

## REVIEW ARTICLE

# Roles of EphA1/A2 and ephrin-A1 in cancer

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The biological functions of the Eph/ephrin system have been intensively investigated and well documented so far since its discovery in 1987. Although the Eph/ephrin system has been implicated in pathological settings such as Alzheimer's disease and cancer, the molecular mechanism of the Eph/ephrin system in those diseases is not well understood. Especially in cancer, recent studies have demonstrated that most of Eph and ephrin are up- or down-regulated in various types of cancer, and have been implicated in tumor progression, tumor malignancy, and prognosis. However, they lack consistency and are in controversy. The localization patterns of EphA1 and EphA2 in mouse lungs are very similar, and both knockout mice showed similar phenotypes in the lungs. Ephrin-A1 that is a membrane-anchored ligand for EphAs was co-localized with EphA1 and EphA2 in lung vascular endothelial cells. We recently uncovered the molecular mechanism of ephrin-A1-induced lung metastasis by understanding the physiological function of ephrin-A1 in lungs. This review focuses on the function of EphA1, EphA2, and ephrin-A1 in tumors and an establishment of pre-metastatic microenvironment in the lungs.

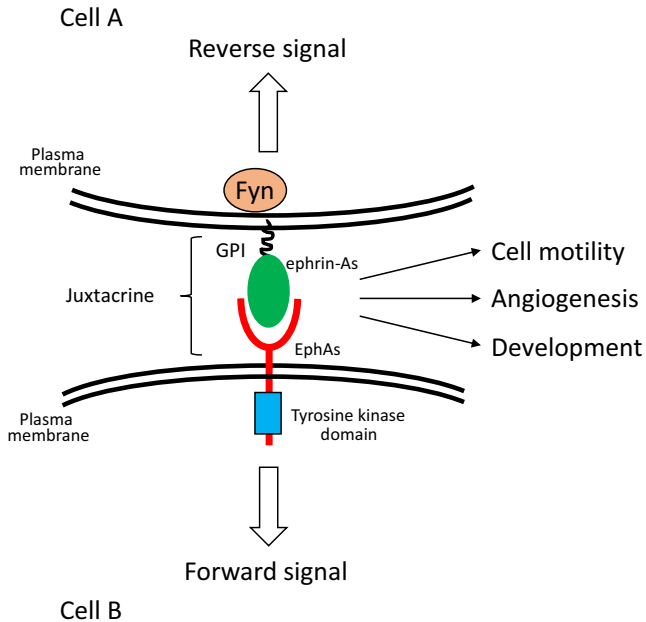
**KEYWORDS**

ADAM, Eph, ephrin, metastasis, S100A8

## 1 | INTRODUCTION

The Eph receptor tyrosine kinase was identified from a hepatocellular carcinoma (HCC) cell line in 1987.<sup>1</sup> The Eph receptor comprises the largest family in receptor tyrosine kinases and can be divided into two groups based on their structures and receptor-ligand specificity. EphA (pronounced "eff-A") consists of 9 type-A Eph receptors (EphA1-8, and EphA10) and 5 type-B Eph receptors (EphB1-4, 6, pronounced "eff-B").<sup>2,3</sup> Ephrins (pronounced "efrin") are also divided into two subfamilies, ephrin-A (ephrin-A1-6) and ephrin-B (ephrin-B1-3) based on their structures. Ephrin-A1, a ligand for the EphA receptors, was identified as an immediate early response gene to TNF $\alpha$ .<sup>4</sup> Ephrin-A1 is a plasma membrane protein anchored by glycosylphosphatidylinositol (GPI) although ephrin-A1 was originally identified as a soluble factor.<sup>4</sup> The EphA receptors transmit their

downstream signals mediated by their tyrosine kinase activities to receptor-expressing cells upon ephrin-A1 binding by a juxtacrine fashion defined as forward signal, and then ephrin-As simultaneously transmit their signals to a ligand-expressing cell termed as reverse signal mediated by the Fyn tyrosine kinase activity<sup>5</sup> (Figure 1). A recent study reported that Progranulin is a functional ligand for EphA2.<sup>6</sup> Progranulin is ubiquitously expressed and has been implicated in cancer progression.<sup>7</sup> The interaction between EphA2 and Progranulin modulates capillary formation in vitro and MAPK signaling pathway.<sup>6</sup> While the EphA1/A2 and ephrin-A1 system plays roles in physiological settings,<sup>8-10</sup> it is implicated in pathological circumstances.<sup>11-13</sup> EphA1 and EphA2 have the highest protein homology (approximately 65% in human) among EphAs. The localization patterns of EphA1 and EphA2 in mouse lungs were almost same. Moreover, both EphA1- and EphA2-deficient mice showed



