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# Ephrin-A1 expression induced by S100A8 is mediated by the toll-like receptor 4

Katsuaki Ieguchi *, Tsutomu Omori, Akiko Komatsu, Takeshi Tomita, Atsuko Deguchi, Yoshiro Maru *

*Department of Pharmacology, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku, Tokyo 162-8666, Japan*

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ABSTRACT

The deregulation of Eph/ephrin protein expression has been shown to lead to tumor development and progression. Both mRNA and protein expression analyses using clinical samples have demonstrated that ephrin-A1 is over-expressed in various cancers and positively correlates with a poor prognosis for cancer patients. The prognosis of cancer patients depends on metastasis to distant organs. We previously demonstrated that ADAM12 metalloproteinase cleaved ephrin-A1 and ADAM12-cleaved ephrin-A1 enhanced vascular permeability by degrading VE-cadherin and the EphA2 receptor at the plasma membrane. An increase of soluble ephrin-A1 levels in the serum facilitated tumor cell recruitment to the lungs, which resulted in lung metastasis. We also found that ephrin-A1 was overexpressed in 3LL tumors, a highly metastatic tumor, in mice and TNFα, an authentic positive regulator of ephrin-A1, was not elevated in the tumors, whereas S100A8 was. Moreover, S100A8 induced ephrin-A1 expression mediated by the toll-like receptor 4 (TLR4). S100A8 is known to be an endogenous ligand for TLR4 and its expression was shown to be increased in the lungs at the premetastatic phase. Thus, S100A8 and ephrin-A1 contribute to lung metastasis. Therefore, elucidating the regulation mechanism of ephrin-A1 overexpression is of importance and may lead to the development of therapeutic drugs against tumor growth and metastasis.

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## 1. Introduction

Ephrin-A1 is a ligand of type-A Eph receptor tyrosine kinases and the Eph/ephrin system has been shown to play important roles in physiological events such as the nervous system, tissue patterning and cell adhesion through receptor–ligand interactions and intracellular signaling [1–3]. Therefore, dysfunctions of the Eph/ephrin system lead to various diseases. Previous studies using clinical samples showed that ephrin-A1 expression was up-regulated in colorectal cancer and hepatocellular carcinoma and positively correlated with a poor prognostic value [4,5]. We recently demonstrated that ephrin-A1 expression was higher in 3LL, a high metastatic tumor, than in LLC, a low metastatic tumor, in mice and the cleavage of ephrin-A1 by a disintegrin and metalloproteinase 12 (ADAM12) resulted in an increase of soluble ephrin-A1 in the serum. Soluble ephrin-A1 was previously shown to enhance lung vascular permeability and lung metastasis [6,7]. An increase of soluble ephrin-A1 in the blood stream has been associated with lung metastasis. The prognosis of cancer patients is known to depend on metastasis. Therefore, elucidating the mechanism for the up-regulation of ephrin-A1 expression is of importance and may lead to the development of therapeutic drugs for cancer and metastasis.

Ephrin-A1 was originally identified as an immediate early response gene to tumor necrosis factor α (TNFα), and was subsequently purified as a soluble factor in tumor conditioned medium [8,9]. Moreover, ephrin-A1 expression was recently shown to be up-regulated by S100A4, S100A8 and S100A9, mediated by receptor for advanced glycation end (RAGE) or CD147, also known as Emmprin [10–12]. However, the mechanism up-regulating the expression of ephrin-A1 in primary tumors remains unknown, and ligand–receptor specificity has not yet been investigated because previous reports did not perform loss-of-function experiments.

S100A8 and S100A9, also known as Myeloid-related protein-8 (MRP-8) and Myeloid-related protein-14 (MRP14), are calcium-binding proteins with EF-hand motif and are one of endogenous damage-associated molecular patterns (DAMPs), also referred to as alarmins. S100A8 and S100A9 were shown to be necessary for initiating the immune response to non-infectious inflammation [13], are abundantly expressed in myeloid lineage cells such as neutrophils, and are mostly released as heterodimers from

*Abbreviations:* Eph, erythropoietin-producing hepatocellular; LLC, Lewis lung carcinoma; MD-2, myeloid differentiation protein 2; SAA3, serum amyloid A3; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

* Corresponding authors. Fax: +81 3 5269 7417.
*E-mail addresses:* ieguchi@research.twmu.ac.jp (K. Ieguchi), ymaru@research.twmu.ac.jp (Y. Maru).

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dying cells or are secreted from immune cells activated by pathogen-associated molecular patterns (PAMPs) [14]. Previous studies demonstrated that S100A8 and S100A9 were increased in the serum due to rheumatoid arthritis and inflammatory bowel disease, and are known to be not only biomarkers in the inflammatory process for these diseases [15,16], but also play a pivotal role in amplifying inflammation. The complex of S100A8 and S100A9 was previously shown to bind to RAGE or CD147 and plays a crucial role in the inflammatory process. Although S100A8 and S100A9 are involved in amplifying the inflammatory process due to infection or inflammatory diseases, they also have been characterized as positive regulators of cancer development and tumor cell dissemination [17–19]. We and other groups have reported that S100A8 and S100A9 physically interacted with the TLR4/MD-2 complex, and this association was confirmed by Surface Plasmon Resonance (SPR) with $K_D$ values of approximately 10 and 2 nM, respectively [19,20]. We also previously demonstrated that VEGF-A, TGF-β, and TNFα induced S100A8 expression in pre-metastatic lungs, and a TLR4-mediated S100A8-SAA3 paracrine system established a pre-metastatic niche in the lungs, leading to lung metastasis. The inhibition of proteins that establish a pre-metastatic niche in the lungs with neutralizing antibodies was shown to decrease the incidence of lung metastasis [19]. Consistent with our previous findings, S100A8 was shown to play an important role in lung metastasis.

