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).

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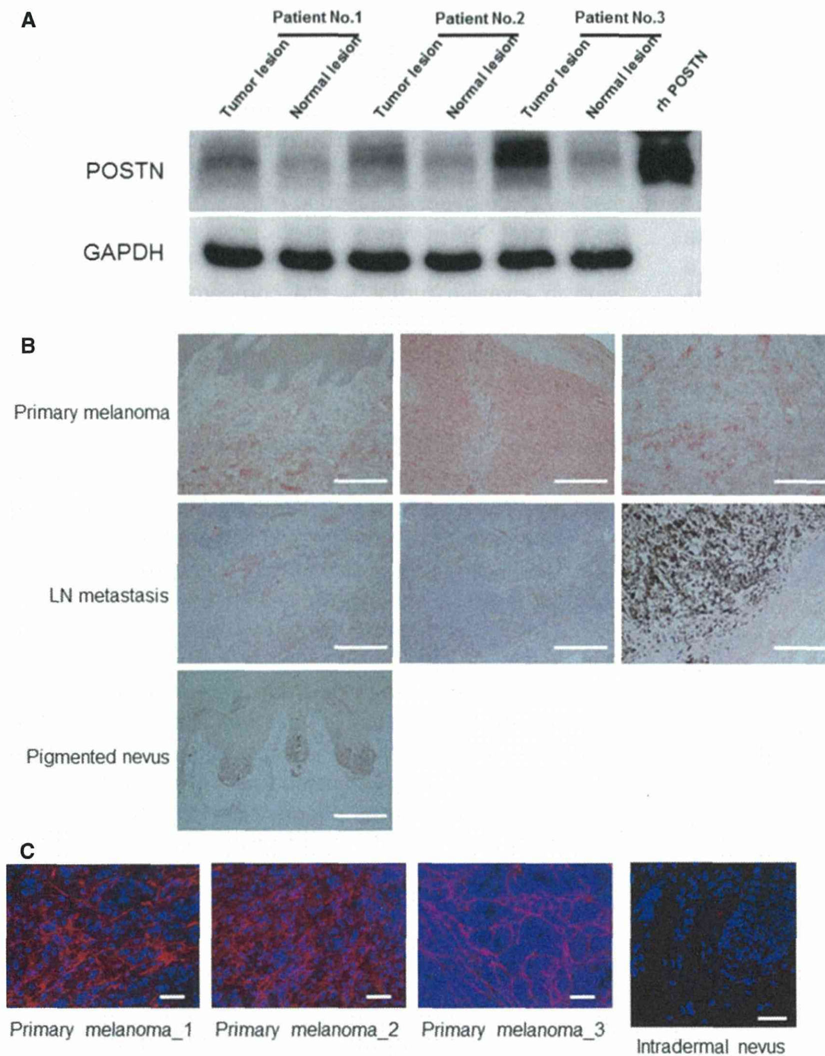

Figure 1. POSTN expression is much higher in melanoma tumor tissues compared with normal lesions. POSTN is predominantly expressed in the stroma of the melanoma tumors not melanoma cells. POSTN was slightly expressed in the in situ melanoma tissues (patient No. 1, No. 2) and highly expressed in the invasive 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).