**FIGURE 1** Activation mechanism of the Eph/ephrin system. Ephrin-A5 anchored by GPI to the plasma membrane bind to EphA5 in adjacent cell. This type of activation mechanism is called juxtacrine. EphA5 and ephrin-A5 simultaneously transmit their signals into each cell termed as forward and reverse signal, respectively

similar phenotypes in the lungs. Collectively, we suppose that EphA1 and EphA2 share their functions in lungs. We described “EphA1 and EphA2” as EphA1/A2 in this review.

## 2 | CLINICOPATHOLOGICAL ANALYSIS OF EPHA1/A2 AND EPHRIN-A1 IN CANCER

Accumulating evidence has showed that EphA1/A2 and ephrin-A1 are correlated with tumor malignancy and prognosis. EphA1/A2 and ephrin-A1 are over-expressed or down-regulated in various types of cancer.<sup>14</sup> For instance, there is a complementary expression of ephrin-A1 and EphA2 in human breast cancer cell lines. Ephrin-A1 was down-regulated in MDA-MB-231, an invasive breast cancer cells as compared to MCF-10A, a benign breast cancer cell. On the other hand, EphA2 was overexpressed in MA-MB-231 and down-regulated in MCF-10A.<sup>15</sup> These expression profiles were also observed in glioma,<sup>16</sup> and expression levels of EphA2 and ephrin-A1 in tumor tissues would be a potential diagnostic and prognostic marker. Some studies have demonstrated that higher ephrin-A1 expression is positively correlated with worse prognosis in liver and colorectal cancer.<sup>17,18</sup> However, among stage I non-small cell lung cancer patients, higher expression levels of EphA2 and ephrin-A1 improved their prognosis.<sup>19</sup> In advanced gastric cancer, analysis of mRNA expression showed that *EPHA1-EPHA4* were overexpressed in most of patients, and high EphA1 and EphA2 were significantly associated with poor prognosis<sup>20</sup>

In human glioblastoma multiforme (hGBM), comparison between EphA2<sup>low</sup> and EphA2<sup>high</sup> populations indicated that the EphA2<sup>high</sup> population has an ability to maintain self-renewal property and tumorigenicity. In an orthotopic murine xenograft model, mice with tumors of high EphA2 expression exhibited shorter survival than those of low EphA2 expression. Moreover, down regulation of EphA2 expression in hGBM by Fc-ephrin-A1 stimulation resulted in loss of self-renewal ability and a decreased proliferating activity in vitro and tumor growth in vivo.<sup>21</sup> Overexpression of EphA3 have showed similar results as observed in EphA2-overexpressing hGBM. Tumors with high EphA3 expression also showed more aggressive and undifferentiated phenotypes.<sup>22</sup> These data suggest that EphA2 and EphA3 seem to be required for the maintenance of self-renewal ability in hGBM. Moreover, ephrin-A1 seems to be a key molecule to decrease self-renewal property of hGBM and prolong survival of cancer patients. However, there is no study to support that high ephrin-A1 expression in hGBM shows much better clinical outcome.

