Gap Junction Coupling Is Required for Tumor Cell Migration Through Lymphatic Endothelium

Natalie O. Karpinich, Kathleen M. Caron

**Objective**—The lymphatic vasculature is a well-established conduit for metastasis, but the mechanisms by which tumor cells interact with lymphatic endothelial cells (LECs) to facilitate escape remain poorly understood. Elevated levels of the lymphangiogenic peptide adrenomedullin are found in many tumors, and we previously characterized that its expression is necessary for lymphatic vessel growth within both tumors and sentinel lymph nodes and for distant metastasis.

**Approach and Results**—This study used a tumor cell-LEC coculture system to identify a series of adrenomedullin-induced events that facilitated transendothelial migration of the tumor cells through a lymphatic monolayer. High levels of adrenomedullin expression enhanced adhesion of tumor cells to LECs, and further analysis revealed that adrenomedullin promoted gap junction coupling between LECs as evidenced by spread of Lucifer yellow dye. Adrenomedullin also enhanced heterocellular gap junction coupling as demonstrated by Calcein dye transfer from tumor cells into LECs. This connexin-mediated gap junction intercellular communication was necessary for tumor cells to undergo transendothelial migration because pharmacological blockade of this heterocellular communication prevented the ability of tumor cells to transmigrate through the lymphatic monolayer. In addition, treatment of LECs with adrenomedullin caused nuclear translocation of β-catenin, a component of endothelial cell junctions, causing an increase in transcription of the downstream target gene C-MYC. Importantly, blockade of gap junction intercellular communication preventedβ-catenin nuclear translocation.

**Conclusions**—Our findings indicate that maintenance of cell–cell communication is necessary to facilitate a cascade of events that lead to tumor cell migration through the lymphatic endothelium. *(Arterioscler Thromb Vasc Biol. 2015;35:1147-1155. DOI: 10.1161/ATVBAHA.114.304752.)*

**Key Words:** connexins ■ endothelial cells ■ gap junctions ■ neoplasm metastasis

Lymphatic vessels serve as a conduit by which tumor cells can escape to colonize distant organs. Seminal studies implicating the lymphangiogenic growth factors vascular endothelial growth factor-C and vascular endothelial growth factor-D in promoting tumor metastasis through enhanced lymphangiogenesis have provided insight into how lymphatics promote cancer progression.1-3 For example, vascular endothelial growth factor-C was shown to activate the lymphatic endothelium leading to vessel destabilization and thereby enabling tumor cell entry into the lymphatic vessel.4 In addition to vascular endothelial growth factor family members, other processes such as chemotraction,5 lymph flow,6,7 enhanced adhesion,7 and lymph node (LN) lymphangiogenesis8,9 are also implicated in promoting entry of tumor cells into the lymphatic vasculature and facilitating metastasis. However, the precise mechanisms of how this occurs at the critical interface between the tumor cells and lymphatic endothelial cells (LECs) remains to be elucidated.

One mechanism by which neighboring cells interact to communicate and exchange information is through the formation of gap junction channels. Oligomerized connexin hexamers termed connexons from juxtaposed cells form channels that allow for cytoplasmic exchange of small signaling molecules, such as metabolites, ions, and secondary messengers. Establishment of gap junctional intercellular communication (GJIC) through these channels is critical to maintain homeostasis during development and in adulthood.10 Of particular relevance, proper development of the lymphatic vasculature, specifically the lymphatic valves, is dependent on precise regulation and expression of certain members of the connexin protein family such as connexins (Cx) 37 and Cx43.11 In fact, missense mutations in the gap junction gene GJC2 (encoding Cx47) have been identified in families with dominantly inherited lymphedema.12 This finding is significant because it links impaired lymphatic activity with a mutation that alters gap junction function. These defects emphasize the critical role that connexins play in lymphatic function and disease.13

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Connexins seem to play diverse roles in cancer. Some studies suggest that expression of connexins confers a tumor suppressor function. Along these lines, mice heterozygous for Cx43 (Cx43+/−) had an increased susceptibility to urethane-induced lung tumors. More recent evidence, however, proposes that connexins are dynamically regulated depending on the stage of tumorigenesis, and therefore elevated levels may be important in promoting angiogenesis and invasion. These data suggest that increased connexin expression in later stages of tumorigenesis enables tumor cells to penetrate the vessels and thus promote colonization of distant tissues. Moreover, connexin proteins also have channel-independent functions such as serving as adhesion sites, which can mediate the invasion of glioma cells through the parenchyma.