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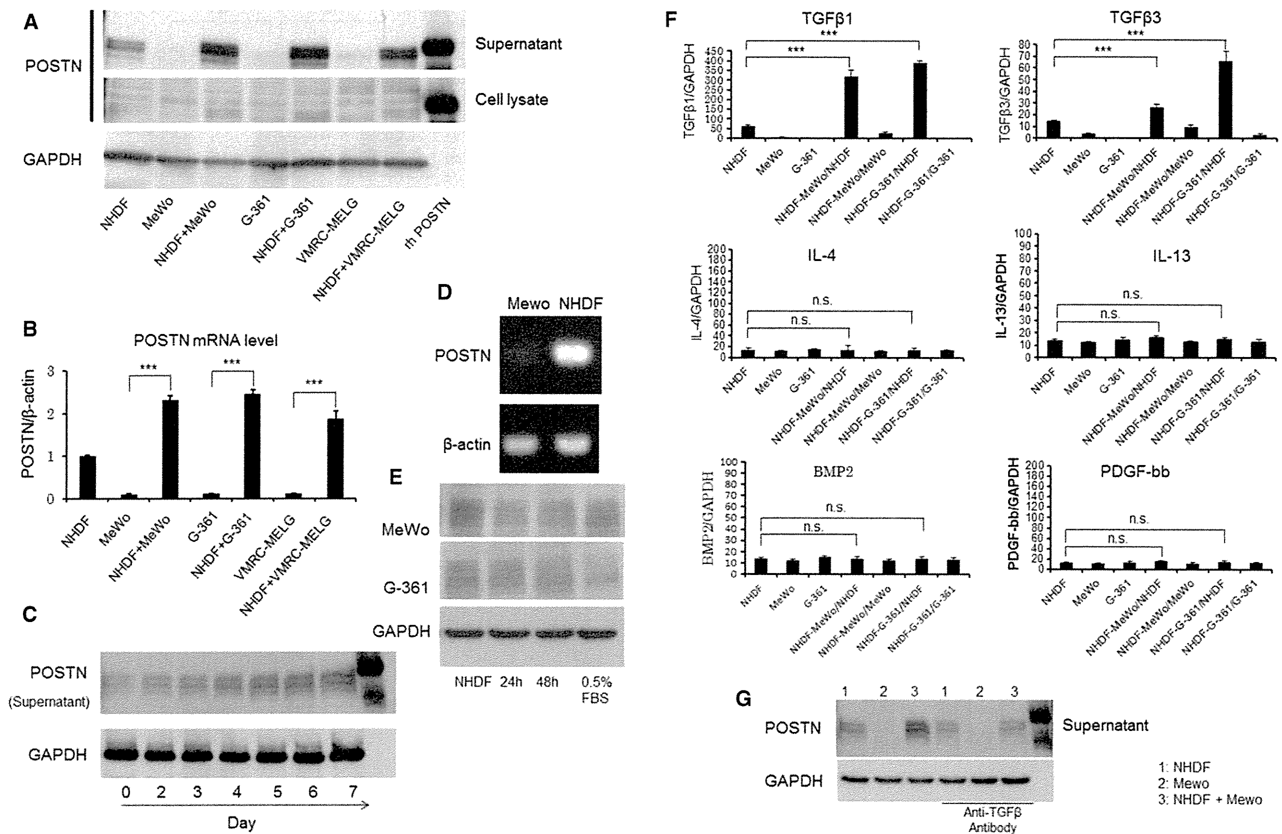

Figure 2. NHDFs secret POSTN during the coculture with human melanoma cells. The POSTN protein was upregulated in the supernatant after the coculture of NHDFs with human melanoma cells as determined by a Western blot analysis (A). POSTN mRNA was upregulated in the cell lysates after the coculture of 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