## 3 | ROLES OF THE EPH/EPHRIN SYSTEM IN TUMOR ANGIOGENESIS

It has been reported that EphB4 and ephrin-B2 determined arterial and venous specification during vasculogenesis by regulating cell adhesion and migration of endothelial cells.<sup>23,24</sup> Moreover, ephrin-B2 is essential for VEGF-induced receptor internalization and signalings.<sup>25,26</sup> However, roles of EphB4 and ephrin-B2 have not been fully understood in pathological settings. In case of colorectal cancer (CRC), expression analysis using clinical samples demonstrated that EphB4 was overexpressed in the plasma membrane of tumor cells but not in normal colon mucosa. Expression of EphB4 was positively associated with TNM stages in CRC, and overexpression of EphB4 resulted in an increase of microvascular density in a xenograft mouse model. ShRNA-mediated knocked down of EphB4 decreased tumor growth and tumor angiogenesis.<sup>27</sup> An inhibition of EphB4/ephrin-B2 ligation by extracellular domain of EphB4 reduced tumor growth and angiogenesis.<sup>28,29</sup> A recent study demonstrated that genetic deletion of ephrin-B2 showed more aggressive phenotypes on tumor growth and invasion than those of controls.<sup>30</sup> Therefore, roles of EphB4 and ephrin-B2 in tumor growth and angiogenesis are still controversial. In pathological conditions, EphA1/A2 and ephrin-A1 have been implicated in tumor angiogenesis that is important for tumor growth to supply O<sub>2</sub> and nutrients.<sup>31,32</sup> However, the detailed molecular mechanisms remain to be elucidated. Activating transcription factor 3 (ATF3) is well known to be induced by various stress including hypoxia as often observed in tumor microenvironment.<sup>33</sup> We found that EphA1 was up-regulated in an ATF3-dependent manner in NP31, a rat endothelial cell line<sup>9</sup> and regulated endothelial tubulogenesis.<sup>9,34</sup> Furthermore, ephrin-A1-induced EphA1 activation promoted SDF-1 secretion and chemotaxis of endothelial progenitor cells to HCC through the SDF-1/CXCR4 signaling pathway.<sup>35,36</sup> Small interfering RNA (siRNA)-mediated inhibition of the EphA1/SDF-1/

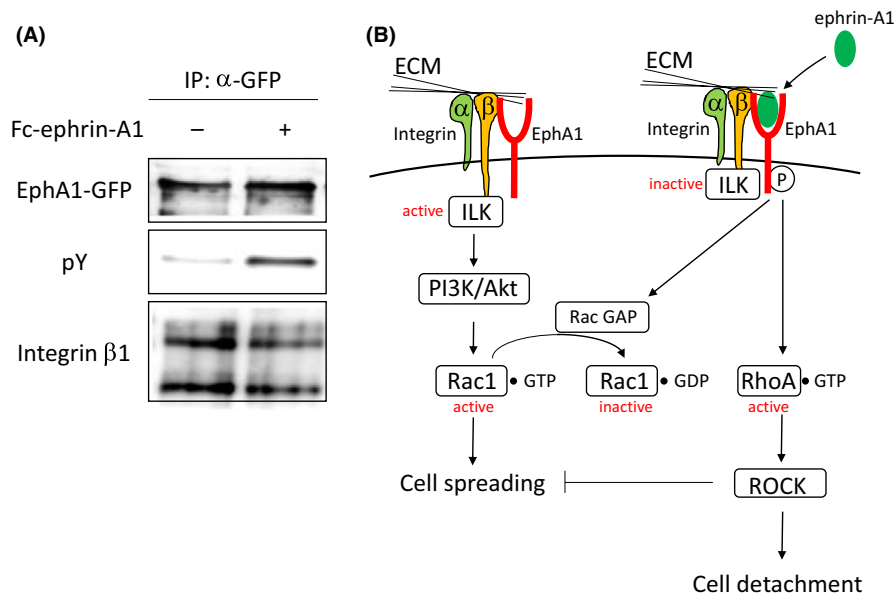
CXCR4 pathway abolished tube formation in vitro and decreased tumor size and angiogenesis due to an inhibition of endothelial progenitor cell homing to the tumor tissue.<sup>36</sup>

