ADRENOMEDULLIN, CHEMOKINE RECEPTORS, AND RECEPTOR ACTIVITY MODIFYING PROTEINS IN LYMPHANGIOGENESIS

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ABSTRACT

Klara Rachel Klein: Adrenomedullin, Chemokine Receptors, and Receptor Activity Modifying Proteins in Lymphangiogenesis
(Under the direction of Kathleen Caron)

Over the past decade, we have begun to appreciate that the lymphatic vascular system does more than simply return plasma back into the circulatory system and, in fact, contributes to a wide variety of normal and disease states. For this reason, much research has been devoted to understanding how lymphatic vessels form and function, with a particular interest in which molecules contribute to lymphatic vessel growth and maintenance. Here, we have focused on a potent lymphangiogenic factor, adrenomedullin, and its known roles in lymphangiogenesis, lymphatic function, and lymphatic disease.

In the course of our studies, we have discovered that the decoy receptor CXCR7 is required as a molecular rheostat for controlling the concentration of AM ligand during cardiac and lymphatic vascular development. Loss of mammalian CXCR7 results in postnatal lethality due to aberrant cardiac development and myocyte hyperplasia. In Part I, we provide the molecular underpinning for this proliferative phenotype by demonstrating that the dosage and signaling of adrenomedullin is tightly controlled by CXCR7. To this end, Cxcr7^−^ mice exhibit gain-of-function cardiac and lymphatic vascular phenotypes which can be reversed upon genetic depletion of adrenomedullin ligand. In addition to identifying a biological ligand accountable for the phenotypes of Cxcr7^−^ mice, these results reveal a previously underappreciated role for decoy receptors as molecular rheostats in controlling the timing and extent of GPCR-mediated cardiovascular development.
In Part II, we investigated whether CXCR7 and related chemokine receptors (CKRs), CXCR4 and CCR5, form protein-protein interactions with one component of the AM signaling system, Receptor Activity Modifying Proteins (RAMPs). BRET studies, confocal microscopy, and fluorogen-activating protein assays indicate CKRs associate with RAMP2 and RAMP3 in vitro, suggesting that a broader group of GPCRs interact with RAMPs than previously thought. Future studies will allow us to characterize the breadth of RAMP interactions, facilitating a shift in the focus of RAMP research to the design of functional assays that help determine whether a given GPCR-RAMP association affects biological activity. A thorough understanding of RAMP effects on biology has the potential to have significant clinical impact, as targeting of the RAMP-GPCR interface could yield specific, high affinity drugs.
To my friends and family, who are endlessly supportive, and especially to my mom, who is my inspiration, my dad, who keeps my feet on the ground, and my husband, who makes every day better than the last.

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LIST OF ABBREVIATIONS

7-TM – seven-transmembrane
ACKR – atypical chemokine receptor
ACTH – adrenocorticotropic hormone
Adm – adrenomedullin gene
AM – adrenomedullin peptide
AMY – amylin
Ang-II – angiotensin II
Assp1 – apoptosis stimulating protein of p53
B₂ADR – beta-2 adrenergic receptor
BEC – blood endothelial cells
BRET – bioluminescence resonance energy transfer
cAMP – cyclic AMP
CaSR – calcium sensing receptor
CFA – complete Freund’s adjuvant
CGRP – calcitonin gene related peptide
CKR – chemokine receptor
CLR – calcitonin receptor-like receptor
CRF – corticotrophin releasing factor receptor
CT – calcitonin
CV – cardinal vein
ER – endoplasmic reticulum
FAP – fluorogen activating protein
GPCR – G protein-coupled receptor
GPER – G protein-coupled estrogen receptor 1
HIV – human immunodeficiency virus
IMD – intermedin
JV – jugular vein
LAD – left anterior descending
LEC – lymphatic endothelial cell
LLC – Lewis lung carcinoma
LPS – lipopolysaccharide
MHC – myosin heavy chain
NSF – N-ethylmaleimide-sensitive factor
NHERF – Na⁺/H⁺ exchanger regulatory factor-1
OVA – ovalbumin
PAM – peptidylglycine alpha-amidating monooxygenase
PDZ – PSD-95/Discs-large/ZO-1
PTH1R – parathyroid 1 receptor
PTH2R – parathyroid 2 receptor
PTHrP – parathyroid related peptide
RAMP – receptor activity modifying protein
Rluc – Renilla luciferase
V2R – vasopressin 2 receptor
VE-Cadherin – vascular endothelial cadherin
VEGF – vascular endothelial growth factor
VPAC1R – vasoactive intestinal peptide 1 receptor
VPAC2R – vasoactive intestinal peptide 2 receptor
ZO-1 – zonulus occludin-1
Chapter 1: Adrenomedullin in Lymphangiogenesis - From Development to Disease

Overview

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

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1 Authors: Klein, K.R. and Caron, K.M.
Introduction

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

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

A multitude of studies in genetic mouse models have also implicated lymphatics in other common diseases. For example, disruption of neolymphangiogenesis in the skin induces salt-sensitive hypertension in mice [6]. This model demonstrates that dermal lymphangiogenesis is required to maintain electrolyte balance and subsequently blood pressure homeostasis [6]. Further, haploinsufficiency of the essential transcription factor Prox1 leads to adult onset obesity due to abnormal fluid leakage from disrupted lymphatic vessels [7]. Examples like these suggest that novel therapeutics designed to modulate the lymphatic vasculature could offer treatment for prevalent diseases like lymphedema, essential hypertension, and obesity.
To this end, a great deal of effort has recently been devoted to identifying factors that participate in lymphangiogenesis and control lymphatic vascular function in adults. The following review will focus on one such factor, adrenomedullin (AM = peptide, Adm = gene), and will address what is currently known about its role in the lymphatic vascular system, and its potential as a novel therapeutic for the treatment of diseases linked to lymphatic dysfunction.

The Adrenomedullin Peptide Family and Its Receptors

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

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

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

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

**Adrenomedullin During Lymphatic Vascular Development**

Initial lymphatic vessel formation begins between e9.5-10.5 with the commitment of endothelial cells of the cardinal vein (CV) to a lymphatic fate (Figure 1-1A) [1]. After polarization of lymphatic progenitor cells in the jugular vein at e11.5-12.5, lymphatic endothelial cells (LECs) begin to migrate away and form a premature lymphatic vessel. Historically, this vessel has been referred to as the lymph sac. Recently however, ultramicroscopy of whole mount mouse embryos has revealed that the lymph sac is comprised of two separate lymphatic structures, a peripheral longitudinal lymphatic vessel and a primordial thoracic duct [32]. These structures are formed by migrating LECs that originate from both the CV and additional venous sources [32]. Failure of these ECs to establish LEC fate, migrate, or proliferate results in severe edema and embryonic lethality. For example, genetic deletion of *Prox1* prevents EC differentiation to lymphatic fate. *Prox1*-null animals are devoid of lymphatic vasculature and die by e14.5 [33-35]. Similarly, though ECs of VEGF-C-null mice can establish LEC fate, LECs fail to migrate away from the CV, lymphangiogenesis is arrested and embryonic edema ensues, demonstrating that VEGF-C is an essential lymphangiogenic factor [36-38]. The severity and timing of the edema and subsequent embryonic lethality in these mouse models provided an essential clue for determining the role of AM in lymphangiogenesis.

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

Consistent with what was known about AM at the time and with the nascent lymphatic vascular field, early studies of these mouse models focused on AM in cardiac and blood vascular development rather than lymphangiogenesis. Indeed, AM was found to have a significant proliferative effect during cardiac development. Adm\(^{-}\), Calcr\(^{-}\), and Ramp2\(^{-}\) mice share a phenotype of small, hypoplastic hearts [14,39,40]. All three genes were also found to be highly expressed in the heart and the vascular endothelium, confounding whether the observed edema could be attributed to cardiac defects or vascular dysfunction.

To solve this problem, Fritz-Six et al. utilized mice expressing an endothelial cell-specific Cre recombinase via expression of a Tie2-Cre transgene [46]. Though Tie2-Cre expression has only been observed in restricted regions of adult lymphatic vessels, Tie2-Cre is expressed in the venous progenitors of lymphatic endothelial cells. Thus, prior to the development of lymphatic-specific Cre drivers, Tie2-Cre mice were widely used to excise genes from venous lymphatic progenitors during embryonic lymphangiogenesis [47-51].

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

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

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

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

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

Likewise, it has become clear that spatial and temporal expression of receptors and their ligands in a microenvironment can affect development of surrounding tissue [59,60]. Because of the close proximity of and complex connections between the lymphatic and blood vasculatures, it is possible that expression of Adm, Calcr1, and Ramp2 by both BECs and LECs has effects not only in their own microenvironments, but also in the adjacent vascular tissue. Recent studies from our lab have substantiated this hypothesis, definitively identifying CXCR7 as a potent modulator of AM signaling and AM-mediated lymphangiogenesis [25].

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

Indeed, as discussed extensively in Chapter 2, consistent with the notion that CXCR7 sequesters AM, $Cxcr7^{-/-}$ mice exhibited enlarged, blood filled lymph sacs. Careful examination of the number of proliferating LECs in the lymph sac demonstrated that the enlarged lymph sacs were hyperplastic (Figure 1-2A). These data suggest that, in the absence of the decoy receptor, an excess of AM leads to hyperproliferation of LECs and subsequently enlarged, poorly formed lymph sacs [25]. Further examination of LECs in vitro and lymphatic beds in vivo confirmed the ability of CXCR7 to affect the lymphatic endothelium. When treated with AM, cultured $CXCR7$ knockdown LECs proliferated more than wildtype LECs [25]. Moreover, dermal and cardiac lymphatic beds were disrupted in $Cxcr7$-null mice and displayed dysmorphic vasculature consistent with hyperplasia [25].

To definitively show that these phenotypes were caused by excessive AM signaling, we genetically titrated AM ligand onto $Cxc7^{-/-}$ mice. Both genetic increase and reduction of AM peptide on the $Cxc7$ null background resulted in striking effects on the lymphatic phenotypes. Genetic reduction of $Adm$ in $Cxc7^{-/-}$ mice restored the lymphatic and cardiac hyperplasia to wildtype levels, indicating that the hyperplasia observed in $Cxc7^{-/-}$ mice resulted from excessive AM signaling [25]. Moreover, intercross of $Cxc7$ mutant mice with $Adm^{hi/hi}$ mice resulted in an exacerbation of the lymphatic enlargement and increased embryonic lethality to the point that it was difficult to maintain a $Cxc7^{-/-}; Adm^{hi/+}$ mouse colony due to embryonic and early post-natal loss [25]. Taken together, these data demonstrate that the dosage of AM is critical for maintaining proper cardiovascular
development and that, without tight control of AM-mediated signaling, proliferation of both myocardial and lymphatic endothelial cells is disrupted (Figure 1-2A).

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

Adrenomedullin Signaling During Adulthood

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

While healthy cornea is largely avascular, lymphangiogenesis occurs in response to inflammation and can ultimately lead to blindness [65]. Though no corneal lymphangiogenesis was reported in Calcr\textsuperscript{fl/fl}CAGGCre-ER\textsuperscript{TM} mice, these mice were only evaluated after one week, which may not allow enough time for neolymphangiogenesis. Given more time, the inflammation in Calcr\textsuperscript{fl/fl}CAGGCre-ER\textsuperscript{TM} may lead to corneal lymphangiogenesis. It is also possible that the absence of the AM receptor would prevent new lymphatic vessels from forming. Regardless, these findings highlight the importance of proper lymphatic function in maintenance of fluid homeostasis in and around the eye.

Indeed, recent studies have demonstrated that the endothelial cells lining Schlemm’s canal express several lymphatic marker genes, are responsive to VEGFC and thereby control the aqueous humor with the anterior chamber of the eye. [66-68]. Thus, modulation of these vessels may allow for either increased hydration or drainage of the eye, allowing for a novel treatment of dry eyes, ocular edema, and glaucoma.

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

To induce edema, complete Freund’s adjuvant (CFA) was injected into the paw of the hindlimb. In contrast to tamoxifen-injected controls, Calcrl$^{fl/fl}$CAGGCre-ER$^{TM}$ mice had prolonged edema [64], suggesting that AM is required for proper reabsorption of interstitial fluid and resolution of edema. As discussed above, previous studies demonstrate that AM treatment stabilizes the lymphatic endothelial barrier (Figure 1-1B) [54]. Therefore, similar to the mesenteric lymphatics, it follows that loss of Calcrl destabilizes the lymphatic endothelium in the peripheral lymphatics, increases vascular permeability, allows for lymphatic fluid leakage back into the interstitium, and prevents proper edema resolution.

These findings (summarized in Figure 1-3) are particularly intriguing because they demonstrate that appropriate AM dosage following development is required for proper lymphatic maintenance and function. Loss of AM signaling results in leaky vessels, failure to resolve edema, and alterations in fat absorption. All of these data suggest that AM may be a reasonable target for the treatment of a variety of diseases for which we currently have limited therapies. Pharmaceuticals that modulate fat absorption could be a useful tool to help prevent or reduce the extent of obesity. Additionally, use of AM agonists could decrease lymphatic vessel leakiness and increase uptake of interstitial fluid, allowing for the partial or complete resolution of lymphatic associated edema.

Adrenomedullin and Lymphedema

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

Recently, AM was implicated as a molecule that might contribute to the onset of secondary lymphedema. Adm haploinsufficiency predisposed mice to developing secondary lymphedema following hind limb injury compared to wildtype mice [52]. Further, systemic injection of AM to Adm<sup>+/−</sup> mice restored proper wound healing, thereby preventing the onset of lymphedema [52]. Here, these data demonstrate the importance of AM dosage in the resolution of edema.

Though lymphedema has yet to be associated with mutations in the AM signaling system in humans, these studies suggest that AM administration has potential as a novel therapeutic for the treatment of secondary lymphedema. The primary cause of secondary lymphedema in western countries is radical axillary lymph node dissection, with 20-30% of patients developing severe lymphedema following surgery [70-72]. The most promising treatment is the generation of new lymphatic vessels [70]. Jin and colleagues demonstrate that AM administration increases lymphatic flow, promotes angiogenesis and lymphangiogenesis, and ultimately decreases tail lymphedema <i>in vivo</i> [73]. The cause of this accelerated healing is likely due to a combination of new blood and lymph vessel
formation, as both angiogenesis and lymphangiogenesis are required for wound healing [73-75]. However, the preferential effect of AM on lymphatic endothelial cells indicates that the primary cause for increased healing is neolymphangiogenesis. Therefore, AM administration following surgical resection of lymph nodes may not only promote wound healing, but also prevent the onset of lymphedema. While there are no current studies using AM infusion to treat lymphedema in humans, the potential of AM to act as a novel therapy for lymphedema is promising.

**Adrenomedullin in Cancer**

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

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

Interestingly, alterations in tumor Adm expression robustly impacted tumor lymphatic vessels. While Adm overexpression had little effect on blood vessel density or BEC
proliferation, Adm overexpression resulted in a 3-fold elevation of LECs in both tumors and sentinel lymph nodes compared to tumors with reduced Adm expression (Figure 1-2B) [83]. Lymphatic vessels of Adm overexpressing tumors were also significantly dilated. Careful evaluation of tumor metastasis revealed that Adm overexpression increased tumor dissemination [83]. These data recapitulate what has been observed in human tumors and suggest that Adm expression can promote metastasis via lymphangiogenesis.