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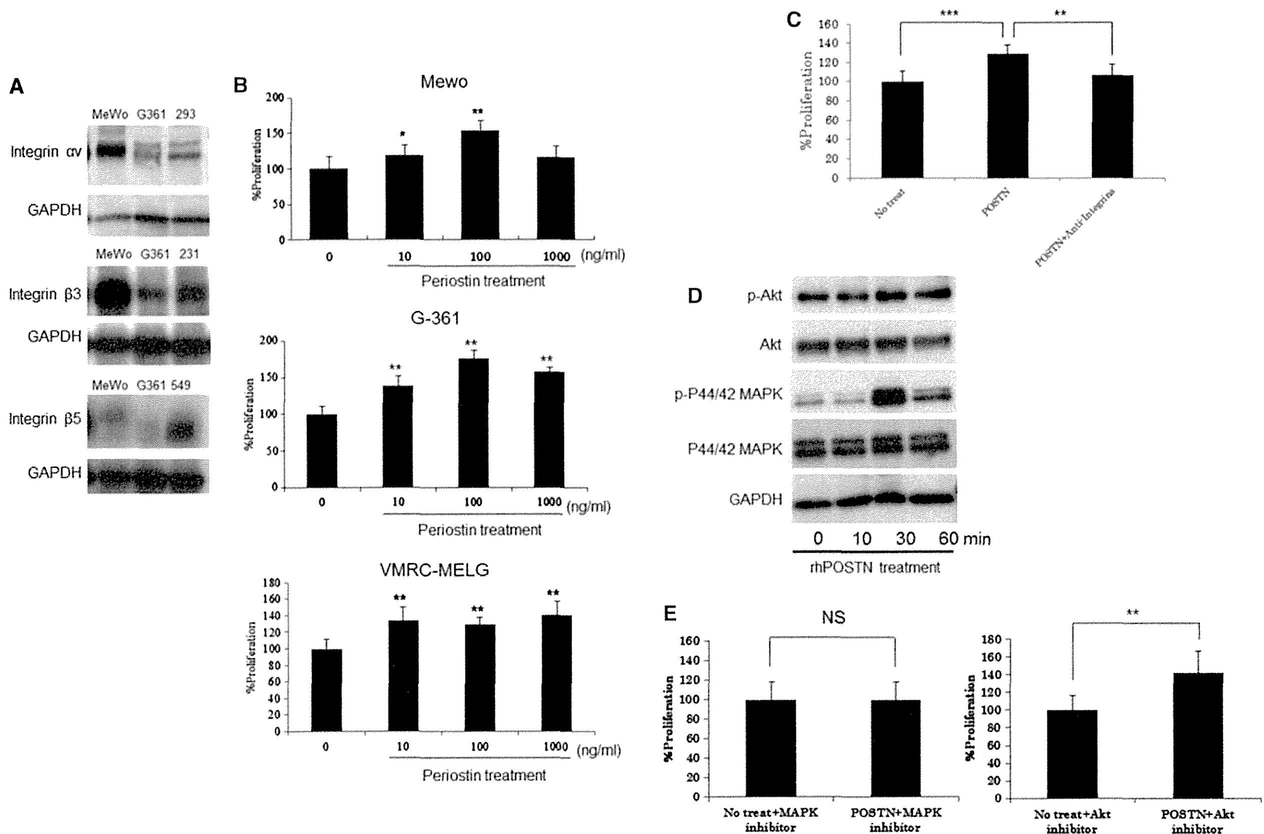

Figure 3. Recombinant POSTN protein accelerates melanoma cell proliferation via the integrin/P44/42MAPK pathway. Integrins αv, β3, and β5 were expressed in human melanoma cell lines (A). Recombinant POSTN increased the proliferation of human melanoma cells (MeWo, G-361, VMRC-MELG) (B). Neutralization of integrins αvβ3 and αvβ5 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

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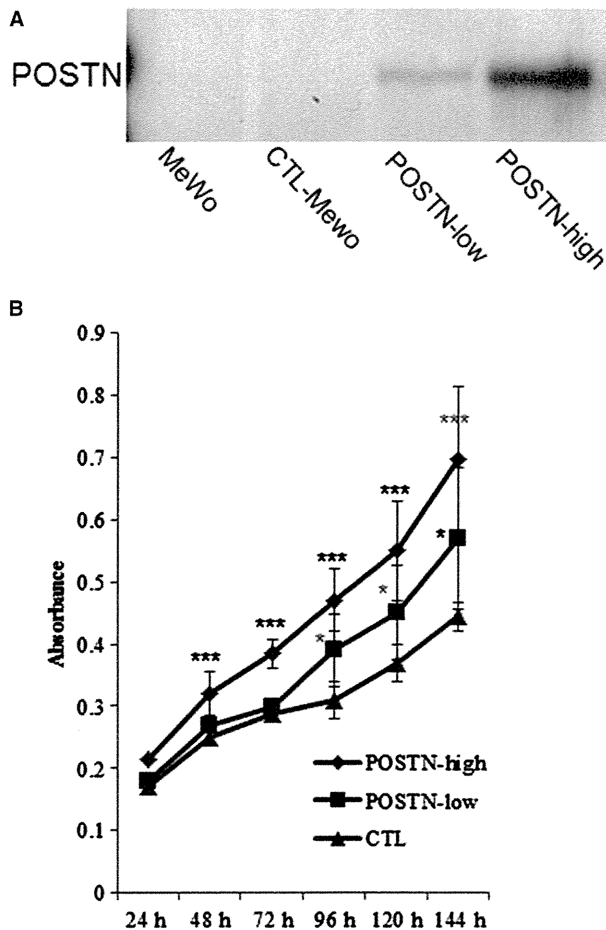