#### 4 | THE SIGNALING CROSSTALK AMONG INTEGRIN, ECM, AND EPHA1

It has been reported that the integrin-extracellular matrix (ECM) axis contributes to tumor angiogenesis. Integrin  $\alpha\beta3$ , an angiogenic marker, is widely expressed in tumors but not in healthy tissues.<sup>37</sup> A monoclonal antibody against either integrin  $\alpha\beta3$  or  $\alpha\beta5$  that inhibits the interaction between integrin and ECM attenuated tumor angiogenesis.<sup>37</sup> However, double-knockout of integrin  $\beta3$  and  $\beta5$  showed an opposite effect on neovascularization in tumors and enhanced tumor angiogenesis.<sup>38</sup> Despite the opposing results, integrins are still attractive target molecules for an inhibition of tumor growth because integrins cooperate with some growth factor receptor signals for cell proliferation.<sup>39,40</sup> Therefore, many researchers devote their intensive works to the development of anti-integrin drugs.<sup>41,42</sup> EphA1 interacted with fibronectin type I repeat and integrin-linked kinase (ILK) mediated by the extracellular and intracellular domain of EphA1, respectively.<sup>8,9</sup> The association of EphA1 and fibronectin was partially involved in VEGF-dependent tube formation in vitro. Although the detailed molecular mechanisms remained unknown, sequestering EphA1 from binding to fibronectin by secretion of Fc-fused EphA1 diminished tumor burden and VEGF-induced angiogenesis.<sup>9</sup> ECM such as collagen and fibronectin is abundant in tumor surroundings. It has been reported that the integrin-ECM signaling

pathway regulates cell motility and cell shape, which is mediated by Rho family small GTPases.<sup>43</sup> We found a linkage among integrin, EphA1, and ECM-related signaling pathways. EphA1-induced signaling pathway in tumors inhibited cell spreading mediated by the ECM-integrin and ECM-EphA1 pathways.<sup>8</sup> EphA1 interacted with integrin  $\beta1$  without affecting tyrosine phosphorylation state (Figure 2A). ILK is recruited to the SAM domain of EphA1 in response to ephrin-A1 stimulation and integrin  $\beta1$  in the presence of integrin-ECM interactions.<sup>8</sup> ECM-integrin signal maintains cell spreading and adhesion by ILK and Rac1 GTP-bound form.<sup>44</sup> When EphA1 is stimulated with ephrin-A1, ILK and Rac1 are inactivated, and thereby RhoA/Rock pathway turns to be activated resulting in an inhibition of cell spreading.<sup>8</sup> Loss of cell-cell adhesion and spreading defect could render tumor cell to easily get off from the tumor mass (Figure 2B).

SiRNA-mediated knockdown of ephrin-A1 attenuated migratory activity of endothelial cells in vitro and tumor angiogenesis in vivo using 4T1, a mammary adenocarcinoma cell.<sup>45</sup> Moreover, an intravenous injection of 4T1 cells in which ephrin-A1 expression was abrogated significantly suppressed a number of metastatic foci in lungs suggesting that ephrin-A1 contributes to migration and/or regrowth in metastatic microenvironment. Taken together, overexpressed-ephrin-A1 also plays an important role in tumor angiogenesis and metastasis. In our knowledge, membrane-anchored ephrin-A1 has no ability to induce tyrosine phosphorylation of EphA1/A2 and maintains cell-cell contacts via association between EphA1/A2 and ephrin-A1 at cell boundaries. Accordingly, membrane-anchored ephrin-A1 needs to be released from the plasma membrane to activate the EphA1/A2 receptor. We identified a disintegrin and metalloproteinase 12 (ADAM12)



