S100A9 is a novel ligand of EMMPRIN that promotes melanoma metastasis

Toshihiko Hibino1*, Masakiyo Sakaguchi2, Shoko Miyamoto1, Mami Yamamoto1,3, Akira Motoyama1, Junichi Hosoi1, Tadashi Shimokata3, Tomonobu Ito3, Ryoji Tsuboi3, and Nam-ho Huh2

1Shiseido Innovative Science Research & Development Center, 2-12-1 Fukuura, Kanazawaku, Yokohama 236-8643, Japan
2Department of Cell Biology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Shikatacho, Kita-ku, Okayama 700-8558, Japan.
3Department of Dermatology, Tokyo Medical University, 6-7-1 Nishi-Shinjuku, Shinjuku-ku, Tokyo 160-0023, Japan

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* Corresponding author:
Toshihiko Hibino, Ph.D.
Shiseido Innovative Science Research & Development Center
2-12-1 Fukuura, Kanazawaku, Yokohama 236-8643, Japan.
Tel: +(81)-45-788-7289; Fax: +(81)-45-788-7277;
E-mail: toshihiko.hibino1@to.shiseido.co.jp

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Abbreviations: EMMPRIN, extracellular matrix protease inducer; RAGE, receptor for advanced glycation end-product; TNFα, tumor necrosis factor-α; TIRAP, toll-interleukin 1 receptor (TIR) domain containing adaptor protein; TRAF, TNF receptor-associated factor; PLA, proximity ligation assay.
Abstract

The calcium binding proteins S100A8 and S100A9 can dimerize to form calprotectin, the release of which during tissue damage has been implicated in inflammation and metastasis. However, receptor(s) mediating the physiological and pathophysiological effects of this damage-associated 'danger signal' are uncertain. In this study, searching for candidate calprotectin receptors by affinity isolation-mass spectrometry, we identified the cell surface glycoprotein EMMPRIN/BASIGIN (CD147/BSG). EMMPRIN specifically bound to S100A9 but not S100A8. Induction of cytokines and matrix metalloproteases (MMPs) by S100A9 was markedly downregulated in melanoma cells by attenuation of EMMPRIN. We found that EMMPRIN signaled utilized the TNF receptor-associated factor TRAF2 distinct from the known S100 binding signaling pathway mediated by RAGE (AGER). S100A9 strongly promoted migration when EMMPRIN was highly expressed, independent of RAGE, whereas EMMPRIN blockade suppressed migration by S100A9. Immunohistological analysis of melanomas revealed that EMMPRIN was expressed at both the invasive edge of lesions as well as the adjacent epidermis, where S100A9 was also strongly expressed. In epidermal-specific transgenic mice, tail vein-injected melanoma accumulated in skin expressing S100A9 but not S100A8. Together, our results establish EMMPRIN as a receptor for S100A9 and suggest the therapeutic utility in targeting S100A9-EMMPRIN interactions.
Introduction

There is a well-established link between chronic inflammation and cancer development (1-3). A number of molecules have been identified as contributing to this process; these include tumor necrosis factor-α (TNFα), interleukin-1 (IL-1), interleukin-6 (IL-6), chemokines, matrix metalloproteases (MMPs), angiogenic factors, and anti-apoptotic proteins. We have shown that many of these molecules are up-regulated in cultured keratinocytes stimulated with S100A8/A9(4). Furthermore, S100A8/A9-induced cytokines and chemokines, in turn, stimulate keratinocytes to synthesize and secrete S100A8/A9, suggesting a positive feedback mechanism between S100A8/A9 and these pro-inflammatory factors. In addition to the inflammatory processes, recent investigations have revealed that S100 proteins play important roles in malignancy, especially in cancer cell metastasis(5-8).

Regarding receptors, RAGE (receptor for AGE, advanced glycation end-products) is known to be a general and inflammation-related S100 protein receptor (9-11). However, RAGE is a multi-ligand receptor of the immunoglobulin superfamily of cell surface proteins that acts as pattern recognition receptors. Ligands of RAGE include AGE, S100/calgranulins, high mobility group box-1 (HMGB1), amyloid beta peptide, and beta-sheet fibrils. In addition, S100A9, S100A11, S100A12, S100A13 and S100P were also thought to interact with RAGE and transduce signals(12, 13).

Although this variety of ligands implicates RAGE in a wide spectrum of pathophysiological conditions, it would be inappropriate to postulate that all the S100-mediated reactions are totally dependent on their interaction with RAGE. Indeed, some S100 proteins seem to signal by engaging not only RAGE but non-RAGE receptors. Other putative S100-binding proteins include CD36(14), heparan sulfate
proteoglycan (15), and carboxylated glycans (16).

In the present study we tried to identify the S100A8/A9 receptor involved in the positive feedback mechanism of inflammation. We employed LC/MS/MS-based shotgun proteomics and succeeded in identifying the extracellular matrix metalloprotease inducer (EMMPRIN) as a novel receptor for S100A9. Interestingly, EMMPRIN is a well-known cell surface molecule that is associated with cancer cell malignancy (17, 18). We explored the S100A9-EMMPRIN relationship in the context of melanoma cell metastasis in vitro and in vivo. Our data provide new and important insights into the molecular mechanism of S100A9-EMMPRIN-mediated inflammation and cancer cell metastasis.
Materials and Methods

**Liquid chromatography coupled with electrospray tandem mass spectrometry (LC/MS/MS).** Proteins were identified via a shotgun-type protein identification approach described previously (19, 20). Briefly a lab-made nano-flow liquid chromatography-mass spectrometry platform consisting of a Nanospace SI-2 high-performance liquid chromatograph (Shiseido Co. Tokyo, Japan), a Yates lab-designed nano-electrospray ionization source, and an LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific, Inc) were used to obtain tandem mass spectra of tryptic peptides. The resulting tandem mass spectrometry spectra were analyzed using the SEQUEST algorithm against a non-redundant human protein database (NCBI, Feb 2007) for putative protein identifications.

