Adrenomedullin Signaling in the Growth and Function of Adult Lymphatic Vessels

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Abstract

SAMANTHA LYNN HOOPES: Adrenomedullin Signaling in the Growth and Function of Adult Lymphatic Vessels (Under the direction of Dr. Kathleen Caron)

The highly conserved peptide, adrenomedullin (Adm=gene; AM=peptide), is known to play a role in the development and permeability of the lymphatic vascular system. AM signals through a G-Protein coupled receptor, calcitonin receptor-like receptor (Calcrl), associated with a receptor activity modifying protein (Ramp2/3). To determine the role of AM signaling during adulthood, several studies were undertaken using mice with temporal deletion of Calcrl and mice haplo-insufficient for Adm. Temporal loss of Calcrl in adult mice resulted in multi-organ lymphangiectasia associated with increased lymphatic permeability and disrupted lymphatic function. These mice also exhibited reduced body weight due to impaired lipid absorption and protein-losing enteropathy. Therefore, AM signaling is required for preservation of normal lymphatic function and permeability. An ear wound assay was performed on mice haplo-insufficient for Adm to stimulate lymphangiogenesis as well as angiogenesis. Genetic reduction of Adm impaired lymphangiogenesis in response to the wound, but angiogenesis was unaffected. AM is highly expressed in all endothelial cells, but from these studies as well as previous developmental studies we have found that there is an enhanced response to AM signaling in lymphatic endothelial cells that is imparted by increased levels of receptor expression. To further understand the molecular differences between lymphatic and
blood endothelial cells that could contribute to the different responses to AM signaling, we explored the expression and function of the serum binding protein for AM, complement factor H. Previous studies showed that AM activity is enhanced by complement factor H and previous microarray data revealed that the complement factor H gene is upregulated in lymphatic endothelial cells. Here we show that complement factor H protein expression is increased in lymphatic endothelial cells and it enhances the AM-induced migration response to a scratch assay in lymphatic endothelial cells. Overall, results from these studies established the importance of AM signaling in lymphatic vessels during adulthood and identified a factor contributing to the different responses to AM in lymphatic and blood endothelial cells.
To my loving and supportive husband, Robby
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GPCR (G-Protein coupled receptor)
CGRP (calcitonin gene-related peptide)
AM (adrenomedulin peptide)
Adm (adrenomedullin gene)
CLR (calcitonin receptor-like receptor protein)
Calcrl (calcitonin receptor-like receptor gene)
RAMP (receptor activity modifying protein protein)
Ramp (receptor activity modifying protein gene)
CFH (complement factor h protein)
Cfh (complement factor h gene)
PAMP (proadrenomedullin N-terminal 20 amino acid peptide)
LEC (lymphatic endothelial cell)
BEC (blood endothelial cell)
vSMC (vascular smooth muscle cell)
HUVEC (human umbilical vein endothelial cell)
HIF-1 (hypoxia inducible factor-1)
NO (nitric oxide)
eNOS (endothelial nitric oxide synthase)
iNOS (inducible nitric oxide synthase)
Chapter I. Introduction: Adrenomedullin Function in Vascular Endothelial Cells: Insights from Genetic Mouse Models

Overview

Adrenomedullin is a highly conserved peptide implicated in a variety of physiological processes ranging from pregnancy and embryonic development to tumor progression. This chapter highlights past and current studies that have contributed to our current appreciation of the important roles adrenomedullin plays in both normal and disease conditions. There is a particular emphasis on the functions of adrenomedullin in vascular endothelial cells and how experimental approaches in genetic mouse models have helped to drive the field forward.

The Multifunctional Adrenomedullin Peptide

Adrenomedullin (gene=Adm; protein=AM) is a highly conserved multifunctional peptide that is implicated in a wide variety of physiological processes including angiogenesis and cardiovascular homeostasis [2]. For over a decade, the association of ~2-fold elevations in plasma levels of AM peptide with a wide variety of cardiovascular disease conditions has prompted intense inquiry into

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understanding the functions and roles of AM in human disease (Figure 1-1).

Moreover, the recent development of highly precise methods for the quantitation of midregional proadrenomedullin (MR-proADM) as a reliable surrogate of mature AM plasma levels [3], has paved the way for the introduction of AM as a clinically useful biomarker for the staging of adverse cardiovascular events, including myocardial infarction, sepsis and community acquired pneumonia [4-7]. While it is clear that AM can elicit powerful effects on vascular smooth muscle cells and thus acutely modulate vascular tone, numerous studies in the past 5 years have elucidated essential functions of AM on vascular endothelial cells. In the following sections information is summarized pertaining to the multi-faceted role of AM in endothelial cells during development, how perturbations in AM signaling may lead to vascular pathologies, and recent discoveries regarding AM that have contributed in substantial ways to the broader field of vascular biology. Much of these discoveries have been unraveled through the use of sophisticated genetic animal models (Tables 1 and 2), and so special emphasis has been placed on describing the merits and shortcomings of these approaches and also highlighting current questions that are of predominant interest to the field today.

**Adrenomedullin GPCR-Mediated Signaling in Endothelial Cells**

G-protein coupled receptors (GPCRs) are widely expressed proteins that span the cell membrane 7 times and respond to a variety of stimuli including peptides, proteins, small organic compounds, lipids, amino acids, and cations. AM binds and signals through the GPCR calcitonin receptor-like receptor (gene=Calcrl;
protein=CLR). The discovery of a novel class of GPCR associated proteins called receptor activity-modifying proteins (gene=Ramp; protein=RAMP) [8] provided insight into how GPCRs signal. The RAMPs are single-pass transmembrane accessory proteins that regulate the translocation of GPCRs to the plasma membrane as well as provide ligand specificity to these receptors. The tissue specific and temporal expression pattern of RAMPs determines the responsiveness of GPCRs to particular ligands. For example, AM binds to the CLR receptor when CLR is associated with either RAMP2 or RAMP3. However, co-expression of CLR with RAMP1 changes the ligand specificity to another potent vasodilator called calcitonin gene-related peptide (CGRP), a related family member of the AM peptide. The ability of CLR to bind multiple ligands provides a unique mechanism by which the receptor can initiate a variety of signaling pathways. Since the AM receptor CLR and the 3 mammalian RAMPs are highly expressed in the vasculature, this cell signaling paradigm is being intensely investigated to determine how it can be exploited for the potential treatment of conditions such as pulmonary hypertension [9], cardiovascular disorders [10], and the inhibition of cancer metastasis [11].

The binding of AM to its receptor CLR results in a myriad of downstream effects including modulation of endothelial cell survival, proliferation, and vessel permeability. For example, AM-induced proliferation and migration of lymphatic endothelial cells is mediated in part by cAMP and downstream MEK/ERK pathways [12]. Similar results were shown using cultured HUVECs. AM-mediated induction of HUVEC proliferation and migration through activation of PKA, PI3K, and focal adhesion kinase were observed and then further substantiated in whole animal
studies [13,14]. AM induced the proliferation and migration of cultured human umbilical vein endothelial cells (HUVECs) [13] and numerous studies have shown a direct role for AM in endothelial growth and survival [15-17].

Using \textit{in vitro} experiments, AM was found to regulate the permeability and migration of HUVECs [18]. Previous studies indicated that adult \textit{Ramp2}^{+/−} mice had increased vascular permeability and overexpression of \textit{Ramp2} in BECs resulted in reduced permeability [19]. AM also reduces the permeability of HUVECs and pulmonary artery endothelial cells treated with permeabilizing agents including hydrogen peroxide and thrombin [20]. AM has been shown to regulate the transport of molecules across the blood brain barrier in cerebral endothelial cells by modulating permeability [21]. In cerebral endothelial cells, AM regulated various functions of the blood brain barrier including increasing transendothelial electrical resistance, reducing fluid-phase endocytosis, and reducing permeability for sodium fluorescein which indicate that the cerebral endothelial cell junctions are tightened by AM [21]. Also in an \textit{in vivo} model, AM treatment reduced lung vascular permeability resulting from ventilator use in a mouse model where prolonged mechanical ventilation was administered [22]. Overall, these data provide evidence for the role of AM as a junctional tightening factor to help regulate endothelial cell permeability.

Although AM functions to promote endothelial cell growth and proliferation in both the blood and lymphatic vasculatures, Fritz-Six \textit{et al.} have shown that there is an enhanced effect of AM on lymphatic endothelial cells (LECs) as compared to blood endothelial cells (BECs) [23]. This biological distinction in AM function is based upon the finding that LECs are enriched in the expression of AM and its
receptor components, *Calcrl* and *Ramp2* [23-25]. This increase in *Calcrl* expression is mediated in part by induction of the transcriptional regulator of lymphatic specification, *Prox1* [23]. It is therefore not surprising that loss of any component of the AM signaling axis (*Adm, Calcrl*, or *Ramp2*) results in embryonic lethality associated with profound lymphatic vascular defects [23]. Furthermore, several *in vitro* and *in vivo* experiments reveal that AM controls lymphatic permeability and flow through reorganization of junctional proteins ZO-1 and an adherens protein VE-Cadherin, independent of changes in junctional protein gene expression [26]. Administration of AM to a monolayer of LECs resulted in tightening of the lymphatic endothelial barrier by reorganization of a tight junction protein at the plasma membrane to form continuous cell-cell contacts. Through the use of *in vivo* tail microlymphography, local administration of AM in a SvEv129/6 mouse tail resulted in decreased velocity of lymph uptake from the interstitial space and movement through the lymphatic dermal capillaries in the tail [26]. Thus, it becomes critically important to consider the pleiotropic effects of AM not just on blood endothelial cells, but also on neighboring lymphatic vessels—a dynamic that may ultimately help resolve the complex functions of AM peptide in cardiovascular disease, tumor progression and inflammation.

While activation of GPCRs typically leads to induction of classical second messenger signaling systems, it is now appreciated that more complex levels of regulation exist [27,28]. Therefore, it is not surprising that pathway cross-talk is one mechanism through which AM modulates certain endothelial cell functions. For example, Yurugi-Kobayashi *et al.* describe a novel embryonic stem cell
differentiation system to study mechanisms of arterial-venous specification. They demonstrated that coordinated signaling of AM/cAMP, VEGF, and Notch induces arterial endothelial cell differentiation from vascular progenitors [29]. Furthermore, GPCR-induced transactivation of receptor tyrosine kinases is another mechanism that allows interaction between signaling molecules. Evidence exists that AM and VEGF pathways are likely to interact in endothelial cells. Although an earlier study claimed that AM-induced capillary tube formation in HUVECs was independent of VEGF activation [15], a more recent study by Guidolin et al. demonstrated that VEGFR2 inactivation inhibited AM-mediated angiogenesis in HUVECs [30]. This latter finding suggests that the pro-angiogenic effects of AM require transactivation of the receptor tyrosine kinase VEGFR2. Although controversy still exists regarding the degree of cooperation between pathways, it is certainly intriguing to consider that regulation of endothelial cell biology may very likely involve coordination of multiple signaling molecules. We now must begin to unravel these complexities and elucidate whether these interactions occur differentially in blood and lymphatic endothelial cells and identify the intermediate molecular players involved in pathway cross-talk in the vasculature.

Development

*Endothelial Adrenomedullin Signaling is Essential for Embryonic Development*

Work by multiple independent groups has established the importance of AM signaling during development. The use of gene targeted mouse models clearly indicates that functional AM signaling is essential for embryonic survival. The
genetic ablation of *Adm* [31-33], *Calcr* [34], *Ramp2* [19,23,35] or the enzyme responsible for functional AM amidation, *peptidylglycine alpha-amidating monooxygenase (PAM)* [36] all result in midgestational lethality associated with severe interstitial edema and cardiovascular defects. The conserved phenotypes between these knockout (KO) mice is compelling genetic evidence that the CLR/RAMP2 complex is the main receptor of AM during development, and also is the first *in vivo* confirmation that RAMP2 functionally interacts with CLR [23].

Although the overt phenotypes of these KO mice are conserved, the physiological cause of edema and lethality is still debated. One possible hypothesis is that loss of AM signaling causes developmental cardiac abnormalities that lead to heart failure, thus resulting in edema and death that is similar to previously characterized KO mice with developmental heart failure [37-39]. Supporting this line of thought, our lab showed that *Adm*/*", *Calcr"*, and *Ramp2"* mice have smaller hearts due to decreased myocyte proliferation and increased apoptosis. Additionally, they have increased left ventricle trabecularization, which leads to decreased ventricular volume [23,31,34]. However, an alternative hypothesis is that vascular defects are responsible for the phenotypes, since *Adm* [31], *Calcr* [34], and *Ramp2* [19] are abundantly expressed in the developing endothelium and vascular smooth muscle cells (vSMC). To help resolve between the two hypotheses, we generated an endothelial-specific *Calcr"* mouse using a *Tie2* promoter to drive *Cre recombinase* expression which recapitulated the phenotype observed in global KO mice [23], indicating that AM signaling in endothelial cells is essential for embryonic development. A remaining caveat to this conclusion is the
fact that Tie2-Cre mediated excision also occurs in developing endocardial cells. Therefore, to definitively determine if cardiac abnormalities contribute to this phenotype the reverse experiment using Cre lines specific to cardiac myocytes would be beneficial.

Although vascular defects are responsible for the edema in these KO mice, it remained unclear whether defects in the blood or lymphatic endothelium were the principle cause of the phenotypes. Given the role of AM in regulating vascular permeability, it seems reasonable that loss of AM signaling could lead to increased vascular permeability and a resulting build up of interstitial fluid. In support of this idea, the KO mice have thinner aorta and carotid artery walls due to a decrease in vSMC proliferation [19,31,34], although the endothelium lining the aorta appeared to be normal [34]. There are reported abnormalities in endothelial basement membranes and a down-regulation of junctional proteins in Adm−/− and Ramp2−/− embryos that may lead to increased vascular permeability and hemorrhage [19,32], but these phenotypes were observed in a small proportion of animals and not conserved in all studies. In addition, the severity of the edema and their survival beyond e10.5 does not resonate with other knockout mouse models with established vascular permeability defects [40-42]. In contrast, the onset (Calcrl=E13.5, Adm=E14.5, Ramp2=E15.5) and severity of the phenotype closely resembles other genetic mouse models that delete genes essential for lymphatic development, including Prox1[43], Sox18 [44], and Vegfc [45]. To determine whether lymphatic vasculature defects may contribute to the edema observed in AM signaling KO animals, we performed a comprehensive study of AM signaling expression and
function during lymphatic vascular development [23], which is further described in Chapter II. It is most likely that a combination of both blood and lymphatic defects leads to the edema and lethality in the KO mice given the integrated physiology between the two vasculatures. However, more specialized genetic assays are required to resolve the relative contributions of each vasculature within these KO mice [46].

An alternative approach to assess the role of AM signaling in development would be to use transgenic mouse models that overexpress Adm, Calcr, or Ramp2. Interestingly, no developmental phenotypes have been reported in gain-of-function mouse models of AM signaling, either by vascular Adm overexpression [47] or vSMC-specific Ramp2 overexpression [48], though these models displayed adult cardiovascular phenotypes. Given the essential nature of AM signaling within the endothelium, it would be interesting to over-express Calcr or Ramp2 specifically in the endothelium, which to our knowledge, has not yet been reported.

**Adrenomedullin vs. Proadrenomedullin**

One potential caveat with the majority of Adm\(^{-/}\) studies is that the gene targeting strategies delete the entire Adm coding sequence [31,32], which results in the genetic KO of two functionally active peptides, AM and proadrenomedullin N-terminal 20 amino acid peptide (PAMP) [49]. PAMP is small peptide that is produced during post-transcriptional splicing of preproadrenomedullin and has numerous actions to complement or antagonize AM signaling [49-52]. For two of the reported Adm deficient mouse lines, the design of the targeted allele could not rule
out whether the observed phenotypes in the KO animals were due to loss of AM, PAMP, or both [31,32]. This controversy was partially resolved using a third independent Adm\(^{-/-}\) mouse, which left PAMP intact, and illustrated that loss of AM alone was enough to recapitulate embryonic lethality [33]. However, these mice lacking only AM had a milder phenotype (less edema and no cardiovascular abnormalities) when compared to KO mice lacking both peptides. This inconsistency in phenotypes could be attributed to differences in mouse strain and/or gene targeting approach [33]. However, a more intriguing hypothesis, which remains to be vigorously experimentally addressed, is that AM and PAMP may have non-redundant functions during cardiovascular development [53].