Figure 4. There is a significant increase in the proliferation of *POSTN*-transfected MeWo cells. *POSTN* gene transfection into MeWo cells (A). *POSTN* gene transfection upregulates the proliferation of MeWo cells (B). * and *** indicate P-value <0.05, <0.001, respectively.

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 $\alpha v\beta3$ and $\alpha v\beta5$/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

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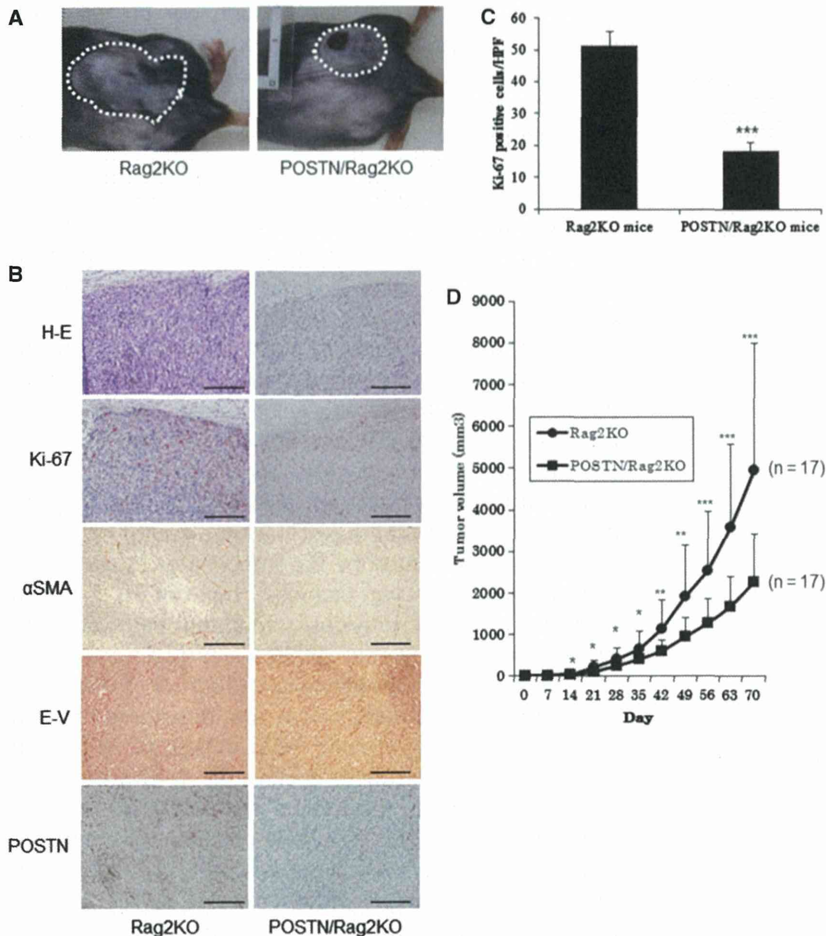

Figure 5. Melanoma tumorigenesis is significantly suppressed in *POSTN* gene knockout mice. Macroscopic feature of the *Rag2* KO and *POSTN/Rag2* KO mice on the day 56 after human melanoma cell implantation (A). There was decreased expression of Ki-67, αSMA, and collagen in the tumors of *POSTN/Rag2* 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).

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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 $P < 0.05$.

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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.

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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