**Preparation of expression vectors.** We prepared various expression vectors for soluble (sol) EMMPRIN, dominant negative (dn) EMMPRIN, cytoplasmic domains of N-terminal (RAGE-cyt)(13) and C-terminal (Emmp.-cyt), EMMPRIN variants (mut-traf and mut-C), TIRAP, MyD88, TRAF2, TRAF6, dnTRAF2, dnTRAF6, S100A8 and S100A9. Details of constructions were provided in the Supplemental methods.

**Co-immunoprecipitation analysis.** Transient transfection of each plasmid to the HEK293 cells was performed using FuGENE-HD (Promega BioSciences, San Luis Obispo, CA). After 48 hours, conditioned medium was subjected to co-immunoprecipitation experiments, using anti-HA tag (clone HA-7) Agarose (Sigma-Aldrich, St Louis, MO) and anti-Myc tag (clone 1G4) Agaroses (MBL, Nagoya,
Japan). The tag-Agarose beads were mixed with conditioned mediums and incubated for 3 h at 4 °C. The precipitated proteins were subjected to SDS-PAGE and detected by subsequent Western blotting using mouse anti-HA tag (clone 6E2; Cell Signaling Technology, Beverly, MA) and mouse anti-Myc tag (clone 9B11; Cell Signaling Technology) antibodies. GTP-bound form of cdc42 was also determined using a Rac/cdc42 Activation Assay Kit (Millipore, Billerica, MA).

**S100-EMMPRIN binding assay.** Recombinant EMMPRIN was immobilized on a 96-well ELISA plate by incubation at 4°C overnight. After washing with PBS containing 0.1% Tween20 (PBS-T), the plate was incubated with PBS containing 5% BSA at 37°C for 1h for blocking. Various concentrations of recombinant S100A9 were added to each well, and the plate was incubated at 37°C for 1h. After washing with PBS-T, anti-S100A9 antibody (1:2000 in PBS) was added and bound S100A9 was detected with HRP-conjugated anti-rabbit antibody (1:2000 in PBS). Color was developed using TMB Peroxydase EIA Substrate Kit (Bio-Rad Laboratories) and read at 630 nm.

**Melanoma cell lines.** SK-MEL-2 (HTB-68), SK-MEL-3 (HTB-69), SK-MEL-5 (HTB-70), MeWo (HTB-65), and A2058 (CRL-11147) were purchased from American Type Culture Collection (ATCC) (Manassas, VA) at 2002, and after expanded culture, 1 x 10^6 cells/tube were kept in liquid nitrogen. B16–BL6 cells (1), a highly invasive variant of a mouse melanoma B16 cell line, was obtained from Dr. Isaiah J. Fidler (M. D. Anderson Cancer Center, Houston, TX). All cell lines were used from these stocks or within three passages. Melanoma cells were cultured with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum.
Establishment of stable clones. For isolation of clones stably expressing dnEMMPRIN, B16-BL6 cells were subjected to conventional transfection method with the plasmids (pDNR-CMVIII-dnEmmp and pMSCV-puro; Clontech TAKARA) using the FuGENE-HD reagent (Promega BioSciences) and then selected with puromycin.

Microarray Analysis. Whole human genome (4x44K) Oligo Microarray Kit (Agilent) was used. We prepared the target RNA from SK-MEL-2 and primary keratinocytes in the presence or absence of recombinant S100A8, S100A9, or S100A8/A9 (10 µg/ml each) for 3h or 24 h as described. cDNAs from total RNA (1.65 µg starting material) isolated from cell culture harvests were used for generation of Cy3-labeled cRNA by in vitro transcription. The cRNA was fragmented before hybridization, and a hybridization cocktail was prepared. Microarrays were hybridized, scanned and image analysis was performed as described by the manufacturer.

Real-time quantitative reverse transcription-PCR (qRT-PCR). Real-time PCR was performed with an ABI 7900HT Real time PCR system (Applied Biosystems) using the Syber Green PCR mix (Invitrogen). Samples were cycled at 95 °C for 10 min, 40 cycles of: 95 °C for 15s, 60 °C for 1 min. Primer sets used were listed in the Supplementary Materials and Methods.

Melanoma tissues. Human skin specimens were obtained under informed consent from the patients with melanoma. A total of 20 melanoma tissues were analyzed immunohistochemically. The study was approved by the Institutional Review Board
of Tokyo Medical University and the Shiseido Committee on Human Ethics.

**Knockdown of EMMPRIN by siRNA silencing.** Recombinant S100A8 and S100A9 were prepared as previously described(4). SK-MEL-2 cells were plated at 50–60% confluency, and transfected with 50 nM siRNA against EMMPRIN or nonspecific control (Santa Cruz, CA) using Lipofectamine RNAi MAX (Invitrogen). After 24 h post transfection, 10 µg/ml recombinant S100A8, S100A9, or S100A8/A9, were added and incubated for an additional 3 h or 24 h.

**In vitro migration assay.** Migration of human melanoma cells was assayed using a 96-well Disposable Chemotaxis System (8 µm pore size; Funakoshi, Tokyo, Japan). The lower wells of the chamber were loaded with DMEM containing recombinant S100A8, S100A9, or S100A8/A9 (10 µg/ml). Melanoma cells (10000 cells per 25 µl in DMEM) were placed in the upper wells. After incubation for 48 h at 37°C, cells on the lower surface of the filter were stained with CellTracker Red CMTPX (5 µM, Molecular Probes, Eugene, OR), and fluorescence intensity was measured and compared with that of the medium control.