**Developmental Role of RAMP2 vs. RAMP3**

While Ramp2\(^{-/-}\) mice recapitulated the Adm\(^{-/-}\) and Calcrt\(^{-/-}\) phenotypes, it appears that RAMP3, another RAMP that associates with CLR and binds AM, is not essential for embryonic survival since Ramp3\(^{-/-}\) mice develop normally to adulthood. There also appears to be no functional redundancy between RAMP2 and RAMP3 in development, since there is no transcriptional compensatory mechanism of either RAMP in response to loss of the other [19,35]. Although RAMP3 has been implicated in receptor trafficking [8,54,55], the functional role of the AM/CLR/RAMP3 signaling complex is not well understood *in vivo.*
**New Developmental Insights of Adrenomedullin Pathway**

A recent study by Nicoli *et al.* expanded our knowledge regarding the role of CLR during embryonic vascular development using a zebrafish model. By knocking down *crlr* they showed drastic vascular defects due to decreased expression of *vegf*. While *vegf* appears to be the critical mediator in the vascular development since overexpression of *vegf* is able to rescue the *crlr* knockdown phenotype, it still appears that *crlr* is essential for appropriate levels of *vegf*. This study provides *in vivo* evidence that *crlr* is downstream of *sonic hedgehog*, but upstream of *vegf* and *notch* signaling in arterial differentiation and development [56]. Modulation of *vegf* levels by AM signaling were previously reported in mice [57] but a complete characterization of AM and VEGF interactions is not well understood. It is novel that *sonic hedgehog* appears to regulate *crlr* expression and further dissection of this pathway in animal models would improve our understanding of how CLR is regulated during development. The zebrafish model system has recently been used to study lymphatic development [58-60] and it would be interesting if phenotypes seen in *Adm<sup>−/−</sup>*, *Calcrl<sup>−/−</sup>* , and *Ramp2<sup>−/−</sup>* mice could be recapitulated in zebrafish.

**Physiology and Pathophysiology**

*Adrenomedullin Signaling in Pregnancy*

AM signaling is known to be a critical component for initiation and progression of normal pregnancy. By the third trimester of a normal pregnancy, plasma levels of AM increase 4- to 5-fold [61-66]. AM is highly expressed in all vascular tissues which include the placenta and uterus [67,68]. Our previous studies in *Adm<sup>+/−</sup>* female
mice expressing 50% less adrenomedullin revealed that there is disrupted fertility, placentation, uterine receptivity, and fetal growth resulting from reduced AM expression [69]. AM signaling components are also expressed in the trophoblast cells [70-75], which take on an endothelial-like function during the process of decidual maternal spiral artery remodeling during pregnancy. The trophoblast giant cells deriving from the trophectoderm invade and replace the vascular wall by inducing a loss of endothelial cells and smooth muscle cell coverage to allow for higher blood flow to the fetus through the spiral arteries. Failure of this remodeling process to occur is a hallmark feature of pre-eclampsia. Further research needs to be performed to determine the extent to which AM signaling affects trophoblast cells in the process of maternal spiral artery remodeling during pregnancy.

**Adrenomedullin Signaling and Cardiovascular Biology**

AM has been reported to be upregulated in various cardiovascular conditions [2,76,77] and is a potent angiogenic factor as well as a cardioprotective factor [2]. Plasma AM increases 2-fold in conditions such as essential hypertension, renal failure and congestive heart failure [78,79] (Figure 1-1). Previous studies with gene-targeted KO mice for *Adm* and *Calcrl* indicated that AM signaling is important for cardiovascular development [23,31,34]. Genetic reduction of *Adm* results in enhanced cardiovascular damage including increased cardiac hypertrophy in male *Adm*+/- mice [80] and marked perivascular fibrosis, coronary artery intimal hyperplasia and oxidative stress with AngII/high-salt treatment [33]. AM protects the heart from hypertrophy and fibrosis during cardiovascular stress such as
hypertension and cardiac hypertrophy, myocardial infarction, heart failure and atherosclerosis [81,82], but the exact mechanisms of AM-mediated cardioprotection have not been fully elucidated. A comprehensive review of the cardioprotective function of AM during hypertension and heart failure has recently been provided by several groups [10,83].

Endothelial dysfunction is characterized by reduced endothelium-dependent vascular relaxation which is associated with most forms of cardiovascular disease. It is partially impacted by reduced nitric oxide and upregulation of adhesion molecules to result in a proinflammatory and prothrombotic state [84]. Research also suggests that endothelial dysfunction may act as an early marker of atherosclerosis [85]. One study indicated that Adm and its receptor components, Calcrl and Ramps were upregulated in the aorta of apolipoprotein E-deficient (ApoE/−) mice [86]. Loss of apoE ultimately results in a mouse model of spontaneous atherosclerosis because apoE is important in the removal of circulating lipoproteins [87]. When these mice were fed an atherogenic diet and treated with AM, the appearance of atherosclerotic lesions was reduced [86]. This study further indicates that AM may help to protect against the progression of atherosclerosis, but the exact mechanism for this action remains to be understood. Expression of adhesion molecules in LECs [88] as well as liver sinusoidal endothelial cells [89] were reduced in response to AM treatment. Similar results were seen with VEGF-treated HUVECs [90]. Thus, AM may impact endothelial dysfunction partially by modulating adhesion molecule expression. With respect to endothelium-dependent vascular relaxation, AM is known to induce vasodilation which is mediated partially by endothelium-derived nitric oxide [91-95].
Also, in a rat model of sepsis induced by cecal ligation and puncture, administration of AM and AM-binding protein (AMBP-1 also known as complement factor H) were shown to prevent against endothelial cell dysfunction and decreased endothelium-dependent vascular relaxation in thoracic aorta [96]. These studies implicate AM as having a protective role in cardiovascular disease and endothelial dysfunction, but further research needs to be performed to investigate how AM directly impacts on the cardiac endothelial cells to regulate their function.

The Role of Adrenomedullin Signaling in Response to Injury, Vascular Dysfunction and Wound Healing

Endothelial proliferation and angiogenesis are known to be impacted by AM signaling. In a hind-limb ischemia model, AM promotes endothelial cell proliferation and capillary formation and conversely, Adm+/− mice showed reduced blood flow and capillary development [57]. Other whole animal studies using matrigel plugs to assess vascular growth demonstrated the role of AM in vascular regeneration because AM increased blood flow and capillary densities through PKA- and PI3K-dependent pathways [13,14]. AM also induced tube-formation of HUVECs cultured on matrigel [15]. Another study pertaining to RAMP2 expression also revealed similar findings. An aortic ring assay and matrigel plug assay with adult Ramp2+/− mice revealed that with decreased RAMP2 expression there was reduced neovascularization in response to growth factor stimulation [19]. Collectively, these studies indicate the importance of AM in endothelial cell proliferation and angiogenesis in adult mice.
AM signaling is known to impact the blood and lymphatic vasculature in other physiological processes and pathological conditions. In a pathological mouse model of subcortical vascular dementia (chronic cerebral hypoperfusion), AM was shown to promote arteriogenesis and angiogenesis as well as inhibit oxidative stress and preserve white matter in the brain [97]. AM signaling can also induce anti-apoptotic and anti-inflammatory effects in response to injury. In the sinusoidal endothelial cells of the liver, AM helps to protect these cells from cold injury during the process of cold preservation for a liver transplant by decreasing endothelial cell apoptosis and inflammation [89]. Conversely, in Adm+/− and Ramp2+/− mice there is increased apoptosis of the sinusoidal endothelial cells in the liver after cold injury [89] further indicating that AM signaling helps to regulate apoptosis. Wound healing is an essential physiological process that requires angiogenesis and lymphangiogenesis for proper healing. Since AM is a known angiogenic factor and lymphangiogenic factor [23], it is not surprising that AM signaling is necessary in the wound healing process. In an ischemia/reperfusion mouse model of a pressure ulcer, AM administration reduced the wound area and accelerated angiogenesis as well as lymphangiogenesis [98]. Also in a wounded HUVEC monolayer, AM promoted vascular regeneration via activation of endothelial Akt in a PKA- PI3K- dependent manner [13]. Lymphedema is a hallmark condition of lymphatic dysfunction resulting in the swelling of one or more limbs due to accumulation of interstitial fluid. In Balb/C mice with tail lymphedema, AM treatment improved lymphedema and increased the number of lymphatic and blood vessels near the injury site [12]. Taken together, these data indicate that AM is an essential component for proper
endothelial cell function in both physiological and pathological states to regulate apoptosis, inflammation, and lymphangiogenesis as well as angiogenesis.

An important issue to still address is to determine the exact role of AM signaling during adulthood by using temporal and spatial KO mice for components of the AM signaling system to evaluate physiology and function of the vascular beds in these mice. Previous studies with genetic KO mice for the AM signaling system reveal an enhanced impact of AM on lymphatic vascular development relative to blood vascular development [23]. It has also been shown that the gene expression of AM receptor components, *Calcrl* and *Ramp2*, are enhanced in LECs compared to BECs [24,25]. Due to these known differences of AM signaling between BECs and LECs, it would be interesting to determine whether there is also an enhanced effect of AM on the lymphatic vasculature in adult physiology and pathology. The underlying mechanisms through which AM impacts the lymphatic vasculature, blood vasculature as well as the more specialized cardiac tissue during adulthood also needs to be identified.

*Adrenomedullin Expression in Tumor Progression*

The AM peptide was initially isolated from a human adrenal tumor (pheochromocytoma) due to its platelet cAMP elevating activity [77]. Since this discovery almost 20 years ago, investigation into the role of AM in tumors has greatly expanded. Early studies noticed elevated levels of AM in lung and brain tumors [99,100] and a comprehensive survey of human tumor cell lines from lung, breast, brain, ovary, colon, and prostate substantiated those reports [101]. AM has
been implicated in a variety of pro-tumor functions including acting as an autocrine growth factor [101-103], apoptosis survival factor [16], promoter of tumor cell motility and invasion [103-105], and molecular intermediate to enhance communication between tumor cells and immune cell infiltrates [106]. Furthermore, it has been suggested that the presence of AM in tumors may signify a more aggressive tumor phenotype due to correlation between Adm gene expression and histological tumor grade [103,107].

The mechanism(s) by which Adm gene expression is transcriptionally regulated in tumors remains unclear. It is likely that AM can be both an autocrine and paracrine factor [108] by providing tumor cells a growth advantage in addition to acting on surrounding endothelial cells to promote proliferation and changes in vessel permeability to perhaps facilitate metastasis. Moreover, it has been suggested that hypoxia may play a role in AM production [9,109]. Tumors often develop hypoxic zones in areas where blood flow is inadequate to supply the necessary oxygen required for the growing tumor cells. As a result of this unfavorable state, hypoxia inducible factor-1 (HIF-1) is activated which in turn upregulates a number of genes to compensate for the reduced oxygen microenvironment. Interestingly, a HIF-1 dependent mechanism was found to increase the expression of Adm in hypoxic human tumor cell lines [110]. Furthermore, Adm and Calcr were found to be upregulated in microvascular endothelial cells cultured under low oxygen conditions [111]. Together, these results show that both tumor cells and surrounding endothelial cells are responsive to
hypoxic conditions and may provide a mechanism for elevated AM levels in a tumor setting.

Although the precise role of AM in tumor development and progression is still unresolved, significant progress has been made to better understand how AM affects not only a tumor cell, but also the endothelial cells in the surrounding microenvironment. Analysis of immunohistochemical staining of human ovarian cancer found that in addition to tumor cells, AM was also localized to the endothelial cells of the surrounding stroma [107]. Furthermore, an in vitro co-culture system found that HUVECs became activated upon exposure to tumor cells and consequently increased transcriptional activity of Adm, among other factors [112]. Since AM directly impacts endothelial cell proliferation and permeability, AM induced modulation of vessels may affect the spread of cancer cells to distant sites via blood or lymphatic vasculature. Research groups have been performing the in vivo studies necessary to confirm that AM promotes tumor progression through its known angiogenic properties. Several reports have shown that inhibition of AM action by neutralizing antibodies or AM antagonist AM\textsubscript{22-52} have reduced the growth of tumor xenografts in vivo by suppressing vascular development [57,113,114].

While much of the focus in understanding the process of tumor lymphangiogenesis and angiogenesis has been upon the VEGF protein family, the contribution of AM to this process should not be underappreciated. Clearly, the studies described above point to AM as a valid target for potential cancer therapies although more research is necessary. Generation and validation of preclinical mouse models that are able to rigorously test AM as a target are greatly needed.
Since the embryonic lethal phenotype of Adm\textsuperscript{+/−} mice makes studying this signaling pathway more complicated, novel genetic mouse models (Table 1) using conditional alleles \cite{19,23,115} and vascular endothelium specific Cre animals are a starting point for such tumor studies. Furthermore, these mouse models will be needed to refine our understanding of the metastatic process. Given the knowledge that AM can act on both the blood and lymphatic endothelium, a key question that remains to be answered is by what mechanisms do tumor cells disseminate into the blood and/or lymphatic vessels.

**Summary and Future Directions**

The use of genetic animal models in the field of AM research has produced significant contributions toward understanding the biology of this pleiotropic molecule, with a renewed appreciation for its critical regulation of endothelial cells function during development and vascular diseases. To date, AM has been implicated in lymphatic vascular development, in proper functioning of blood and lymphatic endothelial cells and in a variety of conditions such as pregnancy, cardiovascular disease, and tumor progression (Figure 1-2). Despite the strides that have been made, there is much more to learn regarding the mechanisms mediating AM function and regulation. With the generation of additional sophisticated molecular biology tools such as genetic mouse models, we are poised to refine our current knowledge as well as discover other novel roles for this peptide and signaling partners in normal and disease physiology.
Author Contributions

All co-first authors including NOK, SLH, and DOK contributed equally to the manuscript entitled “Adrenomedullin Function in Vascular Endothelial Cells: Insights from Genetic Mouse Models.” The design and layout of the chapter was drafted by NOK, SLH, and DOK. The idea for the mouse model figure was designed by NOK, SLH, and DOK and prepared by DOK. NOK contributed sections in the chapter related to adrenomedullin GPCR-mediated signaling in endothelial cells and tumor pathology. DOK contributed the sections on development. SLH contributed the sections on adrenomedullin in pregnancy, cardiovascular biology, injury/wound healing of endothelial cells, and the tables related to mouse models and vascular assays pertaining to adrenomedullin signaling. PML contributed the graph of AM plasma levels. KMC helped design and edit the review article.
<table>
<thead>
<tr>
<th>Mouse Model</th>
<th>Development or Adulthood</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adm&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Development</td>
<td>Embryonic lethal (e14.5), edema, smaller hearts, reduced myocyte proliferation and increased apoptosis, increased left ventricle trabecularization, thinner aorta and carotid artery walls, increased vascular permeability, hypoplastic jugular lymph sac</td>
<td>[23,31-33]</td>
</tr>
<tr>
<td>Adm&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>Adulthood</td>
<td>Pregnancy: Disrupted fertility, placentation, and fetal growth -Cardiovascular: Increased damage including hypertrophy, reactive oxygen species (ROS), and fibrosis -Liver cold injury: increased apoptosis of the sinusoidal endothelial cells</td>
<td>[33,69,80,89]</td>
</tr>
<tr>
<td>Adm&lt;sup&gt;-/−&lt;/sup&gt;/TubulinTa-1-Cre&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Adulthood</td>
<td>High anxiety, hyperactive, impaired motor coordination</td>
<td>[115]</td>
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<tr>
<td>Calcrl&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Development</td>
<td>Embryonic lethal (e13.5), similar phenotype as Adm&lt;sup&gt;-/-&lt;/sup&gt; mice</td>
<td>[23,34]</td>
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<tr>
<td>Calcrl&lt;sup&gt;loxP/−&lt;/sup&gt;/Tie2Cre&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Development</td>
<td>Embryonic lethal (e16.5) and recapitulation of Adm&lt;sup&gt;-/-&lt;/sup&gt;, Calcrl&lt;sup&gt;-/-&lt;/sup&gt;, and Ramp2&lt;sup&gt;-/-&lt;/sup&gt; phenotype</td>
<td>[23]</td>
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<tr>
<td>Ramp2&lt;sup&gt;-/-&lt;/sup&gt; and Ramp2&lt;sup&gt;fl/fl&lt;/sup&gt;/CAG-Cre&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Development</td>
<td>Embryonic lethal (e15.5), similar phenotype as Adm&lt;sup&gt;-/-&lt;/sup&gt; mice</td>
<td>[19,23,35]</td>
</tr>
<tr>
<td>Ramp2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Adulthood</td>
<td>Increased vascular permeability and decreased neovascularization -Liver cold injury: increased apoptosis of the sinusoidal endothelial cells</td>
<td>[19,89]</td>
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<td>PAM&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Development</td>
<td>Embryonic lethal (e14.5) and phenocopy of Adm&lt;sup&gt;-/-&lt;/sup&gt;, Calcrl&lt;sup&gt;-/-&lt;/sup&gt;, and Ramp2&lt;sup&gt;-/-&lt;/sup&gt; mice due to loss of amidation of AM peptide</td>
<td>[36]</td>
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Adrenomedullin (Adm); Calcitonin receptor-like receptor (Calcrl); Receptor activity modifying protein (RAMP); Peptidylglycine alpha-amidating monooxygenase (PAM)
<table>
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<th>Assay</th>
<th>Result</th>
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<td>Atherogenic Model</td>
<td>Atherogenic diet and AM treatment in <em>ApoE</em>&lt;sup&gt;−/−&lt;/sup&gt; mice resulted in reduced formation of atherosclerotic lesions</td>
<td>[86]</td>
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<tr>
<td>Tail microlymphography</td>
<td>AM injected mice showed reduced permeability of dermal lymphatic capillaries</td>
<td>[26]</td>
</tr>
<tr>
<td>Matrigel plug</td>
<td>- AM increased vascular regeneration - <em>Ramp2</em>&lt;sup&gt;−/−&lt;/sup&gt; mice exhibited reduced neovascularization</td>
<td>[13,14,19]</td>
</tr>
<tr>
<td>Aortic ring</td>
<td><em>Ramp2</em>&lt;sup&gt;−/−&lt;/sup&gt; mice exhibited reduced neovascularization in response to growth factor stimulation</td>
<td>[19]</td>
</tr>
<tr>
<td>AngII/high-salt</td>
<td>AM&lt;sup&gt;−/−&lt;/sup&gt; mice exhibited increased reactive oxygen species (ROS), vascular fibrosis, and intimal thickening</td>
<td>[40]</td>
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<tr>
<td>Prolonged mechanical</td>
<td>AM treatment reduced lung vascular permeability resulting from ventilator use</td>
<td>[22]</td>
</tr>
<tr>
<td>hypoperfusion</td>
<td>AM promoted arteriogenesis and angiogenesis</td>
<td>[97]</td>
</tr>
<tr>
<td>Hind-limb ischemia</td>
<td>AM promotes endothelial cell proliferation and capillary formation - <em>Adm</em>&lt;sup&gt;−/−&lt;/sup&gt; mice showed reduced blood flow and capillary development</td>
<td>[57]</td>
</tr>
<tr>
<td>Wound healing</td>
<td>AM reduced wound area and increased angiogenesis and lymphangiogenesis</td>
<td>[98]</td>
</tr>
<tr>
<td>(Pressure Ulcer - Ischemia</td>
<td>-AM improved lymphedema and increased number of lymph and blood vessels</td>
<td>[12]</td>
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<tr>
<td>reperfusion model)</td>
<td>Block AM signaling results in reduced vascular development</td>
<td>[57,113,114]</td>
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<tr>
<td>Tail lymphedema</td>
<td>-AM improved lymphedema and increased number of lymph and blood vessels</td>
<td>[12]</td>
</tr>
<tr>
<td>Tumor xenografts</td>
<td>-Blocking AM signaling results in reduced vascular development</td>
<td>[57,113,114]</td>
</tr>
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Table 2: Vascular assays for studying adrenomedullin function
**Figure 1-1. Fold Change in Plasma Adrenomedullin Levels in a Variety of Human Conditions.**