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

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

**Adrenomedullin-induced Lymphangiogenesis in the Reproductive System**

Perhaps the most exciting and novel aspect of AM-mediated lymphangiogenesis is its involvement in the lymphatic vasculature of the reproductive system. Our lab and others have shown that AM is critical in healthy pregnancy [9]. In a normal human pregnancy, AM increases 3-5 fold [89]. Dysregulation of this physiologic increase in both humans and mouse models has been associated with significant complications, most notably preeclampsia [90-92]. Additionally, haploinsufficiency of *Adm* in female mice results in reduced fertility due to implantation defects [93]. The essential role of AM in the normal reproductive system has therefore become increasingly apparent and clinically relevant over the past two decades. As such, there has been a rapidly growing interest in the role of AM-mediated angiogenesis and lymphangiogenesis in the reproductive system.

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

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

**Concluding remarks**

The essential role for AM in the development, maintenance, and function of the lymphatic vasculature has been clearly demonstrated over the past decade. Genetic models have greatly contributed to our understanding of how AM modulates lymphangiogenesis and underscored the importance of proper AM dosage: Complete loss of AM is incompatible with life. *Adm* haploinsufficiency may predispose individuals to lymphedema, and overexpression of AM in cancer models correlates with severity of disease. Clearly, aberrant AM expression has the potential to lead to significant lymphatic associated pathologies, and tight control of AM dosage from development through adulthood is critical for proper lymphatic function.
Future studies will continue to elucidate the mechanisms of AM signaling. Whether the AM system interacts with other signaling cascades during lymphangiogenesis, such as Notch and VEGFR3, will be of particular interest, as previously published work has shown AM is capable of activating Notch and transactivating VEGFR2 [57,99,100]. These studies will expand our understanding of how AM impacts lymphatic physiology and offer the potential of identifying a G protein-coupled receptor target for the pharmacological modulation of the lymphatic vasculature in human disease.
Figure 1-1. Early development of the lymphatic system and the effects of AM on LECs. (A) Development of the lymphatic system begins between e10.5-11.5 when the master regulators of lymphatic fate, Prox1 and Sox18, turn on. By e13.5, the lymph sac is fully separated from the jugular vein and activation of the AM system allows LEC proliferation. (B) Additionally, AM acts to stabilize the lymphatic endothelial barrier by reorganizing the tight junction protein, ZO-1, and the adherens junction protein, VE-cadherin, thereby decreasing lymphatic vessel permeability. (AM = adrenomedullin; BEC = blood endothelial cell; LEC = lymphatic endothelial cell)
Figure 1-2. Proper AM dosage is required for normal lymphangiogenesis during development and in tumor biology. (A) Precise AM concentration is required during development to regulate LEC proliferation. Absent AM signaling results in small, hypoplastic lymph sacs. Conversely, loss of the decoy receptor, CXCR7, results in excessive AM signaling and enlarged, hyperplastic lymph sacs. (B) Similarly, overexpression of Adm in tumor models results in enhanced proliferation of LECs, dilated lymphatic vessels, and increased tumor metastasis.
Figure 1-3. Loss of AM signaling in adulthood results in disruption of many lymphatic beds. Here, we provide a summary of the findings when AM signaling is disrupted following embryonic development. Conditional knockdown of *Calcrl* in adult mice results in significant alterations in the ocular, intestinal, dermal, and peripheral lymphatic vessels. Additional studies that suggest AM is critical for lymphangiogenesis during adulthood are highlighted in purple.
REFERENCES


Chapter 2: Decoy Receptor CXCR7 Modulates Adrenomedullin-Mediated Cardiac and Lymphatic Vascular Development

Overview

Atypical 7-transmembrane receptors, often called decoy receptors, act promiscuously as molecular sinks to regulate ligand bioavailability and consequently temper the signaling of canonical G protein-coupled receptor (GPCR) pathways. Loss of mammalian CXCR7, the most recently described decoy receptor, results in postnatal lethality due to aberrant cardiac development and myocyte hyperplasia. Here, we provide the molecular underpinning for this proliferative phenotype by demonstrating that the dosage and signaling of adrenomedullin (Adm = gene, AM = protein)—a mitogenic peptide-hormone required for normal cardiovascular development—is tightly controlled by CXCR7. To this end, Cxcr7<sup>-/-</sup> mice exhibit gain-of-function cardiac and lymphatic vascular phenotypes which can be reversed upon genetic depletion of adrenomedullin ligand. In addition to identifying a biological ligand accountable for the phenotypes of Cxcr7<sup>-/-</sup> mice, these results reveal a previously underappreciated role for decoy receptors as molecular rheostats in controlling the timing and extent of GPCR-mediated cardiac and vascular development. A graphical abstract is included in Figure 2-A1.

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Introduction

The precise spatiotemporal dosage of mitogenic and chemotactic factors is critical for the proper organization and development of organ systems. While the concentration of ligands often differs between tissues and developmental stages, the bioavailability of ligands within local microenvironments must also be controlled at a cellular level. Thus, cells can express molecular sink receptors, which in an autocrine or paracrine manner, sequester ligand away from canonical signaling receptors, thereby driving important developmental processes like neurogenesis, angiogenesis, chemotaxis and cellular proliferation [2,3].

Molecular sink receptors include atypical chemokine receptors, also known as decoy receptors, which belong to the larger family of 7-transmembrane receptors. Decoy receptors act as molecular sinks by binding, internalizing, and degrading a wide range of ligands independent of G-protein coupling (Graham, 2012). CXCR7 is the most recently described decoy receptor and has been extensively studied for its role as a CXCL12/SDF-1 receptor [4-6], particularly in tumor cell migration and cancer progression [7,8].

However, prominent roles for CXCR7 during normal development and physiology have also been recently appreciated. In zebrafish, the expression and molecular sink functions of CXCR7 in trailing cells of the posterior lateral line primordium allows for a CXCL12 chemotactic gradient to be established and sensed by the leading primoridal germ cells which express CXCR4, the canonical SDF-1/CXCL12 receptor [9-12]. In this instance, CXCR7 exerts its decoy activities over a wide region to help coordinate and guide the migration of multicellular tissue structures.

However, the decoy activities of CXCR7 can also occur in a cell-autonomous fashion. For example, the co-expression of CXCR7 within migrating cortical neurons allows for the continued sensitization and chemotactic signaling of CXCR4--rather than receptor desensitization and downregulation that would typically occur within an environment of high CXCL12 ligand [13,14]. Recently, CXCR7 expression in endothelial cells has also been
shown to regulate circulating levels of ligands, suggesting that CXCR7 expression in vessels may not only affect signaling events in a microenvironment, but systemically as well [15].

Due to these well-described roles in the CXCL12/CXCR4 signaling axis, Cxcr7−/− mice were expected to exhibit phenotypes that might resemble gain-of-function mutations for the CXCL12/CXCR4 signaling axis. However, Cxcr7−/− mice have unexpected phenotypes including cardiomyocyte hyperplasia and postnatal lethality associated with gross cardiac enlargement and cardiac valve defects [16-18]. Because decoy receptors typically bind and sequester multiple ligands, it has been difficult to discern which ligand may be causally related to the developmental cardiac defects of Cxcr7−/− mice. In this regard, we appreciated that CXCR7 was originally identified as RDC1—a putative receptor for adrenomedullin (Adm = gene, AM = protein), a 52 amino acid mitogenic peptide hormone critical for cardiac and lymphatic vascular development [19-22]. AM binds RDC1/CXCR7 with a Kd of 1.9 x10−7 M, similar to CLR when associated with RAMPs [23]. Importantly, we have recently shown that genetic overexpression of AM ligand in Admhi/hi mice results in gross cardiac enlargement due to cardiac hyperplasia during embryogenesis [24], which closely phenocopies the dysmorphic cardiac hyperplasia of Cxcr7−/− mice.

We therefore sought to address whether a principal function of CXCR7 may involve controlling the dosage of AM ligand during development, first focusing our attention on the cardiac hyperplasia. In the course of our studies, we also discovered lymphatic vascular defects in Cxcr7−/− mice, which are consistent with the prominent role that AM signaling plays in driving normal lymphangiogenesis [19,20,25,26]. In addition to identifying a biological ligand that is causally associated with the Cxcr7−/− phenotypes, the results described here elucidate a role for decoy receptors as molecular rheostats that control normal cardiac and lymphatic vascular development.
Results

Gene expression of Cxcr7 and Adm are coupled in the heart and lymphatic endothelium

Historical ligand binding data [23] and more recent findings showing a down-regulation of Adm gene expression in Cxcr7−/− mice [17], strongly support the existence of this ligand-receptor pair. Considering the well-established function of CXCR7 as a decoy receptor, we predicted that expression levels of Cxcr7 may homeostatically increase under conditions of increased AM peptide. To further evaluate whether this interaction exists, we measured the expression of Cxcr7 in hearts of Adm hi/hi mice which have a genetically engineered, 3-fold increase in Adm gene expression [24]. Indeed, utilizing qRT-PCR, we identified a potent 2.5-fold upregulation of Cxcr7 gene expression in Adm hi/hi cardiac tissue compared to that of wildtype littermates (Figure 2-1A). Conversely, loss of Adm expression in isolated endothelial cells resulted in a nearly 5-fold reduction in Cxcr7 expression (Figure 2-1A).

We also found that Cxcr7 is expressed at high levels in isolated, adult lymphatic vessels—a tissue where AM peptide plays important roles (Figure 2-S1A). Consistently, microarray analysis of cultured, human lymphatic endothelial cells (LECs) showed that expression of the human CXCR7 gene (aka ACKR3 or CMKOR1) was one of the ten most significantly induced genes within 1 hour of 10nM AM treatment (p=2.5E-07) (Figure 2-1B and Table 2-S1). This finding was further confirmed by qRT-PCR, revealing a 4-fold increase in CXCR7 gene expression following 1- and 24- hours of AM treatment (Figure 2-1C). Pretreatment with AM22-52, a CLR/R2 antagonist, significantly reduced this AM-mediated increase (Figure 2-1C), demonstrating that the upregulation of CXCR7 gene expression is modulated through the canonical AM receptor. Collectively, these data indicate that CXCR7 and ADM gene expression levels are coupled within tissues where AM peptide plays important developmental and physiological roles. Since excess AM, either by genetic overexpression in vivo or exogenous treatment in vitro, triggered an increase in
CXCR7 expression, we next tested directly the hypothesis that CXCR7 serves as a decoy receptor to modify AM concentration.

**CXCR7 scavenges AM peptide and dampens canonical AM signaling**

Using a classical scavenger assay, CXCR7-expressing HEK293T cells were treated with biotinylated-AM1-52, and aliquots of media were collected over time to determine the remaining levels of AM peptide within the media. While the levels of biotinylated-AM1-52 in the media of vector-transfected control cells remained unchanged, CXCR7-expressing cells rapidly and steadily depleted AM peptide from the media to levels that were statistically lower than control cells by the conclusion of the time course (Figure 2-1D, E). These data demonstrate the ability of CXCR7 to modulate AM ligand concentrations exogenously in a controlled in vitro system.

To determine whether this scavenging of AM peptide by CXCR7 was conserved in vivo, we compared AM staining in cardiac tissue of wildtype mice and Cxcr7−/− mice, which harbor an insertional GFP reporter within the targeted allele. Firstly, we noted that expression of the GFP reporter was enriched within the epicardium and the endocardium surrounding the trabeculae and weakly expressed in the compact zone (Figure 2-1F). Secondly, the staining pattern of receptor expression was spatially juxtaposed and/or overlapping with the most prominent sites of AM peptide expression, including the epicardium and trabeculae (Figure 2-1G and [24]). Remarkably, we found a significant 30% increase in the relative staining intensities of AM peptide in the epicardium and trabeculae of Cxcr7−/− mice compared to wildtype littermates (Figure 2-1H-K), but no changes within the compact zone where levels of Cxcr7 reporter expression were modest (Figure 2-1L). These findings indicate that spatially juxtaposed and/or overlapping expression of CXCR7 and AM during cardiac development is essential for scavenging AM peptide in cardiac tissue in vivo.
Activation of the canonical AM-receptor complex, CLR and receptor activity modifying protein 2 (RAMP2), elicits an increase in cAMP and subsequent downstream activation and phosphorylation of ERK [19]. Utilizing a highly-sensitive bioluminescence resonance energy transfer (BRET) reporter system [27,28], we found that HEK293 cells that overexpress CXCR7 failed to accumulate cAMP upon AM stimulation—a finding consistent with the lack of G-protein coupling by decoy receptors (Figure 2-S1B). As expected, AM treatment of CLR/RAMP2-expressing cells resulted in a potent accumulation of cAMP (Figure 2-S1B) and pERK:tERK upregulation (Figure 2-1M, N and Figure S1C).

Importantly, while AM did not elicit a pERK:tERK upregulation in cells transfected with CXCR7 alone, the CLR-RAMP2 mediated activation of pERK:tERK was markedly abrogated when cells were co-transfected with CXCR7 (Figure 2-1M, N). These in vitro signaling assays demonstrate that CXCR7 can act as a cell-autonomous molecular rheostat to dampen canonical AM pERK:tERK signaling.

The effects of CXCR7 on dampening pERK:tERK signaling were also confirmed in vivo, where we noted significant accumulation of pERK staining in dermal lymphatic vessels of postnatal day 1 Cxcr7−/− tail skin compared to wildtype littermates (Figure 2-S2A-D). Furthermore, we also observed a significant increase in the pERK staining in the lymph sac of e13.5 Cxcr7−/− embryos compared to wildtype animals (Figure 2-S2E-J). These in vivo data from a genetic loss-of-function model aptly reciprocate the findings from the in vitro gain-of-function experiments and furthermore demonstrate that loss of Cxcr7 expression influences ERK phosphorylation on a tissue level.

**CXCR7 is dynamically expressed in lymphatic endothelium during development**

Previous studies have reported that nearly one third of adult dermal lymphatic vessels express CXCR7 [29], but the spatiotemporal expression of CXCR7 during developmental lymphangiogenesis has yet to be described. Using the GFP-targeted
Cxcr7<sup>+/−</sup> reporter allele, we found Cxcr7 expression co-localized with the lymphatic makers LYVE1 (Figure 2-2A-C), Prox1 (Figure 2-2G-I) and podoplanin (Figure 2-2I-L). At e11.5, lymphatic progenitor cells are arranged in a stereotypically-polarized fashion within the jugular vein (JV) and express Cxcr7 (Figure 2-2D-F, white arrows, Figure 2-2G, H). Interestingly, we often noted that Cxcr7 expression is temporarily reduced as the lymphatic progenitors begin to migrate away from the JV (Figure 2-2F, I asterisks)– underscoring the dynamic expression of the decoy receptor in areas of active cell migration. As lymphatic cells coalesce to form the lymph sacs (LS) between e11.5-e13.5, Cxcr7 was again expressed in some lymphatics, which were identified by LYVE1 and podoplanin co-labeling (Figure 2-2J-O). Cxcr7 was also persistently expressed in the JV cells directly adjacent to the LS (Figure 2-2P-R, white arrowheads), consistent with recently published studies demonstrating a paracrine function for decoy receptors [12,30]. In summary, Cxcr7 is highly and dynamically expressed within lymphatic progenitors and early lymphatic vessels at the time of nascent lymphangiogenesis, which also spatiotemporally correlates with the proliferative effects of AM during lymphatic development.

