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REVIEW

Adrenomedullin in lymphangiogenesis: from development to disease

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Abstract Over the past decade, we have begun to appreciate that the lymphatic vascular system does more than simply return plasma back into the circulatory system and, in fact, contributes to a wide variety of normal and disease states. For this reason, much research has been devoted to understanding how lymphatic vessels form and function, with a particular interest in which molecules contribute to lymphatic vessel growth and maintenance. In the following review, we focus on a potent lymphangiogenic factor, adrenomedullin, and its known roles in lymphangiogenesis, lymphatic function, and human lymphatic disease. As one of the first, pharmacologically tractable G protein-coupled receptor pathways characterized in lymphatic endothelial cells, the continued study of adrenomedullin effects on the lymphatic system may open new avenues for the modulation of lymphatic growth and function in a variety of lymphatic-related diseases that currently have few treatments.

Keywords Lymphedema · Calcitonin receptor-like receptor (CLR = protein; *Calcrl* = gene) · Receptor activity modifying protein (RAMP) · CXCR7

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Introduction

The lymphatic system is a vascular network in parallel with blood vessels that penetrates every tissue in the body, with the exception of bone marrow and the central nervous system [1]. Over the past dozen years, our understanding of how the lymphatic system develops, functions, and contributes to disease has markedly improved [2]. It has become apparent that this complex system is more than just a simple conduit for returning interstitial fluid to blood. The lymphatic system not only participates in maintaining fluid homeostasis, but also mediates fat absorption and provides a highway for immune cell trafficking to distant sites. In fact, the lymphatic system has now been recognized for its contribution to a wide variety of normal and pathophysiological states [3, 4].

Lymphedema, the most common and poorly treated lymphatic disease, affects 140–250 million people worldwide. Characterized by debilitating swelling of one or more limbs, lymphedema is a lifelong condition that can lead to inflammation, fibrosis, infection, subcutaneous fat accumulation, and decreased mobility and function [1, 5]. Despite its prevalence and morbidity, no pharmacological agents exist for the management of lymphedema.

A multitude of studies in genetic mouse models have also implicated lymphatics in other common diseases. For example, disruption of neolymphangiogenesis in the skin induces salt-sensitive hypertension in mice [6]. This model demonstrates that dermal lymphangiogenesis is required to maintain electrolyte balance and subsequently blood pressure homeostasis [6]. Further, haploinsufficiency of the essential transcription factor *Prox1* leads to adult onset obesity due to abnormal fluid leakage from disrupted lymphatic vessels [7]. Examples like these suggest that novel therapeutics designed to modulate the lymphatic

vasculature could offer treatment for prevalent diseases like lymphedema, essential hypertension, and obesity.

To this end, a great deal of effort has recently been devoted to identifying factors that participate in lymphangiogenesis and control lymphatic vascular function in adults. The following review will focus on one such factor, adrenomedullin (AM = peptide; *Adm* = gene), and will address what is currently known about its role in the lymphatic vascular system, and its potential as a novel therapeutic for the treatment of diseases linked to lymphatic dysfunction.

The adrenomedullin peptide family and its receptors

AM, a 52-amino acid peptide hormone, is classified as a member of the calcitonin gene-related peptide (CGRP) family due to its shared secondary structure and overlapping biological activity with other peptide family members [8, 9]. The four peptides of this family besides AM include amylin, calcitonin, intermedin, and CGRP. Each of these peptides has essential roles in normal physiology [10]. Similarities in their secondary structure allow for overlap in the pharmacology of the binding sites of the CGRP family and, consequently, cross-reactivity between their receptors [8, 11]. Therefore, discerning which biological activities are distinct to AM proved to be challenging until the discovery of a class of single-pass transmembrane proteins called receptor activity modifying proteins (RAMPs) [12]. These proteins bind to and confer ligand specificity for G protein-coupled receptors (GPCRs) and allow cells to distinguish between members of the CGRP family.

When bound to a GPCR, RAMPs dictate ligand specificity, downstream signaling, and receptor recycling [9, 12, 13]. The three identified RAMPs, RAMP1, -2, and -3 share 30 % sequence identity, with their nonhomologous domains modulating specificity [8]. For example, when calcitonin receptor-like receptor (CLR) associates with RAMP2 or RAMP3, the receptor binds AM with high affinity. When complexed with RAMP1, on the other hand, CLR performs as a high affinity CGRP receptor. Thus, specific tissue and temporal expression of the RAMPs determine whether a cell will respond to AM or CGRP [13]. As will be discussed below, recognition of the role of RAMPs in the responsiveness of AM allowed for the design of gene-targeted knockout mouse models that helped determine the physiological role of AM. The participation of RAMP2 with CLR was found to be absolutely necessary for AM response during development, as genetic deletion of *Ramp2* phenocopied *Adm* knockout models [14]. This unusual signaling offers a unique opportunity for specific targeting without interrupting the activity of other, closely related ligand–receptor pathways.