**Generation of S100A8 and S100A9 transgenic mice.** Human S100A8 and S100A9 were PCR-cloned from keratinocyte cDNA. Each S100 cDNA was fused to the involucrin promoter (21) (a generous gift from Dr. Taichman) and transgenic (Tg) mice were generated using BDF1 mice. In order to obtain the hairless phenotype, the involucrin-S100 Tg mice were crossed with HR-1 mice. S100A8 and S100A9 Tg mice were used for metastasis assays after three passages.
In vivo metastatic assay. The SK-MEL-2 and SK-MEL-5 cell lines were injected into the tail vein of Tg mice (1 × 10^6 cells in 0.25 ml/mouse) and littermate wild mice. Six mice each were injected per melanoma cell line. The control group without melanoma cells were injected serum-free DMEM. After four weeks of injection, skin was excised and stained for the melanoma markers. Metastases were examined using LSM5 PASCAL (ZEISS).

Immunohistochemical procedures. Primary antibodies used were listed in the Supplementary Materials and Methods. Horse radish peroxidase-conjugated, goat anti rabbit IgG or anti mouse IgG (Nichirei, Tokyo, Japan) were used as secondary antibodies and reacted with diaminobenzidine (DAB). For double detection, Alexa Fluor 555 or 488 (Molecular Probes Inc., Eugene, OR) was used. DAPI (4′, 6′-diamidino-2-phenylindole; Molecular Probes) was used to visualize nuclei.

Statistical analysis. Data are expressed as the mean ± s.d. We employed simple pair-wise comparison with the Student’s t test (two-tailed distribution with two-sample equal variance). p <0.05 was considered significant.
Results

**EMMPRIN is identified as an S100A8/A9 binding protein.** We carried out affinity purification-mass spectrometry using keratinocyte membrane fractions, since S100A8/A9 is strongly up-regulated in the epidermis in inflammatory skin disorders (22). We analyzed interactions between two kinds of membrane extracts (from proliferating and differentiated cells) and glutathione transferase (GST)-fused S100 proteins. Overlapping proteins interacting with GST were removed and finally we identified total of 13 proteins from the analyses ([Table 1](#)). S100A9 showed the highest probability and glutathione S-transferase followed next in each sample, indicating high fidelity of these analyses. Interestingly we found two membrane proteins, leucine zipper-EF-hand transmembrane protein 1 (LETM1) and basigin. Since LETM1 serves as an anchor protein for complex formation with the mitochondrial ribosome(23), a cell membrane protein, basigin, was selected as a candidate molecule. Basigin is also known as EMMPRIN or CD147 (24). EMMPRIN was found to be an S100A9 interactor in extracts from both proliferating and differentiated cells.

**S100A9 specifically binds with EMMPRIN.** Extracellular domain of EMMPRIN (soluble form), S100A8, and S100A9 were expressed in HEK293 cells and immunoprecipitated with appropriate antibodies. We found that S100A9 but not S100A8 was co-immunoprecipitated with EMMPRIN, suggesting that EMMPRIN binds to S100A9 ([Fig. 1a](#)). In addition, when S100A8 and S100A9 were co-expressed and pulled down with EMMPRIN, both proteins were detected in the precipitated fraction. Thus it is likely that the S100A8/A9 heterodimer (calprotectin) is also capable to bind with EMMPRIN. Binding of S100A9 was further confirmed with
EMMPRIN-immobilized ELISA assays. S100A9 bound with immobilized soluble form of EMMPRIN in a dose-dependent manner (Fig. 1b). We next examined EMMPRIN expression in five melanoma cell lines and primary human keratinocytes. The highest expression was seen in SK-MEL-2, which was 3.3 times higher compared to SK-MEL-5 (Fig. 1c). The gene expression profile of SK-MEL-2 cells was investigated after stimulation with S100A8, S100A9, or S100A8/A9 (Table 2). We used 10 μg/ml of each S100 protein, since this concentration showed the highest stimulation of cytokine induction in keratinocytes (Supplementary Fig. S1a). Only 16 genes were up-regulated more than two-fold at 3 h in S100-stimulated SK-MEL-2 cells, including CXCL1, CXCL2, CXCL3, IL8, TNFα, BIRC3 (cIAP2), and EFNA1 (ephrinA1). Components of NFκB as well as associated factors were also induced by these S100 proteins. Interestingly, most of these factors are related to inflammation and cell survival. Our previous study showed that similar genes were also up-regulated in keratinocytes stimulated with S100A8/A9(4), indicating common and essential features of S100A9 function. Since we already knew that MMP10, MMP1, MMP2 and MMP9 were induced at 24 h after S100A8/A9 stimulation in keratinocytes (see Supplementary Table 1), we tested expression of these MMPs in SK-MEL-2, a high EMMPRIN expresser MMP1 was always up-regulated after stimulation with S100A9, S100A8 or S100A8/A9 (Fig. 1d, 1e), although induction of MMP9 and MMP2 was inconsistent in SK-MEL-2 (data not shown).

To elucidate the possible involvement of EMMPRIN in these changes, we employed two approaches, siRNA-mediated knockdown of EMMPRIN (Supplementary Fig. S1b) and treatment with a soluble form of EMMPRIN. Under conditions of EMMPRIN mRNA knockdown, induction of cytokines as well as MMPs was significantly
suppressed compared with the control (Fig. 1d). Induction by S100A8 and S100A8/A9 was also suppressed. Since S100A8 and S100A9 are thought to make a stable heterodimer \textit{in vivo} (25, 26), these results may at least in part be dependent on binding with EMMPRIN via S100A9. Furthermore, addition of soluble EMMPRIN to the medium down-regulated the induction of cytokines and MMPs in SK-MEL-2 (Fig. 1e, Supplementary Fig. S1c). Collectively these results suggest that S100A9 is a novel ligand of EMMPRIN and that S100A9-EMMPRIN interaction is the key to inducing proinflammatory factors and MMPs both in keratinocytes and melanoma cells.