Bars indicate average fold change in circulating AM levels in various disease categories or conditions based on published human clinical data. The dashed horizontal line at 2.33 represents the average fold increase in plasma AM levels across all conditions depicted. Number above each bar indicates the number of published observations assessing plasma AM levels in each category. The clinical papers that were used for our analysis are listed according to the following broad categories: cancer [116-121], cardiovascular [5,116,118,122-149], hepatic and renal [129,130,132,133,150-161], pulmonary [7,130,162-167], infectious & autoimmune [168-174], normal pregnancy [62,63,65,66,175-179], and pregnancy complications [62,66,179-191].
Figure 1-2. Adrenomedullin Signaling in Development and Vascular Biology. (A.) Loss of AM signaling causes embryonic lethality due to severe edema associated with impaired lymphatic vascular development. (B.) In the adult, AM is an angiogenic, lymphangiogenic, and a cardioprotective factor that also regulates vascular permeability and inflammation. Expression of AM is also implicated in pregnancy and tumor progression. (LEC = lymphatic endothelial cell)
Chapter II: The Lymphatic Vascular System

Function of the Lymphatic System

The lymphatic vascular system is a network of blind-ended microvessels that is critical for regulating and maintaining fluid homeostasis. Tissue fluid, proteins, lipids, and cells are unidirectionally transported through the lymphatic vessels from the interstitial space back to the circulatory system via the thoracic duct. White blood cells and antigen-presenting cells are transported through the lymphatic vessels to the lymphoid organs. The initial lymphatic capillaries, lacking a basement membrane, consist of a single layer of overlapping endothelial cells that stretch apart to uptake interstitial fluid and proteins. The initial lymphatics lead to the larger collecting lymphatics covered by lymphatic vascular smooth muscle cells that generate lymph flow primarily through peristaltic-like contractions. In the intestine, lipids are absorbed by the enterocytes and packaged in chylomicrons [192,193]. The chylomicrons then enter the lacteals, the initial lymphatics of the small intestine, and are moved through the lymphatic system and returned to the blood stream [192,193]. In pathological conditions, the lymphatic system plays a critical role in inflammation, scarring, and tumor metastasis [194-196].
Lymphatic Specification and Sprouting

Only in the past few years has there been an increase in the understanding of the development of the lymphatic vascular system with the advent of genetically engineered mouse models. From characterizing the phenotypes of numerous mouse models, several genes have been found to be involved at sequential stages in the development of the lymphatic system as described in several recent comprehensive review articles [197-199] (Figure 2-1). The initial lymphatic endothelial cells (LECs) are derived from a subset of venous endothelial cells. This subset of cells begins to express Sox18 at E9.0 [44] that then activates the expression of Prox1 at E9.5, the transcription factor that is the master genetic regulator of LEC fate, in a polarized manner [200].

In order for LECs to bud away from the venous endothelial cells, VEGFR3 expression becomes localized to the polarized area of LECs [201]. The vascular smooth muscle cells (vSMCs) and mesenchymal cells near the LECs begin to express VEGFC, the ligand for VEGFR3 [202]. Then, LECs begin to respond to VEGFC via VEGFR3 to sprout away from the cardinal vein initiating lymph sac formation.

Lymph Sac Formation and Proliferation

After budding away from the cardinal vein, LECs migrate and proliferate to form a primary lymph sac from E10.5-E11. The origination of the lymphatic system is considered to begin with the development of the primary lymph sacs [203]. The LECs continue proliferating and sprouting to form the primary lymphatic plexus.
between E11-E14.5. Numerous genes have been found to play a role in the formation of the lymph sacs and primary lymphatic plexus including **Adm**, **Calcrl**, **Ramp2**, **Spred-1/2**, **Slp-76**, **Syk**, and **Plcγ2**.

Platelets are known to be important in the development of the lymphatic vascular system because of their ability to activate the hematopoietic SKY-SLP-76 signaling pathway. The C-type lectin receptor on platelets binds to podoplanin, expressed on LECs, to activate the SKY-SLP-76 signaling [204]. A study by Bertozzi, et al showed that disruption of this process in lymphatics results in aberrant vascular connections and blood-lymphatic mixing [204]. Therefore, platelets and SYK-SLP-76 signaling are necessary for proper embryonic blood-lymphatic vascular separation.

The Caron Lab identified **Adm**, **Calcrl**, and **Ramp2** as a novel G-protein coupled receptor (GPCR) pathway that is a critical regulator of lymphatic vascular development [23]. **Adm** is temporally and spatially expressed on the endothelium of the jugular vein in a polarized fashion towards the budding primary lymph sac *in vivo*, which is identical to the lymphatic-specific transcriptional regulator, **Prox1** [43,205,206]. Moreover, **Calcrl** and **Ramp2** are preferentially up-regulated in LECs, partially under the control of the transcription factor, **Prox1** [23]. Gene knockout mice for **Adm** [31], **Calcrl** [34], and **Ramp2** [23,35] all exhibit mid-gestation embryonic lethality characterized by hydrops fetalis due to defects in the development of the lymphatic vascular system. While loss of AM signaling does not affect the differentiation and migration of LECs to form the primary lymph sac or dermal lymphatics, these mice have hypoplastic jugular lymph sacs due to
decreased LEC proliferation [23]. These results indicate that AM is necessary for proper morphological development and proliferation of the embryonic lymphatics.

**Lymphatic Plexus Remodeling and Maturation**

After formation of the primary lymphatic plexus, maturation of the lymphatic vascular network occurs with the involvement of various genes, some of which include *Neuropilin-2, Foxc2, Lyve-1, Ephrin B2, Angiopoietin-2, Fiaf*, and *Integrin-α9*. The maturation process continues several days postnatally to generate the different types of LECs from small capillaries to larger collecting vessels with valves and vSMC coverage. Interestingly, a recent study has implicated the interaction of vSMCs with LECs to be important in the development of the larger collecting lymphatic vessels [207]. This study showed that recruitment of vSMCs to LECs enhanced signaling of an extracellular matrix glycoprotein, Reelin, that is expressed from LECs and regulates lymphatic development and function [207]. After development of the lymphatic system is completed, the vessels are typically quiescent unless growth is triggered in response to a stimulus, such as a wound/injury.

**Summary**

The studies presented in this dissertation focused on exploring various aspects of the lymphatic system and AM signaling. The role of AM in the lymphatic system during adulthood is characterized with emphasis on understanding the function of lymphatic vessels as well as growth in response to injury. Temporal
deletion of *Calcrl* during adulthood resulted in overall lymphangiectasia, dilation of lymphatic vessels, with disruption in the function and permeability of the vessels. A second study using an ear wound assay in adult mice haplo-insufficient for *Adm* determined that there is reduced lymphangiogenesis, while angiogenesis is unaffected in response to the wound. The third and last study in this dissertation focused on understanding why there are differential responses to AM signaling in the lymphatic and blood vascular systems. While both systems express AM receptors and respond to the ligand, there is an enhanced effect of AM signaling on the lymphatic system. This study indicated that complement factor H (CFH), the binding protein for AM, is upregulated in LECs as compared to blood endothelial cells (BECs). CFH enhances the AM-induced migratory response in LECs, which may play a role in the differential responses to AM. Overall, these studies identify a novel and important role for AM signaling in adult lymphatic vessels.
**Figures**

*Figure 2-1. Stepwise process of lymphangiogenesis.* The development of the lymphatic vasculature begins with specification of lymphatic endothelial cells from venous precursors. The chemoattractant and growth promoting properties of numerous growth factors, including VEGFC, causes lymphatic endothelial cells to sprout and separate from veins to form primitive lymph sacs. Proliferation of lymph sacs leads to the formation of a primary lymphatic plexus which is later remodeled into the mature lymphatic vascular system. Below each step is a list of genes for which a functional role has been demonstrated using genetically engineered mouse models. Reprinted with permission from [199].
Chapter III: Characteristics of multi-organ lymphangiectasia resulting from temporal deletion of calcitonin receptor-like receptor in adult mice

Overview

Adrenomedullin (AM) and its receptor complexes, calcitonin receptor-like receptor (Calcrl) and receptor activity modifying protein 2/3, are highly expressed in lymphatic endothelial cells and are required for embryonic lymphatic development.

To determine the role of Calcrl in adulthood, we used an inducible Cre-loxP system to temporally and ubiquitously delete Calcrl in adult mice. Following tamoxifen injection, Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice rapidly developed corneal edema and inflammation that was preceded by and persistently associated with dilated corneoscleral lymphatics. Lacteals and submucosal lymphatic capillaries of the intestine were also dilated, while mesenteric collecting lymphatics failed to properly transport chyle after an acute Western Diet, culminating in chronic failure of Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice to gain weight. Dermal lymphatic capillaries were also dilated and chronic edema challenge confirmed significant and prolonged dermal lymphatic insufficiency. \textit{In vivo} and \textit{in vitro} imaging of lymphatics with either genetic or pharmacologic inhibition of AM signaling revealed markedly disorganized lymphatic junctional proteins ZO-1 and VE-cadherin. The maintenance of AM

signaling during adulthood is required for preserving normal lymphatic permeability and function. Collectively, these studies reveal a spectrum of lymphatic defects in adult $\text{Calcr}^{fl/fl}/\text{CAGGC}re-\text{ER}^{TM}$ mice that closely recapitulate the clinical symptoms of patients with corneal, intestinal and peripheral lymphangiectasia.

**Introduction**

The lymphatic vascular system is a complex vascular network that permeates nearly every organ of the body and plays a critical role in the maintenance of fluid homeostasis, the absorption of intestinal lipids and the trafficking and maturation of immune cells. Despite its pervasive functions, it is surprising that very little is known about the genetic and molecular pathways that regulate lymphatic vascular function in adults[209]. Fortunately, the past dozen years has provided a relative explosion of new and sometimes unexpected genes involved in the development of the lymphatic vascular system, based largely on elegant and exciting embryonic phenotypes uncovered in gene knockout studies in mice and in vertebrate model organisms like zebrafish and xenopus [210]. Some of these discoveries have even paved the way toward the identification and better understanding of human genes in which mutations are causally associated with congenital, primary lymphedema such as $\text{FOXC2}$, $\text{FLT4}$, $\text{SOX18}$, $\text{GJC}2$ and $\text{CCBE1}$. Nevertheless, the development of additional genetic mouse models of lymphatic insufficiency during adulthood is still needed in order to identify novel candidate genes either for genetic testing in families with congenital forms of lymphedema or for therapeutic targeting of lymphatics in disease.
Failure of lymphatic vessels to function properly in adults can result in numerous types of clinical conditions, including primary and secondary lymphedema, which can have a broad range of clinical presentations and associated correlates[211,212]. Some congenital forms of primary lymphedema are associated with lymphangiectasia, which is typically characterized as dilation and enlargement of lymphatic vessels. Interestingly, there are a few organ systems, including the intestine[213], the conjunctiva of the eye[214] and the dermis[215], that are particularly prone to developing lymphangiectasia. While the pathophysiological mechanisms leading to lymphangiectasia are not well understood, it is likely that dilated lymphatic vessels are the result of lymphatic obstruction and improper drainage or lymph stasis. The consequences of persistent lymphangiectasia include, on a cellular level, increased permeability of dilated lymphatic vascular beds, and on a systemic level, protein-losing enteropathy, limb lymphedema and ocular irritation with dryness. Although lymphangiectasia can be associated with a variety of primary, congenital lymphedema syndromes, there is currently no known genetic pathway that directly and predominantly contributes to lymphangiectasia.

Using gene targeting approaches in mice, we have previously discovered and characterized an essential role for adrenomedullin (gene=Adm, peptide=AM) peptide and its receptor complex in lymphatic vascular development. Adrenomedullin, a secreted, multi-functional peptide that is highly expressed in endothelial cells, binds and signals through a G protein-coupled receptor, calcitonin receptor-like receptor (gene=Calcrl; protein=CLR), when the receptor is associated with receptor activity modifying proteins 2 or 3 (RAMP2/3). The complex formed by CLR and RAMP2 is
referred to as the adrenomedullin 1 (AM1) receptor, while the CLR and RAMP3 complex is referred to as the AM2 receptor; both of which bind AM peptide, but differ in their relative binding affinities [216]. Gene knockout mice for Adm[31], Calcrl[34], and Ramp2[23,35] all exhibit mid-gestational embryonic lethality characterized by hydrops fetalis, or marked edema, that is associated with arrested lymphatic vascular development. Conditional deletion of Calcrl in endothelial cells confirmed that AM signaling, and its downstream activation of the MAPK/ERK signaling cascade, is required for normal lymphatic endothelial cell proliferation during development.

AM signaling through Calcrl/Ramp2 also has robust effects on endothelial cell permeability. For example, AM can abrogate the permeabilizing effects of hydrogen peroxide and thrombin on human umbilical vein endothelial cells[20] and it can retard the transport of molecules across the blood brain barrier by tightening the permeability of cerebral endothelial cells[21,217]. Similarly, we have shown that AM can impact the permeability and function of lymphatic endothelial cells (LECs). Treatment of cultured LECs with AM significantly and functionally reduced their permeability by causing a subcellular reorganization of the junctional proteins ZO-1 and VE-Cadherin[26]. Furthermore, in vivo tail microlymphography reinforced these findings since mice injected with AM showed reduced lymph velocity through dermal lymphatic capillaries, indicative of functionally reduced permeability[26].

The apparently biased effects of AM signaling on the embryonic development of lymphatic vessels, versus blood vessels, is likely attributable to the increased expression of Calcrl and Ramp2 in LECs, compared to blood endothelial cells.
Consistent with this notion, continuous administration of AM promoted lymphangiogenesis and ameliorated secondary tail lymphedema in a surgical injury mouse model [12]. Whether the maintained expression of \textit{Calcrl} in adult animals is also required for appropriate lymphatic function remains unclear. To address this question, we used a ubiquitously expressed, tamoxifen-inducible Cre transgenic mouse line (\textit{CAGGCre-ER}^{TM}) to delete a floxed \textit{Calcrl} gene in 3-4 month old animals and thus explore the role of \textit{Calcrl} during adulthood. Our results continue to support a preferential role for \textit{Calcrl} in the lymphatic vasculature and reveal that \textit{Calcrl} expression in adult animals is critical for maintaining the proper function of lymphatic vessels in a wide variety of organs.

\textbf{Methods}

\textit{Animals}

Mice used in these studies were generated from crossing \textit{Calcrl}^{fl/fl} [23] mice (N7-10 on C57BL/6 background) to \textit{CAGGCre-ER}^{TM} mice (The Jackson Laboratory, Bar Harbor, ME 004682, B6.Cg-Tg(CAG-Cre/Esr1)5Amc/J). Male and female adult mice aged 3-4 months were administered tamoxifen (Sigma) consecutively for 5 days (5mg/40g body weight; IP). Mice were genotyped for the floxed and Cre alleles as well as the excised allele after tamoxifen injection. Primer sets (5’-3’) P1: gcggagcatatcaatcacaag, P2: gaaatgtgctgtatgtccaagc, P3: gacgagttctctgagggga, and P4: gaataagttgagctgggcag were used (P1/P2 for wildtype allele; P1/P3 for floxed allele; P1/P4 for excised allele). Mice were routinely anesthetized using 0.2-0.4ml/10g body weight of avertin (2,2,2,-Tribromoethanol, 20mg/ml, Sigma).
For Western Diet studies, mice were fed Teklad Adjusted Calories Diet (TD.88137; 42% from fat; Harlan Laboratories) for 1½ weeks and then housed in metabolic cages for 24 hours during which food intake, urine, and fecal samples were measured. Weights of mice were also recorded before tamoxifen injection, after tamoxifen injection, and after Western Diet.