Building on our previous study, which identified adrenomedullin as a factor which promotes tumor lymphangiogenesis and distant metastasis, we investigated the role of GJIC in this process. By focusing on the tumor cell–endothelial cell interactions, we identified a series of adrenomedullin-induced events that promote the transendothelial migration (TEM) of tumor cells including functional GJIC and subsequent β-catenin nuclear translocation. This study details how tumor cells and LECs physically interact to facilitate tumor spread through the lymphatics, and thus reinforces the often overlooked role that the lymphatic endothelium plays in actively promoting the metastatic process.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Adrenomedullin Promotes the Adhesion of Tumor Cells to the Lymphatic Endothelium and Enhances Their TEM
To test whether adrenomedullin is involved in mediating adhesion of tumor cells to the lymphatic vasculature, we used adrenomedullin-dosed LLC (Lewis Lung Carcinoma) murine tumor cells that either express a 2-fold increase in Adm expression (AM OExp), a 92% reduction in Adm expression (AM RNAi), or maintain basal levels (empty vector control). Importantly, the LLC tumor cells have negligible expression of the adrenomedullin receptor Calcrl; therefore, the tumor cells are insensitive to manipulation of their adrenomedullin levels because the adrenomedullin-induced signal cannot be transduced through CLR. Rather, any resultant biological effects caused by low or elevated adrenomedullin levels can be attributed to adrenomedullin-mediated signaling in other cell types in the microenvironment, such as LECs, which express abundant CLR. The genetically adrenomedullin-dosed cells were labeled with Cell Tracker Green (CTG) dye and added to an LEC monolayer. After 15 minutes of coculture, fluorescence intensity was measured and we detected an adrenomedullin dose-dependent increase in the adhesion of tumor cells to the lymphatic monolayer (Figure 1A and 1B). We confirmed equal CTG fluorescence to ensure that altering Adm dosage does not affect CTG dye labeling (Figure 1C). Next, we used a pharmacological approach to confirm that adrenomedullin was mediating this adhesion. We treated the LEC monolayer with 1 nmol/L murine adrenomedullin peptide and the adrenomedullin receptor antagonist, adrenomedullin22-52, and then added CTG-labeled LLC cells. Again, there was an increased adhesion of tumor cells to LECs in the presence of adrenomedullin, and this adhesion was dramatically reduced in the presence of the adrenomedullin inhibitor (Figure 1D). To corroborate these results, we analyzed the CTG-labeled human tumor cell line MCF-7 (Figure 1E) and similarly found that stimulation of LECs with 10 nmol/L human adrenomedullin (hAM) peptide promoted the adhesion of the MCF-7 cells to the LECs (Figure 1F).

We then wanted to determine whether the adrenomedullin-induced increase in tumor cell-LEC adhesion consequently results in enhanced TEM. Using a standard transwell assay, CTG-labeled adrenomedullin-dosed tumor cells were added to transwell inserts, which contained a confluent monolayer of LECs. Confluency was verified by H&E-stained filters (data not shown). After 6 hours of coculture, the filters from the inserts were removed and the fluorescent threshold area was determined. There was a significant increase in fluorescence as tumor-derived adrenomedullin dose increased, indicative of transmigrated CTG-labeled tumor cells (Figure 1G). To confirm that adrenomedullin was responsible for the increased TEM, we repeated this experiment using a pharmacological approach. First, we pretreated the LEC monolayer with the adrenomedullin inhibitor, adrenomedullin22-52, followed by addition of 10 nmol/L human or 1 nmol/L murine adrenomedullin peptide. After the LEC monolayer was treated with adrenomedullin for 5 minutes, CTG-labeled murine LLC tumor cells or human MCF-7 and SK-MEL-2 tumor cells were added to the transwell inserts. Again, we observed an adrenomedullin-induced increase in TEM in all 3 tumor lines and the adrenomedullin inhibitor reduced the transmigration to levels comparable with vehicle treatment (Figure 1H and 1J). Interestingly, the time course of TEM of the 2 human cell lines ranges from 3 to 24 hours for the SK-MEL-2 and MCF-7 cells, respectively. These data concur with the characterization that SK-MEL-2 cells are a highly metastatic cell line, whereas the MCF-7 cells are reported to be weakly metastatic. In both instances, however, treatment with adrenomedullin promotes TEM. Importantly, comparable with the LLC tumor cells, the MCF-7 and SK-MEL-2 cell lines also had negligible expression of the adrenomedullin receptor Calcrl (C, values =32, data not shown). Therefore, adrenomedullin stimulates the LECs, rather than the tumor cells, to promote TEM.
LECs Form Functional Gap Junctions Upon Adrenomedullin Treatment