\textit{Signal transduction pathways are distinct between EMMPRIN and RAGE.} Since RAGE is a well-known receptor of multiple S100 proteins(12), we compared signaling pathways for EMMPRIN and RAGE. First we identified adaptor molecules for signal transduction. A motif search for EMMPRIN (http://elm.eu.org/) indicated a TRAF binding site in its cytoplasmic domain (Supplementary Fig. S2). Furthermore, our previous work(13) suggested that RAGE required MyD88 and TIRAP for the signal transduction. Thus we include MyD88, TIRAP, TRAF2 and TRAF6 as candidate molecules. Each cytoplasmic domain of EMMPRIN or RAGE tagged with HA was expressed in HEK293 cells. Co-expression analysis indicated that cytoplasmic domain of EMMPRIN only bound with TRAF2, whereas that of RAGE trapped MyD88, TIRAP and TRAF6, suggesting different signal transduction pathways between EMMPRIN and RAGE (Fig. 2a). Mutation of TRAF-binding motif in EMMPRIN (Supplementary Fig. S2) abolished the binding ability with TRAF2 (Fig. 2b). We confirmed that inherent TRAF2 was recruited by the stimulation with externally added S100A9 (Fig. 2c). We next examined whether EMMPRIN and RAGE were able to
form heterodimer each other. **Fig. 2d** clearly showed that they were unable to make up heterodimers, but present as homodimers. Overexpression of dominant negative TRAF2 in SK-MEL-2 cells showed significant reduction of S100A9-induced cdc42 activation (**Fig. 2e**), further supporting the involvement of TRAF2 in S100A9-EMMPRIN signaling pathway.

**Immunohistochemical analysis shows that EMMPRIN and S100A9 are differentially expressed.** We next examined the localization of S100A9 and EMMPRIN. These proteins were hardly detectable in normal human skin (**Fig. 3a**) or in benign pigmented nevi (**Fig. 3b**). In contrast, heavy staining of S100A9 was always observed in the epidermis adjacent to HMB-45-positive melanoma-burdened skin (**Fig. 3c**, Supplementary Fig. S3a). Interestingly, EMMPRIN was detected at the invasive edge of melanoma lesions (**Fig. 3c, 3d**), but was absent in the mass of melanoma tissue (Supplementary Fig. S3b). In the epidermis, EMMPRIN was also present in the upper layer of melanoma tissue (**Fig. 3c, 3d**). Proximity ligation assay (PLA) using anti-S100A9 antibody and anti-EMMPRIN antibody indicated a positive reaction at the invasive edge of melanoma cells, as well as the upper epidermal tissue exposed to the melanoma (Supplementary Fig. S4). These results suggest co-localization of these molecules and possible interaction *in vivo*. For melanoma cell metastasis, it is necessary to degrade the basement membrane at the dermal-epidermal junction. MMP2 and MMP9 are thought to play an important role in the degradation of basement membrane components (27). Loss of the basement membrane structure was obvious at the invasive edge of melanoma as verified by the absence of type VII collagen (28) (**Fig. 3e, 3f**). Immunohistochemical data indicate that melanoma cells and keratinocytes
respond to each other strongly at the invasive edge, where S100A9-EMMPRIN interaction takes place.

**Chemotactic activity of S100A9 is dependent on EMMPRIN expression.** Some S100 family members are known to function as chemoattractants to neutrophils (29) and cancer cells (30). We analyzed the chemotactic activity of S100A8 and S100A9 on melanoma cell lines. S100A9 showed a strong effect on SK-MEL-2 migration (Fig. 4a). S100A8 and S100A8/A9 also showed chemotactic activity on SK-MEL-2 to some extent. Knockdown of EMMPRIN markedly suppressed the chemotactic activity of S100A9, as well as S100A8 and S100A8/A9 on SK-MEL-2. Quantitative analysis further supported the above results (Fig. 4b). Either S100A9 or S100A8 had little effect on the migration of SK-MEL-3 and SK-MEL-5, low EMMPRIN expressers (Fig. 4c). These results indicate that chemotactic activity of S100A9 is closely related to the expression level of EMMPRIN (Fig. 1c).

In addition, we established a clone of B16-BL6 melanoma cells that constitutively expresses the dominant negative (dn) form of EMMPRIN (dnEMMPRIN B16) with 1,400 times higher than intact EMMPRIN. This clone lacks the cytoplasmic domain of EMMPRIN, and thus is unable to transduce S100A9 signals. S100A9 showed significant chemotactic activity on control B16 melanoma cells (Fig. 4d). On the other hand, S100A9 did not show any effect on the migrating activity of dnEMMPRIN B16. Quantitative analysis clearly showed strong effect of S100A9 on B16 cell migration (Fig. 4e). In order to assess the involvement of RAGE in melanoma metastasis, we examined expression of RAGE in these cell lines. Expression of RAGE was 40-fold higher in dnEMMPRIN B16 compared to B16 control, although that of mouse
EMMPRIN is rather suppressed in dnEMMPRIN B16 (Fig. 4f). We also checked expression levels of RAGE in human melanoma cell lines. qPCR analysis showed it was 1.5 times higher in SK-MEL-2 compared to SK-MEL-5 (Fig. 4g). Collectively these data suggest that S100A9-induced cell migration is strongly dependent on EMMPRIN.