All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee of The University of North Carolina Chapel Hill and all efforts were made to minimize suffering.

Cell Culture

Human adult dermal lymphatic endothelial cells (HMVEC-dLyAd-Der Lym Endo Cells, Lonza) of 8 passages or less were maintained using EGM-2MV media with bullet kit (Lonza). Cells were seeded in 6 well plates at 100,000 cells/well and grown on acid washed coverslips until monolayers formed. Treatment conditions included no treatment (control), 10nM AM (American Peptide Co., Inc.), 1µM AM22-52 (AM antagonist; American Peptide Co., Inc.) or AM+AM22-52. Cells were treated for 15 minutes and in the condition with AM+AM22-52, cells were pre-treated with AM22-52 for 30 minutes. Cells were rinsed with HBSS, fixed with 1% PFA, rinsed 3x5 minutes with PBS, and then blocked for 20 minutes with 2% normal donkey serum/0.1% Triton X in PBS. The cells were then incubated overnight at room temperature with primary antibodies (VE-Cadherin=1:200, goat polyclonal; sc-6458, Santa Cruz Biotechnology, Santa Cruz, CA; ZO-1=1:200, monoclonal rat α mouse; clone R40.76, Millipore, Billerica, MA), rinsed 3x5 minutes with PBS, blocked
with 2% normal donkey serum (NDS) for 10 minutes, followed by incubation with secondary antibody for 1 hour at room temperature, rinsed 3x5 minutes with PBS and then mounted on slides using Mowiol.

**Immunohistochemistry and Immunofluorescence**

Tissues were dissected, fixed with 4% PFA overnight and embedded in paraffin or protected in 30% sucrose and embedded in OCT (Tissue-Tek) for sectioning. Sections were permeabilized using 0.1% Triton X-100 (in 0.01M PBS; pH 7.2; 15 minutes), blocked with 5% NDS (in 0.1% Triton X-100; 30 minutes), incubated overnight in primary antibodies, PBS rinsed (3x5 minutes), blocked with 5% NDS (30 minutes), incubated with secondary antibodies (2 hours), rinsed with PBS and coverslipped with Mowiol. Primary antibodies included: LYVE-1 (1:200; polyclonal rabbit α mouse; Fitzgerald, Acton, MA), podoplanin (1:200, Syrian hamster α mouse, Developmental Studies Hybridoma Bank, Univ. Iowa), ZO-1 (1:200, monoclonal rat α mouse; clone R40.76, Millipore, Billerica, MA) and VE-Cadherin (1:200, goat polyclonal; sc-6458, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies included Alexa Fluor 594, Alexa Fluor 488 and Cy3 (1:200, Jackson Immunoresearch) and nuclear marker DAPI (1:1000, bisbenzimide 33258; Sigma, St. Louis, MO). TUNEL staining was performed using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (S7110, Chemicon International) according to the manufacturer’s protocol.
**Tonometry**

Tonometry was performed in anesthetized adult mice using a TonoLab tonometer (Colonial Medical Supply) as described previously [219,220]. After avertin injection, a drop of tetracaine hydrochloride 0.5% (Alcon) was placed on the eye as a local anesthetic. Eyes were lubricated throughout testing with TEARS Naturale FORTE (Alcon). At least six readings were recorded per eye and averaged.

**Tail Microlymphography and Vessel Diameter**

Three to four months post tamoxifen injection, adult mice were used for tail microlymphography as described previously [26] with several modifications. FITC-conjugated dextran (200kDa; 1µl; Molecular Probes, Invitrogen Detection Technologies) was injected intradermally into the mouse tail using a 5µl Hamilton syringe fitted with a 30 gauge needle. Images were taken every minute for 15 minutes and image analysis was performed using Adobe Photoshop 7.0 and Image J.

**Lymphatic and Blood Permeability Assays**

An ear lymphatic permeability assay was performed as previously described [221] with minor modification. Ears of anesthetized mice were injected intradermally with 2µl of 0.5% Evan’s Blue dye (in saline) with a 10ul Hamilton syringe. Images were taken immediately after injection and 5 minutes after injection. A blood permeability assay was performed as previously described with slight
modifications to the protocol [222]. Anesthetized mice were retro-orbitally injected with 200µl 0.5% Evan’s Blue dye (in saline). After 30 minutes, the mice were perfused with saline and the liver, lung, adductor muscle, spleen, intestine, heart, and brain were harvested. Tissues were weighed and desiccated overnight at 55ºC followed by formamide extraction (55ºC, overnight) and 100µl was used for absorbance reading at 600nm.

**Acid Steatocrit/Lipase/Triglyceride Measurements**

Fecal samples collected after Western Diet were examined by testing for fecal steatocrit and fecal lipase as previously described[223] with recent modifications[224]. Fecal specimens were powdered and mixed with 1N perchloric acid and 0.5% oil red O and placed in a capillary tube and centrifuged. Steatocrit was calculated as 100 x {length of fatty layer/(length of solid layer+length of fatty layer)} . Fecal lipase and serum triglycerides were analyzed at the Animal Clinical Chemistry and Gene Expression Labs (UNC-CH).

**Dot Blot Assay**

A dot blot assay using digested fecal samples was performed as previously described [225] with slight modification. In short, mice were fed Western Diet for 1½ weeks and fecal samples were collected and stored at -20ºC. TBS with 5% nonfat dry milk (with protease inhibitors) was added to the fecal samples (20µl/mg). The samples were vortexed and sonicated then centrifuged at 16,000g for 10 minutes after which the supernatant was collected. Three microliters of each supernatant
(1:2000 dilution) was dotted onto a nitrocellulose membrane. The membrane was blocked (TBS+3% nonfat dry milk) for 2 hours at room temperature, rinsed with TBST (1x5 minutes), then incubated overnight with primary antibody (mouse anti alpha-1 antitrypsin-1:500; Novus and mouse anti-actin-1:10000; Sigma) in TBST+3% nonfat dry milk at 4°C. The membrane was rinsed with TBST (3x10 minutes), incubated with secondary antibody in TBST (HRP goat anti-mouse; 1:2000; Upstate) for 45 minutes at room temperature followed by TBST (3x5 minutes) rinses and a final TBS (1x5 minutes) rinse. The membrane was then developed with film (GeneMate) using WesternBright ECL reagents (Advanssta). Analysis of integrated density was performed using Image J.

**Edema Formation Assay**

Anesthetized mice were injected with 10µl of 4µg/µl Complete Freund’s Adjuvant (CFA) in one hind paw. The other hind paw served as an internal control. Paw thickness was measured with calipers before injection of CFA and every other day after injection up to 21 days.

**RNA and qRT-PCR**

Lung and heart tissue were collected in RNAlater Solution (Ambion). RNA was extracted from tissue using TRIZOL (Ambion, Life Technologies) isolation followed by DNAse treatment (Promega) and cDNA preparation. Quantitative RT-PCR was performed on a Stratagene Mx-3000p machine (La Jolla, CA) using TAQMAN GEx Master Mix (Applied Biosystems). *Calcr* expression was assessed
using Assay-on-Demand for Calcr (Mm00516986_m1; Applied Biosystems). The comparative quantitation (\( \Delta\Delta C_T \)) method was used to determine the relative level of Calcr expression in the tissues compared to mouse embryo total RNA calibrator (Ambion). All assays were repeated at least three times and run in duplicate.

**Statistical analysis**

All experiments were repeated at least 3 times and data are expressed as means with SEM values. Student t tests (tails=2, type=3) and two-way ANOVA were performed and \( P \leq 0.05 \) was considered significant.

**Results**

*Temporal deletion of Calcr results in acute onset eye phenotype with enlarged corneoscleral lymphatic vessels*

Tamoxifen injection resulted in a significant reduction of Calcr gene expression in Calcr\(^{fl/fl}\)/CAGGCre-\( \text{ERTM} \) animals compared to Calcr\(^{fl/fl}\) animals and to Calcr\(^{fl/fl}\)/CAGGCre-\( \text{ERTM} \) non-injected animals (control animals) as indicated by qRT-PCR of lung and heart tissue (Figure 3-S2). Within 7 to 10 days of tamoxifen injection, the majority of Calcr\(^{fl/fl}\)/CAGGCre-\( \text{ERTM} \) mice, but none of the control Calcr\(^{fl/fl}\) tamoxifen-injected mice, developed distinct graying of their eyes and the surface of their corneas appeared rough and coarse (Figure 3-1 A,B). The rapid onset of this phenotype prompted us to determine whether it was associated with glaucoma, since a previous study by Ittner et al. indicated that overexpression of Calcr in smooth muscle of mice resulted in a phenotype similar to glaucoma[226].
To this end, TUNEL staining indicated no difference in retinal ganglion cell death between $Calcr^{fl/fl}/CAGGCre-ER^TM$ and $Calcr^{fl/fl}$ control mice (Figure 3-S1A and 2-S1B). We also found no significant histological differences in the optic nerve of $Calcr^{fl/fl}/CAGGCre-ER^TM$ mice compared to $Calcr^{fl/fl}$ control mice (data not shown). Finally, we found no significant difference in the intraocular pressure of $Calcr^{fl/fl}/CAGGCre-ER^TM$ and $Calcr^{fl/fl}$ control mice when compared either before injection or after injection of tamoxifen (Figure 3-S1C) and all intraocular pressure measurements were within the normal range for C56BL/6 mice[219]. Taken together, these data rule out the possibility that the acute-onset eye phenotype in $Calcr^{fl/fl}/CAGGCre-ER^TM$ mice is associated with classical features of glaucoma.

However, hematoxylin and eosin staining of eyes revealed marked changes in histology of the $Calcr^{fl/fl}/CAGGCre-ER^TM$ corneas relative to those of $Calcr^{fl/fl}$ control mice. The corneas of $Calcr^{fl/fl}/CAGGCre-ER^TM$ mice were thickened and edematous and often showed a disrupted and damaged epithelial lining (Figure 3-1C,D, arrow). We also observed pronounced inflammation in the anterior chamber and cornea of $Calcr^{fl/fl}/CAGGCre-ER^TM$ mice (Figure 3-1 E,F, arrowhead).

Based on the well-established role of $Calcr$ in lymphatic vascular development[23], the edema and inflammation in the corneas of $Calcr^{fl/fl}/CAGGCre-ER^TM$ mice suggested to us that there may be problems with the lymphatic vessels of the eyes, particularly those within the corneoscleral junction, which is analogous to the conjunctival lymphatics in humans. Specifically, we found podoplanin-positive and LYVE-1-positive staining in the ciliary body and in vessels at the corneoscleral junction (Figure 3-1 G,H, arrow). Interestingly, the lymphatic vessels at the
corneoscleral junction of Calcrl<sup>fl/fl</sup>/CAGGCre-ER<sup>TM</sup> mice were significantly dilated and twice the size of corneoscleral lymphatics in Calcrl<sup>fl/fl</sup> control mice (Figure 3-1 I,J,K), similar to the phenotype observed in humans with conjunctival lymphangiectasia. More importantly, the eyes of Calcrl<sup>fl/fl</sup>/CAGGCre-ER<sup>TM</sup> mice that did not present with the overt corneal pathology (approximately 1/3<sup>rd</sup> of the mice), either because they failed to develop the phenotype or they were euthanized prior to the onset of the phenotype, still showed significantly dilated lymphatics at the corneoscleral junction. Taken together, these data demonstrate that an abnormal lymphatic vessel phenotype precedes the onset of acute corneal pathology in Calcrl<sup>fl/fl</sup>/CAGGCre-ER<sup>TM</sup> mice.

Calcrl<sup>fl/fl</sup>/CAGGCre-ER<sup>TM</sup> mice exhibit enlarged submucosal lymphatic vessels and lacteals in the intestine with dysfunctional mesenteric collecting lymphatic vessels.

Since dilated lymphatic vessels were observed at the corneoscleral junction in the Calcrl<sup>fl/fl</sup>/CAGGCre-ER<sup>TM</sup> mice, we wanted to assess the morphology and function of lymphatic vessels in other lymphatic vascular beds, for example, within the intestine. The overall histology of the intestines of Calcrl<sup>fl/fl</sup>/CAGGCre-ER<sup>TM</sup> mice was normal when compared to that of Calcrl<sup>fl/fl</sup> control mice under normal conditions (Figure 3-2A,B). Lymphatic vessels within the intestine were identified with LYVE-1 and podoplanin staining, showing co-localization within the lacteals and the submucosal lymphatics (Figure 3-2C-E). Interestingly, the submucosal lymphatics and lacteals of the jejunum were also markedly dilated in the Calcrl<sup>fl/fl</sup>/CAGGCre-ER<sup>TM</sup> mice (Figure 3-2F,G), as evidenced by the visibly larger diameter of the
submucosal lymphatics and a greater proportion of villi sections revealing enlarged, LYVE-1-positive lacteals. Once again, these dilated lymphatics vessels are reminiscent of the dilated lymphatics observed in human patients with intestinal lymphangiectasia.

Intestinal lymphatics are required for normal lipid absorption, and patients with intestinal lymphangiectasia often present with weight loss as a result of lipid malabsorption [227]. Therefore, the function of these vessels was evaluated by placing Calcrl$^{fl/fl}$/CAGGCre-ER$^{TM}$ and Calcrl$^{fl/fl}$ control mice on a short term Western Diet following an overnight fast. After 1½ hours of Western Diet, the Calcrl$^{fl/fl}$/CAGGCre-ER$^{TM}$ mice exhibited chyle-filled mesenteric lymphatic vessels which were not visible in the Calcrl$^{fl/fl}$ control mice (Figure 3-2H,I). Chyle-filled submucosal lymphatic vessels were also visibly distinguishable in Calcrl$^{fl/fl}$/CAGGCre-ER$^{TM}$ mice and contributed to the whitened appearance of the intestine (Figure2-2I), and some animals additionally exhibited chyle leakage into the mesenteric space. Importantly, the lymphatic valves of Calcrl$^{fl/fl}$/CAGGCre-ER$^{TM}$ mice appeared normal and were present at regular intervals along the mesenteric collecting lymphatics (Figure 3-2I, arrows and inset). Therefore, although the intestinal lymphatic vessels of Calcrl$^{fl/fl}$/CAGGCre-ER$^{TM}$ mice were present and appeared overtly normal, the collecting vessels were significantly dysfunctional in their transport of chyle as compared to control mice.
Reduced body weight and impaired lipid absorption with protein-losing enteropathy in Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice

We next wanted to assess the impact of a longer term high fat diet on intestinal lipid absorption in the Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice. There were no significant differences in body weights between 3-4 month old, male or female Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice and Calcrl\textsuperscript{fl/fl} control mice before the injection of tamoxifen (Figure 3-3A,B). However, 3-4 months after the injection of tamoxifen, we found that the Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice weighed significantly less than their control counterparts (Figure 3-3A,B), indicating that the tamoxifen-induced loss of Calcrl contributes to a failure of Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} to gain weight and thrive. The failure of Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice to gain weight and thrive was significantly exacerbated (Figure 3-3A,B) and visibly apparent (Figure 3-3C) when the mice were fed a Western Diet for 1½ weeks.

Moreover, fecal acid steatocrit levels, representative of lipid excretion levels, were significantly elevated in Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} animals maintained on a Western Diet for 1½ weeks compared to similarly fed Calcrl\textsuperscript{fl/fl} control animals (Figure 3-3D), demonstrating reduced lipid absorption. Consistently, fecal pancreatic lipase levels were also increased in Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} animals, likely due to the compensatory effects of pancreatic lipase conversion of triglycerides into monoglycerides and free fatty acids during periods of reduced lipid absorption (Figure 3-3E). Importantly, levels of circulating triglycerides were unchanged in the Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice compared to Calcrl\textsuperscript{fl/fl} control animals (Figure 3-3F), indicating that overall metabolism is not compromised in Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM}
mice and supporting the conclusion that their failure to gain weight is due to abnormal lipid absorption in the intestine. Finally, fecal samples of Western Diet-fed *Calcr*^{fl/fl}/CAGGCre-ER^{TM} mice contained a significantly elevated level of alpha-1 antiprtrysin—a clinical diagnostic marker for protein-losing enteropathy—compared to similarly fed *Calcr*^{fl/fl} control mice (Figure 3-3G,H).