To assess whether gap junction formation is enhanced by adrenomedullin treatment, we performed a scrape-loading dye transfer assay using the gap junction permeable dye Lucifer Yellow. Treatment of LECs with 10 nmol/L hAM for 30 minutes resulted in a dramatic spread (3-fold increase) of Lucifer Yellow between the cells indicative of gap junction coupling (Figure 2). Importantly, this dye transfer was abrogated by pretreatment with the adrenomedullin inhibitor, adrenomedullin22-52, confirming that this was an adrenomedullin-mediated effect. Furthermore, to verify that the spread of Lucifer Yellow

Figure 1. Adrenomedullin (AM) promotes the adhesion and transendothelial migration (TEM) of tumor cells to lymphatic endothelial cells (LECs). A, AM-dosed LLC (Lewis Lung Carcinoma) cells were labeled with Cell Tracker Green (CTG) dye and incubated with a monolayer of LECs. After 15 minutes, nonadhered cells were aspirated and fluorescence of adhered cells was measured. B, Representative images of CTG-labeled tumor cells (black arrows) adhered to an LEC monolayer. Magnification: ×10. Scale bars, 150 μm. Phase-contrast images are an optical zoom of the area surrounded by a white box. C, Graph depicts equal CTG labeling of the AM-dosed LLC cells. D, LECs were treated with vehicle or 1 nmol/L murine AM with or without a 30-minute pretreatment with the AM inhibitor AM22-52 (1 μmol/L) followed by the addition of CTG-labeled LLC tumor cells for a 15-minute incubation. A and D, Fluorescence readings indicated an AM dose-dependent increase in tumor cell adhesion to the LEC monolayer. Reduced AM levels through either RNAi depletion of Adm in tumor cells or pretreatment with the AM inhibitor AM22-52 prevented the increased adhesion. E, A standard curve of CTG-labeled LLC and MCF-7 cells. F, CTG-labeled MCF-7 cells also exhibit an AM-induced increase in adhesion to LECs after a 2-hour incubation; filters were removed from the transwell inserts. Image threshold analysis of 4 fields/ filter revealed that AM promoted TEM. H, Using a pharmacological approach, a confluent LEC monolayer was treated with vehicle or 1-nmol/L murine AM+/− AM22-52 followed by the addition of CTG-labeled unmodified LLC cells similarly showed that AM promoted TEM. Analysis of 2 additional tumor cell lines, CTG-labeled MCF-7 (I) and SK-MEL-2 (J) cells showed similar trends of AM-enhanced TEM within 24 and 3 hours, respectively. G–J, Magnification: ×10. Scale bar, 100 μm. The data shown are mean±SE and are representative of 3 independent experiments that yielded similar results.
dye was through gap junctions rather than through nonspecific dye transmission, we treated cells with the gap junction inhibitor carbenoxolone\(^2\) and subsequently detected no transfer of dye to adjacent cells (Figure 2). Taken together, these results suggest that adrenomedullin treatment enhances gap junction-mediated intercellular communication in LECs.

**Figure 2.** Adrenomedullin (AM) promotes the formation of gap junctions in lymphatic endothelial cells (LECs). Transfer of Lucifer yellow dye into neighboring LECs was determined after scrape-loading. Cells were pretreated with the AM inhibitor AM\(_{22-52}\) (1 \(\mu\)mol/L) or the gap junction inhibitor carbenoxolone (CBX; 100 \(\mu\)mol/L) for 30 minutes, followed by treatment with 10 nmol/L human AM for 10 minutes. The percentage of dye coupled cells was quantified by dividing the number of Lucifer yellow-positive cells by the total number of cells in the field. Data represent mean±SE (n=3). Magnification: ×10. Scale bar, 100 \(\mu\)m.