Suppression of migration activity caused by knockdown of EMMPRIN or blocking of signal transduction may be due to cell aggregation (Supplementary Fig. S5a). dnEMMPRIN B16 showed a very compact cell shape and formed dense cell aggregates, although control B16 or vector-transfected cells grew with a rather uniform spread and an elongated cell shape.

**Overexpression of S100A9 induced melanoma cell migration in vivo.** Lastly, we tested whether S100A8 or S100A9 contributed to the metastatic ability of cancer cells. For this purpose, we generated involucrin promoter-driven S100A8 and S100A9 Tg mice (Fig. 5a). We chose the involucrin promoter, since these molecules were expressed in the upper epidermis of inflammatory skin. We used SK-MEL-2 and SK-MEL-5 for the metastasis assay as high and low EMMPRIN expressers, respectively. SK-MEL-2 or SK-MEL-5 cells were injected into the tail vein of wild-type and Tg mice, and their presence was examined in the skin after four weeks. Skin specimens were scanned with a confocal microscope in an area 1 cm x 2 cm, or immunostained with HMB-45 and MART-1 for the detection of melanoma cells. In SK-MEL-2-injected S100A8 Tg mice, we found very few HMB-45 positive cells (n=6) (Fig. 5b). However, considerable numbers of positive cells were always detected in the skin of SK-MEL-2-injected S100A9 Tg mice (n=6) (Fig. 5c). Presence of melanin in the skin...
of both types of mice was confirmed with Fontana-Masson staining (Fig. 5d). Immunohistochemical analyses, showed that these cells were strongly positive for HMB-45 and MART-1 (Fig. 5e). Many melanoma cells were found scattered around an artery that were verified with anti-CD31 and anti-smooth muscle antibodies (Fig. 5e and supplementary Fig. S6). In some case, melanoma cells were embedded within the vessel wall. Relatively large numbers of melanoma cells were also found near hair follicle-like structures (Fig. 5e). HMB-45-positive cells were not found in the skin of SK-MEL-5-injected S100A8 or S100A9 Tg mice (Supplementary Fig. S7).

In order to elucidate further involvement of EMMPRIN in cancer cell metastasis, we carried out limited attempts using highly invasive melanoma cell line, B16-BL6 and dnEMMPRIN B16. When these cells were injected via tail vein, only the control B16 cells metastasized to lung in both wild and S100A9 Tg mice after 6 weeks observation (Supplementary Table S2 and Fig. S5b). We could not find any incidence for dnEMMPRIN B16 cell metastasis in the lung. Interestingly we observed two tumor nodules in the skin of S100A9 Tg mouse that were tail vein-injected with B16-BL6 cells (Supplementary Fig. S5c).
Discussion

There have been many reports that both S100 proteins and EMMPRIN are involved in malignancy, especially in cancer cell metastasis (7, 31, 32). To date, the metastatic ability of these proteins is considered to be an independent phenomenon. In the present study, we clarified for the first time the ligand-receptor relationship between S100A9 and EMMPRIN. Binding of S100A9 to EMMPRIN was confirmed with 1) affinity isolation-mass spectrometry analysis, 2) immunoprecipitation study in a cellular level, and 3) dose-dependent binding to immobilized EMMPRIN. EMMPRIN’s activity as a functional receptor for S100A9 was further demonstrated by the fact that induction of both proinflammatory cytokines and MMPs was markedly suppressed in the EMMPRIN knockdown cells. In vivo association between S100A9 and EMMPRIN was also demonstrated with PLA at the invasive edge of melanoma and the adjacent epidermis.

EMMPRIN, also known as CD147, is able to induce production of several MMPs (33). It is over-expressed in various cancerous tissues (18, 34, 35). Although induction of MMPs has been regarded as evidence of the homophilic interaction between EMMPRIN molecules, including a soluble form, our results clearly show that the soluble EMMPRIN is able to bind to S100A9 and prohibits S100A9-mediated MMP induction. Thus, MMP induction by EMMPRIN is, at least in part, caused by the binding of the S100A9 or S100A8/A9 heterodimer to EMMPRIN. In malignant melanoma, both MMP-9 and EMMPRIN were variably expressed in the invasive radial growth phase but not in the vertical growth phase, and early invasion of melanoma is associated with de novo expression of MMP-9 and EMMPRIN(36). Our observation
also supports these findings and suggests that peripheral keratinocyte-derived S100A9 promotes MMP production in neighboring cancer cells via EMMPRIN signal transduction. Apparently MMP production is required for efficient cancer cell metastasis by degrading basement membrane and surrounding matrix proteins. We demonstrated the disappearance of the basement membrane just in the area where these proteins are co-expressed.

Some cancer cells, including melanoma cells, seem to influence distant tissues to establish a premetastatic niche before metastasis occurs (8). Primary tumor cells secrete pro-inflammatory factors such as VEGF-A, TGFβ and TNFα, which induce selective expression of chemoattractants S100A8 and S100A9, thereby making it easier for tumor cells to home in on the premetastatic sites. Our metastasis assays using S100A8 and S100A9 Tg mice revealed that only the high EMMPRIN-expressing SK-MEL-2 cells migrated into the S100A9-expressing skin. Low EMMPRIN expresser (SK-MEL-5), EMMPRIN-knocked down SK-MEL-2, and dnEMMPRIN B16 all failed to metastasize or to migrate in response to S100A9. Furthermore, S100A9-EMMPRIN interaction induced cdc42 activation (Fig. 2e). cdc42 is known to promote filopodia formation, cell polarity and migration(37). This may be the reason why EMMPRIN is expressed at the invasive edge of melanomas and not in the denser mass of melanoma cells. Indeed, when we used the established B16 clone that over-expressed dnEMMPRIN failed to migrate after S100A9 stimulation. Our limited attempts to assess the metastatic ability in vivo also showed that only the EMMPRIN-expressing B16 cells, but not dnEMMPRIN B16, metastasized to lung in mice. These lines of evidence further suggest the critical role of EMMPRIN in melanoma cell metastasis.
It is thought that S100 proteins actively participate in the modulation of inflammation and immune responses by signaling via RAGE (32, 38, 39). On the other hand, EMMPRIN is also known as a signaling receptor for cyclophilines A and B (40). Recent studies have demonstrated a role for cyclophilin-EMMPRIN interactions in the regulation of inflammatory responses (41). Although RAGE and EMMPRIN seem to have multiple ligands, S100A9-EMMPRIN interaction is of particular relevance to cancer cell biology.