Prior to the discovery of RAMPs, there were two additional putative AM receptors reported in the literature [15]. In 1995, Kapas and Clark suggested that two orphan GPCRs, RDC-1 and L1, served as AM receptors [16, 17]. An inability to reproduce these findings led to significant controversy regarding their ability to bind AM. While L1, now known as GPR182, remains an orphan receptor, RDC-1 has been identified as an atypical chemokine receptor, a promiscuous receptor that binds several ligands including SDF-1/CXCL12 [18], CXCL11 [19], and intermediate opioid peptide [20]. RDC-1 has since been renamed CXCR7 or atypical chemokine receptor 3 (ACKR3). The role of CXCR7 in AM signaling continues to evolve. Mounting evidence in the literature over the past decade suggests that RDC-1/CXCR7/ACKR3 does indeed associate with adrenomedullin [15, 21–24]. Although the discovery of RAMP-guided CLR responsiveness to AM overshadowed the role of these other receptors in AM biology, as will be discussed later in this review, recent studies demonstrate that AM-mediated downstream signal is indeed modulated through CXCR7 [25, 26]. The identification of a second AM receptor is especially exciting, as it offers another avenue for drug discovery for the treatment of AM-mediated pathologies.

AM has been shown to be involved in a wide variety of human diseases, including sepsis, myocardial infarction, and preeclampsia [13, 27, 28]. In particular, as a potent vasodilator, the AM signaling system has garnered interest as a potential biomarker and therapeutic target for cardiovascular diseases [29, 30]. Preliminary studies suggest that, when used as an adjunct therapy, intravenous AM can improve cardiovascular outcomes such as wall motion and infarct size [31]. Modulation of the AM signaling system, therefore, could prove to be a viable and safe treatment option not only for cardiovascular diseases, but also for other diseases in which AM plays a role. In particular, genetic models have uncovered an additional role for AM in the development and regulation of the lymphatic vascular system.

Adrenomedullin during lymphatic vascular development

Initial lymphatic vessel formation begins between e9.5 and 10.5 with the commitment of endothelial cells of the cardinal vein (CV) to a lymphatic fate (Fig. 1a) [1]. After polarization of lymphatic progenitor cells in the jugular vein at e11.5–12.5, lymphatic endothelial cells (LECs) begin to migrate away and form a premature lymphatic vessel. Historically, this vessel has been referred to as the lymph sac. Recently, however, ultramicroscopy of whole mount mouse embryos has revealed that the lymph sac is

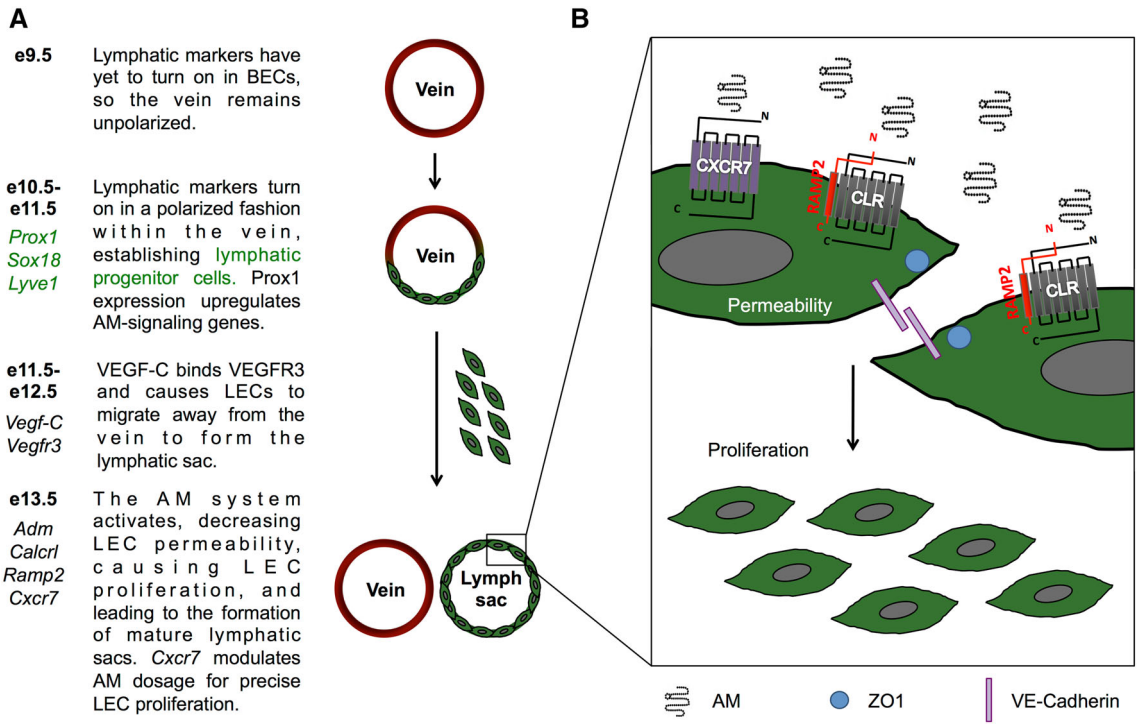

Fig. 1 Early development of the lymphatic system and the effects of AM on LECs. **a** Development of the lymphatic system begins between e10.5 and 11.5 when the master regulators of lymphatic fate, Prox1 and Sox18, turn on. By e13.5, the lymphatic sac is fully separated from the jugular vein and activation of the AM system allows LEC proliferation. **b** Additionally, AM acts to stabilize the lymphatic endothelial barrier by reorganizing the tight junction protein, ZO-1, and the adherens junction protein, VE-cadherin, thereby decreasing lymphatic vessel permeability. *AM* adrenomedullin, *BEC* blood endothelial cell, *LEC* lymphatic endothelial cell

comprised of two separate lymphatic structures, a peripheral longitudinal lymphatic vessel and a primordial thoracic duct [32]. These structures are formed by migrating LECs that originate from both the CV and additional venous sources [32]. Failure of these ECs to establish LEC fate, migrate, or proliferate results in severe edema and embryonic lethality. For example, genetic deletion of *Prox1* prevents EC differentiation to lymphatic fate. *Prox1*-null animals are devoid of lymphatic vasculature and die by e14.5 [33–35]. Similarly, though ECs of VEGF-C-null mice can establish LEC fate, LECs fail to migrate away from the CV, lymphangiogenesis is arrested and embryonic edema ensues, demonstrating that VEGF-C is an essential lymphangiogenic factor [36–38]. The severity and timing of the edema and subsequent embryonic lethality in these mouse models provided an essential clue for determining the role of AM in lymphangiogenesis.