As a step towards understanding the role of the overexpression of ephrin-A1 in primary tumors, we herein elucidated the mechanism that up-regulated the expression of ephrin-A1.

## 2. Materials and methods

### 2.1. Materials

Antibodies against ephrin-A1 (sc-911) and p65 (sc-372) were purchased from Santa Cruz (Dallus, TX). The antibody against actin was purchased from Millipore (Billerica, MA), that against phosphor p65 (#3033) antibody was purchased from Cell Signaling Technology (Danvers, MA), and that against mouse TLR/MD-2 antibody (13-9924) and the isotype control (13-4321) were purchased from eBioscience. FLAG agarose beads and mouse TNFα recombinant protein were purchased from WAKO laboratories (Osaka, Japan). LPS was purchased from Sigma–Aldrich (St. Louis, MO).

### 2.2. Plasmids

C-terminal FLAG-tagged full-length mouse S100A8 was subcloned into the *Kpn*I site of the pEBmulti-Hyg vector (WAKO).

### 2.3. Cell culture

Lewis lung carcinoma (LLC), Raw264.7, HEK293T and E0771 cells were cultivated in Dulbecco's modified Eagle's medium (WAKO) supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. HUVEC were obtained from Takara Bio (Shiga, Japan) and cultivated in human endothelial-SFM basal growth medium (Life Technologies, Carlsbad, CA) supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin and low serum growth supplement (Life Technologies). 3LL tumor cells were cultivated as described previously [7]. F2 cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, 100 mg/ml streptomycin and GlutaMax-I (Life Technologies).

### 2.4. Protein purification of mS100A8 protein

pEBmulti-mS100A8–FLAG was used for transfection into HEK293T cells. To isolate the S100A8 protein, transfected cells ($5 \times 10^8$) were harvested and then lysed with 10 ml of extraction buffer (50 mM Tris–HCl, [pH 7.7], 150 mM NaCl, 1% Nonidet P40, and 0.5% sodium cholate). After brief centrifugation, the supernatant was passed through a filter unit with 0.45-μm pores and loaded onto 1 ml of anti-FLAG tag beads (WAKO). The column was washed with 5 ml of extraction buffer followed by washing with 10 ml of PBS. To remove the endotoxin, the beads were extensively washed with PBS/0.1% Triton X114 followed by PBS, which eliminated residual Triton X-114. S100A8–FLAG was eluted with 1 ml of FLAG peptide solution (0.1 mg/ml in PBS, Wako), and the effluent was concentrated with a centrifugal filter unit and dialyzed against PBS. The endotoxin levels of the purified mS100A8 protein were assessed by the Pyrochrome (Seikagaku, Tokyo, Japan) LAL method. Purified mS100A8–FLAG protein containing less than 0.01 EU/μg endotoxin was used for various assays.

### 2.5. Immunoblotting

Immunoblotting was performed as described previously [21]. To detect phosphorylated p65, cells were starved with DMEM(-) without FBS for 14 h followed by S100A8 or LPS stimulation. Protein concentrations were determined in the cell lysates using the BCA protein assay kit (Fisher Thermo Scientific, Waltham, MA). Twenty micrograms of the whole cell lysates were analyzed by SDS–PAGE and transferred onto a 0.45-μm pore-size polyvinylidene fluoride membrane (Millipore).

### 2.6. Immunofluorescence microscopy

Immunofluorescence staining was performed as described previously [21]. Images were obtained using a confocal laser scanning microscope (LSM710, Carl Zeiss, Oberkochen, Germany) and processed by Zen2011 imaging software.

### 2.7. Quantitative PCR

Total RNA was purified from tumor samples or cultured cells using ISOGENE II (Nippon Gene, Toyama, Japan). Complementary DNA was synthesized with ReverTra Ace reverse transcriptase (TOYOBO, Osaka, Japan) using 0.5 μg of total RNA with oligo dT and random primers. Quantitative PCR (qPCR) was performed using KAPA SYBR Green master mixture (Kapa Biosystems, Woburn, MA) with StepOne Plus (Life Technologies). Each mRNA level was normalized against β-actin. qPCR was performed using the following primer sets as shown in Supplementary Table 2.

### 2.8. RNA interference

Predesigned sequences were synthesized (mouse tlr4: TRCN0000065787) and ligated into the pLKO.1 lentivirus vector (Addgene plasmid 8453) to knockdown *tlr4* in LLC or E0771 tumor cells. LLC and E0771 cells infected with the lentivirus were selected with 2 μg/ml puromycin (InvivoGen, San Diego, CA).

### 2.9. Animal study

Animal studies were performed described previously [7].

### 2.10. Statistical analysis

Data are expressed as means ± S.D. Comparisons between two groups were performed with the two-tailed, paired Student's

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