In the present study, we clearly showed that EMMPRIN and RAGE have distinct properties in S100A9-induced signal transduction. EMMPRIN recruited TRAF2 as an adaptor molecule in its signaling pathway, whereas RAGE required MyD88, TIRAP and TRAF6 (Fig. 2). The fact that EMMPRIN failed to form a heterodimer with RAGE further supports the difference in signal transduction and physiological functions in these molecules.

Because of the involvement of EMMPRIN in the invasion and metastasis processes of many types of cancers, its down-regulation would seem to be a logical method of preventing cancer cell invasion (42, 43). Indeed this idea is supported by reports that transfection of EMMPRIN cDNA into breast cancer cells resulted in increased incidence of metastasis (44), and that EMMPRIN-targeting siRNA inhibited the metastatic activity of malignant melanoma in a nude mouse model of pulmonary metastasis (35). However, S100A9 and EMMPRIN are both pleiotropic molecules which play a critical role in various physiological reactions. S100A9 also works as an anti-infective and anti-inflammatory factor (45), although it is a Janus-faced molecule functioning as a potent amplifier of inflammation in autoimmunity as well as in cancer development and tumor spread (46). EMMPRIN is also involved in fetal development,
retinal function, nervous system development, and plaque formation in Alzheimer’s disease (33). Thus, although knockdown of these molecules might be an effective therapeutic option, there might be limitations to directly targeting S100A9 or EMMPRIN. Interfering with the S100A9-EMMPRIN interaction would be a novel strategy to block metastatic activity of malignant melanomas.

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FIGURE LEGENDS

Figure 1. S100A9 specifically binds with EMMPRIN. (a) Immuno-precipitation study showing binding of S100A9 with EMMPRIN in HEK293 cells. S100A8 and S100A9 were expressed with HA-tag. Soluble form of EMMPRIN was expressed with both HA-tag and Myc-tag. Pull-down with anti-HA antibody showed sufficient expression of these proteins (lanes 1-3). When S100A8 or S100A9 were expressed together with EMMPRIN and pulled down with anti-Myc antibody, only S100A9 was seen to bind with EMMPRIN as verified with anti-HA antibody. Co-expression of S100A8 and S100A9 resulted in the detection of both proteins. (b) ELISA assay showing S100A9 binding with extracellular domain of EMMPRIN in a dose-dependent manner. (c) EMMPRIN mRNA expression in five melanoma cell lines, and normal human keratinocytes. (d) Effect of EMMPRIN knockdown on the expression of S100-induced TNFα, IL8 and MMP1. S100 proteins were added to the SK-MEL-2 culture and total RNA samples were obtained 3h after incubation. (e) Effect of soluble EMMPRIN (sol. EMMPRIN) on the expression of S100-induced TNFα, IL8 and MMP1 in SK-MEL-2. *P<0.05, **P<0.01, ***P<0.001

Figure 2. Signal transduction pathways are distinct between EMMPRIN and RAGE. (a) Identification of adaptor molecules for signal transduction. HA-tagged cytoplasmic domain of EMMPRIN (EMMPRIN-cyt) or RAGE (RAGE-cyt: S391E, phospho-mimic form) as well as each adaptor molecule with Myc-tag were co-expressed in HEK293 cells. Interacting molecules in cell lysate were immuno-precipitated with anti-HA antibody and analyzed with Western blot using anti-Myc antibody. (b) TRAF2 binds with cytoplasmic domain of EMMPRIN. Mutation was introduced into the cytoplasmic domain of EMMPRIN in the TRAF binding motif (Mut Traf) and the C-terminal end (Mut C-ter). They were co-expressed with EMMPRIN-cyt in HEK293 cells and binding with inherent TRAF2 was analyzed with Western blot. (c) Recruitment of TRAF2 to the cytoplasmic domain of EMMPRIN. Full length EMMPRIN was expressed in HEK293 cells and interaction with inherent TRAF2 was investigated in the presence of 1 or 10 nM GST and S100A9. (d) EMMPRIN forms homodimer. Dimer formation between EMMPRIN and RAGE was analyzed after co-expression of HA-tagged and Myc-tagged EMMPRIN and
RAGE. Immuno-precipitation was carried out using anti-HA antibody and verified with anti-Myc antibody. (e) TRAF2 but not TRAF6 mediates signal transduction in S100A9/EMMPRIN system. Dominant negative form of TRAF2 (dn TRAF2) or TRAF6 (dn TRAF6) was overexpressed in SK-MEL-2 cells and effect of cdc42 activation was monitored after S100A9 stimulation.