**Figure 3.** Connexin expression and localization in response to adrenomedullin (AM). A, Gene expression analysis revealed that treatment of lymphatic endothelial cells (LECs) with 10 nmol/L human AM (hAM; 4 hours) caused an increase in GJA1, a decrease in GJA4, and no change in expression of GJC2. B and C, Western blot analysis confirms these trends in expression after AM treatment (24 hours). D and E, LECs were pretreated with inhibitors (1 \(\mu\)mol/L AM\(_{22-52}\) or 100 \(\mu\)mol/L carbenoxolone [CBX]) followed by a 15-minute treatment with 10 nmol/L hAM and then stained for Cx43. Immunofluorescence analysis revealed that AM and AM+CBX treatments caused a distinct linearization of Cx43 at cell–cell contacts when compared with a more punctuate pattern in vehicle and AM inhibitor treatments. G and H, AM treatment of LECs failed to cause a change in localization of Cx37 and Cx47. Magnification: ×40. Scale bar, 50 \(\mu\)m.
Connexins Assemble to Form Gap Junctions Upon Adrenomedullin Treatment

We assessed the expression of connexins 37, 43, and 47 (Cx37, Cx43, and Cx47, respectively) given their importance in lymphatic vascular development. Using real-time quantitative polymerase chain reaction, we found that 10 nmol/L hAM treatment of LECs caused an increase in GJA1, a decrease in GJA4, and no change in expression of GJC2 (Figure 3A–3C). These dynamic changes in mRNA transcript were verified by Western blot analysis of adrenomedullin-treated LECs (Figure 3D and 3E). Using immunofluorescence analysis, we found that vehicle-treated LECs displayed punctate Cx43 staining throughout the cytoplasm with only minimal localization to the cell membranes (Figure 3F). By contrast, LECs treated for 15 minutes with 10 nmol/L hAM had Cx43 localized to regions of cell–cell contacts (Figure 3F). Analysis of Cx37 and Cx47 revealed no changes in localization after hAM treatment (Figure 3G and 3H). The striking adrenomedullin-induced membrane localization of Cx43 was prevented by the treatment with the GJ inhibitor carbenoxolone before addition of hAM (Figure 3F). Interestingly, LECs pretreated with the GJ inhibitor carbenoxolone before addition of hAM still had localization of Cx43 to regions of cell–cell contact (Figure 3F). This suggests that gap junction coupling itself is not required for adrenomedullin-induced Cx43 membrane localization. Collectively, these data show that stimulation of LECs with adrenomedullin causes an upregulation of Cx43 expression and changes its subcellular localization to areas of cell–cell contact.

Adrenomedullin-Overexpressing Tumors Have Increased Expression of Cx43 in Lymphatic Vessels

Studies have reported that levels of Cx43 mRNA or protein are dynamically regulated and, for example, are elevated in estrogen-stimulated endometrium and metastatic tissue. To determine whether adrenomedullin can similarly modulate Cx43 expression in vivo, we subcutaneously injected mice with 1×10⁶ adrenomedullin-dosed LLC cells and harvested tumors after 14 days. Lymphatic vessels were identified by LYVE1 staining, and interestingly, in the presence of high tumor-derived adrenomedullin (AM OExp), integrated density analysis revealed that the lymphatic vessels had increased expression of Cx43 (Figure 4). Furthermore, tumors expressing low levels of adrenomedullin had only minimal Cx43 staining in the lymphatic vessels (Figure 4). These data demonstrate that in vivo tumor lymphatic vasculature responded to high levels of tumor-derived adrenomedullin by upregulating Cx43 expression.