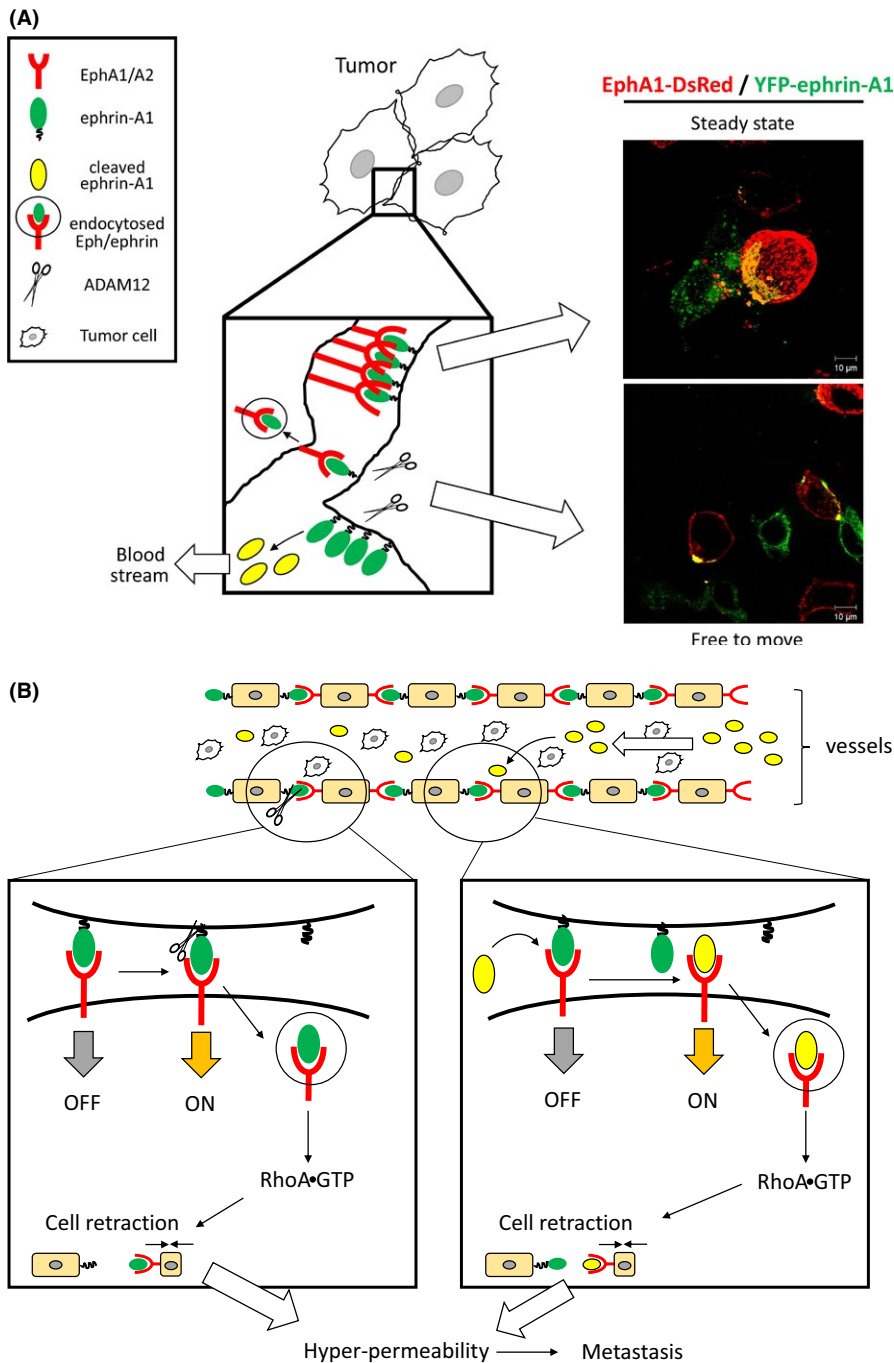
**FIGURE 2** Regulation mechanism of ECM/integrin/EphA1-mediated cell adhesion. A, Co-immunoprecipitation of EphA1 and integrin  $\beta1$ . GFP-fused EphA1-expressing HEK293 cells were stimulated with Fc-ephrin-A1 (2  $\mu$ g/mL). EphA1 was co-immunoprecipitated with integrin  $\beta1$  without affecting tyrosine phosphorylation status of EphA1. B, Regulatory signaling pathway of ECM/integrin/EphA1-mediated cell adhesion. In the absence of ephrin-A1 stimulation, ILK is active. ECM/integrin signal provides cell spreading mediated by Rac1 activation. Once EphA1 is stimulated with ephrin-A1, ILK is inactivated. Subsequently, the Rac1 GTP-bound form is hydrolyzed by Rac1 GTPase activating protein followed by RhoA activation, which in turn induces cell retraction

as a binding partner of EphA1 and EphA2 by the yeast two-hybrid screening using extracellular domain of EphA1. ADAM12 cleaves membrane-anchored ephrin-A1 in trans.<sup>46</sup> Cleaved ephrin-A1 may be capable of activating EphA1/A2 by a paracrine and/or an autocrine fashion. Ephrin-A1-induced EphA1 activation showed cell spreading defect as described above. ADAM12-deficient mice in MMTV-PyMT mouse breast cancer model showed a delayed tumor progression and spontaneous lung metastasis by unknown mechanisms.<sup>47</sup> The other study suggested that ADAM12 localized at the invadopodia and regulated migration and invasion.<sup>48</sup> ECM deposition and loss of cell-cell contacts render tumor cell to metastasize to distant organs. ADAM12 shows matrix metalloproteinase

activity toward ECM such as fibronectin.<sup>49</sup> Taken together, we suppose that, ADAM12 contributes to degradation of fibronectin and loss of cell-cell contacts via cleavage of ephrin-A1 unbound to the receptor in primary tumors (Figure 3).