**Temporal deletion of *Calcr* results in increased dermal lymphatic capillaries with exacerbated and prolonged edema.**

The dermal lymphatic capillaries of *Calcr*^{fl/fl}/CAGGCre-ER^{TM} mice also exhibited significant dilation and dysfunction. Specifically, intradermal injection of a large molecular weight (200kDa) FITC-dextran into the subdermal area of the tail tip revealed significantly enlarged dermal capillaries in *Calcr*^{fl/fl}/CAGGCre-ER^{TM} mice compared to *Calcr*^{fl/fl} control mice (Figure 3-4A,B,C). Despite this dermal lymphangiectasia, we noticed that at the basal or quiescent state, *Calcr*^{fl/fl}/CAGGCre-ER^{TM} mice did not exhibit pronounced edema in their extremities (Figure 3-5F, day 0). Thus, we injected the hindpaws of *Calcr*^{fl/fl}/CAGGCre-ER^{TM} and *Calcr*^{fl/fl} control mice with (CFA) in order to challenge the lymphatic vascular system with localized edema. Both *Calcr*^{fl/fl}/CAGGCre-ER^{TM} mice and *Calcr*^{fl/fl} control mice exhibited a rapid and significant increase in hindpaw thickness within 1 day of CFA injection (Figure 3-4D). However, unlike the *Calcr*^{fl/fl} control mice which immediately began to resolve their edema by day 3, the *Calcr*^{fl/fl}/CAGGCre-ER^{TM} mice developed exacerbated and prolonged edema that peaked between days 9-11, and only began to show slight improvement after two weeks following CFA injection.
(Figure 3-4D). These data demonstrate that the expression of \textit{Calcrl} is required for maintaining highly effective lymphatic function under conditions of edema and inflammation.

\textit{Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM}} mice exhibit increased lymphatic capillary permeability with no apparent disruption of blood vascular permeability.

To better evaluate the permeability of lymphatic and blood vasculatures in \textit{Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM}} and \textit{Calcrl\textsuperscript{fl/fl}} mice, we used the small molecular weight Evan’s blue dye which can freely penetrate in and out of dermal capillaries. Injection of 0.5\% Evan’s blue dye intradermally in the ear showed rapid uptake of the dye by dermal lymphatics in both \textit{Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM}} and \textit{Calcrl\textsuperscript{fl/fl}} mice (Figure 2-5A,B). However after 5 minutes, \textit{Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM}} mice exhibited increased leakage of the dye from the lymphatic vessels, as evidenced by the diffuse spreading of the dye and poorly demarcated lymphatics throughout the ear region compared to the \textit{Calcrl\textsuperscript{fl/fl}} control mice (Figure 3-5C,D). To determine whether this lymphatic permeability defect was impacted or perhaps confounded by a permeability defect in the blood vasculature, we also measured relative blood vascular permeability in mice receiving a venous injection of Evan’s blue dye. Absorbance readings of Evan’s blue dye showed no statistically significant differences between \textit{Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM}} mice and \textit{Calcrl\textsuperscript{fl/fl}} control mice for multiple tissues including liver, lung, adductor muscle, spleen, intestine, heart and brain (Figure 2-5E). Based on these data, we conclude that temporal deletion of
Calcrl in adult animals results in increased lymphatic capillary permeability with no overt or functional changes in blood vascular permeability.

Inhibition of AM signaling results in disorganization of lymphatic endothelial cell junctions.

To elucidate the molecular mechanisms contributing to the lymphatic dysfunction in Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice, we evaluated VE-Cadherin expression and localization in mesenteric lymphatic vessels of Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} and Calcrl\textsuperscript{fl/fl} control mice that had been fed a high fat diet for 1½ hours. VE-Cadherin expression was visibly disrupted in lymphatic vessels of Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice (Figure 3-6B,D) compared to control mice (Figure 3-6A,C). More specifically, while the relative expression levels of VE-cadherin appeared similar between genotypes, the VE-cadherin in mesenteric lymphatic vessels of Calcrl\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} appeared as punctate lobules throughout the cells and was not localized to well-defined cell boundaries, as seen in the Calcrl\textsuperscript{fl/fl} control mice.

To better characterize the effects of inhibiting Calcrl-mediated signaling in cultured lymphatic endothelial cells (LECs) we used an adrenomedullin-specific peptide inhibitor, AM22-52. As we have previously described, treatment of LECs with AM peptide resulted in a marked reorganization of junctional proteins, VE-Cadherin and ZO-1, from a jagged, zipper-like configuration to a cohesive and stabilized cell-cell barrier that is functionally associated with reduced permeability (Figure2- 6E,F and[26]). In contrast, treatment with the Calcrl antagonist, AM22-52, either alone or in combination with AM, abolished the effects of AM peptide and
resulted in highly disorganized and jagged junctional protein configurations (Figure 3-6G,H). Taken together, these results demonstrate that in vitro and in vivo inhibition of Calcrl signaling, either by antagonist treatment or by genetic deletion, results in a profound loss of junctional protein organization, likely resulting in increased permeability of lymphatic endothelial cell barriers.

Discussion

These studies demonstrate that temporal loss of murine Calcrl in adulthood causes lymphatic insufficiency in a wide range of organs, representing functional similarities to the sequelae observed in patients with a variety of lymphangiectasia conditions. Consistently, the lymphatic vessels in the eye, intestine and skin of Calcrlfl/fl/CAGGCre-ERTM mice were dilated, had irregular junctional protein organization and were dysfunctional when challenged with either fat absorption or edema and inflammation. Taken together, these data identify an important new role for AM signaling as a potent regulator of lymphatic vascular drainage and permeability in adult animals.

The rapid-onset eye phenotype in Calcrlfl/fl/CAGGCre-ERTM mice provides novel and clinically relevant insights to the potential role of lymphatic vessels in the eye. Several recent studies have shown that lymphatic markers are expressed in the human eye [228,229], but it is still unclear whether and how these lymphatic vessels contribute to fluid homeostasis of the eye. Our staining of lymphatic markers in the eye correlates well with these previous studies, since we showed robust LYVE-1 and podoplanin staining in the ciliary body and distinct LYVE-1-
positive lymphatic vessels in the corneoscleral junction. Most importantly, we found that temporal deletion of Calcr resulted in dilated corneoscleral lymphatic vessels that preceded and were associated with the formation of corneal edema and inflammation. Therefore, it is likely that appropriate fluid homeostasis and hydration of the cornea, which is an important physiological feature to consider in terms of dry eye disease, corneal surgeries or conjunctival lymphangiectasia, is modulated by lymphatic vessels. Since AM peptide can be clinically administered [230] and the Calcr/Ramp interface is pharmacologically tractable [231,232], the potential of harnessing these targets for the therapeutic modulation of fluid homeostasis in the eye may prove to be an exciting avenue.

The intestinal lymphatic phenotype of Calcr^{fl/fl}/CAGCre-ERTM mice also correlates well with the clinical presentation of intestinal lymphangiectasia in humans. Under a short-term Western Diet, Calcr^{fl/fl}/CAGCre-ERTM mice showed signs of lymphatic insufficiency because their intestinal mesenteric lymphatic vessels failed to transport chyle as effectively as similarly fed control mice. Because Calcr^{fl/fl}/CAGCre-ERTM mice are significantly leaner than age-matched control mice 3-4 months post tamoxifen injection, it is likely that the collecting mesenteric lymphatics of these animals function at a consistently reduced capacity. In this regard, it is important to note that weight loss is often associated with lymphangiectasia in the form of protein-losing enteropathy[212] and lipid malabsorption. Consistently, the Calcr^{fl/fl}/CAGCre-ERTM mice also exhibit elevated alpha-1 antitrypsin in fecal samples after Western diet, which is indicative of protein-losing enteropathy, similar to the clinical phenotype that is frequently observed in
humans with intestinal lymphangiectasia. While the mechanism of lipid absorption through lymphatic lacteals is not completely understood, it is thought to involve both active and passive transport mechanisms through lymphatic endothelial cells[19]. Our data demonstrate that AM signaling through Calcr/Ramp2 is required for normal intestinal lipid uptake and junctional protein organization in intestinal lymphatic capillaries. Whether the maintenance of the lymphatic permeability barrier and loss of Calcr is connected with the active and/or passive transport mechanism of lipid absorption within the lacteal will be an important future area of study that may have bearing on better understanding the functional underpinnings of intestinal lymphangiectasia.

It is notable that lymphangiectasia is commonly associated with limb edema. When placed under challenge, we found that the hindpaw of Calcr\textsuperscript{fl/fl}/CAGGCre-\textsuperscript{ER\textsuperscript{TM}} mice had significantly exacerbated edema that resolved over a longer time period than similarly challenged wildtype animals. These results correlate with the results from the high fat diet in that the lymphatic system of the Calcr\textsuperscript{fl/fl}/CAGGCre-\textsuperscript{ER\textsuperscript{TM}} mice does not respond effectively as that of control mice to different stresses indicating that there are dysfunctional lymphatic vessels in the Calcr\textsuperscript{fl/fl}/CAGGCre-\textsuperscript{ER\textsuperscript{TM}} mice.

Importantly, the Calcr\textsuperscript{fl/fl}/CAGGCre-\textsuperscript{ER\textsuperscript{TM}} mice do not exhibit overt edema in the basal state and we found no significant effects of Calcr loss on blood vascular permeability. Studies by T. Shindo and colleagues using Ramp2 gene targeted mice suggested that loss of Ramp2 led to a reduction in the expression of junctional proteins and a loss of blood vascular integrity[19]. Using an independent line of
Ramp2 gene targeted mice, we have demonstrated that Ramp2\(^{-/-}\) mice also have arrested lymphangiogenesis[23]. Because Ramp2 associates with multiple G protein-coupled receptors beyond Calcrl, it is likely that the expanded vascular phenotypes of Ramp2\(^{-/-}\) mice can be attributed to additional signaling pathways, and this notion is further supported by the extensive endocrine phenotypes of Ramp2\(^{+/+}\) mice compared to Calcrl\(^{+/+}\) mice[233]. Taken together, these data continue to support a predominant and preferential role for Calcrl in the lymphatic vasculature compared to the blood vasculature[46], which may be partially explained by the fact that Calcrl and Ramp2 are expressed at higher levels in lymphatic endothelial cells compared to blood endothelial cells[24,25,218].

Calcrl also serves as a receptor component for the neuropeptide, calcitonin gene related peptide (CGRP), when the receptor is associated with RAMP1. Therefore, we cannot formally exclude the possibility that the phenotypes from temporal loss of Calcrl are not partially attributable to loss of CGRP signaling. For example, other studies have indicated that adult αCGRP knockout mice fed a high fat diet do not gain as much weight as control mice. However, in contrast to the Calcrl\(^{fl/fl}\)/CAGGCre-ER\(^{TM}\) mice, the αCGRP knockout mice eat more and have higher levels of energy expenditure compared to controls [234]. Also, in our colony, αCGRP knockout mice and RAMP1 knockout mice have never exhibited the visible eye phenotype that is hallmark of the Calcrl\(^{fl/fl}\)/CAGGCre-ER\(^{TM}\) mice. Finally, the αCGRP knockout mice [235-237] are not embryonic lethal and no vascular defects have been reported in these mice. In contrast, many similarities exist between the phenotypes of Calcrl knockout mice and those of AM and RAMP2 knockout mice,
with a primary defects in the vasculature. Therefore, while some implication of CGRP signaling cannot be excluded in the Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{Tm} mice, the phenotypes revealed are more consistent with a predominant attribution to AM signaling. Nevertheless, additional characterization of Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{Tm} mice for phenotypes more closely associated with the physiological functions of CGRP, like pain perception, will be an interesting future direction.

Ultimately these studies indicate functional similarities between temporal loss of Calcr in adult mice and human lymphangiectasia, but the mechanistic relationship remains elusive and will be an interesting focus for future studies. The underlying cause of lymphatic insufficiency in Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{Tm} mice is likely attributable to various mechanisms including insufficient lymph transport and disrupted lymphatic vessel permeability. The Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{Tm} mice do not respond sufficiently to stress on the lymphatic system indicating the lymphatic network is dysfunctional. Moreover, our permeability findings are consistent with previous studies showing that addition of AM both \textit{in vitro} and \textit{in vivo} results in decreased permeability of LECs and lymphatic vessels through reorganization of the junctional proteins VE-cadherin and ZO-1[26]. In the blood vasculature, Rap1 (Ras-related protein 1), a small GTPase, plays a predominant role in regulating cell adhesion and cell junction organization in response to cAMP/Epac/ERK signaling pathways[238]. Since the major downstream effectors of AM signaling in LECs are cAMP/Epac/ERK, it will be interesting in future studies to determine whether similar or identical Rap1 mechanisms contribute to the lymphatic permeability phenotypes of Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{Tm} mice.
Author Contributions