**Cxcr7<sup>−/−</sup> mice have enlarged, blood filled lymphatic sacs**

We have previously established that AM signaling is required for normal LEC proliferation at e13.5 [19]. Thus, we investigated whether loss of Cxcr7, which we hypothesize to be a molecular rheostat for AM, disrupts lymphangiogenesis at this point during embryogenesis. Indeed, approximately 10% of Cxcr7<sup>−/−</sup> mice exhibited visible interstitial edema upon dissection at mid-gestation (Figure 2-3A, white arrows, Figure 2-3B, C, black arrows). Histological evaluation further revealed that approximately 10-15% of Cxcr7<sup>−/−</sup> mice displayed interstitial edema, particularly within the thoracic regions surrounding the developing jugular lymphatics. Additionally, we noticed abnormal LS morphology, including markedly enlarged and dysmorphic LS in Cxcr7<sup>−/−</sup> embryos compared to wildtype
littermate embryos (Figure 2-3D-F). Utilizing computerized morphometry to calculate LS and JV area, we found that the LS:JV ratio of null mice was increased 4-fold compared to Cxcr7+/+ mice (Figure 2-3G). Some sections revealed failure of the LS to separate from the JV, with prominent platelet thrombi (Figure 2-3F, black arrows). Moreover, Cxcr7−/− lymphatic vessels exhibited remarkable blood (Figure 2-3E, asterisks) and proteinaceous deposits (Figure 2-3E, arrowheads), which are phenotypes commonly ascribed to pathologic lymphangiogenesis and lymph stasis in several mouse models [31,32]. A scoring rubric to assess the severity of lymphatic defects showed that LS of Cxcr7−/− embryos had significantly more blood and protein accumulation compared to control mice (Figure 2-3H, I). Taken together these results demonstrate that loss of Cxcr7 during embryonic development results in aberrant LS formation.

To determine whether the blood accumulation in the LS was due to improper development or structure of the lymphovenous valves, we stained frontally sectioned embryos with the lymphatic markers Prox1 and podoplanin. We observed no structural differences between Cxcr7+/+ and Cxcr7−/− lymphovenous valves (Figure 2-S3A, B), with both wildtype and mutant animals exhibiting characteristic high-Prox1 staining on the valve leaflet. We next considered whether the blood accumulation in Cxcr7−/− lymph sacs might be associated with precocious development of the lymphatic sac. As expected, e11.5 wildtype embryos exhibited polarization of LYVE1+ lymphatic progenitors within the jugular vein. However, some Cxcr7−/− littermate embryos exhibited premature migration of LECs from the jugular vein and precocious formation of enlarged, blood-filled lymph sacs (Figure 2-S3C, D)—a process that typically occurs 1-2 days later in development. Thus, we reasoned that the likely cause of blood accumulation in the Cxcr7−/− mutants is precocious lymph sac formation prior to proper separation of the blood and lymphatic vascular systems.
Loss of CXCR7 enhances LEC migration in vivo and in vitro

We next sought to determine whether loss of Cxcr7 affected lymphangiogenesis in other lymphatic vascular beds. At e18.5, staining of Cxcr7−/− cardiac tissue revealed increased LYVE1+ vessels on the surface of the heart (Figure 2-4A). This increase might be expected due to the cardiac hyperplasia in Cxcr7−/− embryos. Nevertheless, when normalized to total surface area of the heart, null animals exhibited a 20% increase in cardiac lymphatic vessels on the ventral surface of the heart (Figure 2-4B). Higher power examination of the cardiac lymphatic vessels of Cxcr7−/− animals revealed a disruption of branching complexity and lacunae number (Figure 2-S4A-F), as well as an extensive network of lymphatic vessels in the curvature of the outflow tract on cardiac dorsal-surface (Figure 2-S4C, arrows) which were not present in wildtype mice. Additionally, these LYVE1+ vessels tended to extend farther down the apex of the heart in Cxcr7−/− embryos, suggesting that Cxcr7 also affects LEC migration (Figure 2-4A, C).

To elucidate whether loss of CXCR7 is directly involved in enhancing AM-mediated downstream signaling pathways and cellular migration, we utilized shRNA lentiviral vectors to achieve 80% knockdown of CXCR7 in human neonatal-LECs (Figure 2-4D). We first used a scavenger assay to confirm that CXCR7 shRNA-infected LECs scavenged less AM than control cells, resulting in increased AM available to interact with the signaling receptor, CLR/R2 (Figure 2-S5A, B). Next, we showed that knockdown of endogenous CXCR7 in LECs results in an increase in AM-mediated ERK phosphorylation, with CXCR7 shRNA-infected LECs exhibiting a potent upregulation in pERK:tERK ratios in response to AM treatment, whereas control cells did not (Figure 2-S5C).

These CXCR7 knockdown cells were then used to evaluate whether loss of CXCR7 enhances AM-mediated LEC migration. Using an in vitro scratch assay, we showed that AM promotes LEC migration, since AM-treated control cells migrated 41% more than vehicle-treated cells (Figure 2-4E, G). On the other hand, AM treatment of CXCR7 knockdown cells...
caused the cells to migrate into the scratch 66% more than vehicle-treated cells (Figure 2-4F, H). Moreover, percent migration of AM-treated CXCR7 knockdown LECs was significantly increased above all other conditions (Figure 2-4I). These scratch assay findings were fully recapitulated using a transwell migration assay (Figure 2-4J). Finally, the effect of CXCR7 on AM-mediated cell migration was corroborated using an overexpression model. As expected, AM treatment of CLR/RAMP2-expressing HEK293T cells resulted in increased migration across a transwell. However, this migration was abrogated when cells were co-transfected with a CXCR7 expression plasmid (Figure 2-4K). Collectively, these data show that CXCR7 expression modulates AM-mediated downstream signaling activity, with knockdown of endogenous CXCR7 increasing and overexpression of CXCR7 reducing AM-mediated cellular migration.

Dermal lymphatic vessels of Cxcr7−/− animals are enlarged, with less branching complexity

We also observed morphological changes in the dermal lymphatic vessels of Cxcr7−/− mice. Interestingly, although there was stochastic expression of Cxcr7 in dermal lymphatic vessels (Figure 2-S6), we consistently observed Cxcr7 expression in blood vessels, again suggesting that non-cell autonomous expression of Cxcr7 can affect lymphangiogenesis. While the dermal lymphatics of wildtype animals formed a highly-structured lattice network, those of Cxcr7−/− embryos failed to extend and connect to neighboring vessels, resulting in fewer ring-like structures or lacunae (Figure 2-5A, B asterisks). For example, control lymphatic capillary networks comprised between 6-8 lacunae per image, but the lymphatic network of Cxcr7−/− skin consisted of only 4-5 lacunae per image (Figure 2-5C). Quantitation of the number of branch points also revealed a significant reduction in branching complexity, from 23.2 to 16.3 per image, between control and Cxcr7−/− dermal lymphatics (Figure 2-5D). (Image area measured 132800 µm².) Additionally, Cxcr7−/− dermal lymphatic vessels were enlarged compared to littermate controls (Figure 2-5E-I, yellow dashed line). The junctional
Previously published studies demonstrate that this type of increase in vessel diameter and decreased branching complexity is consistent with a hyperplastic phenotype [33,34], suggesting that loss of CXCR7 results in hyperproliferation of LECs. We therefore sought to determine if this hyperplastic phenotype of Cxcr7\(^{-/-}\) dermal lymphatics could be attributed to increased AM-mediated LEC proliferation—a biological effect of AM which has been demonstrated by our group and others [19,35,36]. Using CXCR7 knockdown LECs, we observed a 50% increase in proliferation in AM-treated CXCR7 knockdown LECs compared to AM-treated control cells (Figure 2-5K). Collectively, these data demonstrate that loss of CXCR7 promotes AM-mediated LEC lymphangiogenesis by enhancing both migration and proliferation \textit{in vivo} and \textit{in vitro}.

\textit{Genetic titration of Adm changes Cxcr7\(^-\) survival}

We next sought to determine whether the phenotypes of Cxcr7\(^{-/-}\) embryos could be causally associated with AM ligand concentration \textit{in vivo}. To test this, we employed a genetic approach, depicted in Figure 2-6A, C, which allowed for the generation of Cxcr7 gene-targeted mice on a titrated background of AM ligand that ranges from 50\% to 300\% wildtype levels. Adm\(^{+/+}\) mice express 50\% of wildtype levels of Adm mRNA and peptide and exhibit exacerbated cardiovascular damage, reduced female fertility, and defective lymphatic function [22,37,38]. Adm\(^{hi/hi}\) animals survive to adulthood, but exhibit profound cardiac hyperplasia during development [24]. Therefore, we were confident that the effective dosage of AM peptide achieved with the AM “gene titration” mice was within a range that would have significant biological impact on lymphatic and cardiac development.

First, because we noted some embryonic lethality in Cxcr7\(^{-/-}\) animals in the genetic reduction experiment, we asked whether Adm haploinsufficiency might improve Cxcr7\(^{-/-}\)
embryo survival during mid-gestation. At e13.5 we observed equivalent numbers of Cxcr7−/−;Adm+/− and Cxcr7+/−;Adm+/− animals from compound heterozygous intercrosses (Figure 2-6B, e13.5). However, at e14.5 the expected 1:1 ratio of Cxcr7+/−;Adm+/−:Cxcr7−/−;Adm+/− genotype being offset by a disproportionate survival of Cxcr7+/−;Adm+/− embryos (Figure 2-6B, e14.5). Thus, haploinsufficiency of Adm improves Cxcr7+− embryo survival during mid-gestation. It is also noteworthy that embryonic lethality was significantly increased in the Cxcr7+/−;Adm+/− cross, which is likely due to the critical role of AM in female reproductive physiology [37,39-42]. This finding suggests that disruption of the parental CXCR7-AM axis likely contributes to the severity of the phenotypes.

Next, we bred Cxcr7+/− mice to Admhi/hi animals in order to evaluate whether AM overexpression might exacerbate Cxcr7−/− gestational loss or influence survival. Interestingly, we found that postnatal survival of compound heterozygous pups was poor, with nearly 30% postnatal lethality, making maintenance of the Cxcr7+/−;Admhi/+ mouse colony very challenging. Whereas we had not previously observed Cxcr7+/− pup lethality in other genetic crosses, Cxcr7+/− animals that expressed a single Adm+ allele were more susceptible to death. Moreover, homozygosity for the Admhi allele resulted in a striking 65% lethality of Cxcr7+/−;Admhi/hi mice (Figure 6D). Collectively, these data demonstrate that titration of the endogenous AM ligand is causally associated with profound changes in the survival of Cxcr7 gene targeted animals.

Haploinsufficiency of AM normalizes lymphatic and cardiac hyperproliferation of Cxcr7−/− mice

Based on the improved embryonic survival of Cxcr7+/−;Adm−/+ embryos, we next performed phenotypic characterization of lymphatic and cardiac development in these animals, with the expectation that haploinsufficiency for AM ligand might normalize the hypertrophic cardiovascular phenotypes of Cxcr7 null embryos. Importantly,
haploinsufficiency of AM had no effect on the LS:JV ratio in Cxcr7\(^{+/+}\) animals (Figure 2-6E, F). Consistent with Figures 3E-G, the podoplanin-positive lymph sacs of e13.5 Cxcr7\(^{-/}\);Adm\(^{+/+}\) embryos showed a significant 4-fold enlarged LS:JV ratio compared to wildtype littermates (Figure 2-6G, I). However, this lymph sac enlargement was normalized in Cxcr7\(^{-/-}\);Adm\(^{+/+}\) mice, resulting in LS:JV ratios that were statistically indistinguishable from wildtype animals (Figure 2-6H, I). Additionally, we used Ki67 staining to quantitate the number of proliferating LECs and found a direct correlation between the proliferation of LECs and the LS:JV ratios and genotypes. While Cxcr7\(^{-/-}\);Adm\(^{+/+}\) mice had significantly more proliferating cells in the LS compared to wildtype animals (Figure 2-6G, J), haploinsufficiency for AM (Cxcr7\(^{-/-}\);Adm\(^{+/+}\)) allowed this hyperproliferation to revert to levels that were equivalent to those observed in wildtype animals (Figure 2-6H, J). Taken together, these findings demonstrate that the increase in LS:JV ratio in CXCR7 null mice is due to an increase in LEC proliferation which can be reversed by genetic reduction of AM ligand.

Similar effects were observed in the developing heart. Using BrdU incorporation assays, we quantitated the amount of proliferating cells in e11.5 cardiac tissue from the AM genetic titration animals. Percent proliferation of cardiac cells in Cxcr7\(^{+/+}\);Adm\(^{+/+}\) animals was equivalent to wildtype animals, demonstrating that Adm haploinsufficiency alone does not affect heart size (Figure 2-7A-D). Consistent with other studies [18], we noted a statistically significant increase in proliferation of Cxcr7\(^{-/-}\)cardiac tissue compared to wildtype (Figure 2-7E, F, I). Importantly, this aberrant cardiac hyperproliferation was normalized to wildtype levels in Cxcr7\(^{-/-}\);Adm\(^{+/+}\) embryos (Figure 2-7G, H, I). We noted increased proliferation particularly within the epicardium and trabeculae (Figure 2-7F-H, arrows and arrowheads respectively)--regions of the heart where CXCR7 is highly expressed (see Figure 2-1F). Furthermore, 2 days after the AM-mediated peak in myocyte proliferation, many of the hearts of Cxcr7\(^{-/-}\);Adm\(^{+/+}\) embryos appeared phenotypically normal and similar in size to wildtype animals (Figure 2-7J-L). We therefore conclude that genetic reduction of AM
peptide alleviates the pathological cardiac hyperproliferation of Cxcr7 null animals and ultimately impacts cardiac size during development.

Though difficulty maintaining the line precluded extensive timed matings, we observed even more precocious development of the LS of Cxcr7-/-;Adm^{hi/hi} embryos. At e11.5, Cxcr7-/-;Adm^{hi/hi} embryos often had fully formed lymph sacs that were dramatically enlarged, and blood filled (Figure 2-S3E). This further exacerbated lymphatic development confirmed our hypothesis that Cxcr7-/- LS develop precociously. Hearts of Cxcr7-/- on the Adm^{hi/hi} background also tended to be enlarged with thickened compact zones and significant blood accumulation in the ventricles and atria (data not shown).

Discussion

The pleiotropic consequences of CXCR7 loss have made it difficult to discern which ligand(s) are responsible for a given phenotype. In this study, using both loss-of-function and gain-of-function animal models, we demonstrate that several of the essential functions of CXCR7 during cardiovascular development can be attributed to its decoy activities for the ligand, AM. Haploinsufficiency of AM in a Cxcr7-/- animal effectively reversed cardiac and lymphatic hyperproliferation, demonstrating that CXCR7 is required as a molecular rheostat for controlling AM ligand availability during development. While other atypical chemokine receptors are known to bind multiple ligands, only recently has the repertoire of CXCR7 ligands been expanded beyond the chemokines CXCL12 and CXCL11. For example, a very recent study has elegantly demonstrated that proteolytic peptide fragments of the adrenal neuropeptide proenkaphalin A interact with CXCR7 and thereby mediate responses to glucocorticoid secretion and anxiety behaviors in mice [43]. Structure-function studies of the proenkaphalin A-derived peptides, along with our current findings on the AM peptide, further highlight the ability of CXCR7 to bind to several classes of small peptidergic ligands, which also happen to be particularly enriched in the adrenal gland. Whether
adrenomedullin—which as it name implies, is also highly expressed in the adrenal gland—is also implicated in the CXCR7-mediated glucocorticoid secretion and anxiety behaviors has yet to be determined.