In the early 2000s, an elegant series of gene-targeted knockout mouse models identified AM as an essential factor for proper lymphatic vascular development [13, 14, 39]. Several labs observed that mice globally lacking *Adm* exhibited profound edema, known as hydrops fetalis [40–42]. Similarly, knockouts of *Calcrl,* the gene for CLR, [39], or *Ramp2* [14, 43, 44], which make up the canonical receptor for AM, have the same lethal phenotype. Furthermore, *peptidylglycine alpha-amidating monooxygenase* (PAM) knockout mice [45], an enzyme required for the amidation and function of AM, are also embryonic lethal due to extreme edema [27]. These models clearly demonstrate that AM is essential for life; however, the root cause of this edema was not understood.

Consistent with what was known about AM at the time and with the nascent lymphatic vascular field, early studies of these mouse models focused on AM in cardiac and blood vascular development rather than lymphangiogenesis. Indeed, AM was found to have a significant proliferative effect during cardiac development. $Adm^{-/-}$, $Calcrl^{-/-}$, and $Ramp2^{-/-}$ mice share a phenotype of small, hypoplastic hearts [14, 39, 40]. All three genes were also found to be highly expressed in the heart and the vascular endothelium, confounding whether the observed edema could be attributed to cardiac defects or vascular dysfunction. To solve this problem, Fritz-Six et al. utilized mice expressing an endothelial cell-specific Cre recombinase via expression of a *Tie2-Cre* transgene [46]. Though *Tie2-Cre* expression has only been observed in restricted regions of adult lymphatic vessels, *Tie2-Cre* is expressed in the venous progenitors of lymphatic endothelial cells. Thus, prior to the development of lymphatic-specific *Cre* drivers, *Tie2-Cre* mice were widely used to excise genes

from venous lymphatic progenitors during embryonic lymphangiogenesis [47–51].

Using this *Tie2-Cre* model, Fritz-Six et al. [14] found that specific deletion of *Calcrl* in the venous lymphatic progenitors results in extreme hydrops fetalis, suggesting that AM-mediated effects in endothelium, not the heart, were responsible for the edema. Because *Tie2-Cre* is expressed in progenitors that lead to both blood and lymphatic endothelial lineages [27], this model could not definitively distinguish whether blood or lymphatic vessel dysfunction led to the hydrops fetalis. However, as mentioned above, the onset of edema in the AM signaling-disrupted mice between e12.5 and e14.5 coincides with the separation of the lymphatic sac from the jugular vein and the beginning of lymphatic flow, suggesting that the edema could be lymphatic in origin.

Fritz-Six et al. [14] surveyed the AM system effects on developmental lymphangiogenesis by deleting *Adm*, *Calcrl*, and *Ramp2*. Each gene-targeted knockout model exhibited the same edematous phenotype, while no problems with blood vascular leakage were observed [14]. Quantitative PCR of both lymphatic and blood endothelial cells further revealed that *CALCRL* and *RAMP2* were enriched in the lymphatic endothelium compared to the blood endothelium, a finding that has since been confirmed by other groups [14, 52]. Additionally, transfection of cultured LECs with a *PROX1* expression plasmid led to a threefold increase in endogenous *CALCRL* expression, demonstrating that the genes required for AM signaling are inducible by this master lymphatic fate regulator [14]. Later studies have also shown that AM treatment of LECs causes an upregulation of *PROX1* [53], demonstrating that not only are the genes required for AM signaling upregulated in LECs leading to preferential AM action, but that AM activity in LECs feeds back positively on *PROX1* expression. Consistent with this notion, Fritz-Six et al. [14] found that prior to embryonic lethality, the lymphatic sacs of AM signaling-null mice were significantly smaller when compared to those of wild-type littermates. Careful study of proliferation in LECs revealed that loss of AM signaling results in hypoplasia of the lymphatic sacs. These hypoplastic lymphatic sacs failed to collect extravasated fluid and resulted in aberrant accumulation of interstitial fluid in mutant embryos. This finding was consistently observed in $Adm^{-/-}$, $Calcrl^{-/-}$, and $Ramp2^{-/-}$ embryos.

Furthermore, the lymphatic vessels of AM signaling-null mice are hyperpermeable, thus perpetuating and contributing to edema formation. Previous work has shown that VEGF-A treatment of cultured blood endothelial cells (BECs) and LECs increases permeability to trypan blue-labeled albumin [54, 55]. However, co-treatment with AM and VEGF-A dose dependently prevents VEGF-A-induced permeability [54]. This reduction of permeability is caused by a reorganization of the cell–cell junctions. VEGF-A treatment of LECs disrupts the junctional proteins Zonulus Occludin-1 (ZO-1) and vascular endothelial cadherin (VE-Cadherin), thereby creating characteristic gaps and a zipper-like staining pattern [54]. AM treatment stabilizes ZO-1 and VE-cadherin (Fig. 1b) and abrogates VEGF-A-induced disruption, decreasing lymphatic permeability [54]. Fluorescent microlymphography of adult mice confirms this finding. AM-treated mice have significantly decreased uptake of FITC-dextran when compared to control mice, demonstrating that AM stabilizes the lymphatic endothelial barrier in vivo [54]. Therefore, it follows that mice lacking *Adm, Calcrl,* or *Ramp2* may also have increased lymphatic permeability that exacerbates the edema. Taken together, the evidence strongly suggests that in the absence of AM signaling, edema is caused by aberrant lymph sac formation, failed lymphangiogenesis, and abnormally permeable lymphatic vessels.