Conceived and designed the experiments: SLH and KMC. Performed the experiments: SLH and HHW. Analyzed the data: SLH and KMC. Contributed reagents/materials/analysis tools: SLH, HHW, and KMC. Wrote the paper: SLH and KMC.
Figure 3-1. Acute-onset eye phenotype, eye inflammation, edema, and enlarged lymphatic vessels in Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>M</sup> mice. A,B, Gross eye images indicating normal appearance of the control Calcrl<sup>fl/fl</sup> mice (A) and the distinct color change and disruption of the cornea of Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>M</sup> (B), (scale = 2mm). C,D, Hematoxylin and eosin staining of mouse eyes indicating normal histology in Calcrl<sup>fl/fl</sup> (C) and disruption of the cornea in Calcrl<sup>fl/fl</sup>/CAGG-CreERT<sup>M</sup> mice (D), (4x objective, scale = 500µm). E,F, Higher magnification of histological sections of eyes from Calcrl<sup>fl/fl</sup> mice (E) as compared to Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>M</sup> mice (F) exhibiting corneal edema (arrow) and inflammation (arrowhead) (10x objective, scale=200µm). Gross anatomy and histology images are representative from Calcrl<sup>fl/fl</sup> mice (n=8) and Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>M</sup> mice (n=9). G, Eye diagram indicating the location of components of the eye (l=lens, c=cornea, ac=anterior chamber, cb=ciliary body, i=iris). H, Lymphatic markers expressed in the eye shown by podoplanin(red) and Lyve-1(green) staining in a control mouse eye (20x objective, scale=100 µm). I,J, Visualization of lymphatic vessels at the corneoscleral junction in the Calcrl<sup>fl/fl</sup> (I) and Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>M</sup> mice (J) indicating enlarged lymphatic vessels in Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>M</sup> mice (Lyve-1=green; DAPI=blue; 20x objective, scale=100µm). K, Graph representing increased lymphatic vessel area at the corneoscleral junction in Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>M</sup> mice compared to control mice calculated using Image J software(*p<0.015). Mice used were 3-4 months of age.
Figure 3-2. Dilated lacteals and submucosal lymphatics in \textit{Calcrl}^{fl/fl}/CAGGCre-\textit{ER}^{TM} mice and chyle-filled lymphatics after short-term Western diet. A,B, Hematoxylin and eosin staining of mouse intestine showing normal histology in both \textit{Calcrl}^{fl/fl}(A) and \textit{Calcrl}^{fl/fl}/CAGGCre-\textit{ER}^{TM} mice(B) (6.3x objective, scale=500µm). C,D,E, Lymphatic marker expression in the lacteals and submucosal lymphatic vessels in wildtype mouse. Image was obtained from the jejunum of the intestine. Lyve-1 (C,green) and podoplanin(D,red) colocalize in the lymphatic vessels as seen in the merged image(E) (20x objective; scale=100µm). F,G Lyve-1(green) and DAPI(blue) staining in \textit{Calcrl}^{fl/fl}(F) and \textit{Calcrl}^{fl/fl}/CAGGCre-\textit{ER}^{TM} (G) mice indicating dilated lacteals and submucosal lymphatic vessels with temporal deletion of \textit{Calcrl} in the jejunum of the intestine (4x objective, scale=500µm). Histology and immunofluorescent images are representative from \textit{Calcrl}^{fl/fl} mice (n=7) and \textit{Calcrl}^{fl/fl}/CAGGCre-\textit{ER}^{TM} mice (n=6). H,I, Chyle-filled mesenteric collecting lymphatic vessels in \textit{Calcrl}^{fl/fl}/CAGGCre-\textit{ER}^{TM} mice (I) relative to non-chyle filled vessels in control animals (H). Valves are distinctly visible in \textit{Calcrl}^{fl/fl}/CAGGCre-\textit{ER}^{TM} mice (arrows; inset refers to enlarged image of valve; scale=3mm; n=4 per genotype). Mice used were 6-8 months of age.
Figure 3-3. *Calcrl*<sup>fl/fl</sup>/CAGGCre-ER<sup>TM</sup> mice exhibit reduced body weight due to impaired lipid absorption. A,B, Graphs of female(A) and male(B) body weights before injection of tamoxifen (Pre-TAM; 3-4 months of age), after injection of tamoxifen (Post-TAM; 3-4 months after), and after 1½ weeks on Western Diet (After WD). Both male and female *Calcrl*<sup>fl/fl</sup>/CAGGCre-ER<sup>TM</sup> mice were significantly smaller than *Calcrl*<sup>fl/fl</sup> mice after tamoxifen injection and after Western Diet. C, Image of *Calcrl*<sup>fl/fl</sup> and *Calcrl*<sup>fl/fl</sup>/CAGGCre-ER<sup>TM</sup> mice after 1½ weeks Western Diet. D, Acid steatocrit measurement in fecal samples from *Calcrl*<sup>fl/fl</sup> and *Calcrl*<sup>fl/fl</sup>/CAGGCre-ER<sup>TM</sup> mice after Western Diet for 1½ weeks indicating increased lipid excretion in the experimental mice. E, Lipase measurements in fecal samples from *Calcrl*<sup>fl/fl</sup> and *Calcrl*<sup>fl/fl</sup>/CAGGCre-ER<sup>TM</sup> mice on Western Diet for 1½ weeks indicating increased fecal lipase in experimental mice. F, Total triglyceride levels in *Calcrl*<sup>fl/fl</sup> and *Calcrl*<sup>fl/fl</sup>/CAGGCre-ER<sup>TM</sup> mice. G, Alpha-1 antitrypsin levels in fecal samples from *Calcrl*<sup>fl/fl</sup> and *Calcrl*<sup>fl/fl</sup>/CAGGCre-ER<sup>TM</sup> mice after 1½ weeks Western diet indicating lower levels in *Calcrl*<sup>fl/fl</sup>/CAGGCre-ER<sup>TM</sup> mice. (Integrated density values are scaled and should be multiplied by 10<sup>5</sup>). H, Image of dot blot assay for alpha-1 antitrypsin in *Calcrl*<sup>fl/fl</sup> and *Calcrl*<sup>fl/fl</sup>/CAGGCre-ER<sup>TM</sup> mice fecal samples after Western diet (1:2000 dilution of samples). (*p<0.03; **p<0.002).
Figure 3-4. Dilated dermal lymphatic capillaries with exacerbated and prolonged edema. A, B, Images of dermal lymphatic capillaries in the tail of \textit{Calcrl}^{fl/fl} (A) and \textit{Calcrl}^{fl/fl}/CAGGCre-ER$^{TM}$ (B) mice indicating increased diameter of these lymphatic vessels in \textit{Calcrl}^{fl/fl}/CAGGCre-ER$^{TM}$ mice (scale= 0.5mm). C, Graphic representation of the increase in vessel diameter in the \textit{Calcrl}^{fl/fl}/CAGGCre-ER$^{TM}$ mice with respect to \textit{Calcrl}^{fl/fl} mice (*p$\leq$0.05). D, Edema formation assay using hindpaw injections of CFA (4µg/µl on Day 0). Assessment of paw thickness over 3 weeks (n=5 for \textit{Calcrl}^{fl/fl} and n=4 for \textit{Calcrl}^{fl/fl}/CAGGCre-ER$^{TM}$ mice) indicated enhanced and prolonged edema in \textit{Calcrl}^{fl/fl}/CAGGCre-ER$^{TM}$ mice relative to control mice (**p$<0.01$, *p$<0.001$). Representative images of CFA-injected hindpaws at Day 11 for \textit{Calcrl}^{fl/fl} and \textit{Calcrl}^{fl/fl}/CAGGCre-ER$^{TM}$ mice (scale=3mm). Mice used were 6-8 months of age.
Figure 3-5. Increased lymphatic vascular permeability without change to blood vascular permeability in *Calcr* 
*Calcr* 
A,B,C,D, In vivo lymphatic permeability assay assessing the leakage of Evan’s blue dye from the dermal lymphatic vessels in the ear. Images represent Evan’s blue dye location directly after injection of the dye and 5 minutes post injection. There is an increase in leakage of the dye from the *Calcr* 
*Calcr* 
E, Blood vascular permeability assay indicating there is no difference in permeability between genotypes in the various tissues (n=4 per genotype for each tissue).
Figure 3-6. Inhibition of AM signaling disrupts lymphatic endothelial cell-cell junctions. A,B,C,D, Confocal images of VE-Cadherin (red) and Lyve-1 (green) expression in mesenteric lymphatic vessels of Calcrl^{fl/fl} (A,C) and Calcrl^{fl/fl}/CAGGCre-ER^{TM} mice (B,D) (scale=10µm). Boxed region depicted in C and D. Junctional protein, VE-cadherin, is disorganized in Calcrl^{fl/fl}/CAGGCre-ER^{TM} mice relative to Calcrl^{fl/fl} mice (representative images from n=4 per genotype, age 6-8 months). 
E,F,G,H, Lymphatic endothelial cells stained with VE-Cadherin (green), ZO-1 (red) and DAPI (blue) after various treatments including a no treatment control (A), 10nm AM (B), 1µm AM22-52 (C), AM+AM22-52 (D) (arrow refers to inset region). Disorganization of cell-cell junctions occurs with inhibitor treatment (AM22-52) as compared to AM treatment. (VE-cadherin=red, Lyve-1=green, DAPI=blue, 40x objective, scale=100µm; representative images from 3 independent experiments).
Figure 3-S1: The acute onset eye phenotype with temporal deletion of *Calcrl* is not associated with glaucoma-like characteristics. A,B, TUNEL staining of retinal ganglion cells (arrows) in *Calcrl*^fl/fl^ (A) and *Calcrl*^fl/fl^/CAGGCre-ER^TM^ mice (B) (DAPI=red; TUNEL=green; scale=100µm) C, Tonometry measurements of intraocular pressure in *Calcrl*^fl/fl^ and *Calcrl*^fl/fl^/CAGGCre-ER^TM^ mice before tamoxifen injection and one month post-injection.
Figure 3-S2: *Calcrl* gene expression in lung and heart tissue from *Calcrl*^{fl/fl} and *Calcrl*^{fl/fl}/CAGGCre-ER^{TM} mice. A, qRT-PCR quantitation of relative expression of *Calcrl* normalized to mouse elongation factor in *Calcrl*^{fl/fl} and *Calcrl*^{fl/fl}/CAGGCre-ER^{TM} non-injected control mice relative to *Calcrl*^{fl/fl}/CAGGCre-ER^{TM} mice. There is a significant reduction in *Calcrl* expression in both lung and heart tissue of *Calcrl*^{fl/fl}/CAGGCre-ER^{TM} mice relative to control mice (*p<0.04, **p<0.02).
Overview

Adrenomedullin (AM=protein; Adm=gene) and its signaling components consisting of its G-Protein coupled receptor, calcitonin receptor-like receptor (Calcrl) and its receptor activity modifying protein 2 (Ramp2) have been established to play a role in the development of the lymphatic vascular system. More recent studies have elucidated a role for this signaling system in the maintenance and function of lymphatic vessels during adulthood. Lymphatic vessels are typically quiescent during adulthood and it is not completely understood if adrenomedullin affects these vessels under conditions of induced lymphangiogenesis, such as in response to a wound. Using a minimally invasive ear wound model as a means to stimulate lymphangiogenesis as well as angiogenesis in adult mice, these studies demonstrated that there is reduced lymphangiogenesis in adult Adm+/− mice relative to control mice. There is also an associated reduction in vessel diameter after 28 days of wound healing. These results demonstrate that a modest 50% reduction in adrenomedullin significantly impairs wound-induced lymphangiogenesis.

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Introduction

Adrenomedullin (AM=protein, Adm=gene) is a small peptide vasodilator that is highly expressed in vascularized tissues and particularly in endothelial cells. This signaling system consists of adrenomedullin, its G-Protein coupled receptor calcitonin receptor-like receptor (Calcrl) and its associated receptor activity modifying protein (Ramp2/3). It has been extensively characterized that adrenomedullin plays a role in the blood vascular system. AM is upregulated in various cardiovascular conditions [2,76,77] and is a potent angiogenic factor as well as a cardioprotective factor [2]. Also, AM is known to directly affect endothelial cell proliferation and permeability. In vitro experiments with human umbilical vein endothelial cells (HUVECs) indicated that AM regulates permeability [18], migration [18], and tube-formation [15] of these cells. A hind-limb ischemia model confirmed these results by showing that AM promotes endothelial cell proliferation and capillary formation in vivo [57]. AM can reduce the permeability of HUVECs treated with permeabilizing agents including hydrogen peroxide and thrombin [20]. Also, AM has been shown to regulate the transport of molecules across the blood brain barrier in cerebral endothelial cells by modulating permeability [21]. AM activates endothelial Akt that promotes in vitro and in vivo vascular regeneration [13]. Taken together, these data indicate that AM is an essential component for proper endothelial cell function.

More recently the role of AM signaling in the lymphatic vascular system has begun to be elucidated. The lymphatic vascular system parallels the blood vascular system and primarily functions to regulate and maintain fluid homeostasis in the
body. AM signaling is necessary for the proper development of the lymphatic system. When there is genetic deletion of Adm, the lymphatic system is unable to form properly due to decreased proliferation of lymphatic endothelial cells [23]. However, the implications of adrenomedullin in adult lymphatics are not completely understood. Recent studies indicated that adult mice with temporal deletion of Calcrl, exhibited lymphangiectasia, or dilated lymphatic vessels, in a variety of lymphatic vascular beds [208]. Also, another recent study indicated that tumor derived adrenomedullin stimulates lymph node lymphangiogenesis in adult mice [239]. In a surgical tail injury model, infusion of AM resulted in increased lymphangiogenesis and reduced lymphedema in mice [12]. It is still not completely understood how adrenomedullin impacts lymphangiogenesis in adult mice. Therefore, this study used a minimally invasive ear wound model to stimulate lymphangiogenesis and angiogenesis in wildtype and AM$^{+-}$/mice. The vascular systems are typically quiescent during adulthood, but these vessels have growth potential in response to wounds because a major component of wound healing is neovascularization. Here we reveal that lymphangiogenesis is affected by a genetic reduction of adrenomedullin signifying that adrenomedullin signaling is a critical component for sufficient wound-induced lymphangiogenesis.

**Methods**

**Animals**

Mice used in these studies were generated and maintained by crossing Adm$^+/+$ to Adm$^{+-}$ mice on a SvEv129/6-TC1 background as described previously
Adult male mice (8 weeks old) were used in these studies. Mice were genotyped as previously described [240]. In short, primer sets included: primer 1, 5′-CAGTGAGGAATGCTAGCCTC-3′; primer 2, 5′-GCTTCCTCTTGCAAACCA-3′; and primer 3, 5′-TCGAGCTTCCAAGGAAGACCAGG-3′. Primers 1 and 3 amplified the wildtype 1.8 kb product and primers 2 and 3 amplified the 1.3 kb targeted product.

All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee of The University of North Carolina at Chapel Hill.

**Ear Wound Assay**

Using a 2 mm hole punch (Roboz), a hole was punched out of the ears of adult (8 weeks old)  Adm+/− and  Adm+/+ male mice. The ears were allowed to heal for 28 days. After wound closure, ears were imaged using a Leica dissecting microscope. Images were analyzed in Image J to determine the hole area which was then compared to the initial hole size to determine the percent wound closure.

**Immunofluorescence and Imaging**

Ear tissue was harvested and peeled apart to expose both internal sides of the ear skin. Then, the middle collagen layer was removed from the ear skin. The ear tissue was fixed in 4% PFA overnight and stained for lymphatic vessels using LYVE-1 (1:100; polyclonal rabbit anti-mouse; Fitzgerald, Acton, MA) and blood vessels using PECAM-1 (4µg/ml; mouse anti-rat; BD Biosciences, San Jose, CA). Secondary antibodies included Cy2 (1:100; Jackson Immunoresearch) and Cy3.
(1:250; Jackson Immunoresearch). Once stained, the ears were imaged on a Leica SP2 Laser Scanning Confocal Microscope using the 20x objective. In order to increase clarity of the ear whole mounts, Z series were generated using the confocal microscope. Six different areas in the healing margin of the wound of each ear were imaged.

**Vessel Density and Diameter Analysis**

Images were analyzed using Adobe Photoshop and Image J. The images were first opened in Adobe Photoshop and the region of the image containing the ear hole was excised to exclude this area from the analysis. The images were then analyzed in Image J. The threshold of the image was set to include the vessels in order to calculate the density of vessels relative to the total image size.

**Statistical Analysis**

All experiments were repeated at least 3 times. The data presented in the graphs are the mean values with SEM error bars. Student t tests (two-tailed distribution with two sample unequal variance) were performed and a p value ≤0.05 was considered significant.

**Results**

*Ear wound closure is not affected by a 50% reduction in adrenomedullin.*

In order to stimulate lymphangiogenesis and angiogenesis during adulthood, a minimally invasive ear wound assay was performed in adult *Adm*+/+ and *Adm*+/-.
mice. First, it was important to determine if the difference in Adm genotype impacted the wound closure capability of these mice. The wounds were allowed to heal for 28 days (Figure 4-1A-D) after which the percent wound closure was calculated. It was determined that there was no significant difference in wound closure between the Adm\(^{+/+}\) and Adm\(^{+/-}\) mice (Figure 4-1E).

*Angiogenesis is unaffected in adult Adm\(^{+/-}\) mice relative to wildtype mice.*

In order to determine if haplo-insufficiency for Adm impacted angiogenesis in response to a minimally invasive ear wound, the ears were harvested after 28 days of healing and stained for PECAM-1 (Figure 4-2A-B). Then, the vessel densities in the healing margin of the wound were calculated. There was no significant difference in blood vessel density between genotypes (Figure 4-2C) indicating that angiogenesis in response to ear wounds is unaffected with a 50% genetic reduction of Adm.

*Lymphangiogenesis is reduced in adult mice with a genetic reduction of Adm.*

After determining that there was no effect on ear wound-induced angiogenesis, we wanted to determine if there was an impact on lymphangiogenesis; therefore, the ears were also stained with LYVE-1 to visualize lymphatic vessels (Figure 4-3A-B). In response to an ear wound, there is a significant decrease in the lymphatic vessel density from 13.2% in control mice compared to 8.9% in Adm\(^{+/-}\) adult male mice (Figure 4-3C) signifying that
lymphangiogenesis is affected in an ear wound model with alterations in the level of AM.

*Lymphatic vessel diameter is significantly smaller in Adm+/− mice relative to control mice after wounding.*

To further characterize the lymphangiogenic defect seen in response to ear wounds in Adm+/− mice, the vessel diameter was measured. The largest vessel in each ear image was measured as a general assessment of vessel diameter. Basally there was no difference in vessel diameter between Adm+/+ and Adm+/− mice (Figure 4-4A). However, after the wound healing period mice with a 50% genetic reduction in Adm exhibited significantly smaller lymphatic vessels averaging 17.5 µm in diameter as compared to 21.6 µm in control mice (Figure 4-4B).

**Discussion**

Neovascularization is a critical component of wound healing. Therefore, both the lymphatic and blood vascular systems are integral to the process of wound healing. A wound acts as a trigger to initiate growth of these vessels that are typically quiescent during adulthood. In this study, ear wound closure is unaffected with haplo-insufficiency for Adm allowing this minimally invasive wound model to act solely as a means to stimulate lymphangiogenesis and angiogenesis in the adult mouse. This study revealed that genetic reduction of Adm caused reduced wound-induced lymphangiogenesis associated with reduced lymphatic vessel diameter. These finding are consistent with previously published developmental data stating
that loss of AM signaling results in reduced proliferation of LECs and smaller jugular lymph sacs [23].

Since this study indicated that wound-induced lymphangiogenesis is in part regulated by AM, it is conceivable that this regulation of lymphangiogenesis may have impacts on wound healing when studying more invasive wound models, such as a tail or back wounds, for longer periods of time. Therefore, it could be hypothesized that the disrupted lymphangiogenic response identified in this minimally invasive wound model may contribute to the inability to effectively alleviate edema in a severe wound model.

A recent study with a more invasive hind leg skin wound in mice haplo-insufficient for Adm determined that both the blood and lymphatic vascular systems were impacted by a reduction in AM signaling after 3 days of healing [241]. Since the hind limb wound model and this ear wound model are different and different time courses were used during the experiments, it is difficult to compare results of these studies. However, it is conceivable that the hind limb skin study performed over a 3 day time course only recorded short term-responses, such as inflammatory responses, while this ear wound study performed over a 28 day time period could evaluate responses over a longer time course, such as vessel growth. The blood vascular system, while not as sensitized to AM signaling, may be able to compensate over time. Nevertheless, the hind limb study showed increased lymphedema with haplo-insufficiency for Adm [241], which is consistent with our results, demonstrating a defect in the lymphatic system. Due to these differential findings, important future studies need to be completed to determine the effect of AM
signaling on lymphangiogenic and healing responses to severe wound models over both short and long time periods.

Appropriate levels of AM are necessary to maintain normal wound-induced lymphangiogenesis. The data in this study indicates that the lymphatic system is more responsive to changes in AM, which is also corroborated with our previous research showing that knockout mice for \textit{Adm}, \textit{Calcrl}, and \textit{Ramp2}, primarily exhibit defects in the lymphatic system rather than the blood vascular system. In future studies, it will also be imperative to explore the underlying molecular differences between the lymphatic and blood vascular systems to determine why lymphatic vessels are more responsive to varying levels of adrenomedullin than blood vessels.

**Author Contributions**

SLH designed and performed experiments, analyzed data, and wrote the manuscript. KMC designed experiments and edited the manuscript.
Figure 4-1. Ear wound closure is unaffected by a 50% genetic reduction in Adm. A-D, Images of ears on Day 0 (initial hole size) for Adm\(^{+/+}\) (A) and Adm\(^{+-}\) (B) mice and Day 28 (after healing) for Adm\(^{+/+}\) (C) and Adm\(^{+-}\) (D) mice that were hole punched (scale=2mm). E, Graph of percent wound closure after allowing the ears of Adm\(^{+/+}\) and Adm\(^{+-}\) mice to heal for 28 days.
Figure 4-2. Ear wound-induced angiogenesis is not altered in Adm+/− adult mice.