Likewise, our study does not formally rule out the involvement of CXCL12 in the described cardiovascular hyperplasia phenotypes of Cxcr7/- mice. Genetic deletion of either CXCR4 or CXCL12 has been associated with defective ventricular septum formation [44,45]. However, only recently have studies in chick embryos demonstrated a gain-of-function phenotype for CXCL12 overexpression in neural crest cell migration related to cardiac development [46]. While the effects of CXCL12 overexpression might be anticipated to more closely parallel the effects of CXCR7 loss-of-function, the cardiac phenotypes of these two models are markedly different. CXCL12 mis-expression in chick embryos diverts neural crest cell migration to the heart, while CXCR7 deficiency promotes cardiac myocyte hyperplasia and septal defects. Our current study does not preclude the functional deregulation of CXCL12, however it does definitively demonstrate that genetic reduction in AM ligand is fully sufficient to rescue the Cxcr7/- cardiac hyperplasia. Therefore, although we cannot exclude potential effects of CXCL12 on the Cxcr7/- cardiac phenotypes, we have identified AM as the critical ligand for mediating the cardiac hyperproliferation.

With respect to lymphangiogenesis, genetic studies in zebrafish have also established a central role for CXCL12(SDF-1)/CXCR4 signaling during the stepwise assembly of the lymphatic trunk network [47]. Interestingly, the requirement for CXCL12 in guiding zebrafish LEC migration occurs at developmental time points that are subsequent to LEC sprouting from the posterior cardinal vein. However, our expression studies in mice revealed little expression of CXCR7 in LECs that have migrated away from the jugular vein, and we failed to observe prominent defects in the migration and assembly of lymphatic sacs in Cxcr7/- mice. Although these spatiotemporal differences in receptor-ligand expression patterns may simply be attributed to differences between species, a more compelling
implication of these studies is that dynamic changes in the expression of CXCR4 and CXCR7 may be critical for orchestrating the extent and time frame to which nascent LECs sense and respond to different lymphangiogenic growth factors. Moreover, the dynamic spatiotemporal expression of signaling and decoy receptors likely provides a mechanism for individual LECs and their adjacent tissues to create a localized microgradient of chemotactic or proliferative factors [11,12] to promote the stepwise growth of lymphatic vessels.

Although our current study has focused on the effects of CXCR7 on AM-mediated cardiac and lymphatic development, it is important to recognize that the interaction of CXCR7 with AM may initiate downstream signaling and active processes in other cell types or tissues. For example, several studies have pointed to potential non-decoy functions of CXCR7, such as signaling through β-arrestin, heterodimerizing with other GPCRs in certain tissues, and coupling with G-proteins in astrocytes [23,48,49]. Therefore, while we have currently established CXCR7 as a molecular rheostat for AM signaling during cardiovascular development, future studies may identify potential CXCR7-GPCR complexes that allow for functional AM signaling in other cell types or tissues.

Our findings of increased ERK phosphorylation in dermal lymphatics of Cxcr7−/− mice are consistent with several previously described models with aberrant lymphangiogenesis associated with increased ERK signaling. For example, ex vivo expression of Spred-1/2, negative regulators of ERK activation, suppresses LEC proliferation while double knockout mice exhibit dilated, blood-filled lymphatics—similar to the Cxcr7−/− phenotype described here [50]. Likewise, mice lacking apoptosis stimulating protein of p53 (Aspp1) have increased ERK activation and exhibit similar transient subcutaneous edema with dilated and dysmorphic lymphatics and increases in cardiac LYVE1+ staining [51]. Most recently, a fine-tuned balance between ERK and Akt signaling pathways has been recognized as an essential component for establishing LEC fate determination and differentiation [52,53].
Collectively, these studies identify ERK signaling as a critical regulator of lymphangiogenesis and either loss or excessive ERK signal as a cause of aberrant lymphangiogenesis. Results of this study identify a mechanism, whereby the positioning of a decoy receptor at the junction of lymphatic sprouting and migration serves as a biological rheostat for regulating the migratory and mitogenic effects of lymphangiogenic growth factors that are upstream of ERK activation. Additional studies to determine whether and how other atypical chemokine receptors may influence cardiac and lymphatic development are warranted and may lead to conceptual paradigms about how growth factor gradients and their downstream signaling pathways can be precisely controlled by 7-transmembrane decoy receptors during cardiovascular development.

**Experimental Procedures**

*Mice*

Mice that contain a GFP reporter knocked into the Cxcr7 gene were purchased (C57BL/6-Ackr3<sup>2m1Litt/J</sup>, The Jackson Laboratory). Generation of Adm<sup>+/+</sup> and Adm<sup>hi/hi</sup> mice with a targeted, deletion and overexpression of Adm, respectively, has been previously described. [21,22,40]. For timed pregnancies, Cxcr7<sup>+/+</sup> animals were intercrossed with Cxcr7<sup>+/+</sup> or Cxcr7<sup>+/+</sup>;Adm<sup>+/+</sup> animals. Dams were monitored for vaginal plugs, and the day when the vaginal plug was detected was considered E0.5. Cxcr7<sup>+/+</sup>;Adm<sup>hi/hi</sup> animals were also intercrossed to establish survival. For BrdU incorporation assays, pregnant females were injected with BrdU (0.1 mg/g of BW, Sigma-Aldrich) via intraperitoneal injection two hours prior to dissection. All experiments involving mice were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill.
**Cell culture and RNAi**

Human adult (HMVECdLyAd-Der) and neonatal (HMVEC-d Neo) dermal lymphatic endothelial cells (Lonza) were cultured in EGM-2MV media. HEK293T cells were maintained in DMEM with 10% fetal bovine serum and 1% penicillin streptomycin or gentamicin. Lentiviral particle production and infection were performed according to standard protocol. Briefly, human CXCR7 shRNA pLKO1 vectors (UNC Viral Core) were co-transfected into HEK293T cells with lentiviral packaging vectors psPAX2 and MD2.G (Addgene) using Lipofectamine 2000 (Invitrogen). Viral supernatants were filtered, supplemented with 6μg/mL polybrene, and used to infect LECs for 48 hours before functional assays were performed.

**Gene Expression Analysis**

Agilent human gene expression microarrays were performed on three independent plates of hLECs treated with 10nM AM (American Peptide Company). Analysis was performed using the Significance Analysis of Microarrays (SAM) software (Stanford University). For embryonic endothelial cells, CD31 positive cells were isolated using magnetic beads. Quantitative RT-PCR was performed using primers and probes or hCXCR7 Assays on Demand (Life Technology) after reverse transcription of 2 μg of total RNA. For AM_{22-52} treatment, cells were incubated with 1μM AM_{22-52} (American Peptide Company) for 30 minutes prior to treatment with 10nM AM and 1μM AM_{22-52}.

**Scavenger assay**

HEK293T cells were transfected with CXCR7 or pcDNA3.1 (negative control) using standard calcium phosphate transfection. Cells were treated with biotinylated-AM (Phoenix Pharmaceuticals), and aliquots of media were collected over 8 hours. Biotinylated-AM was detected with IRDye Streptavidin (1:2500, Li-COR).
**ERK phosphorylation**

HEK293T cells were transfected with expression plasmids, serum starved for 20 hours, and treated with vehicle or 10nM AM for 1 minute. Blots were blocked in 5% BSA, probed overnight with monoclonal rabbit anti-mouse pERK and tERK (1:1000, Cell Signaling) and monoclonal mouse anti-GAPDH (1:2000, Novus Biologicals), incubated in appropriate secondary antibody, and imaged on the Odyssey scanner (Li-COR). A blot with 3 independent experiments run on the same gel was used to perform statistical analysis.

**Immunohistochemistry**

Embryo sections and whole mount tissue were permeabilized, blocked with 5% normal donkey serum, and incubated overnight at room temperature with primary antibodies, and then probed with appropriate secondary antibodies. Antibodies are described in the supplemental experimental procedures.

**Quantitation of LS:JV ratio and Blood and Protein Accumulation in LS**

Transverse sections of jugular lymph sacs of wildtype and mutant mice were H&E stained. The area of the LS and JV were measured using ImageJ software (NIH), and sections were graded for blood and protein accumulation in the LS using a scoring rubric. Blood and protein were graded as follows: 0 = no red blood cells (RBC); 1 = 3-10 RBC; 2 = 3-50 RBCs; 3 = >50 RBCs; 0 = no protein; 1 = minimal protein accumulation; 2 = moderate protein accumulation; 3 = extensive protein accumulation. Brightfield images were taken on a Leitz Dialux 20 Microscope.

**In vitro Migration and Proliferation Assays**

**Scratch assay.** CXCR7 knockdown (and negative control) LECs were grown to confluence and then scratched with a pipette tip. LECs were rinsed with PBS to remove non-adherent cells and then treated in 0.5% FBS RPMI with vehicle or 10nM AM. Four
fields per well were imaged at T=0 hrs and at T=18hrs post-scratch using an Olympus IX-81 inverted microscope equipped with a QImaging Retiga 4000R camera at 4X magnification. The percent change in migration was calculated by measuring the open area of the scratch (ImageJ). Results shown are representative of three independent experiments.

Transwell migration assay. HEK293T cells transfected with expression plasmids and LECs with lentiviral induced CXCR7 knockdown were labeled with 5µM Cell Tracker Green (CTG) CMFDA (Life Technologies). Cells (1x10^5) were treated with 10nM AM for 5 min and then seeded onto 8µm transwell inserts (BD Biosciences). After 4 hour incubation, inserts were fixed with 4% PFA, and filters were mounted for analysis. Quantification of transmigrated cells was done by measuring the threshold of CTG-labeled cell fluorescence using ImageJ (NIH).

Proliferation. CXCR7 knockdown and scramble LECs were plated, serum starved for 4 hours, then treated with 10nM AM for 24 hours. Cells counts were assessed using a Countess Automated Cell counter (Life Technology).

Statistical Analysis

Student’s two-tailed t test was used for all comparisons unless otherwise noted in the figure legend.

Supplemental Experimental Procedures

qRT-PCR of thoracic duct and vena cava

RNA was isolated from the thoracic duct and vena cava of adult wildtype mice (Qiagen RNeasy Micro kit and Qiashredder). Quantitative RT-PCR was performed after reverse transcription of 2 µg of total RNA. Primer sequences for mCxcr7 amplification were
5'-GTCAGGAAGGCAAACCACAGC-3’ and 5’-AGGCTCTGCATAGTCAAACAAGTG-3’, and the probe sequence was 5’-FAM-GAAGCCCTGAGGTCACTTGGTCGCTC-TAMRA-3’.

**Gene Expression Analysis**

Quantitative RT-PCR was performed using primers and probes or hCXCR7 Assays on Demand (Life Technology) after reverse transcription of 2 µg of total RNA. Primer sequences for hCXCR7 amplification were 5’-GCAGAGCTCACAGTTGTTGC-3' and 5’GCTGATGTCCGAGAAGTTGC-3’, and the probe sequence was 5’-FAM-AGGTCATTTGATTGCCCGCCTCAGAA-TAMRA-3’.

**Immunohistochemistry**

Embryo sections and whole mount tissue were permeabilized, blocked with 5% normal donkey serum, and incubated overnight at room temperature with primary antibodies and incubated with appropriate secondary antibodies (1:200; Jackson ImmunoResearch Laboratories) and DAPI (1:1000; Sigma-Aldrich). Antibodies included rabbit anti-mouse adrenomedullin (1:100; Novus Biological); chicken anti-GFP (1:500, Aves Lab); rabbit anti-mouse LYVE1 (1:600, Fitzgerald); Syrian hamster anti-mouse podoplanin (1:50; Developmental Studies Hybridoma Bank); rabbit anti-mouse pERK (1:1000, Cell Signaling); mouse anti-BrdU (1:200; Zymed Laboratories); rabbit anti-mouse Ki67 (1:200; Vector Laboratories), and rabbit anti-mouse Prox1 (1:100; Angiobio). For Ki67 and Prox1 staining, antigen retrieval with citrate buffer was performed prior to beginning staining protocol. Images were acquired on a Nikon E800 microscope (Nikon) with a Hamamatsu camera (Hamamatsu Photonics) with Metamorph software (Molecular Devices, Inc.). H&E staining was performed by UNC Histopathology Core.

**Bioluminescence Resonance Energy Transfer (BRET)**

BRET studies to test cAMP accumulation were carried out as described previously. Briefly, the calcium phosphate precipitation method was used to transfect HEK293 cells with
**hCxcr7/hRAMP2, hCLR/hRAMP2** (positive control), and EPAC, a biosensor for cAMP accumulation. Cells were treated with the luciferase substrate coelenterazine H (Promega) for 10 min. Five minutes into coelenterazine H treatment, increasing doses of AM (American Peptide Company) were added. At 10 minutes post-coelenterazine H treatment, BRET signal was read using a Berthold Technologies Mithras LB940 plate reader. BRET signal was calculated as the ratio of light emitted from YFP (530 nm) to light emitted from RLuc (485 nm). YFP/RLuc ratios of PBS-treated cells were used to control for baseline cAMP accumulation. Results were analyzed using GraphPad Prism. Results shown are representative of at least 3 replicate BRET experiments.

**Whole mount immunohistochemistry**

**Whole mount heart.** Cardiac tissue was carefully removed from fixed embryos and fixed again in 4% PFA for 4 hours at RT. Hearts were permeabilized in PBST (0.1% Triton in PBS) at 4°C overnight, washed (5’ in PBST, 5’ in H2O, 7’ in acetone at -20°C, 5’ H2O, and 5’ in PBST) and were blocked in 5% normal donkey serum at 4°C overnight. Samples were then incubated in primary antibody (LYVE1 - 1:200) for 5 days. Hearts were washed and blocked in 5% normal donkey serum (1.5 hours) and incubated in secondary antibody for 48 hours at 4°C.

**Whole mount skin – P1.** Skin was removed from the back of P1 pups, submerged in 20 mM EDTA, and heated in a 37°C oven for 3 hours to remove epidermis. Skin was fixed overnight, incubated with LYVE1 and pERK antibodies, and analyzed for ERK phosphorylation and vessel morphology. A branch point is defined as a vessel with 3 vessels branching away. A lacuna is defined as a space where 3 or more branch points coalesced.
Whole mount skin – e13.5. Skin was removed from e13.5 embryos as previously described (James et al., 2013). Briefly, dorsal skin was carefully removed from fixed e13.5 embryos and blocked in 10% NDS for 2 hours at RT. Samples were then incubated in primary antibody overnight at 4°C. Samples were washed, incubated in secondary antibody at RT for 1 hour, and whole mounted on glass slides with ProLong Gold (Life Technologies). Images were acquired on a Nikon E800 microscope (Nikon) with a Hamamatsu camera (Hamamatsu Photonics) with Metamorph software (Molecular Devices, Inc.).

Scavenger assay and ERK phosphorylation in LECs

Scavenger Assay. Control LECs and LECs with lentiviral induced CXCR7 knock-down were treated with biotinylated-AM (Phoenix Pharmaceuticals), and aliquots of media were collected over 8 hours. Biotinylated-AM was detected with IRDye Streptavidin (1:2500, Li-COR, Lincoln, Nebraska USA). Results shown are representative of at least three separate experiments.