Ichikawa-Shindo and colleagues observed similar edematous phenotypes in *Ramp2* knockout mice [43]. However, while Fritz-Six et al. concluded the edema was predominantly lymphatic in origin, Ichikawa-Shindo et al. reported that *Ramp2*-deficient mice also exhibit significant vascular endothelial cell dysfunction and increased blood vascular permeability due to decreased expression of VE-Cadherin, claudin 5, and type-IV collagen [43, 56]. Disruption of these proteins, which make up tight and adherens junctions and the basement membrane of endothelial cells, results in increased pericellular leakage and edema [43]. While these studies did not examine lymphatic vascular development or function, the effects of loss of RAMP2 on the blood endothelium are consistent with the known role of AM to modulate the development and regulate the function of the blood vasculature [41, 57, 58]. Thus, leaky blood vessels coupled with arrested lymphangiogenesis and poor lymphatic uptake of interstitial fluid likely account for the severity of the edematous phenotype.

Moreover, the lymphatic and blood endothelium are interdependent. Lymphatic vessels develop directly from the venous vasculature and then parallel the blood vascular system throughout the body. These two vascular systems may only physically connect on either side of the body near the jugulo-subclavian junction where lymph is returned to the blood; however, these systems are developmentally, genetically, and molecularly connected in many complex ways [56]. The lymphatic vasculature has effects on the blood vasculature throughout development and beyond, and vice versa. Therefore, although aberrant lymphangiogenesis is the primary cause of AM-associated edema, the effects of AM on the blood vasculature should not be disregarded.

Likewise, it has become clear that spatial and temporal expression of receptors and their ligands in a microenvironment can affect development of surrounding tissue [59,

60]. Because of the close proximity of and complex connections between the lymphatic and blood vasculatures, it is possible that expression of *Adm*, *Calcrl*, and *Ramp2* by both BECs and LECs has effects not only in their own microenvironments, but also in the adjacent vascular tissue. Recent studies from our lab have substantiated this hypothesis, definitively identifying CXCR7 as a potent modulator of AM signaling and AM-mediated lymphangiogenesis [25].

As discussed in the previous section of this review, whether CXCR7, a known non-signaling decoy receptor, acts as an AM receptor has remained unclear for over a decade. The promiscuity of the decoy receptor has also made it challenging to discern which ligand is responsible for a given phenotype. This difficulty was particularly apparent in *Cxcr7*-null mice, which exhibited cardiac enlargement and hyperplasia, a phenotype that was not observed in mutant mice of the canonical ligands, SDF-1 and CXCL11 [21, 61, 62]. However, the cardiac phenotypes closely phenocopied a genetic model of AM overexpression (known as $Adm^{hi/hi}$ mice) which results in gross cardiac hyperplasia during embryogenesis [25, 63]. The similarity in cardiac phenotypes and the historical association of AM and CXCR7 sparked curiosity about whether CXCR7 could behave as a decoy receptor for AM. In this way, loss of the AM decoy receptor would result in a surplus of AM, excessive AM-mediated signal, and a phenocopy of the $Adm^{hi/hi}$ mice. Our lab hypothesized that if CXCR7 does behave as an AM decoy receptor, loss of CXCR7 would also lead to lymphatic vascular defects. Because loss of AM signaling results in small, hypoplastic hearts and lymphatic sacs [14, 39, 40], and AM overexpression results in enlarged, hyperplastic hearts, we predicted that loss of *Cxcr7* would result in enlarged, hyperplastic lymphatic vessels.

Indeed, consistent with the notion that CXCR7 sequesters AM, $Cxcr7^{-/-}$ mice exhibited enlarged, blood-filled lymphatic sacs. Careful examination of the number of proliferating LECs in the lymphatic sac demonstrated that the enlarged lymphatic sacs were hyperplastic (Fig. 2a). These data suggest that, in the absence of the decoy receptor, an excess of AM leads to hyperproliferation of LECs and subsequently enlarged, poorly formed lymphatic sacs [25]. Further examination of LECs in vitro and lymphatic beds in vivo confirmed the ability of CXCR7 to affect the lymphatic endothelium. When treated with AM, cultured *CXCR7* knockdown LECs proliferated more than wild-type LECs [25]. Moreover, dermal and cardiac lymphatic beds were disrupted in *Cxcr7*-null mice and displayed dysmorphic vasculature consistent with hyperplasia [25].