Adrenomedullin Promotes Heterocellular GJIC and Blockade of This Communication Prevents TEM

To address whether adrenomedullin has the ability to promote heterocellular gap junction formation, we performed a dye transfer assay. LLC tumor (donor) cells were loaded with the gap junction permeable dye Calcein, whereas the LECs (acceptor) were loaded with the gap junction impermeable dye DiI. After a 3-hour coculture, we observed ≈80% of hAM-treated LECs (acceptor) were Calcein positive (Figure 5). Treatment with the gap junction inhibitor carbenoxolone completely blocked any transfer of Calcein from the tumor cells to LECs and confirmed that functional gap junction channels were responsible for the observed dye transfer. Next, we wanted to determine the functional consequences of this adrenomedullin-induced heterotypic cellular communication, and we hypothesized that GJIC may be necessary for the adrenomedullin-induced TEM observed in Figure 2. Consistent with these results, we found that adrenomedullin promoted the TEM of CTG-labeled tumor cells and intriguingly, carbenoxolone prevented the ability of tumor cells to migrate...
through an LEC monolayer (Figure 5B). These findings demonstrate that adrenomedullin-induced formation of functional gap junctions between tumor cells and LECs is necessary for tumor cell migration through an LEC monolayer.

**Adrenomedullin-Induced GJIC Causes β-Catenin Nuclear Translocation**

Adrenomedullin was recently identified as a Wnt/β-catenin target, and we examined whether this pathway was implicated in the TEM process by assessing β-catenin localization in LECs after treatment with hAM. In contrast to the jagged staining pattern observed at cell–cell contacts in control cells, 10 nmol/L hAM treatment caused a more linear arrangement of β-catenin (Figure 6A). Given that β-catenin is a part of a complex at the endothelial junction, this finding is consistent with the linearization observed in VE-cadherin staining after adrenomedullin treatment. Furthermore, treatment with adrenomedullin induced nuclear localization of β-catenin in 26% of hAM-treated LECs (Figure 6A and 6B). Importantly, the adrenomedullin inhibitor, adrenomedullin_22-52, prevented β-catenin translocation to the nucleus, confirming that this translocation is triggered by adrenomedullin signaling. The gap junction inhibitor, carbenoxolone, also failed to cause β-catenin accumulation at the nucleus, but interestingly, staining shows that it was aligned at cell–cell contacts as seen in adrenomedullin-treated LECs (Figure 6A). This suggests that functional gap junctions are necessary for β-catenin nuclear translocation, which then leads to an increase in gene transcription of the downstream target C-MYC (Figure 6C). Our model summarizes how adrenomedullin activates the lymphatic endothelium to promote tumor cell dissemination (Figure 6D).

**Discussion**

It has been speculated that tumor cells use similar mechanisms as immune cells to home to and transverse through the...
lymphatic endothelium. For example, gradients of CCR7 and CCL21 allow immune cells to chemoattract toward lymphatic vessels, and integrins have been shown to participate in the transit of immune cells across lymphatic endothelium under inflammatory conditions. In support of these findings, several groups have shown similar chemokine gradients contribute to tumor cell migration and invasion toward lymphatics. Furthermore, Garmy-Susini et al identified integrin α4β1 as a marker of tumor lymphatic endothelium and the downstream signaling cascade it elicits promotes the adhesion and invasion of LECs. We find that adrenomedullin promotes the adhesion of tumor cells to the lymphatic endothelium because blockade of adrenomedullin signaling reduces the ability of tumor cells to adhere to the endothelium and establish functional heterocellular gap junction channels. It remains unclear whether the adhesion is directly mediated by integrins or perhaps through the gap junction protein, connexins that are expressed by both the tumor cells and the vasculature.

In addition to increased peritumoral lymphatics, enhanced lymphatic flow has also been reported to contribute to lymphatic metastasis. In a B16 melanoma tumor model, induction of LN lymphangiogenesis has been associated with a dramatic increase in lymph flow to the tumor-draining LN. We have previously shown that high levels of tumor-derived adrenomedullin induces LN lymphangiogenesis, and literature suggests that the resultant enhanced flow could serve to promote initial tumor cell entry into the lymphatic vessels and then tumor cell transit into and through the draining LN. Intriguingly, disrupted lymph flow due altered gap junction function has been linked to mutations in GJC2 (encodes for the connexin 47 protein). Similarly, conditional knockout mice for the adrenomedullin receptor (Calclr) exhibit dysfunctional lymphatic flow and permeability. These results warrant further investigation to determine whether and how gap junctions facilitate lymph flow and importantly, how compromised lymph flow affects a pathological process like tumor metastasis.