A, B, Ears from Adm+/+ (A) and Adm+/− (B) mice were stained for blood vessels using PECAM-1 (dashed lines indicate ear wound hole; scale=100µm; 20x objective). C, Graph of blood vessel density in Adm+/+ and Adm+/− mice both basally and after allowing the ear wounds to heal for 28 days.
Figure 4-3. Lymphangiogenesis is significantly reduced in response to an ear wound in Adm+/+ mice.  A,B, Ears from Adm+/+(A) and Adm+/- (B) mice were stained for lymphatic vessels using LYVE-1 (dashed lines indicate ear wound hole; scale=100µm; 20x objective).  C, Graph of lymphatic vessel density around the ear wound in Adm+/+ and Adm+/- mice both basally and after allowing the ear wounds to heal for 28 days (*p<0.0001).
Figure 4-4. Lymphatic vessel diameter in the healing margin of an ear wound is significantly smaller in Adm⁺/- mice relative to control adult mice after 28 days. A,B, Graphs of lymphatic vessel diameters for the largest vessel in the ear images of Adm⁺/+ and Adm⁺/- mice both basally (A) and after allowing the ear wound to heal for 28 days (B). (*p<0.004)
Chapter V: Complement Factor H is enriched in lymphatic endothelial cells and increases adrenomedullin-induced migration

Overview

Adrenomedullin (AM=protein; Adm=gene) signaling is a critical component for proper development and function of the lymphatic vascular system. This signaling system consists of AM, its G-Protein coupled receptor, calcitonin receptor like receptor (Calcrl) and associated receptor activity modifying protein (RAMP2/3). While AM also plays a role in the blood vascular system, the lymphatic system is more sensitized to changes in levels of AM. This can be seen in knockout mice for the Adm, Calcrl, and Ramp2, and in adult mice with loss of AM signaling that primarily exhibit defects in the lymphatic vascular system. It is not completely understood what molecular mediators contribute to these differences. One particular molecule of interest is Complement Factor H (CFH), which is known as the serum binding protein for AM that enhances the activity of AM that has yet to be studied in the context of AM signaling in lymphatic endothelial cells (LECs). Therefore, we sought to determine if CFH enhanced AM-induced responses in LECs. This study revealed that CFH expression is enhanced in LECs relative to blood endothelial cells (BECs). Treatment of LECs with CFH enhances AM-induced migration in response

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to a scratch wound suggesting that CFH may be one of the molecules involved in
the differential responses to AM in the blood and lymphatic systems. To further
understand the molecular components involved in the AM-induced migration
response, the role of the small GTPase, Rap1, was explored. Rap1 has previously
been implicated in migratory responses of BECs and is known to be activated by
cAMP, which is a downstream effector of AM signaling. In this study, gene
expression data showed that Rap1 is expressed in LECs. Resulted indicated that
the active form of Rap1 is upregulated in response to AM treatment; therefore, this
molecule may play a role in the AM-induced migratory response in LECs.

Introduction

The lymphatic vascular system exists in parallel to the blood vascular system and
primarily functions to regulate tissue fluid homeostasis. While these two vascular
systems exist in close proximity, their functions and responses to various signaling
pathways are quite diverse. In particular, the small peptide, adrenomedullin (AM), its
G-Protein coupled receptor calcitonin receptor-like receptor, Calcrl, and receptor
activity modifying protein (Ramp2) are highly expressed in all endothelial cells, but
evidence indicates that there are enhanced responses to this signaling system in
lymphatic endothelial cells (LECs) as compared to blood endothelial cells (BECs).

Knockout mice for the Adm, Calcrl, and Ramp2 have reduced lymphatic
endothelial cell proliferation, but interestingly there is not a significant reduction in
venous endothelial cell proliferation [23]. Temporal deletion of the receptor for AM,
Calcrl, in adult mice results in multi-organ lymphangiectasia with decreased
lymphatic function and increased lymphatic permeability, but there are negligible effects on the blood vascular system [208]. Also, injection of tumor cells expressing varying levels of AM into mice resulted in changes in lymph node lymphangiogenesis [239]. Haplo-insufficiency for AM in adult mice results in reduced lymphangiogenesis in response to a minimally invasive ear wound model, while there is not significant impact on wound-induced angiogenesis (Hoopes, SL unpublished data). These data raise the question of why there is a differential response to AM signaling in the lymphatic and blood vascular systems, which then leads to the bigger question in the field of what makes a LEC different from a BEC?

Morphologically and functionally lymphatic and blood vessels are different, but the molecular and genetic differences between LECs and BECs are not completely understood. Several microarray analyses have been published that characterized differential gene expression between LECs and BECs [24,25,218]. Many pro-inflammatory cytokines and chemokines as well as genes regulating cell-cell interactions were some of the main differences found between BECs and LECs [24]. As our data supports, the genes for the receptor for AM, Calcrl and Ramp2, are upregulated in LECs compared to BECs, which sensitizes the LECs to AM signaling because there is more functional AM receptor on LECs [24,25]. This up-regulation can be partially attributed to transcriptional regulation of Calcrl and Ramp2 by the lymphatic transcription factor Prox-1 that genetically reprograms venous endothelial cells to have a LEC fate during development [23].

Another interesting gene, complement factor H (CFH), was shown to be enhanced in LECs compared to BECs [218]. CFH is known as the serum binding
protein for adrenomedullin that enhances AM activity 2-fold [242,243]. Using a readout of cAMP production as a measure of AM activity, it has been shown that Rat-2 fibroblast cells treated with a constant dose of AM and increasing CFH treatment have a dose-dependent increase in cAMP production [243]. Also, in the breast cancer cell line T-47D there is a dose-dependent increase in proliferation with increasing dosage of CFH in the presence of constant AM dosage [243]. Another study indicated that AM and CFH together help protect neural cells from hypoxia-induced apoptosis [244]. Two binding sites for AM were found in CFH; one with high affinity near the carboxy terminal end of CFH and one in the middle of CFH [245]. The main binding site consists of several short consensus repeats (SCR 15-20) suggesting that a specific 3-dimensional structure is also necessary for binding of AM [245].

Currently, the mechanisms that control differential responses to AM in LECs and BECs are not fully understood and it has not been determined whether CFH impacts these responses. Here we present evidence that CFH expression is increased in LECs versus BECs and CFH enhances the AM-induced migration response to scratch assays in LECs. Since results in this study related to migratory responses, we began to explore the downstream molecules involved in this signaling cascade. One particular GTPase of interest was Rap1 because it has been shown to play a role in migration of BECs [246,247]. Here we present results showing that AM can induced the active form of Rap1 in LECs.
Methods

Cell Culture

Human adult dermal lymphatic endothelial cells (HMVEC-dLyAd-Der Lymphatic Endo Cells, Lonza) were maintained using EGM-2MV media with a bullet kit (Lonza). Human umbilical vein endothelial cells (HUVEC, Lonza) were maintained using EGM media with a bullet kit (Lonza). Cells were used for experiments at 8 passages or less.

Western Blot

CFH protein expression in both LECs and HUVECs was analyzed using Western blots. Since CFH is a secreted protein, it was essential to control for both cell number and media volume. A total of $1.5 \times 10^5$ cells were plated in 6-well plates in a total volume of 3 ml media. After 24 hours, the cells were collected and the final cell number was counted using a Countess automated cell counter (Life Technologies). The media was also collected and the final media volume was recorded. The two cell types are cultured in different media; therefore, there is not a sufficient internal control for use on a Western blot. For the Western blot, the total cell numbers were standardized to the final media volumes in order to load the correct volume of media per cell number. Equal volume of 2X protein sample buffer was added to the media samples. Samples were boiled for 10 minutes, loaded onto a 7.5% Tris-HCl gel, and resolved by SDS-PAGE. Protein was transferred to a nitrocellulose membrane and blocked in Casein at room temperature for 1 hour. The Western blot was probed for CFH (1:125; Quidel, San Diego, CA) followed by
incubation with secondary antibody IRDye 800 Donkey α Goat (1:5000; Rockland) and imaged on the Licor Odyssey.

In vivo Scratch Assay

Human lymphatic endothelial cells were plated at 75,000 cells/well on a 24 well plate. After overnight incubation the monolayer was scratched with a P200 pipette tip. Wells were rinsed with PBS to remove non-adherent cells and LECs were then treated in low serum (0.5% FBS) RPMI media with combinations of the following: 1µM AM 22-52 (30min pretreatment; AM antagonist), 1nM human AM, and 75nM CFH. Four fields per well were imaged at T=0hrs and at T=18hrs post-scratch using an Olympus IX-81 inverted microscope equipped with a QImaging Retiga 4000R camera at 4X magnification. The percentage of migration was calculated by measuring the open area using ImageJ. Mean percent migration was determined using at least 3 independent experiments.

Cell Counts and Viability

Following the post-scratch imaging after 18 hours, cell number and viability in each well were determined using a Countess automated cell counter (Life Technologies). Two measurements were averaged per well.

Rap1 Gene Expression

Thoracic ducts from adult mice (C57BL/6; 8 weeks or older) were collected and flash frozen with liquid nitrogen. Tissue was digested using a Precellys 24 bead
homogenizer (Bertin Technologies) followed by RNA extraction using a RNeasy Micro Kit (Qiagen) and cDNA preparation using an iScript Select cDNA Synthesis Kit (Bio-rad). Quantitative RT-PCR was performed on a StepOnePlus system (Life Technologies) using TaqMan Gene Expression Master Mix (Life Technologies). Rap1 gene expression was assessed using assays-on-demand for Rap1a and Rap1b (Life Technologies) and was compared to mouse embryo total RNA calibrator (Ambion). All assays were repeated at least three times and run in duplicate.

**Active Rap1 Detection Assay**

An active Rap1 pull-down and detection assay was performed using a kit from Thermo Scientific (Active Rap1 Pull-Down and Detection Kit; #16120) on adult dermal lymphatic endothelial cells treated with AM (1nM; American Peptide Co., Inc.) and no treatment (control). Cells were grown in 10 cm dishes and treatment conditions were repeated at least 3 times. Following pull-down of the active form of Rap1, a Western Blot was performed with an anti-Rap1 antibody (1:1,000; Thermo Scientific, Rockford, IL) according to the kit protocol. Whole cell lysate was used as a positive control. Anti-actin antibody (1:10,000; Sigma, Saint Louis, MO) was used as a control on the Western Blot. Secondary antibodies included HRP goat α mouse (1:2,000; Upstate; Lake Placid, NY) and HRP goat α rabbit (1:20,000; Rockland, Gilbertsville, PA). The Western Blot membrane was developed on Blue Ultra Autorad Film (GeneMate) after incubation with Western Bright ECL reagents (advansta).
**Statistical Analysis**

All experiments were repeated at least three times. Data are expressed as means with SEM values as error bars. Student *t* tests (two-tailed distribution with two sample unequal variance) were performed and *p*≤0.05 was considered significant.

**Results**

*Blood and lymphatic endothelial cells secrete CFH, but it is more highly expressed from LECs.*

In order to determine if CFH is differentially expressed in LECs and BECs, LECs and HUVECs were cultured in 6 well plates. After 24 hours, the media was removed from the cells and used for Western Blots because CFH is secreted from the cells into the media. CFH was robustly expressed in the LEC media with a 2.5-fold increase in expression as compared to the HUVEC media under which cells were grown (Figure 5-1A,B) indicating that CFH secretion is enhanced in LECs as compared to HUVECs.

*CFH treatment enhances AM-induced migration of LECs after a scratch assay.*

Since CFH was upregulated in LECs, we wanted to determine if addition of CFH to LECs plays a functional role in association with AM signaling. LECs were grown to confluent monolayers and a scratch was administered to the cells in the presence of treatment conditions including vehicle (H₂O), 1 nM AM, 1 µM AM22-52, 1 nM AM + 1 µM AM22-52, 75 nM CFH, 1 nM AM + 75 nM CFH. The percent
migration of LECs was recorded for each treatment condition by comparing the initial scratch area to the final open area of the scratch after 18 hours (Figure 5-2A-E). AM22-52 is an antagonist of AM signaling. This antagonist is used to inhibit the activity of AM in order to determine if the effects seen in the scratch assay were induced by AM. When LECs were treated with 1 µM AM22-52 migration was reduced to 10.5% as compared to 16.6% migration with vehicle (H₂O) treatment (Figure 5-2G). Also, treatment with 1 nM AM in the presence of 1 µM AM22-52 significantly reduced the AM-induced migration response from 23.7% to 11.9% (Figure 5-2G). There was a significant increase in migration with 1 nM AM treatment to 23.7% as compared to vehicle (H₂O) with a 16.6% migration, (Figure 5-2G). Treatment with 75 nM CFH alone did not significantly impact migration with a migration percentage of 18.8 (Figure 5-2G). However, in the presence of both 1 nM AM and 75 nM CFH the migration was increased to 32.6%, which was a significantly increased over AM alone (Figure 5-2G).

*Total number of live cells and cell viability of LECs was not affected after 18 hours of treatment with CFH and AM.*

To specifically determine if the effects seen in the scratch assay were the result of a migratory effect and not in addition to proliferative or apoptotic effects, total live cells and cell viability of LECs were recorded after 18 hours for the same treatments and conditions as the scratch assay (low serum media). It was shown that there was no significant change in live cell numbers (Figure 5-3A) or cell viability.
(Figure 5-3B) across treatments including vehicle (H$_2$O), 1nM AM, 1µM AM22-52, 1nM AM + 1µM AM22-52, 75nM CFH, 1nM AM + 75nM CFH.

*Rap1 is expressed in mouse thoracic duct and with AM treatment there is an increase in active Rap1.*

Since there is an impact on migration after injury in the presence of AM as well as AM+CFH, we wanted to determine what molecular pathway could be regulating this migratory response to AM signaling. Rap1 is a GTPase activated via cAMP, a downstream effector of AM signaling. Rap1 is known to play a role in the migration of BECs. Since Rap1 has yet to be studied in the context of LECs, we sought to determine if Rap1 is expressed in LECs as well as if active Rap1 is impacted by an increase in AM signaling in LECs. qRT-PCR results indicated that both genes, *Rap1a* and *Rap1b*, were expressed in the thoracic duct (LECs) isolated from mice (Figure 5-4A). Human LECs were then treated with 1 µm AM and an active Rap1 pull-down assay was performed. With AM treatment, there was a significant 3.7 fold-increase in active Rap1 expression compared to no treatment control (Figure 5-4B,C).

**Discussion**

The blood and lymphatic vascular systems parallel each other in the body, but the underlying molecular components that allow for different functions in each of these vascular beds is not completely understood. Both vascular systems respond
to AM, but the lymphatic vascular system seems to be more sensitized to changes in the levels of AM as compared to the blood vascular system.

This study showed that CFH protein expression is enhanced in LECs over HUVECs, which further supports previously published microarray data indicating that the \textit{Cfh} gene is upregulated in LECs [218]. From these results, it was necessary to explore the functional role of CFH in LECs with respect to AM signaling. Migration is a critical function of LECs for proper lymphangiogenesis and wound response that is known to be impacted by AM. In order to determine if CFH plays a role in AM-induced migration response, a scratch assay was performed on confluent monolayers of LECs that were treated with AM and AM+CFH. CFH increased the AM-induced migration in LECs. These cells were also treated with an antagonist of AM signaling, AM22-52, to further confirm that the results of this study were specifically due to changes in AM signaling. No significant changes in total live cells and cell viability confirmed that the results were specific to migratory responses rather than cell death or proliferation. While previous studies have indicated that AM does effect LEC proliferation [12,23], the short time course of this experiment as well as the use of low serum media are not ideal conditions for growth of cells. It is plausible that in future studies related to proliferation that CFH may impact AM-induced proliferation over longer time courses.

These studies show that CFH is differentially expressed in LECs and BECs and that it enhances AM-induced effects in LECs. CFH may be one of the molecular mediators enhancing AM signaling effects in the lymphatic system because it is upregulated in the LECs as compared to BECs. Future \textit{in vivo} studies could expand
upon these *in vitro* studies with CFH and AM. Ear and tail wounds could be assessed in adult mice with differential expression of *Cfh* and *Adm* to determine the impact of CFH on AM-induced signaling in conditions of stimulated lymphangiogenesis. Since these studies implicated CFH in enhancing the AM-induced migratory response in LECs, further studies should be performed to analyze the molecular components involved in the AM migration response. This study also showed that active Rap1 is induced by AM in LECs. Since it is known that Rap1 regulates migration in BECs, it would be interesting to further examine the role of Rap1 in the migration response of LECs.