To definitively show that these phenotypes were caused by excessive AM signaling, we genetically titrated AM ligand onto $Cxcr7^{-/-}$ mice. Both genetic increase and reduction of AM peptide on the *Cxcr7* null background resulted in striking effects on the lymphatic phenotypes. Genetic reduction of *Adm* in $Cxcr7^{-/-}$ mice restored the lymphatic and cardiac hyperplasia to wild-type levels, indicating that the hyperplasia observed in $Cxcr7^{-/-}$ mice resulted from excessive AM signaling [25]. Moreover, intercross of *Cxcr7* mutant mice with $Adm^{hi/hi}$ mice resulted in an exacerbation of the lymphatic enlargement and increased embryonic lethality to the point that it was difficult to maintain a $Cxcr7^{+/-}$; $Adm^{hi/+}$ mouse colony due to embryonic and early post-natal loss [25]. Taken together, these data demonstrate that the dosage of AM is critical for maintaining proper cardiovascular development and that, without tight control of AM-mediated signaling, proliferation of both myocardial and lymphatic endothelial cells is disrupted (Fig. 2a).

Interestingly, while the lymphatic endothelium dynamically expresses *Cxcr7,* we observed that the blood endothelium directly adjacent to the lymphatic endothelium persistently expressed *Cxcr7. Cxcr7* was infrequently observed in the dermal lymphatics where $Cxcr7^{-/-}$ mice exhibited phenotypes consistent with dermal lymphatic hyperplasia but was regularly observed in the dermal blood vessels [25]. Consistent with previously published papers, therefore, we concluded that the presence of the scavenging receptor in the adjacent blood endothelium impacts development of the surrounding tissue [25]. These findings again highlight the importance that expression of the ligand and its receptors in *proximity* of (but not necessarily directly within) the lymphatic endothelium has the ability to impact lymphangiogenesis. Taken together, these data demonstrate that *Cxcr7* modulates AM signal and identifies a new paradigm of 7-transmembrane decoy receptors as regulators of lymphatic vascular development.

AM signaling during adulthood

Mid-gestational embryonic lethality due to global knockout of the AM signaling system has made elucidation of the signaling effects of AM on the lymphatic system in adulthood difficult. The recent design of an inducible knockout model, however, has confirmed an important role for AM in lymphatic maintenance and function following normal lymphangiogenesis. Global reduction of *Calcrl* in $Calcrl^{fl/fl}$ animals using a ubiquitously expressed, tamoxifen-inducible Cre transgenic line ($CAGGCre\text{-}ER^{TM}$) resulted in acute and chronic lymphatic dysfunction, leading to ocular inflammation, disrupted fat absorption, and delayed wound healing due to persistent edema [64]. Seven to 10 days following tamoxifen induction of Cre, two thirds of $Calcrl^{fl/fl}CAGGCre\text{-}ER^{TM}$ mice developed overt ocular opaqueness [64]. After ruling out glaucoma,

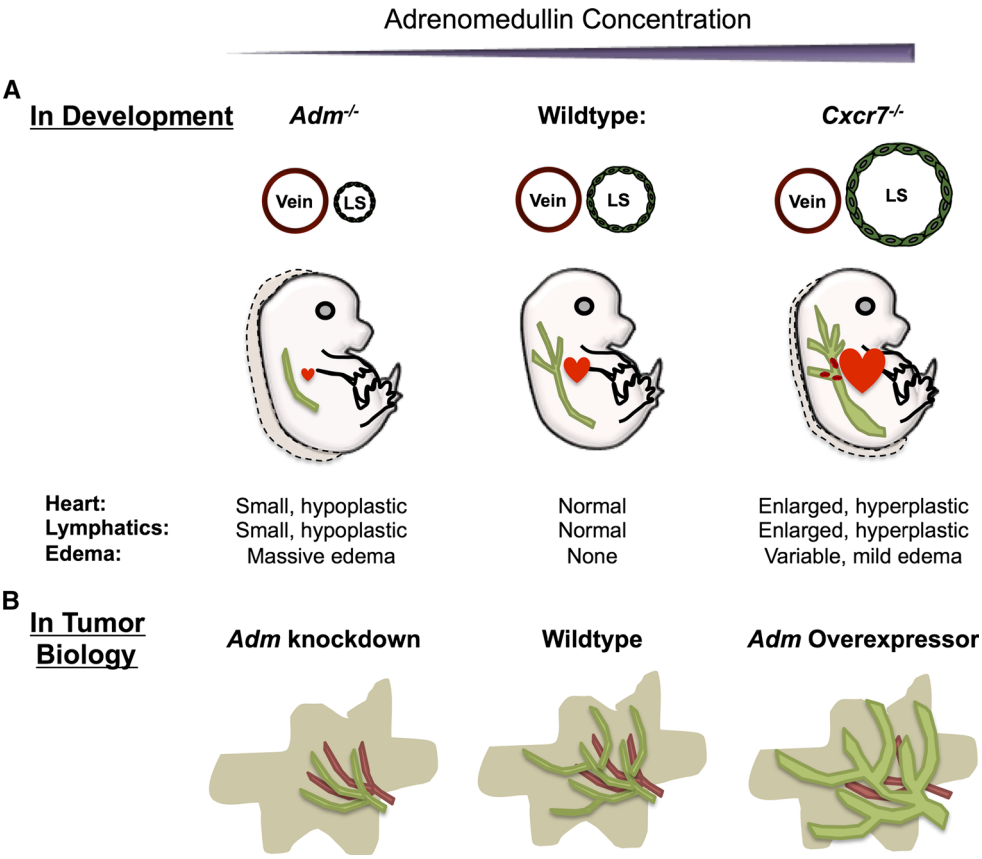

Fig. 2 Proper AM dosage is required for normal lymphangiogenesis during development and in tumor biology. **a** Precise AM concentration is required during development to regulate LEC proliferation. Absent AM signaling results in small, hypoplastic lymphatic sacs. Conversely, loss of the decoy receptor, CXCR7, results in excessive AM signaling and enlarged, hyperplasic lymphatic sacs. **b** Similarly, overexpression of *Adm* in tumor models results in enhanced proliferation of LECs, dilated lymphatics, and increased tumor metastasis

Hoopes et al. reported that *Calcrl*-deficient mice exhibit corneal edema, inflammation, and dilated corneoscleral lymphatic vessels.