The identification of mutations in the connexin gene family, which cause alterations in gap junction–mediated functions begs the question of whether this gene family can be exploited as a possible therapeutic target. The ability of the lymphatic vasculature to form heterocellular gap junctions with tumor cells to facilitate TEM has potentially important implications because of the phenomenon known as the bystander effect. The concept that a signal or molecule is transferred among neighboring cells to elicit a biological response has been used, for example, as a treatment strategy for eradication of brain tumors. The commonly used approach involves retroviral-mediated transfer of the herpes simplex virus thymidine kinase that sensitizes the transduced tumor or endothelial cells to the antiviral ganciclovir to cause cell death. In a 9L brain tumor model using this strategy, researchers discovered a decrease in tumor vasculature and tumor regression. Intriguingly, evidence suggests that gap junction–mediated intercellular

Figure 6. β-catenin nuclear translocation is promoted by adrenomedullin (AM) and is dependent on gap junction intercellular communication (GJIC). A, Treatment of lymphatic endothelial cells (LECs) with 10 nmol/L human AM caused an alignment of β-catenin at the membrane along with its nuclear translocation. These changes were prevented by pretreatment with AM inhibitor AM22-52 (1 μmol/L). The gap junction inhibitor CBX (100 μmol/L) prevented β-catenin nuclear translocation indicating that GJIC is necessary for its translocation. Magnification: ×40. Scale bar, 50 μm. B, Quantification showing the percentage of cells with β-catenin nuclear translocation relative to the total number of cells in the field. Eighty to 120 cells were analyzed for each treatment from 3 independent experiments and expressed as mean±SE. C, RNA isolated from LECs treated with vehicle or 10 nmol/L human AM (4 hours) was analyzed by quantitative polymerase chain reaction for expression of C-MYC. D, The model depicts how high tumor-derived AM promotes increased tumor cell adhesion to LECs, connixin-mediated GJIC, β-catenin nuclear translocation, and subsequent gene transcription to facilitate transendothelial migration.
communication is one mechanism that contributes to bystander cytotoxicity. In fact, some have proposed to target connexins for gene therapy because their expression, localization at the plasma membrane, and assembly into functional gap junctions would improve the efficacy of bystander effect. Our results clearly demonstrate that adrenomedullin signaling promotes the organization of connexins into functional gap junctions to promote intercellular communication and subsequent TEM through a lymphatic monolayer. The development of therapies that rely on functional coupling of cells and subsequent transfer of molecules among adjacent cells could provide a novel therapeutic strategy to disrupting or reducing the ability of tumor cells to escape through the lymphatics.

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Disclosures

None.

References


Lympahetic Intercellular Coupling in Metastasis


### Significance

The lymphatic vascular system contributes to the distant spread of tumor cells, but the molecular mechanism of how this occurs is unclear. We provide evidence that the lymphangiogenic peptide adrenomedullin found to be elevated in many tumors, promotes the adhesion and subsequent connexin-mediated gap junction intercellular communication between tumor cells and lymphatic endothelial cells. This coupling or ability to communicate promotes transendothelial migration, and we identify beta-catenin nuclear translocation as a mediator of this process. This study provides novel insight into how the lymphatic endothelium communicates with tumor cells through gap junction channels to facilitate escape through the lymphatic vasculature.
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Methods and Materials

Cell Culture. Murine Lewis Lung Carcinoma cells (CRL-1642, American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM (30-2002, ATCC) supplemented with 1X penicillin/streptomycin and 10% fetal bovine serum. Human MCF-7 and SK-MEL-2 tumor cells were cultured in MEM alpha media supplemented with 1X penicillin/streptomycin and 10% fetal bovine serum. Generation of genetically dosed adrenomedullin (AM) LLC cells (Adm OExp, Adm RNAi) were described previously by our laboratory¹. Briefly, the complete coding sequence of murine Adm cDNA was cloned into the pcDNA3.1 expression vector and proprietary shRNA sequences (Dharmacon; Thermo Fisher Scientific, Lafayette, CO, USA) spanning the length of Adm were subcloned into the pSUPER vector to generate stable overexpression or knockdown cell lines, respectively. Human dermal lymphatic endothelial cells (hLEC) (CC-2810, Lonza, Basel, Switzerland) were cultured in EGM2-MV media (CC-3202, Lonza). Murine primary dermal lymphatic endothelial cells (mLEC) (C57-6064L, Cell Biologics, Chicago, IL, USA) were cultured in mouse endothelial cell media (M1168, Cell Biologics). The LEC identity of the cultures was confirmed by Prox1 staining. All cells were incubated at 37°C containing 5% CO₂.