## 5 | REGULATORS OF EPHRIN-A1 EXPRESSION

Overexpression of ephrin-A1 has been implicated in poor prognosis and tumor malignancy. The highest risk factor for poor prognosis is whether metastasis occurs. However, the detailed molecular



mechanisms in metastasis were largely unknown. Ephrin-A1 expression had no response to TNF $\alpha$  stimulation in some mouse tumor cells such as Lewis lung carcinoma (LLC) and E0771 although ephrin-A1 expression was significantly enhanced by TNF $\alpha$  stimulation in HUVECs and F2, vascular endothelial cell lines.<sup>50</sup> It has been reported that pattern recognition receptors (PRRs) and their ligands, damage-associated molecular patterns (DAMPs) regulate ephrin-A1 expression. It is well known that inflammatory cytokines and DAMPs are abundant in tumor microenvironment and modulate angiogenesis, tumor growth, and immune responses.<sup>51</sup> The S100A9/EMMPRIN<sup>52</sup> and S100A4/RAGE<sup>53,54</sup> axis have been considered to be involved in the regulation of ephrin-A1 expression. Lipopolysaccharides (LPS), a gram-negative bacteria-derived ligand for the toll-like receptor 4 (TLR4) has been thought to be a positive regulator of ephrin-A1 expression. TLR4 is a PRR and essential for the innate immune system. TLR4 is expressed not only in immune cells but also in some tumor cells and regulates tumor microenvironment.<sup>55</sup> LPS stimulation up-regulated ephrin-A1 expression in LLC.<sup>50</sup> Subsequently, we investigated the effect of S100A8 on ephrin-A1 expression since S100A8 has been proposed as an endogenous ligand for TLR4 and elicits inflammatory actions.<sup>56,57</sup> Knockdown of TLR4 abrogated S100A8-induced ephrin-A1 expression.<sup>50</sup> Hypoxia as often observed inside of tumors is strongly associated with inflammation in various pathological conditions. Nuclear factor-kappa B (NF- $\kappa$ B) is a common downstream target of hypoxia- and inflammation-induced signals, and its expression is up-regulated by hypoxia inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) and inflammatory cytokines such as TNF $\alpha$  and 100A8.<sup>58</sup> It was reported that ephrin-A1 was up-regulated in hypoxic conditions.<sup>59</sup> Taken together, overexpression of ephrin-A1 in tumors is mediated through inflammation- and hypoxia-induced NF- $\kappa$ B activation.