While healthy cornea is largely avascular, lymphangiogenesis occurs in response to inflammation and can ultimately lead to blindness [65]. Though no corneal lymphangiogenesis was reported in *Calcrl*$^{fl/fl}$*CAGGCre-ER*^{TM} mice, these mice were only evaluated after one week, which may not allow enough time for neolymphangiogenesis. Given more time, the inflammation in *Calcrl*$^{fl/fl}$*CAGGCre-ER*^{TM} may lead to corneal lymphangiogenesis. It is also possible that the absence of the AM receptor would prevent new lymphatic vessels from forming. Regardless, these findings highlight the importance of proper lymphatic function in maintenance of fluid homeostasis in and around the eye. Indeed, recent studies have demonstrated that the endothelial cells lining Schlemm's canal express several lymphatic marker genes, are responsive to VEGF-C and thereby control the aqueous humor with the anterior chamber of the eye. [66–68]. Thus, modulation of these vessels may allow for either increased hydration or drainage of the eye, allowing for a novel treatment of dry eyes, ocular edema, and glaucoma.

In addition to the ophthalmic pathology, *Calcrl*$^{fl/fl}$-*CAGGCre-ER*^{TM} mice also failed to match weight gain of their wild-type littermates. Examination of the mesenteric vessels of experimental mice showed dilated lymphatic vessels filled with chyle when fed a high-fat meal. These data suggest that AM signaling is critical for absorption of fat from mesenteric lymphatic vessels. Close examination of the mesenteric lymphatic vessels following high-fat diet revealed disrupted lymphatic junctions, a finding that was consistent with previously published work demonstrating that AM stabilizes the lymphatic endothelial barrier and increases vascular permeability [54]. Indeed, further examination of the lymphatic endothelium revealed disrupted lymphatic permeability. Intradermal injection of Evans blue dye into the ear revealed rapid lymphatic uptake in both experimental and control mice. At five min, however, mice lacking *Calcrl* showed increased leakage and diffuse spreading of the dye [64]. Importantly, no difference in blood vascular permeability was observed, reinforcing that AM loss affects primarily the lymphatic vasculature [64].

To induce edema, complete Freund's adjuvant (CFA) was injected into the paw of the hind limb. In contrast to tamoxifen-injected controls, *Calcrl*$^{fl/fl}$*CAGGCre-ER*^{TM} mice had prolonged edema [64], suggesting that AM is required for proper reabsorption of interstitial fluid and resolution of edema. As discussed above, previous studies demonstrate that AM treatment stabilizes the lymphatic endothelial barrier (Fig. 1b) [54]. Therefore, similar to the mesenteric lymphatics, it follows that loss of *Calcrl*

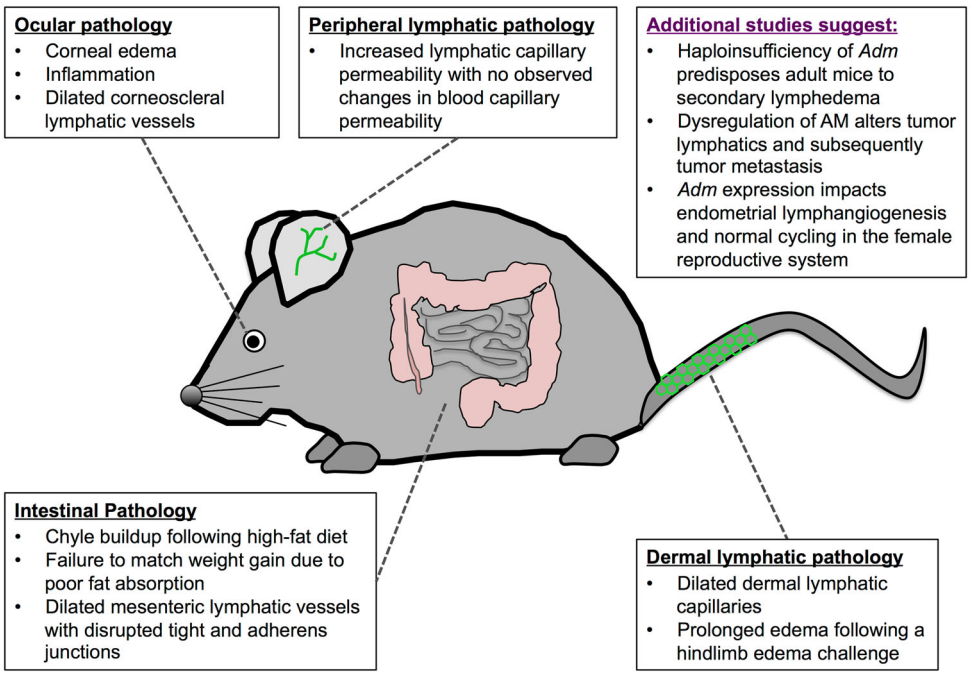

Fig. 3 Loss of AM signaling in adulthood results in disruption of many lymphatic beds. Here, we provide a summary of the findings when AM signaling is disrupted following embryonic development. Conditional knockdown of *Calcrl* in adult mice results in significant alterations in the ocular, intestinal, dermal, and peripheral lymphatic vessels. Additional studies that suggest AM is critical for lymphangiogenesis during adulthood are highlighted in *purple*

destabilizes the lymphatic endothelium in the peripheral lymphatics, increases vascular permeability, allows for lymphatic fluid leakage back into the interstitium, and prevents proper edema resolution.