ERK phosphorylation in LECs. Control LECs and LECs with lentiviral induced CXCR7 knock-down were serum starved in 0.5% FBS for 16 hours with or without 10nM AM. Cells were then treated with vehicle or 10nM AM for 15 minutes. Blots were blocked in 5% BSA, probed overnight with monoclonal rabbit anti-mouse pERK and tERK (1:1000, Cell Signaling), and monoclonal mouse anti-GAPDH (1:2000, Novus Biological), incubated in appropriate secondary antibody, and then imaged on the Odyssey scanner (Li-COR). A blot with 3 independent experiments run on the same gel was used to perform statistical analysis.
In vivo proliferation

Lymphatic endothelial cell proliferation. Paraffin sections of e13.5 embryos were stained for Ki67 and podoplanin. Podoplanin+ and Ki67+ cells were counted and normalized to total number of DAPI cells in the lymph sac to determine percent proliferation.

Cardiac proliferation. Two hours prior to dissection, pregnant females were injected intraperitoneally with BrdU (1 mg/g of BW, Sigma-Aldrich). At e11.5, embryos were carefully dissected and genotypes were determined from DNA extracted from the tail. Paraffin sections of the heart were stained for BrdU, and percent proliferation was determined by quantitation of BrdU+ cells normalized to total number of cells.
Figure 2-A1. Graphical Abstract
Figure 2-1. CXCR7 scavenges AM, dampens AM-mediated ERK phosphorylation in vitro, and reduces AM peptide levels in vivo.

(A) Cxcr7 expression in cardiac tissue of Adm+/+ and Adm+/hi mice (n=6) and isolated endothelial cells of Adm+/+ and Adm+/ mice (n=3)
(B) The nine most significantly upregulated and four most significantly downregulated genes in human LECs (hLECs) treated with 10nM AM for 1 hour.
(C) CXCR7 expression in vehicle and 10nM AM treated hLECs at 15m, 1h and 24h, and AM22-52 AM, and (AM22-52+AM) for 4 hr.
(D) Representative western blots probed for biotin and (E) quantitation of biotinylated-AM1-52 depletion over 8 hours by either CXCR7 or EV expressing cells in three independent experiments.
(F,G) Cxcr7 and AM staining in e13.5 Cxcr7+/− cardiac tissue with epicardial colocalization (white arrows).
(H,I) AM staining of e13.5 Cxcr7+/+ and Cxcr7−/− cardiac tissue. White arrows highlight epicardium, CZ identifies the compact zone, and white arrowheads highlight the cardiac trabeculae. Images were obtained at the same exposure and the amount of AM expressed in the three regions of the heart was assessed by measuring the integrated density of staining using Image J software (n=3-5). Scale bars, 100 µM.
(J-L) Quantitation of AM staining intensity in Cxcr7+/− (n=3) vs. Cxcr7−/− (n=5) animals in 3 regions of the heart, trabeculae, epicardium, and compact zone. Staining intensity is expressed as arbitrary units of integrated density measured by ImageJ.
(M) Representative western blot and (N) quantitation of change in pERK:tERK between vehicle and 10nM AM treated CXCR7-, CLR/R2-, and CLR/R2+CXCR7-expressing HEK293T. Quantitation was calculated using 3 independent experiments run on the same gel.

In (A-L), data are represented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

See also Figures 2-S1, 2-S2, and Table 2-S1.
Figure 2-2. *Cxcr7* is dynamically expressed in lymphatic endothelium during development.

(A-C) *Cxcr7* and LYVE1 colocalize in the JV of e11.5 *Cxcr7<sup>+/−</sup>* animals.

(D-F) Higher magnification of the LYVE1 positive portion of the JV. White arrows highlight areas of *Cxcr7* and LYVE1 colocalization. Asterisk highlights migrating LYVE1 positive, *Cxcr7* negative cells.

(G-I) *Cxcr7* and Prox1 colocalize in the JV of e11.5 *Cxcr7<sup>+/−</sup>* animals. Asterisks highlight migrating Prox1 positive, *Cxcr7* negative cells LECs.

(J-O) *Cxcr7* and lymphatic markers (J, podoplanin; M, LYVE1) colocalize in the LS (white arrows) of e11.5 (J-L) and e13.5 (M-O) *Cxcr7<sup>+/−</sup>* animals.

(P-R) *Cxcr7* is also expressed in cells of the JV directly adjacent to the LS. White arrowheads highlight *Cxcr7* expression in the JV. Scale bars, 50 µM.
Figure 2-3. *Cxcr7*−/− embryos have enlarged blood filled lymphatic sacs and interstitial edema.

(A-C) Some *Cxcr7*−/− embryos exhibit interstitial edema at e13.5 (white (A) and black (C) arrows).

(D-F) *Cxcr7*−/− embryos exhibit enlarged lymph sacs filled with blood (asterisks) and proteinaceous deposits (arrowheads) at e13.5.

(F) In some *Cxcr7*−/− embryos, the LS fails to separate from the JV properly. Platelet thrombi are highlighted by arrowheads.

(G-I) Quantitation of the LS:JV ratio (t test) and blood and protein accumulation (Mann-Whitney U test) in the LS of *Cxcr7*−/− embryos (*n*=10) compared to wildtype controls (*n*=5). A detailed description of the scoring rubric is provided in the methods section. Data are represented as mean ± SEM. *p<0.05, **p<0.01.

See also Figure 2-S3.
Figure 2-4. Loss of CXCR7 enhances cardiac lymphangiogenesis by promoting AM-mediated cellular migration.

(A) View of ventral side of whole mount hearts of e18.5 embryos stained with the lymphatic marker LYVE1.

(B,C) Quantitation of LYVE1 staining normalized to surface area (B) and LEC migration down the apex of the hearts (C) (n=3).

(D) CXCR7 knockdown in LECs by two shRNA lentiviral constructs.

(E-H) Control or CXCR7 knockdown LECs treated with vehicle (E,F) or 10nM AM at 18 hrs (G,H). Migration from the time of scratch (T=0) was measured. Scale bars, 100 µM.

(I) Quantitation of LEC migration in CXCR7 knockdown and control LECs.

(J,K) Quantitation of transwell migration of CXCR7 knockdown LECs treated with vehicle or 10nM AM (J), and empty vector (EV), CXCR7-, CLR/R2-, and CLR/R2+CXCR7-expressing HEK293T cells treated with 10nM AM.

Data are represented as mean ± SEM. *p<0.05, **p<0.01.

See also Figure 2-S4 and 2-S5.
Figure 2-5. Loss of CXCR7 causes enlarged dermal lymphatics with decreased branching complexity in vivo and enhances LEC proliferation in vitro.

(A,B) Cxcr7−/− P1 skin (n=5) exhibit dysmorphic dermal lymphangiogenesis compared to controls (n=4). White asterisks identify a lacuna; yellow dots highlight branch points. Scale bars, 50 µM.

(C,D) Quantitation of the number of lacunae and branch points respectively. Fewer lacunae and decreased branching complexity were observed in Cxcr7−/− animals. A branch point is defined as a vessel with 3 vessels branching away. A lacuna is defined as a space where 3 or more branch points coalesce.

(E-H) Skin of e13.5 Cxcr7−/− embryos. Scale bars, 50 µM.

(I-J) Lymphatic vessels of P1 Cxcr7−/− embryos are dilated, with increased junctional area where vessels coalesce to form a branch point. Yellow dashed and straight line in (G,H) represent the vessel diameter and junctional area, respectively, measured in P1 skin.

(K) Quantitation of LEC proliferation after a 24h treatment with 10nM AM.

In (A-K), data are represented as mean ± SEM. *p<0.05, **p<0.01. See also Figure 2-S6.
Figure 2-6. Genetic titration of Adm influences Cxcr7−/− phenotypes.
(A-B) Schematic of Adm genetic reduction experiment with expected and observed Mendelian ratios. Cxcr7−/−;Adm−/+ mice were bred with Cxcr7+/−;Adm−/+ mice, resulting in the following expected distribution: 25% Cxcr7+/+, 50% Cxcr7−/−, and 25% Cxcr7−− with 50% of each Cxcr7 genotype being AM heterozygous. Observed number of animals was statistically different from expected as judged by a chi-squared test (p=0.02).
(C-D) Schematic of Adm genetic increase experiment with expected and observed percentage of survival of animals, n = 85. Observed number of animals (including resorptions) was statistically different from expected as judged by a chi-squared test (p=0.001).
(E-H) Representative images of JV and LS of e13.5 embryos from the gene reduction experiment stained for podoplanin and Ki67. Scale bars, 100 µM.
(I) Quantitation of the LS:JV ratio in e13.5 embryos (n= 4-8 for each genotype).
(J) Percent proliferating cells in the LS (n= 4-8 for each genotype).
Data are represented as mean ± SEM. *p<0.05, **p<0.01 (One-way ANOVA).
Figure 2-7. Genetic reduction of AM normalizes Cxcr7−/− cardiac proliferation and size. (A-G) (A,C,E,G): BrdU staining of cardiac tissue of e11.5 embryos from the gene titration experiment. Exposure, 750 ms. Scale bars, 100 µM. (B,D,F,H): Arrows highlight proliferating epicardium, and arrowheads highlight proliferating endocardium. Exposure, 250 ms. Scale bars, 100 µM. (I) Percent proliferating cardiac cells in embryos from genetic reduction experiment. (n=3-5 for each genotype). Data are represented as mean ± SEM. *p<0.05 (One-way ANOVA). (J-L) H&E-stained cardiac tissue of e13.5 animals from the genetic reduction experiment. (n=3-5 for each genotype). Scale bars, 100 µM.
Figure 2-S1. Supplemental data related to Figure 2-1

(A) Cxcr7 expression has been established in normal and pathologic blood vasculature [54,55]. To assess whether Cxcr7 is expressed in adult lymphatic tissue, we compared mRNA expression in the vena cava and the thoracic duct. Although we observed a trend for increased expression in the thoracic duct compared to vena cava, this did not reach statistical significance. Data are represented as mean ± SEM. (B) CXCR7-expressing cells do not elicit canonical AM-mediated signal. CLR/R2 expressing HEK293T cells exhibit a robust accumulation of cAMP with increasing concentrations of AM treatment. CXCR7-expressing cells, however, do not elicit the same upregulation of cAMP. (C) Quantitation of pERK blot in Figure 2-1M. This graph represents the integrated density of the representative blot, whereas Figure 2-1N represents quantitation of the change in pERK from baseline levels of each condition in three independent experiments.
Figure 2-S2. LYVE1 and pERK colocalize in lymphatic vessels from Cxcr7⁻/⁻ mice (related to figure 2-1).

(A, B) Minimal colocalization of LYVE1 (red) and pERK (green) was observed in lymphatic vessels (white arrows) from the skin of Cxcr7⁺/⁺ mice. (C, D) LYVE1 (red) and pERK (green) staining of skin from Cxcr7⁻/⁻ mice showed colocalization of pERK in lymphatic vessels (white arrows). Average integrated density of pERK in LYVE1 positive vessels of Cxcr7⁺/⁺ and Cxcr7⁻/⁻ tails was 102762 and 181768, respectively. (E-J) Podoplanin and pERK staining in the JV and LS of Cxcr7⁺/⁺ mice (E-G) and Cxcr7⁻/⁻ mice (H-J). Average integrated density of pERK in LYVE1 positive vessels of Cxcr7⁺/⁺ and Cxcr7⁻/⁻ tails was 87231 and 95660, respectively. The increase of pERK in the LS of Cxcr7⁻/⁻ embryos was statistically significant, p<0.05 (n=3).
Figure 2-S3. Cxcr7\(^{-/-}\) mice have normal lymphovenous valves and precocious lymphatic development (related to figure 2-3). (A, B) e13.5 Cxcr7\(^{+/+}\) and Cxcr7\(^{-/-}\) embryos were frontally sectioned and stained with Prox1 and podoplanin. Prox1 highlights the lymphovenous valves (LVV) (white arrows). Cxcr7\(^{-/-}\) embryos exhibit structurally normal LVV. Abbreviations: Internal Jugular Vein (IJV), Subclavian Vein (SCV). (C, D, E) JV and LS of Cxcr7\(^{+/+}\), Cxcr7\(^{-/-}\), and Cxcr7\(^{-/-}\); Adm\(^{hi/hi}\) at e11.5 stained with lymphatic markers. Sections from (C, D) were stained with LYVE1 and (E) was stained with Prox1 and podoplanin.
Figure 2-S4. Cxcr7+/− embryos have more cardiac lymphatics with decreased branching complexity (related to figure 2-4). (A) Dorsal side of whole mount heart of Cxcr7+/+ embryos. (B) Higher magnification of cardiac lymphatic vessels shows the classical honeycomb patterning of lymphatic vessels in Cxcr7+/+ embryos. (C) Dorsal side of whole mount heart of Cxcr7−/− embryos. White arrows emphasize an extensive network of vessels on the outflow tract of the dorsal side of the heart that is not present in wildtype littermates. (D) Higher magnification of Cxcr7−/− embryos highlights the decreased lymphatic vessel branching complexity. (E, F) Quantitation of the number of branch points and lacunae in the cardiac lymphatics. Data are represented as mean ± SEM. *p<0.05.
Figure 2-S5. Loss of CXCR7 affects AM-signaling in LECs (related to figure 2-5). (A, B) Western blot probed for biotin (A) and (B) quantitation of biotinylated-AM depletion over 6 hours by either control or CXCR7 shRNA infected LECs. (C) Western blot analysis for pERK and total ERK of control or CXCR7 shRNA LECs treated with or without 10nM AM. Integrated density of the pERK/IERK ratio normalized to vehicle treated control cells is included below each blot.
Figure 2-S6. Cxcr7 is stochastically expressed in dermal lymphatic vessels (related to figure 2-5). LECs in the skin of P1 Cxcr7−/− pups reveal stochastic Cxcr7 expression, with high expression in dermal LECs and low expression in valve associated LECs. Gray arrows highlight the dermal lymphatic endothelial cells that express Cxcr7.
Table 2-S1. List of genes significantly changed following 1-hour AM treatment as identified by SAM plot (related to figure 1).

(A) List of genes up-regulated by 1-hour AM treatment
(B) List of genes down-regulated by 1-hour AM treatment

(A) List of up-regulated genes

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Fold change</th>
<th>p-value</th>
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<td>NR4A1, <em>Homo sapiens</em> nuclear receptor subfamily 4, group A, member 1 [NM_002135]</td>
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<td>RGS2, <em>Homo sapiens</em> regulator of G-protein signaling 2, 24kDa [NM_002923]</td>
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<td>DUSP2, <em>Homo sapiens</em> dual specificity phosphatase 2 [NM_004418]</td>
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<td>Description</td>
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REFERENCES


Chapter 3: Conclusions and Future Directions

Summary of Results

We have demonstrated that the decoy receptor CXCR7 (ACKR3) is required as a molecular rheostat for controlling the concentration of AM ligand during cardiac and lymphatic vascular development. Classical scavenging assays and western blot revealed that CXCR7-expressing cells are capable of binding and decreasing AM-mediated downstream signaling. Consistently, immunohistochemical staining of Cxcr7−/− mouse tissue indicated increased AM and pERK staining compared to wildtype controls, suggesting that, in the absence of CXCR7, AM scavenging is disrupted in vivo.

Immunohistochemistry of developing lymph sacs established that CXCR7 is dynamically expressed in the lymphatic progenitors and early lymphatic vessels during embryonic lymphangiogenesis. Importantly, the spatiotemporal expression of Cxcr7 coincided with AM activity. In contrast to Adm−/− animals, which exhibit small, hypoplastic lymph sacs, Cxcr7−/− mice displayed the reciprocal phenotype of enlarged, hyperplastic, blood and protein filled lymph sacs. Enhanced cardiac and dermal lymphangiogenesis was also observed. Though lymphatic vessel expression of Cxcr7 was stochastic, blood vessel expression was consistent. We therefore concluded that CXCR7 affects lymphangiogenesis in both a cell autonomous and non-cell autonomous fashion.