Adhesion Assay. Effect of AM on adhesion was assessed both genetically and pharmacologically. Murine LLC and human MCF-7 cells (5x10⁵) were labeled with 5µM Cell Tracker Green (CTG) CMFDA according to manufacturer’s instructions (C2925, Life Technologies, Grand Island, NY, USA) and added to a confluent monolayer of mLECs or hLECs plated in a 96 well plate. CTG- labeled AM-dosed tumor cells were added atop the mLECs and incubated for 15 minutes. The supernatant was then aspirated to remove non-adhered cells and fluorescence for attached CTG-labeled LLC cells was detected using a Berthold Technologies Mithras (Bad Wildbad, Germany) LB940 plate reader. For the pharmacological experiments, we added CTG- labeled non-modified murine LLC cells to a mLEC monolayer and CTG-labeled human MCF-7 cells to a hLEC monolayer pretreated for 30 minutes with the AM inhibitor AM22-52 (1µM) (22-1-16A, American Peptide Company, Sunnyvale, CA, USA) followed by addition of 1nM murine AM (mLEC) (010-31, Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) or 10nM human AM (hAM) (22-2-10A, American Peptide Company, Sunnyvale, CA, USA). Tissue culture grade water (46-000-CM, Mediatech, Inc., Manassas, VA, USA) was used for vehicle treatment. After 15 minute incubation for LLC cells and 2 hour incubation for MCF-7 cells, fluorescence was measured. Experiments were run in triplicate and performed 3 times. Statistical analysis was performed using the unpaired Student t test.

Transendothelial Migration (TEM) Assay. Effects of AM on TEM were assessed both genetically and pharmacologically. AM-dosed LLC cells (5x10⁵) were labeled with 5µM CTG according to manufacturer’s instructions and then 1x10⁵ cells in 200µl media were added to a confluent monolayer of mLECs plated on 8µm transwell inserts (354578, BD Biosciences, Bedford, MA, USA). Alternatively, the endothelial monolayers were pretreated for 30 minutes with 1µM AM22-52 inhibitor or 100µM carbenoxolone (CBX; C4790, Sigma, Saint Louis, MO) followed by addition of 1nM mAM or 10nM hAM and CTG-labeled LLC, MCF-7, or SK-MEL-2 cells (5x10⁵). Tissue culture grade water was used for vehicle treatment. After incubation with
labeled cells (LLC cells, 6hrs; MCF-7 cells, 24hrs; SK-MEL-2 cells, 3hrs), inserts were fixed with 4% PFA, cells on the top surface of the filter were scraped off with a cotton swab, and the filter was mounted for analysis. Quantification of transmigrated cells was done by measuring the threshold of CTG-labeled tumor cell fluorescence using Image J (NIH). Statistical analysis was performed using the unpaired Student t test.

**Scrape-Loading Dye Transfer.** A confluent monolayer of hLECs grown on glass coverslips was pretreated with the AM inhibitor AM22-52 (1µM) or CBX (100µM) for 30 minutes followed by a 10 minute treatment with 10nM hAM. Tissue culture grade water was used for vehicle treatment. The gap junction permeable fluorescent dye Lucifer Yellow (0.05% final; L0259, Sigma) was added to the cells and the monolayer was scraped with a 27 gauge needle. After 10 minutes, cells were rinsed with PBS and then fixed with 4% PFA for 20 minutes. Slides were mounted and Lucifer Yellow positive cells were counted and expressed as a percentage of total cells in the field. Experiments were run in triplicate and performed at least three times. Statistical analysis was performed using the unpaired Student t test.

