, we investigated the correlation between histamine and the fibrotic factor periostin in primary cultured dermal fibroblasts.

RESULTS

Collagen production is induced by histamine stimulation

To investigate whether histamine influences collagen synthesis, primary cultured murine dermal fibroblasts were stimulated with histamine at concentrations ranging from 0 to 100 μM, as described in our previous report (Murota *et al.*, 2008; Murota and Katayama, 2009).

Histamine-induced type I collagen production was observed with concentrations of histamine from 1 to 100 μM (Figure 1). Compared with nontreated controls, collagen synthesis was significantly increased after histamine treatment

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Abbreviations: AD, atopic dermatitis; CREB, cAMP response element-binding protein; ERK1/2, extracellular signal-regulated kinase 1/2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H1R, histamine receptor 1; WT, wild type

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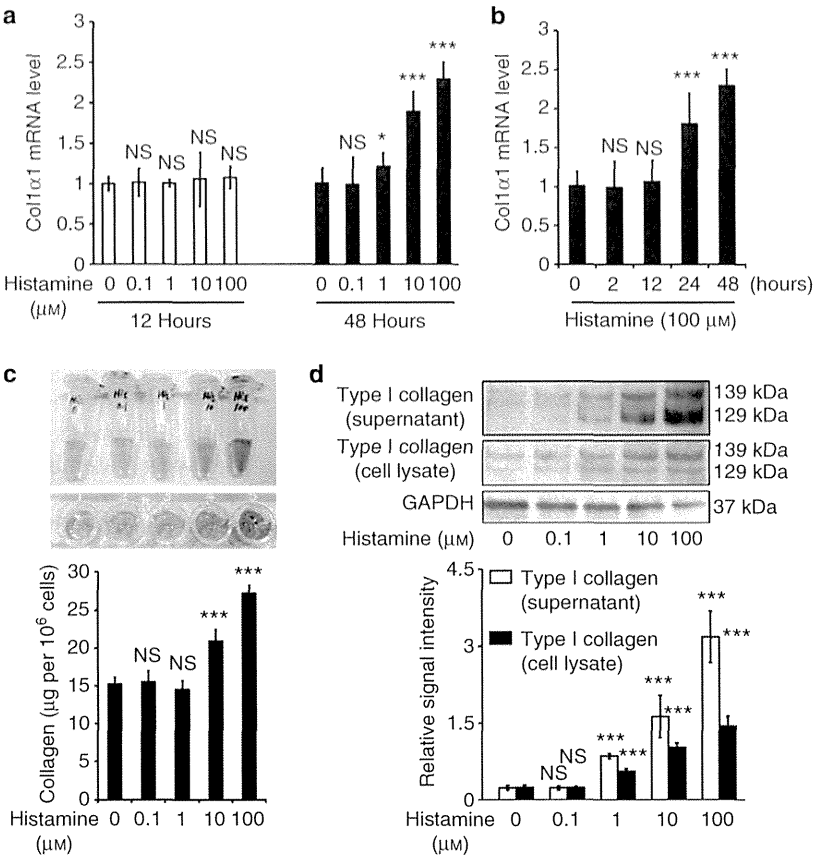

Figure 1. Collagen production is induced by histamine stimulation in cultured wild-type (WT) fibroblasts. (**a**) Effect of histamine on collagen type-I alpha 1 (Col1α1) mRNA expression was assessed by quantitative real-time reverse transcriptase–PCR (qRT-PCR) at 12 hours (white bar) and 48 hours (black bar) after the addition of histamine at the indicated concentration. (**b**) Effect of histamine on Col1α1 mRNA expression after the stimulation with histamine (100 μM) for the indicated time periods. (**c**) Soluble collagen content in the supernatants of WT fibroblasts that had been stimulated with histamine for 48 hours. (**d**) Representative western blotting and quantitative analyses of signal density on blots from three independent experiments analyzing collagen protein expression in response to 48 hours of histamine stimulation (using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control). Values were derived from three independent experiments using WT fibroblast cultures. Values represent the mean ± SD for the three independent experiments in each condition. $^{*}P<0.05$; $^{***}P<0.001$; NS, no significance, compared with control (0 μM histamine) by one-way analysis of variance (ANOVA) followed by Dunnett's test.

in a dose-dependent manner (Figure 1a, c and d). No significant increase was observed at 2 and 12 hours, whereas collagen was markedly induced by histamine 24 and 48 hours after addition (Figure 1a and b). In our previous study (Yang *et al.*, 2012), the mRNA expression of type I collagen was found to be significantly increased in cultured mouse dermal fibroblasts after a 2-hour stimulation with recombinant periostin alone. Therefore, these late responses in the present study may be a result of *de novo* synthesis of certain second messengers.

Periostin is upregulated upon histamine stimulation in dermal fibroblasts

Next, to investigate whether histamine affects the expression level of periostin, we stimulated wild-type (WT) primary dermal fibroblasts with histamine at the indicated concentrations (Figure 2). As expected, two hours of incubation with histamine produced a significant dose-dependent increase of periostin mRNA expression in dermal fibroblasts, as assessed by reverse transcriptase–PCR and quantitative real-time reverse transcriptase–PCR (Figure 2a and b). After 24 hours of incubation with histamine, the periostin protein levels increased in the culture supernatant and cell lysates (Figure 2 c). These results suggest that histamine may directly upregulate the transcription and synthesis of periostin.

Histamine upregulates periostin expression via histamine receptor 1 (H1R)

To identify the histamine receptor subtype responsible for the histamine-induced periostin expression, antagonists for H1R, H2R, and H4R were tested *in vitro*. The effects of histamine on periostin mRNA and protein levels were evaluated at 2 hours or 24 hours, respectively, in dermal fibroblasts after histamine stimulation following preincubation with or without these histamine receptor antagonists (Figure 3a and b). Histamine-induced periostin expression was blocked by H1R antagonist