Finally, we have shown that the cardiac hyperplasia and lymphatic phenotypes observed in Cxcr7−/− animals were causally associated with AM ligand concentration. Using a genetic approach, we generated gene-targeted mice onto a titrated background of AM
ligand that ranged from 50% to 300% of wildtype levels. Haploinsufficiency of Adm improved survival of Cxcr7−/− mice, whereas Adm overexpression resulted in significantly decreased survival. Additionally, while Adm overexpression exacerbated lymphatic phenotypes, haploinsufficiency of Adm was fully sufficient to rescue the cardiac and lymphatic hyperplasia.

Collectively, the findings presented in Chapter 2 clarify the interaction between CXCR7 and AM and identify a novel role for the decoy receptor during lymphangiogenesis. To our knowledge, these data are the first to identify an atypical chemokine receptor as a modulator of lymphatic vascular development. As such, they offer a new conceptual paradigm about how growth factors gradients can be controlled during cardiovascular development.

**Current State of the Field – AM, decoy receptors, and lymphangiogenesis**

Our studies have identified a role for the decoy receptor CXCR7 during developmental lymphangiogenesis. However, much remains to be learned about how this and other atypical chemokine receptors affect lymphatic vasculature during normal and disease states. Additionally, the study of the contribution of the lymphatic system to human pathophysiology is a relatively nascent field [1]. However, data are emerging that implicate the lymphatic vasculature in common diseases, and consequently, the field is expanding. A few relevant and interesting areas of research are highlighted below.

*AM and developmental lymphangiogenesis*

The importance of AM during developmental lymphangiogenesis has been appreciated for nearly a decade, and our results emphasized that precise dosage of this peptide hormone is essential. Our study also suggests that atypical chemokine receptors play a prominent role in the development of the lymphatic vasculature.
Current dogma indicates that lymphatic vessels originate exclusively from venous sprouts [2]. However, recent data has demonstrated that a significant portion of the dermal lymphatic vasculature forms from a non-venous origin [3]. The identity of the progenitors that give rise to dermal LECs is unknown, and the molecular mechanisms and lymphangiogenic factors that dictate their migration and proliferation have not been elucidated. Interestingly, a significant portion of the dermal lymphatic vessels of mice lacking *Ramp2*, an essential component of the AM-signaling complex, was observed to be normal, suggesting that the early development of these vessels does not require AM-signaling [4]. These data indicate that non-venous dermal LECs may behave differently than their venous-derived counterpart. Future research is necessary to characterize these LECs and help determine if known lymphangiogenic factors, like AM and CXCR7, participate in such ‘lymphvasculogenesis’ [3].

*AM and lymphatics in the clinical setting*

Roles also exist for AM outside of embryogenesis. A detailed discussion of current knowledge regarding the role of AM in lymphatic vasculature is presented in Chapter 1. Recently, our group has discovered that AM is important for maintenance of adult lymphatic vessels [5]. Loss of AM-signaling during adulthood disrupts fat metabolism, results in ocular edema, and recapitulates the clinical symptoms of multi-organ lymphangiectasia. Whether this phenomenon depends on precise dosage of AM ligand is unknown. Future studies will aim to determine whether loss of the decoy receptor CXCR7 in adulthood affects lymphatic homeostasis in adulthood. These and future research are significant as the AM signaling system is among the most pharmacologically-tractable molecular players that regulate lymphangiogenesis and have potential for use in the clinic.

*Lymphatics and Lipid Metabolism.* In the past decade and a half, we have learned that the lymphatic vasculature plays critical roles not only in fluid homeostasis, but also in lipid metabolism and immune defense [6]. The lymphatic vasculature within the intestinal
villi (lacteals) absorbs and delivers chylomicrons to the bloodstream [7,8]. Several groups have demonstrated that disruption of the lacteals results in dysregulated fat metabolism. Mice heterozygous for Prox1, a transcription factor that is considered the master-regulator of lymphatic fate, exhibit leaky lymphatic vessels and develop adult-onset obesity and inflammation [9]. In contrast, while they also develop leaky lymphatic vessels, conditional Calcrl−/− mice fail to match weight gain of their wildtype controls [5]. Whereas Prox1+/− mice absorb fat but have dysregulated fat deposition, conditional Calcrl−/− mice exhibit lipid malabsorption. As both of these phenotypes are attributed to the permeability of vessels, these data suggest that additional mechanisms contribute to lipid absorption by the intestinal lymphatics. As such, several research groups are actively trying to understand the interplay between the lymphatic vasculature and lipid metabolism [7,10]. It will be interesting to determine whether polymorphisms in lymphatic associated genes are associated with either leanness or obesity. Moreover, as obesity continues to be a major health issue, there will be much interest in determining whether targeting the intestinal lymphatics for the treatment of weight disorders is feasible. The AM-system, which contains two pharmacologically tractable GPCRs in CLR and CXCR7 may prove to be a promising drug target.

**AM, CXCR7, and lymphangiogenesis in cancer.** Tumor lymphangiogenesis has also received significant attention as it has become clear that lymphatic vessels and lymphangiogenic growth factors contribute to promoting cancer metastasis [11]. Several studies in the early 2000s revealed vascular endothelial growth factor C and D (VEGF-C and VEGF-D) increased tumor lymphangiogenesis and facilitated lymph node metastasis [12-14]. Examination of human cancers confirmed a positive correlation between tumor-induced lymphangiogenesis, lymphangiogenic factors, and the poor prognosis associated with lymph node metastasis [11,15]. As such, a major focus of the lymphatic field is to identify the molecular players and mechanisms by which metastases utilize lymphatic transport.
VEGF-C, its receptors, and additional molecules including AM have been shown to promote lymphatic associated metastasis [16]. Gene-titration studies in Lewis lung carcinoma (LLC) cells revealed that AM leads to distant metastasis by stimulating lymphangiogenesis [16]. LLCs that overexpress AM increase lymphatic proliferation, dilation and result in increases in metastasis to the lung compared to wildtype LLCs. Karpinich and colleagues have further established that AM enhances gap junction coupling, which facilitates transendothelial migration of tumor cells [17]. Inhibition of intracellular gap junction communication prevented tumor cell migration through LECs. The impact of CXCR7 on AM-mediated tumor cell migration remains to be addressed.

Several groups have shown that CXCR7 promotes tumor growth and metastasis [18-20]. These reports are perhaps counter to what would be expected from the described role of CXCR7 as an AM-decoy receptor. In its role as AM scavenger, one might speculate that overexpression of CXCR7 would decrease AM-signaling, thereby decreasing metastasis. However, it has become clear that the cellular milieu impacts CXCR7 behavior dramatically. For example, while CXCR7 is generally thought not to stimulate intracellular signaling, recent reports demonstrate that it does couple to G proteins in astrocytes [21]. It is possible that in tumor cells CXCR7 functions as a signaling AM receptor (a potential mechanism for this will be addressed extensively in Part II of the dissertation), or that its interaction with other peptides overwhelms its ability to modulate AM signaling. Additionally, as AM is often highly expressed in tumors, the capacity of CXCR7 to downregulate AM-signaling may be diminished.

Decoy Receptors, Macrophages, and Lymphangiogenesis

Shortly after our work was published, a second atypical chemokine receptor was identified to modulate lymphangiogenesis. Mice lacking ACKR2, a CCL2 chemokine receptor primarily expressed on the lymphatic endothelium [22], exhibit increased lymphatic
density [23]. Conversely, mice deficient for the CCL2 signaling receptor CCR2 exhibit increased lymphatic density. Misregulation of prolymphangiogenic macrophages in the absence of these receptors is responsible for these reciprocal phenotypes. Loss of the scavenging receptor ACKR2 results in increased macrophage recruitment to the developing vessel network, whereas CCL2-deficient mice have decreased pro-lymphangiogenic macrophages and a lower concentration of lymphangiogenic factors. These findings nicely complement our studies as they identify a second GPCR that regulates pro-lymphangiogenic factor gradients. Here, rather than controlling the ligand concentrations, ACKR2 controls macrophage recruitment.

Two potential mechanisms may explain how macrophages contribute to post-natal lymphangiogenesis: (1) they can serve as a source of VEGF-C, which stimulates LEC proliferation and migration and (2), macrophages can transdifferentiate into LECs and integrate into developing lymphatic capillaries [24]. First discovered in association with tumors, pro-lymphangiogenic macrophages, once stimulated, release VEGF-C and VEGF-D to facilitate tumor lymphangiogenesis and lymph node metastasis [25]. During inflammation-induced lymphangiogenesis, circulating macrophages start to express lymphatic markers and are incorporated into lymphatic vessels [26]. In a corneal wounding model, lymphangiogenesis was abrogated with pharmacological depletion of macrophages, suggesting that macrophages play a central role in neolymphangiogenesis [27].

As such, it is possible that modulation of the lymphangiogenic activity of macrophages may prove useful in disease states. Inhibition of pro-lymphangiogenic macrophages may offer potential to prevent tumor-associated lymphangiogenesis and subsequent metastasis. Additionally, autoimmune disorders, such as inflammatory bowel disease and diabetes, where aberrant lymphangiogenesis contributes to excessive inflammation, may improve with inhibition of neolymphangiogenesis. As many studies have indicated that macrophages migrate towards and express AM, pharmacological inhibition of
AM may have potential as a therapeutic target [25,28-30]. Further research in this arena is warranted.

**Novel Roles for the Lymphatic Vasculature**

Lymphatic vasculature has newly recognized regulatory roles in physiology. The discovery that dermal neolymphangiogenesis is required for electrolyte and blood pressure homeostasis has challenged the long-held notion that renal electrolyte elimination is sufficient for control of systemic pressure [31]. Interestingly, macrophages attracted to the dermis respond to high salt load by expressing the transcription factor TonEBP, a specific membrane “osmomemeter”, which activates the VEGF-C promoter in macrophages [1,32]. Macrophage stimulated neolymphangiogenesis enhanced electrolyte clearance. Inhibition of macrophage recruitment or VEGF-C trapping inhibited this novel lymphatic vessel formation and increased sensitivity to dietary sodium, resulting in increased salt loading and hypertension [1,31].

Recent publications indicate a role for lymphatics in the eye, previously thought to be free of lymphatic vessels [33]. Two groups showed that the cells of Schlemm’s canal, a specialized vascular structure that drains aqueous humor, achieve a lymphatic-like phenotype [34,35]. This lymphatic reprogramming is necessary to maintain drainage through Schlemm’s canal. Suppressing these “lymphatic-fated” or “lymphatic-like” cells can lead to increased intraocular pressure and potentially glaucoma. These newly identified lymphatic-like cells not only provide a potential clinical target for the treatment of glaucoma, but also suggest that vessels can adopt a hybrid fate.

Ongoing work in our lab supports this concept, as we have begun to identify lymphatic-fated cells in the spiral arteries of the placenta – a tissue where both AM and CXCR7 are highly expressed. Here, the spiral arteries remodel to ensure proper blood flow to the developing fetus [36]. In accordance with our preliminary data that cells of the spiral
arteries turn on LEC markers such as Prox1 and Vegfr3, we hypothesize that, like the cells of Schlemm's canal, these cells become lymphatic-fated. Future work will aim to address whether the AM-signaling system governs this lymphatic reprogramming.

**Future Directions**

The data presented in Part I identify a role for decoy receptors as modulators of the timing and extent of GPCR-mediated signaling during cardiovascular development and identified a means for the mechanism to achieve control of AM-signaling. Future studies should expand our understanding of the role of AM and CXCR7 in cardiovascular tissues and continue to emphasize the clinical importance of lymphatic vascular system.

**Future studies: role of AM, CXCR7, in cardiac development**

As discussed in Chapter 2, our excitement about Cxcr7 began with a similar phenotype of cardiac hyperplasia observed in both Cxcr7<sup>-/-</sup> and Adm<sup>hi/hi</sup> mice. Remarkably, haploinsufficiency of AM was sufficient to rescue the cardiac hyperproliferation in Cxcr7<sup>-/-</sup> mice, demonstrating excessive AM-signaling is responsible for the hyperplastic phenotype. Moreover, while our work suggested that cardiac expression of Cxcr7 paralleled Adm expression, we hypothesized that non-cell autonomous CXCR7 expression can affect cardiovascular development. Future studies will be aimed at further elucidation of the mechanisms of CXCR7 modulation of AM-signaling, with a particular focus on characterizing the cell types responsible for scavenging AM.

Recent work from our lab has identified epicardial-derived AM as the primary driver of cardiac proliferation. Immunohistochemistry of AM peptide in Adm<sup>hi/hi</sup> animals showed increased staining in the epicardium and the trabeculae in developing mouse hearts (Figure 3-1). As shown in Figure 2-1F, H, this increase in expression closely paralleled the expression of CXCR7. Additionally, loss of CXCR7 in these regions resulted in increased
AM-staining intensity, suggesting that local levels of CXCR7 are responsible for AM scavenging (Figure 2-1G, I). In support, Cre-mediated depletion of the Adm^{hi} gene from the epicardium of Adm^{hi/hi} animals completely abolished cardiac hyperplasia, definitively demonstrating that local AM secretion from the epicardium is responsible for the cardiac hyperplasia [37]. Similar conditional and cell-specific expression and deletion of Cxcr7 will help determine whether expression of the decoy receptor in specific cell types modulates AM ligand concentrations.

To address this question, Cxcr7^{fl/fl} mice can be intercrossed with Cre lines to generate cell-type specific deletion of Cxcr7. Interestingly, previously reported endothelial specific deletion of Cxcr7 via Tie2-Cre resulted in a similar, but less severe cardiac phenotype compared to global Cxcr7^{−/−} mice [38]. These data suggest that endocardial expressed CXCR7 plays a significant role in AM scavenging. This finding is consistent with our observed increase in AM staining in the trabeculae. However, Tie2-Cre causes recombination in many other cell types, including all cells stemming from venous progenitors. Therefore, vascular expression of Cxcr7 may also contribute to AM scavenging in this model.

Use of additional Cre lines will help parse out which cell types are responsible. Intercross of Cxcr7^{fl/fl} mice with the epicardial specific WT1-Cre will be of particular interest. Based on the Tie2-Cre model, which identifies the endocardium as a major player in the Cxcr7^{−/−} phenotype, loss of CXCR7 exclusively in the epicardium would not be expected to fully recapitulate the cardiac phenotypes. However, because AM expression from the epicardium is essential to cardiac proliferation, if AM production from these cells remains unchecked, it is possible that Cxcr7^{fl/fl};WT1-Cre^{+} could result in a phenotype similar to Adm^{hi/hi} and Cxcr7^{−/−} mice. As we have hypothesized that both cell autonomous and non-cell autonomous mechanisms of AM scavenging, it is also possible that loss of Cxcr7 in any given cell type may not cause a complete phenocopy. As such, regardless of the outcome,
these studies have potential to build upon our studies and extend our understanding of the molecular mechanisms that control AM dosage. Understanding whether Cxcr7 works in a cell-autonomous or non-cell autonomous fashion (or both) will guide future drug development for the treatment of cardiovascular and other diseases.

Future studies: AM, decoy receptors, and cardiac lymphatics

Cardiac lymphatics, which drain the subendocardial, myocardial, and subepicardial areas, have recently been shown to modify heart pathologies [39]. As the function of cardiac lymphatic vessels is dependent on cardiac contraction, many diseases associated with poor myocardial function are also associated with poor lymphatic drainage and myocardial edema. Interstitial edema around the myocardium can lead to poor oxygenation, inflammation, and fibrosis, exacerbating cardiac injury. Yet, despite their association with this significant clinical problem, little is known about how lymphatic vessels might contribute to or resolve cardiac edema.