**Cell dye transfer assay.** Adherent LLC cells were loaded with 5µM calcein AM (C-3099, Molecular Probes, Eugene, OR) for 20 minutes at 37°C. Meanwhile, mLECs plated on glass coverslips were stained with 5µM Dil (V-22885, Molecular Probes, Eugene, OR) for 20 minutes at 37°C. mLECs were rinsed with PBS and treated with 1nM mAM and 100µM CBX for 30 minutes. Tissue culture grade water was used for vehicle treatment. Calcein AM loaded LLCs (3x10^3) were added to the mLEC monolayer and fixed with 4% PFA after a 3 hour co-culture. The number of tumor cells that transferred calcein AM dye to an adjacent endothelial cell was scored and expressed as a percent of dye coupled cells. Statistical analysis was performed using the unpaired Student t test.

**Immunohistochemistry and Immunofluorescence.** Antibodies used include rabbit anti-Cx43 (C6219, Sigma, Saint Louis, MO), rabbit anti-Cx37 (ab101928, Cambridge, MA), rabbit anti-Cx47 (36-4700, Invitrogen, Camarillo, CA), rabbit anti-LYVE1 (70R-LR005, Fitzgerald, Acton, MA), and mouse anti-β-catenin (610153, BD Biosciences, San Jose, CA). For IF, hLECs were grown on glass coverslips and pretreated for 30 minutes with 1µM AM22-52 or 100µM CBX followed by 10nM hAM for 15 minutes. Cells were fixed with 1% PFA, permeabilized with 0.01% Triton X-100 in 2% normal donkey serum (NDS), and incubated overnight with anti-Cx43 (1:200) or anti-β-catenin (1:50) followed by Cy3 secondary antibodies (1:500, Jackson ImmunoResearch Laboratories, West Grove, PA). Tumors generated for IHC analysis were described previously by our laboratory\(^1\). Briefly, 1x10^6 (in 100µl PBS) AM-dosed LLC cells were injected subcutaneously into female C57BL/6 mice and 14 days post-injection mice were euthanized and tumors were harvested. Following routine histological processing, the AM dosed tumor cryo-sections were permeabilized with Triton X-100, blocked with 5% NDS, incubated overnight with anti-Cx43 (1:100) and anti-LYVE1 (1:800) followed by Alexa Fluor 488 or 549 secondary antibodies (1:100, Molecular Probes) and DAPI (1:2000). Images were acquired on a Nikon E800 microscope (Nikon, Tokyo, Japan) with a Hamamatsu camera (Hamamatsu Photonics, Hamamatsu, Japan) with Metamorph software (Molecular Devices,
Inc., Sunnyvale, CA, USA). Color images for the Cx43 and β-catenin IF were converted to black and white and then inverted to better represent staining.

**Western Blot analysis.** After 24 hour treatment of hLECs with 10nM AM or vehicle (TC-grade water), cell extracts were obtained and protein concentrations were determined by bicinchoninic acid assay (23225, Thermo Scientific, Rockford, IL). Equal concentrations of cell lysates were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with mouse anti-Cx43 primary antibody (13-8300, Invitrogen, Camarillo, CA), rabbit anti-Cx37 (ab101928, Cambridge, MA), or rabbit anti-Cx47 (36-4700, Invitrogen, Camarillo, CA), and visualized using the Odyssey imaging scanner (LI-COR, Biosciences, Lincoln, NE). GAPDH (NB300-285, Novus Biologicals, Littleton, CO) was used as a control for equal protein loading. Quantitation of connexin protein expression relative to GAPDH was performed using integrated density analysis (ImageJ). Statistical analysis was performed using the unpaired Student t test.

**Gene expression analysis.** Total RNA was isolated from LECs using Trizol reagent (15596026, Sigma) followed by RQ1 DNase treatment (M610A, Promega, Madison, WI) and cDNA synthesis (28025-013, Invitrogen). qRT-PCR for human C-MYC and GJA1, GJA4, and GJC2 were performed with Assay on Demand (Applied Biosystems, Carlsbad, CA). Graphs are representative of runs performed in triplicate and normalized to GAPDH. Statistical analysis was performed using the unpaired Student t test.

**References**