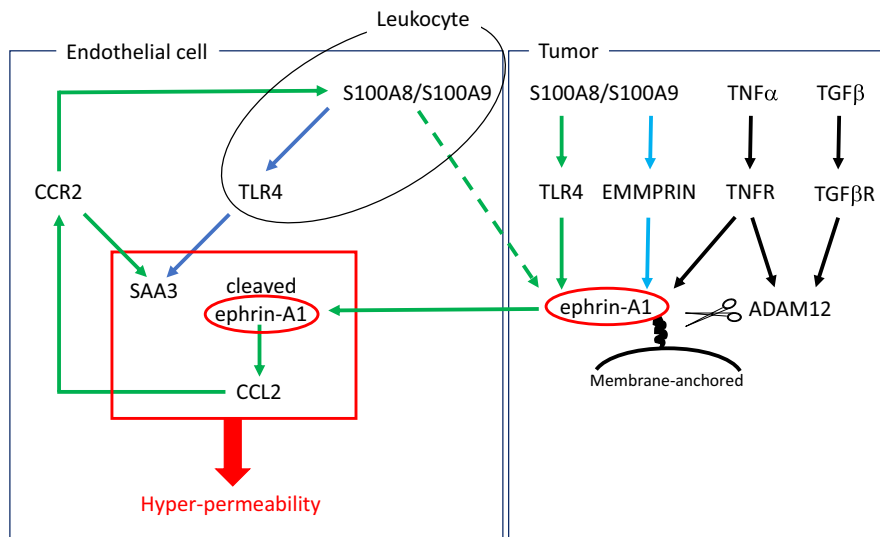
## 6 | MOLECULAR MECHANISM OF EPHRIN-A1-INDUCED LUNG METASTASIS

Interactions between EphA1/A2 and ephrin-A1 at cell boundaries provide cell-cell adhesion (Figure 3A). Co-culture of EphA1-expressing cells and ephrin-A1-expressing HEK293 cells formed large cell aggregations compared to HEK293 cells transfected with mock vector. EphA1/A2 and ephrin-A1 were expressed and co-localized in vascular endothelial cells, and both EphA1 and EphA2 knockout mice showed lung hyper-permeability because of the absence of Eph/ephrin-mediated cell adhesion in vascular endothelial cells.<sup>46</sup> The other group demonstrated that an injection of Fc-fused ephrin-A1 (Fc-ephrin-A1) caused lung hyper-permeability followed by loss of the plasma membrane localization of cell adhesion-related molecules such as ZO-1 and claudin-5.<sup>60</sup> Moreover, soluble forms of ephrin-A1 such as Fc-ephrin-A1 compete for pre-existing EphA2/ephrin-A1 binding at cell boundaries. Stimulation with soluble forms of ephrin-A1 in HUVECs induced endocytosis of EphA2 followed by degradation of VE-cadherin. An intravenous injection of Fc-ephrin-A1 resulted in a significant reduction of EphA2 at the plasma

membrane and enhanced tumor cell recruitment to the lungs.<sup>46</sup> EphA2 is known to be degraded by the c-Cbl-dependent ubiquitination system in response to ephrin-A1 stimulation.<sup>61</sup> These data suggest that the Eph/ephrin system regulates vascular homeostasis in lungs, and breakdown of the Eph/ephrin-mediated cell adhesion in lung endothelial cells causes lung hyper-permeability facilitating lung metastasis. A neutralizing antibody against ephrin-A1 that inhibits the interaction between EphA1/A2 and ephrin-A1 successfully inhibited lung metastasis.<sup>46</sup> Taken together, tumor-derived and ADAM12-cleaved ephrin-A1 enter into blood stream and compete for pre-existing Eph/ephrin binding at cell boundaries. Ephrin-A1-induced lung hyper-permeability render tumor cells easily to extravasate into lungs (Figure 3B). The data suggest that soluble ephrin-A1 would be a metastatic marker and a good candidate as a molecular targeting drug. Moreover, in primary tumors, ADAM12 may contribute to extravasation of tumor cells because ADAM12 cleaves E-cadherin that is an essential molecule for adherence junction.<sup>62</sup> We have demonstrated that ADAM12 cleaves membrane-anchored ephrin-A1 at the plasma membrane, and then cell boundaries organized by the Eph/ephrin interaction were disorganized. Tumor cells in which E-cadherin and ephrin-A1 are cleaved leave tumor mass and become free to move to distant organs (Figure 3A).

## 7 | PERSPECTIVES

Ephs and ephrins are regarded as promising candidates for drug development. However, Eph and ephrin had been considered as undruggable target molecules because the interactions between Eph and ephrin are not specific and promiscuous. Therefore, there are no drugs against the Eph/ephrin families for medical use so far. Dasatinib is the only drugs for medical use that shows an inhibitory effect on EphA2 activity. Dasatinib, a Bcr-Abl tyrosine kinase inhibitor that is clinically used for chemotherapy against chronic myeloid leukemia and acute lymphoblastic leukemia. Pretreatment of dasatinib completely blocked ephrin-A1-induced tyrosine phosphorylation of EphA2 in vitro.<sup>63</sup> However, dasatinib has not been used for any anti-EphA2 therapy so far. As we demonstrated, the Eph/ephrin system regulates tyrosine kinase activity-dependent and -independent physiological functions. Therefore, we suppose that tyrosine kinase inhibitors may not be always effective on the Eph/ephrin system. Based on this idea, some researchers isolated protein-protein interaction (PPI) inhibitors by a typical chemical screening and isolated lithocholic acid (LCA) as a PPI inhibitor of EphA2-ephrin-A1.<sup>64</sup> LCA completely blocked ephrin-A1-induced tyrosine phosphorylation of EphA2 without affecting cell viability and other receptor tyrosine kinase such as EGFR and IGFR. However, LCA showed an inhibitory effect on all Eph-ephrin-A1 and all EphB-ephrin-B1 interactions. This means that LCA is broad spectrum PPI inhibitor for the Eph/ephrin system. In silico optimization of LCA improved the affinity to the Eph receptors. The derivatives called UniPR1331 is orally available and decreased tumor burden and angiogenesis in an orthotopic mouse GBM