Many labs have demonstrated that AM exerts cardioprotective effects in mouse and human [40-43]. Our data in Chapter 2 contribute to a growing literature describing the role of AM in lymphangiogenesis. Whether the cardioprotective effects of AM are exerted through modulation of cardiac lymphatics has yet to be studied. Preliminary data generated by a fellow graduate student in the Caron lab suggest that Adm<sup>hi/hi</sup> mice subjected to left anterior descending (LAD) artery ligation to cause myocardial infarction have increased survival and significantly improved cardiac function compared to wildtype littermates. Immunohistochemical staining of post-infarct cardiac tissues shows increased and more patent LYVE1 (an LEC marker)-positive vessels compared to wildtype controls subjected to the same surgery. These observations suggest that Adm<sup>hi/hi</sup> mice exhibit more abundant and dilated lymphatic vessels following cardiac infarction.
While we have not yet evaluated whether the abundance of lymphatic vessels is due to increased cardiac lymphangiogenesis during development, analysis of lymphangiogenesis in an ear-wounding model suggests that Adm\(^{hi/hi}\) mice do not have enhanced lymphangiogenesis, indicating that Adm\(^{hi/hi}\) likely do not have increased lymphatic density. This absence of increased lymphatic density may be due to an ability to increase Cxcr7 expression to compensate for excessive AM-signaling. Instead, as data suggest that ADM is potently induced during a variety of cardiovascular disease conditions, we expect that the increased lymphatic staining and lymphatic vessel dilatation seen in post-infarct cardiac tissue are due to Adm upregulation following injury [44]. Therefore, these data suggest the cardioprotective effects of AM are at least partially mediated through the increased function and novel formation of cardiac lymphatics in the setting of myocardial ischemia.

To understand the role of AM and CXCR7 during this lymphatic remodeling, careful characterization of the post-injury hearts is essential in order to determine whether the increased cardiac lymphatic vessels resolves myocardial edema. Measurement of myocardial water content 10 days post-surgery will allow for the evaluation of myocardial edema. Here, a portion of the ventricular tissue will be weighed and then dried and reweighed. If overexpression of Adm contributes to the resolution of myocardial edema, the wet:dry ratios will be less than that of their wildtype controls.

Additionally, evaluation of Cxcr7 expression in post-ischemic wildtype and Adm\(^{hi/hi}\) cardiac tissue may prove interesting. Previous characterization of Adm\(^{hi/hi}\) hearts revealed an upregulation of Cxcr7, which we hypothesized was to homeostatically compensate for excessive AM peptide. During myocardial edema, however, where Adm expression is hypothesized to be cardioprotective, expression of the molecular rheostat might be expected to decrease. qRT-PCR of post-ischemic cardiac tissue will elucidate whether regulation of Cxcr7 expression plays a role in cardioprotection.
LAD ligation in Cxcr7⁻/⁻ mice that survive into adulthood will also allow us to determine if increased lymphatic density can ameliorate myocardial edema following cardiac injury. As we have shown in Chapter 2, Cxcr7⁻/⁻ mice exhibit increased lymphangiogenesis during development. We hypothesize that this increased lymphatic density will protect from post-injury cardiac edema and therefore improve survival and cardiac function. Additionally, these mice exhibit increased AM-signaling. Determining whether increased lymphatic density coupled with increased AM ligand allows for survival benefits above Adm<sup>hi/hi</sup> mice will be interesting. Collectively, these data will enhance our understanding of AM-mediated cardioprotection. Perhaps more importantly, this work will also elucidate a novel mechanism that may lead to a treatment for myocardial edema, which often accompanies and worsens chronic heart diseases.

*Future directions: Role of AM and CXCR7 in chemokine signaling*

Part II of my dissertation will address an additional future direction of this body of work. The signaling mechanisms that govern chemokine receptors are complex and poorly understood. The association of CXCR7 with the AM-signaling system has provided a clue into how these receptors might be regulated – via the interaction with Receptor Activity Modifying Proteins. Chapter 4 discusses the biology of RAMPs at length, and Chapter 5 focuses on current studies trying to understand the complexities of chemokine signaling.

*Concluding Remarks*

Taken together, the data presented in Part I support an important role for AM and CXCR7 during lymphangiogenesis and contribute to the expanding field of lymphatic vascular development. Research over the past decade has created a strong argument for pursuing the lymphatic system for the treatment of many conditions. The next decade will see advances in understanding of the molecular mechanisms that govern
lymphangiogenesis in development and adulthood and likely will identify a plethora of conditions to which dysregulation of lymphatic vasculature contributes. Hopefully, research will also identify small molecules to modulate these vessels toward the ultimate goal of treating human disease.
Figure 3-1. AM expression is localized to the developing epicardium and up-regulated in Adm^{hi/hi} mice.\(^\text{1,2}\) Immunofluorescence of AM peptide in heart sections of E13.5 embryos. **A,C.** Adm^{+/+} control mice. **B,D.** Adm^{hi/hi} mice. Images A,B were obtained with a 300 ms exposure, while images C and D were obtained with a 75 ms exposure. Boxes in A and B represent fields shown in panels C and D, respectively. **E.** The amount of AM expressed in the epicardium was assessed from panels C and D by measuring the integrated density of staining using Image J software. Data are expressed as arbitrary units (AU) of integrated density. Colocalization of AM peptide and the epicardial marker, cytokeratin. **F,H.** Adrenomedullin staining. **G,I:** Cytokeratin staining. The white boxes on panels F and G indicate the fields presented in panels H and I, respectively. Filled arrow heads on panels F and H indicate areas of AM staining, while open arrow heads in panels G and I highlight areas positive for cytokeratin staining. TR = trabecular zone; CZ = compact zone. Scale bars = 50 µM. All values are means ± SEM. **p< 0.01

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REFERENCES


Chapter 4: From the dish to the mouse: A glance at receptor activity modifying protein biology

Introduction

Receptor activity modifying proteins (RAMPs) are single-pass transmembrane proteins that heterodimerize with seven-transmembrane (7-TM) G protein-coupled receptors (GPCRs) to modulate GPCR function. GPCRs constitute ~2% of the human genome and activate major signaling pathways in cells [1]. Due to their physiological importance and their cell surface expression, the GPCR family is the most pharmacologically tractable class of proteins known. Approximately 40% of drugs marketed for the clinic target GPCRs [2]. Only a small fraction of GPCRs has been selected for drug development thus far, while the complex biology of the remainder are under intense study [3]. GPCR pharmacology is further complicated by the ability of these receptors to form homodimers and to heterodimerize with different GPCRs and with other proteins, including RAMPs, to form GPCR oligomers. How GPCR oligomers affect signaling and function is of particular interest, as protein-protein interactions may allow for novel drug development with more specific effects. While their interaction with Family B receptors has received the most attention, RAMPs have been shown to oligomerize with GPCRs from Family A (Rhodopsin-like) and C as well. Thus, the RAMP family extends the targets available for modifying clinical disease.

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The association of RAMPs with GCPRs affects ligand specificity, receptor trafficking, signaling capabilities, and receptor desensitization. Since their discovery over fifteen years ago, much has been learned about the effects of RAMPs on their GPCR partners. However, a dearth of knowledge still exists regarding additional GPCR-RAMP pairs, and more importantly, the biological significance of both known and unknown GPCR-RAMP partners. This review will discuss what is currently known about how RAMPs modulate GPCR pharmacology, will focus on how RAMPs regulate physiology in vivo, and, finally, will address what important questions remain.

The discovery of RAMPs

RAMPs were first identified during an effort to understand the regulation of calcitonin gene-related peptide (CGRP) signaling. CGRP belongs to the calcitonin peptide family, which consists of five peptide hormones: CGRP, calcitonin (CT), amylin (AMY), adrenomedullin (AM), and intermedin (IMD). These peptides have significant structural homology, and their often overlapping biological activities include gastric emptying (AMY, IMD), vasodilation (AM, CGRP, IMD), angiogenesis (AM, CGRP), and pain sensation (CGRP, CT) [4,5]. Despite sharing multiple functional targets, the calcitonin family of peptides also mediates unique biological events. This suggests that although the peptide group shares affinity for GPCRs, they also must target specific and distinct receptors [5].

Following the cloning of calcitonin receptor-like receptor (CLR), conflicting data emerged regarding whether the orphan receptor was able to bind CGRP. CLR exhibits 58% homology to the human CT receptor and was predicted to bind one of the CGRP peptides [6]. Previous data suggested that CGRP acted through a G_\text{\alpha}s-coupled GPCR to elicit cyclic AMP (cAMP) production [7]. Transfection of human CLR or the rat homologue into COS-7 cells, however, did not result in cAMP accumulation following CGRP treatment [6,8]. In contrast, when CLR was transfected into HEK293 cells, CGRP treatment produced a 60-fold
increase in cAMP production [9,10]. These data led to the speculation that HEK293 cells endogenously expressed an additional factor that was required to activate the CGRP receptor, while COS-7 cells did not express this factor.

Using an expression cloning approach, McLatchie and colleagues demonstrated that the CGRP receptor is formed from a GPCR heterodimer: CLR and the 148 amino acid protein RAMP1 [11]. Co-transfection of CLR and RAMP1 followed by CGRP treatment resulted in significant upregulation of cAMP, compared to transfection of either protein alone [11]. Further study showed that cells expressing endogenous RAMPs are able to generate cAMP response when transfected with CLR, whereas cell lines without endogenous RAMP1 cannot [6,8,9]. Clearly, the RAMP1-CLR complex alters the affinity of the CLR for CGRP.

Interestingly, two additional RAMPs were identified and found to modify ligand specificity of CLR. While the RAMP1-CLR complex binds CGRP, when RAMP2 or -3 is bound to the GPCR, CLR becomes a high-affinity AM receptor (Figure 4-1A) [11]. These data provided a novel mechanism for altering ligand specificity of GPCRs [7]. As such, the interaction of the RAMP family of proteins with GPCRs illustrated the complexity of CGRP signaling, yielding a paradigm shift in the understanding of GPCR pharmacology [12].

Studies over the past 15 years have identified GPCR-RAMP interactions in three GPCR classes (Table 4-1). Moreover, the three known RAMPs have been shown to play a broader role in the regulation of GPCRs than simply modifying ligand affinity.

**A multitude of effects: RAMP pharmacology**

The three identified RAMPs share a common structure, yet the proteins only have 30% sequence homology, which allows unique effects on their GPCR partners. RAMP1 and -3 are 148 amino acids long, while RAMP2 is considerably larger at 175 amino acids [11]. Each RAMP has a large extracellular N-terminal domain, a single transmembrane domain, and a cytoplasmic C-terminus [11]. These domains are critical in dictating how the RAMP
modulates the GPCR. For example, studies using chimeras that exchange the domains between RAMPs revealed that the N-terminus helps in determining ligand specificity, whereas the C-terminus appears to modify downstream signaling [13,14]. The recently solved crystal structures of the extracellular domains of RAMP1 and -2 confirmed that several intermolecular interactions in the amino terminus of RAMP2 are required for AM binding. These are not conserved in RAMP1, indicating that the N-terminus serves to define the specificity for ligand binding [15,16].

**Ligand Binding and plasma membrane expression.** As discussed above, the ability of RAMPs to modify CLR was essential to their discovery. As such, RAMP interaction with CLR and with the calcitonin receptor (CTR) has been particularly well characterized. In the case of CLR, RAMP1 is required for CGRP binding, and RAMP2 or -3 confer the ability to bind AM [11]. Additionally, RAMPs act as chaperones for CLR, as co-expression of a RAMP is required for cell surface trafficking of CLR. Studies have shown that RAMPs have an endoplasmic reticulum retention signal within the C-terminus [17]. RAMP association with CLR overrides this retention signal, allowing translocation of the CLR-RAMP complex from the endoplasmic reticulum (ER) to the plasma membrane. RAMPs are therefore critical for plasma membrane localization of some GPCRs.

RAMPs, however, can affect binding specificity of GPCRs that successfully express at the plasma membrane independent of RAMP association. Similar to the receptors for CGRP, the receptors for AMY proved challenging to isolate [7]. While AMY appeared to associate with CTR, CTR transfection into cell lines did not consistently reveal convincing AMY ligand binding [7,18]. After the discovery of RAMPs, several groups suspected that these inconsistencies were due to cell endogenous expression of RAMPs, and subsequently demonstrated that CTR interaction with all three RAMPs results in an AMY receptor [19-21]. In contrast to CLR, CTR does not require a RAMP for cell surface localization (Figure 4-1B) [5]. However, the CTR-RAMP interaction in the ER allows for formation of a functional AMY
receptor at the cell surface. CTR interactions with RAMPs 1-3 have been classified as AMY\textsubscript{1-3}, which display varying affinities for AMY [5]. However, the physiological relevance of the specific AMY receptors took several years to understand and demonstrated another important biological effect of RAMPs: the ability to affect post-receptor signal.

\textit{Cellular signaling.} Because the discovery of RAMPs caused a major shift in the understanding of how GPCRs are regulated, there was significant interest in which other receptors interacted with RAMPs. CLR and CT are both family B GPCRs. Given the structural similarities of family B GPCRs and their affinity for peptide ligands, it stood to reason that other members of family B could interact with RAMPs. Using epitope-tagged constructs and immunofluorescence confocal microscopy, the Sexton laboratory discovered four novel RAMP-GPCR interactions [22]. Co-transfection of the glucagon, parathyroid 1 receptor (PTH1R), parathyroid 2 receptor (PTH2R), or vasoactive intestinal peptide 1 receptor (VPAC1R) with RAMPs resulted in cell surface expression of RAMPs, where RAMP-only transfection did not. Interestingly, not all of these receptors interacted with all of the members of the RAMP family. While VPAC1R associated with all three RAMPs, glucagon and PTH1R associated only with RAMP2, and PTH2R only with RAMP3, highlighting the specificity of the RAMP-receptor interaction [22].

Further analysis of VPAC1R revealed that the interaction of a single GPCR with different RAMPs could elicit different downstream signals. VPAC1R is able to couple to multiple G-proteins and thus able to stimulate cAMP accumulation and phosphoinositide hydrolysis [22]. While treatment of cells cotransfected with VPAC1R and RAMP1 or -3 did not alter baseline cAMP accumulation, cotransfection of VPAC1R with RAMP2 caused significant increase in phosphoinositide hydrolysis, suggesting that RAMP2 could modify G-protein coupling [22]. Whether this change in signaling is due to direct modification in G-protein coupling or due to changes in RAMP-receptor localization remains to be determined [23].
Studies of AMY1-3 offer evidence that RAMPs can indeed alter G-protein coupling. Morfis et al. observed that AMY treatment of cells co-transfected with CTR and RAMP1 or -3 (AMY1 and AMY3 respectively) resulted in a 20- to 30-fold increase in cAMP production compared to transfection of CTR alone [24]. Conversely, only a 2- to 5-fold induction of intracellular Ca^{2+} was observed, suggesting that AMY1 and AMY3 preferentially couple to G_{as} versus G_{aq} relative to CTR without a RAMP. Interestingly, while no G proteins altered AMY binding in cells expressing CTR or CTR-RAMP1, overexpression of G_{as} significantly increased AMY binding in CTR-RAMP2-expressing cells, and overexpression of G_{as} and G_{aq} increased binding in CTR-RAMP3-expressing cells [24]. These data suggest that individual RAMPs have the capacity to dictate G-protein coupling compared to RAMP-independent GPCRs.