Figure 3. Immunohistochemical analysis shows that EMMPRIN is expressed in the invasive edge of melanomas. (a) Normal skin showing no expression of S100A9 and EMMPRIN. (b) Pigmented nevus. (c) Immunohistochemistry for S100A9 (red) and HMB-45 (green) in primary melanoma. Nuclear staining with DAPI (blue) is also shown. (d) Magnified view of invasive region of melanoma from boxed area of (c). (e) Immunostaining for collagen type VII (Col VII) of normal human skin. (f) Loss of basement membrane in EMMPRIN-expressing melanoma lesion. An example of melanoma tissue stained with collagen type VII (Col VII, red) and EMMPRIN (green). Dotted line shows the edge of cornified layer. Scale bars: 100 μm.

Figure 4. Chemotactic activity of S100A9 is dependent on EMMPRIN expression. (a) SK-MEL-2 chemotaxis assay. Entire views of lower chemotaxis filter were shown for S100 proteins stimulated SK-MEL-2 and siRNA-treated SK-MEL-2. Scale bars: 1 mm. (b) Quantitative analyses for (a). (c) Results for SK-MEL-3 and SK-MEL-5, low EMMPRIN expressers, were quantified (n=4). (d) B16 chemotaxis assay. Similar experiments were performed for B16 and dnEMMPRIN-B16. Scale bars: 1 mm. (e) Quantitative analysis of chemotaxis assays were shown. (n=4). (f) qPCR analysis of mouse RAGE expression in B16-BL6 and dnEMMPRIN B16. (g) qPCR analysis of human RAGE expression in SK-MEL-2, SK-MEL-5 and keratinocytes.

Figure 5. Overexpression of S100A9-induced melanoma cell migration in vivo. (a) Expression of S100A8 or S100A9 in Tg mouse skin. Involucrin promoter-driven S100A8 (left) and S100A9 (right) Tg mouse skin were immunostained with anti-human S100A8 antibody or anti-human S100A9 antibody, respectively. Scale bars:100 μm. (b) Confocal analysis of the skin with epidermis-specific S100A8 Tg mice. SK-MEL-2 or SK-MEL-5 cells were injected into the tail vein and four weeks later, skin was scanned for 460 μm x 460 μm at a time after HMB-45 staining. Images of bright and merged
fields from Control (Ctrl), SK-MEL-2 injected, and SK-MEL-5 injected, mouse tissue are shown. (c) Confocal analysis of the skin of epidermis-specific S100A9 Tg mice. (d) Mason-Fontana staining of S100A9 Tg mouse skin injected with SK-MEL-2 cells. Magnified view was also shown. (e) SK-MEL-2-injected S100A9 skin stained for H&E, HMB-45 and MART-1. Enlarged figures of the boxed areas were also shown. Scale bars: 100 μm.
Table 1. Summary of LC/MC/MC analysis of S100A8/A9 interacting proteins

<table>
<thead>
<tr>
<th>No</th>
<th>Description</th>
<th>S100A9</th>
<th>S100A8/A9</th>
<th>S100A9</th>
<th>S100A8/A9</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>S100 calcium-binding protein A9 [Homsapiens]</td>
<td>1.00E-302</td>
<td>2.00E-10</td>
<td>1.00E-30</td>
<td>2.26E-11</td>
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<td>2</td>
<td>Glutathione S-Transferase (E.C.2.5.1.18)</td>
<td>4.00E-13</td>
<td>7.66E-14</td>
<td>4.11E-14</td>
<td>4.03E-13</td>
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<tr>
<td>3</td>
<td>Xeroderma Pigmentosum Group E Complementing</td>
<td>7.29E-13</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>4</td>
<td>Glutathione transferase [Homo sapiens]</td>
<td>1.21E-12</td>
<td>2.84E-11</td>
<td>6.98E-11</td>
<td>9.91E-13</td>
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<td>5</td>
<td>Tubulin alpha 6 [Homo sapiens]</td>
<td>1.27E-08</td>
<td>6.63E-09</td>
<td>N.D.</td>
<td>N.D.</td>
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<td>6</td>
<td>Acyl-CoA synthetase long-chain family</td>
<td>5.94E-08</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>7</td>
<td>Leucine zipper-EF-hand containing transmembrane</td>
<td>1.07E-07</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>8</td>
<td>B Chain B, Human Glutathione S-Transferase</td>
<td>2.93E-07</td>
<td>8.76E-07</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>9</td>
<td>Eukaryotic translation elongation factor 1 beta 2</td>
<td>1.19E-05</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>10</td>
<td>Basigin isoform 1 [Homo sapiens]</td>
<td>1.62E-05</td>
<td>N.D.</td>
<td>3.16E-05</td>
<td>N.D.</td>
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<tr>
<td>11</td>
<td>Ribosomal protein P2 [Homo sapiens]</td>
<td>2.71E-05</td>
<td>N.D.</td>
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<td>N.D.</td>
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<tr>
<td>12</td>
<td>Myosin, light polypeptide 6, alkali, smooth muscle</td>
<td>3.86E-04</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
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</table>

*Stringent search criteria: Sf score > 0.85; peptide probability < 0.001; number of top matches: 1; precursor mass tolerance: < 5 ppm; minimum number of peptides to identify proteins: 1; enzyme specificity: half-tryptic or fully tryptic peptides only.\)
Table 2.  Summary of microarray analysis for S100-stimulated SK-MEL-2 at 3 h