These findings (summarized in Fig. 3) are particularly intriguing because they demonstrate that appropriate AM dosage following development is required for proper lymphatic maintenance and function. Loss of AM signaling results in leaky vessels, failure to resolve edema, and alterations in fat absorption. All of these data suggest that AM may be a reasonable target for the treatment of a variety of diseases for which we currently have limited therapies. Pharmaceuticals that modulate fat absorption could be a useful tool to help prevent or reduce the extent of obesity. Additionally, use of AM agonists could decrease lymphatic vessel leakiness and increase uptake of interstitial fluid, allowing for the partial or complete resolution of lymphatic-associated edema.

Adrenomedullin and lymphedema

As mentioned above, lymphedema is the most common lymphatic disease, affecting hundreds of millions of people worldwide. Lymphedema is categorized as primary or secondary. Primary lymphedema often results from congenital abnormalities. Mutations in nineteen genes have been associated with primary lymphedema, which have been extensively reviewed recently by Brouillard and colleagues [69]. Many of these mutations disrupt genes critical for downstream signaling in LECs, including mutations in the genes encoding *VEGFR3, VEGFC,* and *CCBE1* [69]. Additionally, disruption in several transcription factors required for LEC differentiation and specification (for example, *GATA2*, *FOXC2*, and *SOX18*) result in syndromic forms of primary lymphedema [1, 69]. Although no mutations in the AM signaling cascade have yet been identified in human primary lymphedema, as whole exome sequencing studies continue, it is possible that AM will be found to associate with primary lymphedema. Secondary lymphedema, on the other hand, stems from disruption of the normal lymphatic vasculature by infection or iatrogenic intervention [5]. Despite its prevalence and significant effect on quality of life, currently no cure exists for lymphedema. Additionally, it is not well understood why some individuals are predisposed to developing lymphedema.

Recently, AM was implicated as a molecule that might contribute to the onset of secondary lymphedema. *Adm* haploinsufficiency predisposed mice to developing secondary lymphedema following hind limb injury compared to wild-type mice [52]. Further, systemic injection of AM to $Adm^{+/-}$ mice restored proper wound healing, thereby preventing the onset of lymphedema [52]. Here, these data demonstrate the importance of AM dosage in the resolution of edema.

Though lymphedema has yet to be associated with mutations in the AM signaling system in humans, these studies suggest that AM administration has potential as a novel therapeutic for the treatment of secondary lymphedema. The primary cause of secondary lymphedema in western countries is radical axillary lymph node dissection, with 20–30 % of patients developing severe lymphedema

following surgery [70–72]. The most promising treatment is the generation of new lymphatic vessels [70]. Jin and colleagues demonstrate that AM administration increases lymphatic flow, promotes angiogenesis and lymphangiogenesis, and ultimately decreases tail lymphedema in vivo [73]. The cause of this accelerated healing is likely due to a combination of new blood and lymph vessel formation, as both angiogenesis and lymphangiogenesis are required for wound healing [73–75]. However, the preferential effect of AM on lymphatic endothelial cells indicates that the primary cause for increased healing is neolymphangiogenesis. Therefore, AM administration following surgical resection of lymph nodes may not only promote wound healing, but also prevent the onset of lymphedema. While there are no current studies using AM infusion to treat lymphedema in humans, the potential of AM to act as a novel therapy for lymphedema is promising.

Adrenomedullin in cancer

Originally isolated from pheochromocytoma, a rare neuroendocrine tumor, elevated AM has been associated with neoplasms [76, 77]. Many cancer subtypes overexpress *Adm* [15], and plasma AM levels often correlate with metastasis, invasion, and poor survival [78, 79]. How AM modulates cancer progression is not well understood. Many experts have speculated that AM contributes to tumor survival and progression by increasing blood flow to a hypoxic tumor environment [15, 76, 80]. However, the importance of lymphangiogenesis and lymphatic vessel remodeling to cancer biology [81, 82] has also delineated a role for AM-mediated lymphangiogenesis in promoting distant metastasis.

Using a Lewis lung carcinoma (LLC) cell line, Karpinich et al. [83] generated a series of tumor cells that stably over- or under-expressed *Adm* and demonstrated a regulatory role for AM in tumor lymphangiogenesis. The LLC model was particularly attractive because LLCs do not express the canonical AM receptor, *Calcrl* and *Ramp2*. Consequently, alterations in *Adm* expression did not affect tumor cell proliferation [83]. Therefore, following injection into mice, AM-mediated increases in tumor size would not confound differences in tumor metastasis.

Interestingly, alterations in tumor *Adm* expression robustly impacted tumor lymphatic vessels. While *Adm* overexpression had little effect on blood vessel density or BEC proliferation, *Adm* overexpression resulted in a threefold elevation of LECs in both tumors and sentinel lymph nodes compared to tumors with reduced *Adm* expression (Fig. 2b) [83]. Lymphatic vessels of *Adm*-overexpressing tumors were also significantly dilated. Careful evaluation of tumor metastasis revealed that *Adm* overexpression increased tumor dissemination [83]. These data recapitulate what has been observed in human tumors and suggest that *Adm* expression can promote metastasis via lymphangiogenesis.