**Author Contributions**

SLH designed and performed experiments related to CFH protein expression and Rap1. SLH also analyzed data, designed the figures, and wrote the manuscript. NOK performed experiments and analyzed data for migration, cell counts, and viability experiments. KMC designed experiments and edited the manuscript.
Figure 5-1. CFH excretion is significantly enhanced in human LECs as compared to HUVECs. **A,** Western Blot of CFH in control media samples as well as media under which cells (LECs or HUVECs were grown). **B,** Quantification of CFH expression in the Western Blot (A) expressed relative to the average of the control samples (*p<0.005).
Figure 5-2. CFH enhances AM-induced migration of LECs in response to a scratch wound. A, Scratch wound images of LEC monolayers after 18 hours of wound closure for the treatment conditions: vehicle (A), 1nM AM (B), 1µM AM22-52 (C), 1nM AM + 1µM AM22-52 (D), 75nM CFH (E), 1nM AM + 75nM CFH (F) (scale=300µm). G, Graph of percent migration of LECs after 18 hours of wound closure relative to initial wound area for the multiple treatment conditions (*p<0.04, **p<0.0002, ***p<0.03).
Figure 5-3. CFH treatment in the presence of AM does not affect LEC proliferation or cell viability after 18 hours. A,B, Graph of live cells (A) and cell viability (B) after 18 hours of treatments: vehicle, 1nM AM, 1µM AM22-52, 1nM AM + 1µM AM22-52, 75nM CFH, 1nM AM + 75nM CFH.
Figure 5-4. *Rap1* is expressed in mouse thoracic duct and AM treatment induces active Rap1 protein expression in human LECs. **A,** qRT-PCR of *Rap1a* and *Rap1b* gene expression in mouse thoracic duct normalized to mouse *elongation factor* gene expression. **B,** Western blot of active Rap1 after treatment conditions: no treatment, and 1µM AM (*p<0.02*).
Chapter VI: Conclusions and Future Directions

Summary

In summary, these studies, results and conclusions describe how AM impacts the lymphatic vascular system during adulthood. These mouse models extend developmental finding because AM also preferentially impact the lymphatic vascular system during adulthood. Another major aspect of this dissertation was to explore the mechanisms that underlie differences between the lymphatic and blood vascular systems. Results from these studies implicated AM-binding protein as a molecular mediator potentially important in the differential responses to AM signaling in the two vascular systems. These studies aid in the understanding of the role of AM in lymphatics, which in a broader sense may help with the understanding of lymphatic disease and open avenues for AM signaling as a pharmacological target of the lymphatic vascular system.

Current State of the Lymphatic Field

Function of the Lymphatic Vessels and Lymph Valves

The studies presented here have advanced the understanding of how AM regulates lymphatic function and growth during adulthood. Recent research in the field has begun to focus on understanding the pumping activity of larger collecting lymphatic vessels as well as the general mechanics controlling the flow of lymph...
fluid through the lymphatics. This new direction has expanded due to recent antibody development and the advancement of imaging and experimental techniques.

A main component of lymphatic vessels that allows for lymph flow and prevention of backflow in larger collecting vessel are the lymphatic valves. Until recently, little was known about their formation or the mechanics of how they function. The valves consist of two leaflets within the lumen of the vessel comprised of two layers of endothelial cells with extracellular matrix and connective tissue between the two layers [248-250].

In order to assemble the extracellular matrix within the valve leaflets, integrin-alpha9 expression is important because it allows for the deposition of fibronectin-EIIIA in the extracellular matrix that regulates fibronectin fibril assembly [248]. Connexins 37 and 43 are also expressed on the upstream and downstream side of LECs in lymphatic valves and are critical for proper development of the lymphatic valves. Loss of these gap junction proteins results in impaired valve formation, lymphedema and chylothorax [251]. Mechanotransduction, PROX-1, and FOXC2 work to regulate the expression of connexin37 as well as calcineurin/NFAT signaling during lymphatic valve formation, which function to determine the lymphatic valve territory during valvulogenesis [252]. Loss of calcineurin/NFAT signaling in newborn mice results in regression/degeneration of valves, implying that this signaling pathway is necessary for the maintenance of lymph valves [252]. Data also shows that valves usually form at vessel biforcations [253] and where there is disrupted flow [254] suggesting that oscillatory shear stress may induce valve formation. Also,
when LECs are challenged with oscillatory shear stress there is induction of connexin37 expression and nuclear accumulation of NFATc1 [252].

Several recent studies showed that the axonal guidance molecule semaphorin3A, through its interaction with its receptor neuropilin-1 and plexinA1, is necessary for lymph valve development. Loss or inhibition of the neuropilin-1 signaling system in mice results in lymphatic valve defects [255,256]. Recent review articles have nicely summarized some of this work on lymphatic valves as well [257,258]. It will be interesting to see how this research unfolds to give us a better understanding of how the valves form and function in various mouse models. Results from my studies showed that $Calcr^{fl/fl}/CAGGCire-ER^{TM}$ mice exhibited normal morphology of their mesenteric lymphatic valves, but there was disrupted flow through the lymphatic vessels. Further research needs to be done to determine if valve dysfunction is contributing to the phenotype of these mice.

**Lymphatic Flow**

Recent studies have also made advancements in understanding movement of lymph fluid through the lymphatic vessels. Studies testing an increase in afterload in isolated rat mesenteric lymphangions resulted in adaptation of the lymphatic muscle by increasing contractility, similar to the cardiac pump when afterload is elevated [259]. This action of the lymphatic vessel would be important in conditions where edema is present to allow for the vessel to maintain their function and remove excess fluid. Nitric oxide (NO) has been shown to be important in lymphatic pumping because its concentration as well as the concentration of endothelial nitric
oxide synthase (eNOS) is enhanced in the valve regions of lymphatic vessels, which works to limit the flow of lymph fluid by decreasing the frequency and stroke volume of lymph vessel contractions [260].

Another study also indicated that eNOS is required for proper lymphatic contraction under physiological conditions, but in conditions of inflammation inducible nitric oxide synthase (iNOS)-expressing CD11b(+)Gr-1(+) cells reduced lymphatic vessel contraction and also reduced the response to antigens [261]. These results indicated that in an inflammatory condition iNOS production impairs lymphatic contraction resulting in immunosuppression [261].

As briefly discussed in the introduction, a recent study indicated the importance of the recruitment of SMCs to LECs to enhance Reelin signaling allowing for proper development and function of collecting lymphatic vessels [207]. This is an interesting avenue of emerging research that could be explored to determine the effects of SMCs on lymphatic pumping other than the active process of SMCs contracting to allowing for pumping to occur. The interesting pumping capability has risen to the forefront of lymphatic research with the advancement of techniques to isolate individual mesenteric collecting lymphatics from rats and mice and techniques to assess particular flow dynamics in these vessels.

**Blood and Lymphatic Vascular Systems**

While the research and techniques in the lymphatic system still lag behind that of research on the blood vascular system, overall the field of lymphatic research is growing at a rapid pace. There have been increases in the understanding of how
these vessels form and how they are different from the blood vessels. Advancements have been made pertaining to how lymphatic vessels in various regions of the body are different from each other. There is also current emphasis on the mechanical understanding of how lymphatic vessels function to pump and allow for fluid flow as well as lipid absorption.

While research is constantly underway to determine molecular differences between BECs and LECs, there is also recent evidence that there are molecular differences between LECs in the various lymphatic vascular beds. A microarray analysis performed comparing human intestinal and dermal lymphatics indicated that numerous genes (206 genes) were differentially expressed in the two types of LECs [262]. One gene in particular was highly elevated in intestinal LECs known as PAR protein-tyrosine phosphatase-interacting protein, liprin β1, and knockdown of this gene in tadpoles resulted in impaired lymphatic function [262]. There is still little understood as to how different genes regulate the different functions of lymphatic endothelial cells in different areas of the body.

**Future Directions**

*Eye-Related Experiments*

There are still a variety of avenues that need to be explored further relating to AM signaling and its role in the lymphatic vascular system based on the findings in this dissertation. In order to expand upon the studies in this dissertation, experiments addressing the temporal loss of Calcrl could be performed to further understand the eye phenotypes in these mice. While we have established that the
Calcrl mice have dilated lymphatic vessels at the corneoscleral junction and do not exhibit a glaucoma-like phenotype, there are still areas to explore with respect to the eye phenotype.

Recently, several studies showed that lymphatic markers are expressed in the human eye [228,229] and there may be lymphatic-like flow in the eye, but there is substantial debate on whether the well-studied conventional aqueous humor outflow pathway or the unconventional/uveoscleral outflow pathway is a lymphatic-like outflow pathway. Excess fluid in the eye is drained from eye tissues to the venous system in the form of aqueous humor. Interestingly, the aqueous humor also functions to transport immune cells, including antigen-presenting cells, which are typically transported through the lymphatic vessels in other parts of the body [263-265]. The well described conventional pathway [266] consists of the aqueous humor, generated in the ciliary body, traveling into the anterior chamber and draining out of the eye segments through the trabecular meshwork and Schlemm’s Canal. Alternatively, the less well-understood unconventional outflow pathway [267-269] is thought to consist of the aqueous humor, produced in the ciliary body and also draining out of the ciliary body.

Results from a recent study by Birke K, et al showed that Lyve-1 was expressed in dendriform cells in the ciliary body and podoplanin stained all cells of the trabecular meshwork, but no vessel structures were stained [228]. Along with the lymphatic markers, the authors characterize the expression of chemokine ligands, such as Ccl21 [228]. Ccl21 and podoplanin also stained along the anterior iris surface and the anterior chamber angle [228]. These results suggest that there
may be a chemokine gradient that guides chemokines and antigen-presenting cells through the conventional aqueous humor outflow pathway [228].

Another study by Yucel YH, et al identified Lyve-1-positive and podoplanin-positive lymphatic channels in the ciliary body of the human eye [229]. This group also injected fluorescent nanospheres into the anterior chamber of sheep eyes and after a period of time traced their location to the LYVE-1 positive channels in the ciliary body [229]. Iodine-125 radiolabeled human serum albumin was injected into the sheep eye and it was later traced to lymph nodes draining the head and neck region indicating that some fluid leaving the eye is drained through the lymphatic system [229]. From these results, Yucel YH, et al suggest that there are lymphatics in the ciliary body of the eye and that to some extent fluid backflows through this “uveolymphatic” system through the ciliary body [229]. More studies will need to be done on this subject to further gain an understanding of this newly emerging concept of lymphatics in the eye.

Since an eye phenotype has been identified with temporal loss of AM signaling in adult mice, it would be interesting to pursue some of these experiments in the $\text{Calcr}^{\text{fl/fl}}/\text{CAGGCre-ER}^\text{TM}$ mice to determine if this potential lymphatic-like flow is disrupted in these mice. Expression of chemokine ligands can be visualized in the eyes of these mice. While the tracer experiments presented in the study above were done in sheep, the anterior chamber of mouse eyes can easily be injected [270]. The eyes could be injected with a large molecular weight FITC dextran that preferentially moves through lymphatic vessels to determine if lymphatic-like flow is visualized in the eye and if the dextran will drain to lymph nodes. Since these mice
exhibit gray colored eyes with disruption of the cornea and there is a lymphatic defect at the corneoscleral junction, it would be interesting to determine whether they exhibit dry eye characteristics and vision defects. Dry eye characteristics could be tested using fluorescein corneal staining and an aqueous tear production assay as previously described [271]. Vision defects could be assessed through the administration of a visual acuity Morris water maze test [272]. Loss of vision would significantly diminish the capability to navigate the water maze. These results will help us further understand the eye phenotype in these mice with respect to overall physiology and the lymphatic system.

*Experiments Pertaining to the Intestine and Intestinal Lymphatics*

Further studies could be performed to determine the direct effect of loss of *Calcrl* in intestinal LECs and to determine the molecular components involved in the reduced lipid absorption seen in these mice. In order to specifically determine if there is direct involvement of LECs in the intestinal lymphangiectasia and lipid phenotypes in the *Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup>* mice, we will characterize the previously developed lymphatic-specific tamoxifen inducible Cre mouse line, *Prox<sup>CreER</sup>* [273] for intestinal phenotypes as was performed with the *Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup>* mice. We can also further characterize these *Calcrl<sup>fl/fl</sup>/Prox<sup>CreER</sup>* mice by stressing them with high fat diet to determine the effect of loss of AM signaling on either paracellular or transcellular lipid transport pathways. Currently, there is controversy as well as little understanding in the field as to how lipids move into the lymphatic system. The
movement of lipids into the lymphatic vessels could be captured using transmission electron microscopy.

From these high fat diet experiments, we could also determine if there is physiological and genomic homeostatic compensation in response to loss of AM signaling and to high fat diet in intestinal LECs and intestinal enterocytes. Isolated intestinal LECs can be used for microarray analysis to uncover the mechanistic basis for altered lymphatic permeability with loss of AM signaling. Isolated intestinal enterocytes could be used for gene-specific profiling for the expression of genes involved in enterocyte lipid transport such as CD36, GLUT2, NPC1L1, and ABCA1, which will allow us to gain information pertaining to any feedback communication that may exist between lacteals and enterocytes for lipid absorption, particularly under conditions of intestinal lymphangiectasia.

The Calcrl<sup>fl/fl</sup>/Prox<sup>CreER</sup> as well as our Calcrl<sup>fl/fl</sup>/CAGGCre-ER<sup>TM</sup> mice could also be used to determine whether loss of AM signaling affects regenerative lymphangiogenesis following mucosal injury and inflammation. There are several models of intestinal injury. Ischemia-reperfusion of the superior mesenteric artery [274] and indomethacin treatment [275] are preferred since they rapidly induce acute-onset intestinal injury. After recovery, epithelial damage and hemorrhage, tissue enlargement via histology and wet:dry weight ratios, mucosal edema formation, immune cell infiltration, myeloperoxidase activity as an index of neutrophil accumulation, and local and systemic chemokine profiling will be performed to assess how loss of AM with the subsequent reduction in lymphangiogenic capability impacts intestinal recovery. Depending on the results of the above studies, it may
also be beneficial to explore how changes in the fluid mechanical forces influence the process of lipid transport and metabolism.

**Experiments Pertaining to the Role of CFH in the Lymphatic System**

Future experiments could be performed to further investigate the role of CFH in the lymphatic system. LECs could be transfected with CFH siRNA to look at loss of CFH and how that affects AM-induced migration response to a scratch assay. *In vivo* studies should also be performed with genetic alterations in both *Adm* and *Cfh*. An ear wound experiment could be performed on *Cfh<sup>−/−</sup>*/*Adm<sup>+/+</sup>* adult mice to determine if the loss of CFH exacerbates the impaired lymphangiogenesis phenotype seen in ear wounds of *Adm<sup>+/−</sup>* mice. An invasive tail or back wound could be used to assess edema resolution and wound healing in these mice.

Since CFH not only enhances the activity of AM, but both AM and CFH actually impact the bioactivity of each other [243] it is conceivable that genetic changes in *Adm* may also affect CFH phenotypes. Two year old *Cfh*-deficient mice exhibit age related macular degeneration characteristics in the eye including retinopathy with retinal abnormalities and visual dysfunction [272]. *Cfh<sup>−/−</sup>*/*AM<sup>+/−</sup>* mice could be assessed to determine if this phenotype presents at an earlier age or is exacerbated in these mice. Retinal abnormalities can be assessed by observing changes in the distribution of retinal pigment epithelial cell organelles and disorganization of the rod photoreceptors. Visual dysfunction can be determined by using a visual acuity water maze task similar to the experiments by Coffey PJ et al
These experiments would determine if AM and CFH are affecting each other’s functions \textit{in vivo}.

\textit{Migration and Rap1-Related Experiments}

Lastly, studies could be done to investigate the migration response to a scratch assay in LECs treated with AM in order to further explore the role of Rap1 in this process. Rap1, a small GTPase, has been shown to regulate migration of BECs. In HUVECs that were transfected with Rap1GAP, the Rap1-GTPase activating protein that downregulates Rap1, there was reduced proliferation, tube formation, and migration with a significant reduction in pAKT and pERK expression [246]. Another study indicated that Rap1 becomes activated at the leading edge of the endothelial cells during migration occurring as a result of chemotaxis and wound healing [247]. Active Rap1 and its associating molecule RAPL, which is localized on microtubules, have been shown to contribute to the directional migration of the vascular endothelial cells [247]. These studies have implicated a role for Rap1 in the migration of BECs. The studies in this dissertation determined that Rap1 is expressed in the lymphatic endothelium and that AM treatment induces Rap1 activation in human LECs.

Since there is a migration response to AM treatment in LECs and AM treatment activates Rap1, it would be reasonable to hypothesize that Rap1 may play a role in the migratory process in LECs. Similar experiments as described in the papers indicating a role for Rap1 in BECs should be performed to determine its precise role in LECs. LECs could be transfected with Rap1GAP and assays could
be performed to determine if this impacts typical AM signaling responses such as proliferation, migration, tube formation, and phosphorylation of ERK and AKT. Also, a scratch assay could be performed on a confluent monolayer of LECs treated with AM and on LECs transfected with Rap1GAP. After completing the scratch assay, staining could be done to look at expression of Rap1 and RAPL. RAPL is located on microtubules and active Rap1 initiates the dissociation of RAPL from the microtubules [247]. The staining could reveal if activation of Rap1 or loss of Rap1 alters scratch assay-induced migration of LECs.

These studies would further our understanding of lymphatic physiology in adulthood. The role of AM in the lymphatic vascular system with relevance to multiple lymphatic vascular beds and multiple models of lymphatic growth induction would be assessed. These future experiments would further characterize the role of Rap1 in the lymphatic system and characterize the role of CFH and AM together in vivo to enhance our understanding of molecules involved in AM signaling and the lymphatic system.
References