**FIGURE 4** A proposed paracrine loop to establish pre-metastatic microenvironment in lungs. Ephrin-A1 expression is enhanced by the TLR4/S100A8 axis. ADAM12 is up-regulated by inflammatory cytokines such as TNF $\alpha$  and TGF- $\beta$ 1. Cleaved ephrin-A1 enters into blood stream and stimulates lung vascular endothelial cells leading to an elevation of CCL2 secretion. The CCR2/CCL2 axis contributes to an increase of SAA3 and S100A8/S100A9 expression. The paracrine loop increases permeability factors enclosed by red box, and then tumor metastasis are facilitated in the lungs. Up-regulated S100A8/A9 expression in endothelial cells and leukocytes may increase ephrin-A1 expression mediated by TLR4 and/or EMMPRIN in primary tumors

model.<sup>65</sup> Pasquale and her colleagues successfully isolated some antagonistic and agonistic peptides with micromolar affinity that interact with ligand binding pocket of the Eph receptors by phage display.<sup>66</sup> Isolated peptides are very specific and bind to the only single Eph receptor. Some modifications in those peptides gave stability in blood and made them possible to be used for SOD<sup>G93A</sup> transgenic mice, an ALS mouse model.<sup>67</sup> The effect of antagonistic peptides against EphA4 on ALS therapy is still under investigation. EphA2 agonistic peptides conjugating to paclitaxel markedly decreased tumor growth in prostate cancer and renal cell carcinoma models compared to sole paclitaxel use.<sup>68</sup> Therefore, the Eph/ephrin system is currently no longer undruggable target. We suppose that anti-Eph/ephrin drugs will come soon.

We have demonstrated that soluble form of ephrin-A1 would be a good therapeutic target and biomarker for metastasis. In our study, soluble form of ephrin-A1 was increased in serum of tumor-bearing mouse compared to that of healthy mice<sup>46</sup> and also in HCC patients.<sup>69</sup> Furthermore, partial ephrin-A1 peptides were found in human urine derived from kidney disease patients,<sup>70</sup> and we found that immuno-reactive bands around 25 kDa by using anti-ephrin-A1 antibody by western blotting. The size of immuno-reactive bands were almost same as that of membrane-anchored ephrin-A1 and commercially available recombinant ephrin-A1 (19aa-182aa) (unpublished data). Therefore, we suppose that the immuno-reactive urinary ephrin-A1 is nearly intact. Urinalysis is the best investigation method because it is much less harmful and expensive compared to others such as biopsy for cancer patients. Accordingly, urinary ephrin-A1 would be a good biomarker for some diseases such as cancer and kidney diseases. We need

further investigations to use urinary ephrin-A1 as a biomarker and/or a therapeutic target.

Lastly, we underline basic research in this system. It was reported that C-C motif chemokine ligand 2 (CCL2, also known to MCP1) is up-regulated by ephrin-A1 stimulation in endothelial cells.<sup>71</sup> An enhanced expression was observed in hyper-permeable regions in tumor-bearing mouse lungs compared to non-permeable regions. The CCR2/CCL2 axis promoted S100A8/A9 expression leading to up-regulation of SAA3 mediated by TLR4.<sup>72</sup> S100A8/A9 up-regulated by the CCR2/CCL2 axis induces ephrin-A1 and SAA3 expression, and then ephrin-A1 enhanced CCL2 expression. Collectively, the paracrine system by the ephrin-A1-CCL2-S100A8/A9 axis may exist in endothelial cells in the hyper-permeable regions in tumor-bearing mouse lungs, and the paracrine system may increase each protein expression levels and establish fertile soil in the pre-metastatic lungs (Figure 4). An inhibition of the paracrine loop would be a good approach to inhibit lung metastasis.

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#### CONFLICT OF INTEREST

The authors have no conflict of interest.

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