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Factor of Increase</th>
<th>+S100A8</th>
<th>+S100A9</th>
<th>+S100A8/A9</th>
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<tbody>
<tr>
<td>CXCL3</td>
<td>7.30±0.81</td>
<td>14.77±0.56</td>
<td>12.57±0.78</td>
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<tr>
<td>IL8</td>
<td>6.20±0.68</td>
<td>14.36±2.12</td>
<td>10.69±0.47</td>
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<tr>
<td>CCL2</td>
<td>5.35±0.85</td>
<td>13.62±0.62</td>
<td>10.03±0.35</td>
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</tr>
<tr>
<td>BIRC3</td>
<td>5.14±0.69</td>
<td>12.74±0.97</td>
<td>10.41±0.15</td>
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<tr>
<td>ZC3H12A</td>
<td>4.08±0.43</td>
<td>7.81±0.37</td>
<td>6.82±0.22</td>
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<tr>
<td>CXCL2</td>
<td>4.03±0.49</td>
<td>7.37±0.52</td>
<td>6.55±0.15</td>
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<tr>
<td>TNF</td>
<td>3.12±0.26</td>
<td>5.92±0.29</td>
<td>4.86±0.39</td>
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<tr>
<td>NFKBIA</td>
<td>2.83±0.38</td>
<td>4.94±0.24</td>
<td>4.88±0.24</td>
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<tr>
<td>C10orf10</td>
<td>2.26±1.0</td>
<td>4.58±0.21</td>
<td>4.10±0.21</td>
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<tr>
<td>CXCL1</td>
<td>3.36±0.17</td>
<td>4.28±0.18</td>
<td>4.21±0.07</td>
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<tr>
<td>RELB</td>
<td>2.18±0.37</td>
<td>4.07±0.15</td>
<td>3.78±0.12</td>
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<tr>
<td>EFNA1</td>
<td>1.91±0.24</td>
<td>4.04±0.29</td>
<td>3.38±0.16</td>
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<tr>
<td>TIFA</td>
<td>1.84±0.42</td>
<td>3.74±0.25</td>
<td>3.17±0.07</td>
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<td>CEBPD</td>
<td>2.07±0.24</td>
<td>2.79±0.07</td>
<td>2.68±0.04</td>
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<td>IER3</td>
<td>1.94±0.32</td>
<td>2.78±0.18</td>
<td>2.69±0.13</td>
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<tr>
<td>BCL3</td>
<td>1.96±0.24</td>
<td>2.74±0.05</td>
<td>2.59±0.09</td>
<td></td>
</tr>
</tbody>
</table>

Expressed as mean values ± s.d. from N=3 microarray analysis.
Figure 1

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(a) Transfection

- Cell lysate
- WB: HA
- WB: Tubulin

- Culture medium
- WB: HA

- Bound proteins
- IP: myc
- WB: myc

(b) Absorbance at 630 nm

<table>
<thead>
<tr>
<th>Amount of S100A9 (μg/ml)</th>
<th>0</th>
<th>0.63</th>
<th>1.25</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance</td>
<td>0.05</td>
<td>0.3</td>
<td>0.25</td>
<td>0.35</td>
<td>0.3</td>
<td>0.35</td>
</tr>
</tbody>
</table>

(c) EMPRIN expression

(d) Fold Increase

- TNFα
- IL-8
- MMP1

- Control
- siEMP
- siEMP+S100A9
- siEMP+S100A8
- siEMP+S100A9-A8
- siEMP+S100A8-A9

(e) Fold Increase

- TNFα
- IL-8
- MMP1

- Control
- A9 (1nM)
- A9 (10 nM)
- Control
- A9 (1nM)
- A9 (10 nM)

+sol.EMMPRIN
**Figure 2**

**a**

- **EMMPRIN-cyt**
  - Control
  - MyD88
  - TIRAP
  - TRAF2
  - TRAF6

**Cell lysate**

**IP: anti-HA**

**b**

**transfection**

- control
- Wt
- Mut Traf
- Mut C-ter.

**Cell lysate**

**IP: anti-HA**

**c**

**EMMPRIN (transfected)**

<table>
<thead>
<tr>
<th>stimulation</th>
<th>GST</th>
<th>S100 A9</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 10 nM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Cell lysate**

**IP: anti-HA**

**d**

**transfection**

- +EMMPRIN (myc) +RAGE (myc)

**IP: anti-HA**

- RAGE
- EMMPRIN

**e**

- +S100A9

**transfection**

- Control
- none
- dnTRAF2
- dnTRAF6

**Cell lysate**

- cdc42 (Active)
- cdc42 (Total)
Figure 3

a. S100A9 and EMMPRIN staining in different conditions. 

b. S100A9 with DAPI staining in different conditions. 

c. Hematoxylin and Eosin (HE) staining of tissue sections. 

d. EMMPRIN and HMB45 staining with DAPI merge. 

e. Collagen VII (Col VII) staining with EMMPRIN and DAPI. 

f. Collagen VII (Col VII) staining with EMMPRIN and DAPI merge.
Figure 4

Hibino et al

(a) SK-MEL-2 and SK-MEL-2+ siEMMPRIN

(b) Relative intensity (fold increase)

(c) SK-MEL-3 and SK-MEL-5

(d) B16 control and dnEMMPRIN-B16

(e) Relative intensity (fold increase)

(f) Fold increase for mRAGE

(g) hRAGE/G3PDH

Note: The images and data represent the experimental results of the study by Hibino et al., showing the expression levels of different proteins in various cell lines under different conditions.
Figure 5

(a) S100A8 and S100A9 staining in SK-MEL-2 and SK-MEL-5 cells.

(b) HMB45 and Bright Field images of Ctrl, SK-MEL-2, and SK-MEL-5 cells with S100A8 Tg merge images.

(c) HMB45 and Bright Field images of Ctrl, SK-MEL-2, and SK-MEL-5 cells with S100A9 Tg merge images.

(d) H&E staining images of SK-MEL-2/S100A8 Tg.

(e) MART1 staining images of SK-MEL-2/S100A9 Tg.
S100A9 is a novel ligand of EMMPRIN that promotes melanoma metastasis

Toshihiko Hibino, Masakiyo Sakaguchi, Shoko Miyamoto, et al.

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