Importantly, these findings extend beyond the LLC model. Studies in cervical cancer have also found associations between AM-mediated lymphangiogenesis and severity of disease. Huang et al. [84] report that loss in the tumor stromal endothelium of miR-126, a microRNA that maintains vessel integrity during development, results in significant upregulation of *Adm* and strongly associates with invasive carcinomas. Upregulation of AM peptide also coupled with increases in CD31-positive endothelium (5.3 % in tumors with high levels of miR-126 expression vs. 71 % in invasive tumors with low miR-126 expression), suggesting that AM may promote disease progression through angiogenesis [84]. However, because lymphatic and blood vessels both stain CD31-positive, it is unclear whether this increase in vessel density is due AM-mediated effects on the blood or lymphatic endothelium. Taken together with previous in vivo and in vitro studies that demonstrate that AM has preferential effects on the lymphatic endothelium, it is expected that much of the increase in vessel density is due to AM-mediated lymphangiogenesis. Consistently, the authors found significant co-localization of AM peptide with LYVE1-positive vessels [84].

These findings make a compelling argument for an association between increased *Adm* expression and tumor invasion. As such, modulation of *Adm* expression via AM inhibitors may prove to be an exciting chemotherapeutic target. VEGF inhibitors, which target tumor angiogenesis, are being utilized in the clinic at present and have been found to increase patient survival in certain cancer types [85, 86]. However, the survival increase is modest, often measured only in months rather than years [86]. The benefit of VEGF inhibitors could be strengthened by co-administration with AM inhibitors [87]. This hypothesis has already been tested in mouse models of prostate cancer, where anti-AM antibodies were found to disrupt tumor vasculature, decrease lymphatic vessel density, increase LEC death, and suppress tumor growth [88]. Though this anti-AM antibody has not been fully characterized or utilized in other studies, this finding supports the hypothesis that AM inhibition may improve cancer-related outcomes.

Adrenomedullin-induced lymphangiogenesis in the reproductive system

Perhaps the most exciting and novel aspect of AM-mediated lymphangiogenesis is its involvement in the lymphatic vasculature of the reproductive system. Our lab and others

have shown that AM is critical in healthy pregnancy [9]. In a normal human pregnancy, AM increases three- to fivefold [89]. Dysregulation of this physiologic increase in both humans and mouse models has been associated with significant complications, most notably preeclampsia [90–92]. Additionally, haploinsufficiency of *Adm* in female mice results in reduced fertility due to implantation defects [93]. The essential role of AM in the normal reproductive system has, therefore, become increasingly apparent and clinically relevant over the past two decades. As such, there has been a rapidly growing interest in the role of AM-mediated angiogenesis and lymphangiogenesis in the reproductive system.

Consistent with the finding that AM promotes tumor progression, several studies report increased lymphangiogenesis and metastasis in pregnant women with melanoma when compared with non-pregnant melanoma patients [94, 95]. The direct cause of the increased lymphangiogenesis remains unknown. However, it stands to reason that the significant increase in *Adm* during pregnancy may be responsible. The physiologically higher levels of AM during pregnancy may interact with the tumor endothelium, thereby promoting LEC proliferation and lymphangiogenesis. This hypothesis differs from previous reports suggesting that AM originates from the tumor cell. Here, increased plasma concentration of AM originates from the host and impacts the tumor environment. It will be interesting to tease out whether levels of AM originating outside of the tumor impact cancer progression via lymphangiogenesis. *Adm* overexpression models may prove useful in determining host AM status on tumor progression [63].

Despite the focus on AM as a tumor-promoting peptide, increased *Adm* expression also plays important roles during the normal reproductive cycle. Recent studies have suggested that endometrial lymphangiogenesis is required for normal menstrual cycling and repair of damaged blood vessels during menstruation [96]. Examination of *Adm* during various stages of the menstrual cycle reveals that *Adm* expression is elevated during stages of endometrial repair [97]. Also, AM treatment results in increased lymphatic endometrial endothelial cell growth [97, 98]. Taken together, these findings suggest that AM-mediated lymphangiogenesis facilitates endometrial repair and allows progression through the menstrual cycle. Dysregulation of endometrial *Adm* expression may lead to improper repair of damaged blood vessels and prolonged or heavy menstrual bleeding [97]. These findings again suggest that modulation of the AM signaling system could be utilized clinically for the treatment of common diseases that significantly affect quality of life.

Concluding remarks

The essential role for AM in the development, maintenance, and function of the lymphatic vasculature has been clearly demonstrated over the past decade. Genetic models have greatly contributed to our understanding of how AM modulates lymphangiogenesis and underscored the importance of proper AM dosage: complete loss of AM is incompatible with life. *Adm* haploinsufficiency may predispose individuals to lymphedema, and overexpression of AM in cancer models correlates with severity of disease. Clearly, aberrant AM expression has the potential to lead to significant lymphatic-associated pathologies, and tight control of AM dosage from development through adulthood is critical for proper lymphatic function. Future studies will continue to elucidate the mechanisms of AM signaling. Whether the AM system interacts with other signaling cascades during lymphangiogenesis, such as Notch and VEGFR3, will be of particular interest, as previously published work has shown AM is capable of activating Notch and transactivating VEGFR2 [57, 99, 100]. These studies will expand our understanding of how AM impacts lymphatic physiology and offer the potential of identifying a G protein-coupled receptor target for the pharmacological modulation of the lymphatic vasculature in human disease.

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