Finally, a recent study demonstrated that RAMPs associate with vasoactive intestinal peptide 2 receptor (VPAC2R) and corticotrophin releasing factor receptor (CRF) and alter G-protein coupling of both receptors [25]. Here, co-expression of RAMP1 and RAMP2 with VPAC2R in HEK293T and CHO-K1 cells significantly increased basal coupling to G_{i/o/t/z}, compared with VPAC2R alone [25]. Similarly, co-expression of RAMP2 with CRF significantly enhanced G_{i/o/t/z} and G_{q/11} coupling, leading to enhanced Ca^{2+} elevation in response to agonists [25]. Taken together, these data demonstrate the potential of RAMPs to alter downstream signaling, suggesting that an evaluation of the signaling profile is important to understanding the physiology of GPCR-RAMP interactions.

Receptor Trafficking. Finally, as mentioned briefly above, heterodimerization of RAMPs with CLR is required for cell surface expression of the GPCR. RAMPs have been identified for the first time to play a similar role for a family C receptor, calcium sensing receptor (CaSR). When transfected alone into COS-7 cells, CaSR is retained in the ER [26]. Co-expression of RAMP1 and RAMP3 with CaSR resulted in plasma membrane expression of CaSR, indicating that the RAMP-CaSR interaction promotes forward trafficking of CaSR.
to the membrane [26]. Although this finding elucidated a mechanism for the regulation of CaSR, these data in and of themselves were not groundbreaking. However, this was the first non-family B receptor identified to associate with RAMPs, opening the door to the possibility that RAMP effects are more pervasive than originally thought. A recent study has expanded this even further by identifying an association of RAMP3 with a class A receptor, GPR30, now known as G protein-coupled estrogen receptor 1 (GPER) [27]. This study will be discussed in detail in the following section addressing the biological effects of RAMPs.

RAMPs also have the capacity to alter receptor internalization. Early studies demonstrated that CLR-RAMP2 internalization was β-arrestin- and dynamin-mediated [7,28,29]. Later BRET studies indicated that, for CLR, agonist-mediated β-arrestin recruitment was dependent on the presence of RAMP1 [30]. Whether this was unique to RAMP1 was not investigated.

More recent studies have suggested unique protein trafficking roles for RAMP3. The C-terminal tail of RAMP3 contains a type 1 PSD-95/Discs-large/ZO-1 (PDZ) domain that is not present in RAMP-1 or -2. The PDZ domain allows for additional protein-protein interactions that alter receptor trafficking following receptor internalization. In contrast to the degradative pathway seen with RAMP1 and -2, Bomberger and colleagues showed that interaction of N-ethylmaleimide-sensitive factor (NSF) with the PDZ domain of RAMP3 causes CLR to be recycled back to the cell surface, allowing for rapid receptor resensitization [31]. Additionally, interaction of the PDZ domain with the Na+/H+ exchange regulatory factor (NHERF) tethers the CLR-RAMP3 complex to the actin cytoskeleton and inhibits the internalization of CLR [32]. In contrast, NHERF had no effect on CLR-RAMP1 or CLR-RAMP2 complexes that lack this PDZ domain.

These data not only highlight another important role for RAMPs but also the unique effects that RAMPs have on receptor processing. Many GPCRs interact with several RAMPs. Therefore, it is important to define the individual ligand affinity, signaling profile, and
trafficking pathway of each RAMP-GPCR complex. It is also worth noting that many additional RAMP effects remain undefined, so while the breadth of RAMP effects already seems widespread, we likely have only begun to uncover the capacity of RAMPs to alter GPCR physiology.

Figure 4-2 summarizes what is currently known about RAMP effects on GPCR pharmacology. In vitro biochemical studies have not only enabled the identification of novel GPCR-RAMP pairs but also elucidated much about how RAMPs affect GPCR biology. GPCRs are pharmacologically tractable themselves, but the unique GPCR-RAMP interface may prove to be even more advantageous for the design of highly selective drugs [3]. However, in order to harness their potential as drug targets for disease, we must first understand how RAMPs affect physiology and what roles they play in physiological dysfunction in vivo.

RAMP function in vivo

In general, our knowledge of RAMP biochemistry far exceeds our understanding of their biological effects. However, over the past decade, utilization of mouse models has taught us enormous amounts about RAMP biology in vivo. When evaluating genetic models of RAMPs, it is important to keep in mind that the ability of RAMPs to interact with multiple receptors is a major caveat; their promiscuous nature complicates the interpretation of RAMP knockout models. Therefore, conclusions suggesting any particular RAMP-GPCR interaction should be made with caution. Furthermore, the ability of GPCRs to interact with several RAMPs may allow for alternate RAMPs to compensate for the loss of another RAMP. Nonetheless, knockout and transgenic overexpression models of RAMPs demonstrate their importance to normal and disease physiologies. Our lab has previously reviewed the RAMP mouse model literature [33]; however, in the short time since its
publication, many additional studies utilizing genetically engineered RAMP mouse models have been published to enhance our understanding of how RAMPs affect physiology.

**RAMP1 and the cardiovascular system**

CGRP, a 37 amino acid neuropeptide with potent vasodilatory activity, binds the CLR-RAMP1 complex. It was therefore expected that RAMP1-null mice would display cardiovascular phenotypes. Indeed, since the generation of RAMP1-null mice, several groups have shown a variety of cardiovascular defects. In 2007, the first Ramp1−/− model was published [34]. Global deletion of Ramp1 resulted in mice that were viable and overtly normal but that did not exhibit a decrease in blood pressure in response to CGRP infusion, thus confirming loss of the functional CGRP receptor [34]. However, though serum CGRP levels remained similar, the basal blood pressure of Ramp1−/− mice was significantly higher than their wildtype controls and increased with age, suggesting that CGRP action through CLR-RAMP1 helps maintain low basal blood pressure. Interestingly, Ramp1−/− mice exhibited a mild but significant suppression of the hypotensive response to AM administration, suggesting that AM exhibits some of its vasodilatory effects through CLR-RAMP1 [34]. Therefore, while drugs that target the CLR-RAMP1 interface may be effective for treatment of cardiovascular disease, they may also interfere with AM-mediated effects.

Overexpression models have identified a protective role for RAMP1 in vascular function [35]. Angiotensin II (Ang-II) is an important mediator of hypertension in humans. Chronic exposure to Ang-II in mice increases cardiovascular risk by causing increases in sympathetic drive and inhibition of baroreflex-mediated decreases in vessel tone and heart rate. Interestingly, overexpression of hRAMP1 abrogated Ang-II-mediated decreases in baroreceptor reflex and increases in blood pressure [35]. Pressor response to a CGRP antagonist during Ang-II treatment was also enhanced in hRAMP1-expressing mice, suggesting that overexpression of RAMP1 induces hypersensitivity to CGRP stimulation.
These data indicate that CGRP activity is limited by the availability of RAMP1 and suggest that CGRP association with RAMP1 may be cardioprotective.

ChriSSobolis et al. utilized a global RAMP1 overexpression model to demonstrate that RAMP1 is also vasoprotective [36]. Ang-II treatment normally leads to diminished endothelial vasodilatory response to acetylcholine. Whereas wildtype mice failed to respond to acetylcholine following Ang-II treatment, RAMP1-overexpressing mice maintained baseline acetylcholine-induced relaxation responses in carotid arteries, indicating that RAMP1 overexpression protects against endothelial dysfunction [36]. Since endothelial dysfunction leads to significant increase in cardiovascular risk, these data suggest that development of drugs that target RAMP1 interactions with GPCRs may be useful for treating vascular disease [36].

Moreover, recent data has emerged that has identified a new role for RAMP1 in neovasculogenesis during wound healing. Kurashige et al. noted that healing of skin wounds was significantly delayed in Ramp1-null mice [37]. Experimental mice displayed significantly decreased microvessel and lymphatic vessel density during wound healing, suggesting that RAMP1 has a role in promoting new vessel formation after injury. Notably, the authors did not find RAMP1-positive vessels in granulation tissues, suggesting RAMP1 activation does not play a direct role in neovascularization [37]. Instead, it appears that CGRP activation in macrophages causes an increase in pro-angiogenic and pro-lymphangiogenic factors.

CGRP treatment of wildtype macrophages resulted in increased VEGF-A, a pro-angiogenic glycoprotein, while macrophages of Ramp1-/- mice had no response. VEGF-C, a lymphangiogenic factor, was also upregulated by CGRP and along with its receptor, VEGFR3, was observed to be decreased in Ramp1-/- animals. Bone marrow transplant from Ramp1-/- to wildtype mice recapitulated this phenotype, suggesting that CGRP activity in macrophages recruited from the bone marrow is critical for normal wound healing. These
data suggest that in the absence of RAMP1, proper angiogenesis does not occur, resulting in delayed wound healing. While AM and the CLR-RAMP2 complex are well known to participate in lymphangiogenesis (Klein and Caron, unpublished), particularly during embryogenesis [38], these data provide some of the first in vivo evidence that CGRP and RAMP1 also play an important role during wound healing. Further, that this effect of RAMP1 may be mediated through macrophages is particularly interesting, as RAMP1 has been found to have important roles in the immune system.

**RAMP1 and inflammation**

Outside of the cardiovascular system, CGRP and RAMP1 also participate in the regulation of inflammation. When the first Ramp1\(^{-/-}\) mouse model was generated, lipopolysaccharide (LPS) induced inflammation was examined. Again, basal CGRP levels were normal, but LPS administration induced a significant increase in serum CGRP levels in Ramp1\(^{-/-}\) mice compared to controls [34]. Ramp1\(^{-/-}\) mice also expressed high serum levels of pro-inflammatory cytokines and chemokines. CGRP, which is known to suppress the production of cytokines in bone-marrow derived dendritic cells, does not yield this suppression in Ramp1\(^{-/-}\) mice [34]. These data suggest that CGRP action through CLR-RAMP1 has anti-inflammatory properties. Recent data from our lab demonstrate the converse in the context of the ovalbumin (OVA) induced allergy [39].

To determine the effect of RAMP1 on allergic asthma, Ramp1\(^{-/-}\) mice were OVA sensitized. Interestingly, methylcholine treatment of OVA-sensitized Ramp1\(^{-/-}\) mice results in significantly attenuated airway resistance in comparison to wildtype controls [39]. Similar findings are observed in mice that are haploinsufficient for CLR (Calcrl\(^{+/+}\)) mice, indicating that both of the components of the CGRP receptor complex are involved in airway hyperresponsiveness. Mice deficient in either RAMP1 or CLR have better outcomes following OVA sensitization. Smooth muscle-specific loss of CLR did not attenuate airway
resistance in response to methacholine, indicating that CGRP action on smooth muscle cells of the airway is not the primary cause of the airway hyperresponsivity. However, both $Ramp1^{-/-}$ and $Calcr^{-/-}$ mice had significantly reduced IL-4 concentrations from bronchoalveolar lavage fluid, suggesting that CGRP, acting through the CLR-RAMP1 complex, increases inflammatory mediators during allergic asthma and may worsen outcomes. In contrast to the previous study discussed, this study suggests that CGRP and the CLR-RAMP1 complex also may have a pro-inflammatory role.

Studies using a neuronal-specific RAMP1 overexpression model also indicate a role for RAMP1 during inflammation in neuronal tissue. Injection of CGRP into transgenic mice expressing $hRAMP1$ exhibited increased plasma extravasation and inflammation in subcutaneous tissue compared to control mice [40]. Co-treatment with CGRP antagonists abrogated this response. Again, overexpression of RAMP1 led to an increased response to a given dose of CGRP, suggesting that CGRP action in neurons is also limited by the availability of RAMP1. This finding is particularly interesting because many studies have suggested that CGRP plays a major role in migraine [41]. This study raises the possibility that elevated RAMP1 expression in neurons may predispose individuals to CGRP-induced migraines. In fact, several small molecules antagonists of CLR-RAMP1 have been developed and tested in clinical trials with reasonable efficacy [42]. Still, the development of an orally available small molecule with few off-target effects has been challenging [42]. Drug design specifically at the CLR-RAMP1 interface could prove to be the gold standard for migraine treatment.

Though these studies together do not paint a clear picture of whether RAMP1 has pro-inflammatory or anti-inflammatory roles, it is likely that the effects differ based upon tissue. It is possible that tissue-specific targeting of the CLR-RAMP1 interface has potential for relieve symptom of a variety of diseases, including asthma and migraine. Off-target effects could be significant given the diverse physiological roles of CLR-RAMP1.
Regardless, more work needs to be done to understand the roles of RAMP1 during inflammation. Furthermore, the majority of Ramp1 mouse model studies have focused on CGRP and the CLR-RAMP1 interaction. As discussed previously, biochemical studies have identified several additional GPCR partners for RAMP1. Consequently, future work should determine how loss of RAMP1 affects the biology of other peptides.

**RAMP2 in the cardiovascular system**

The importance of RAMP2 in vascular biology was recognized with the generation of the first Ramp2−/− animal. In contrast to RAMP1 and RAMP3, RAMP2 is required during embryogenesis [38,43]. Comparative phenotyping of AM (gene = Adm), CLR (gene = Calcrl), and RAMP2 knockout mice revealed a conserved phenotype between the three: extreme edema and mid-gestational lethality [38]. Our lab suspected that defective lymphangiogenesis was the root cause of the edema. Indeed, examination of jugular lymph sacs of Ramp2−/−, Adm−/−, Calcrl−/− mice revealed that mutant lymph sac to jugular vein ratios were significantly smaller than their wildtype controls [38]. BrdU-incorporation assays indicated that the lymphatic endothelial cells of AM-signaling-null mice were hypoproliferative, resulting in abnormally small lymphatic sacs. Electron microscopy revealed that lymphatic endothelial cells of Ramp2−/− mice were thin and often necrotic, though the junctional barrier between the blood and lymphatic endothelium remained intact [38]. Together, these data demonstrate that Ramp2 is necessary during embryonic lymphangiogenesis, and loss of RAMP2 is incompatible with survival.

Interestingly, a second, independent Ramp2 knockout model was developed at the same time [43]. Ichikawa-Shindo et al. described a similar edematous phenotype and embryonic lethality. They also described occasional hemorrhage and defects in the blood endothelial barrier in this model of Ramp2−/− mice [43]. The authors concluded that the
edema was caused by paracellular leakage from blood vessels. Lymphangiogenesis was not evaluated.

These two studies described the first $Ramp2$-null mice. Though they did not come to the same conclusion, they both highlight a critical role for RAMP2 during embryonic vasculogenesis and maintenance of endothelial barriers. Moreover, while biochemical and more recent $in vivo$ data demonstrate that RAMP2 has many GPCR partners, the work from Fritz-six et al. specifically highlight the importance of the CLR-RAMP2 complex during development, as neither RAMP1 nor RAMP3 can compensate for the loss of RAMP2.

In the past five years, the Shindo lab has published numerous papers utilizing tissue-specific deletion of $Ramp2$. Several of these models are tamoxifen-inducible in order to circumvent embryonic lethality and evaluate the role of RAMP2 during adulthood. Yoshizawa and colleagues suggest that tamoxifen-induced cardiomyocyte deletion of RAMP2 ($C-Ramp2^{−/−}$ mice) using a myosin heavy chain (MHC)-MerCreMer transgenic mouse results in a dilated cardiomyopathy-like heart failure in adult mice with enlargement of cardiac ventricles and diminished systolic function [44]. Immunohistochemical analysis of $C-Ramp2^{−/−}$ hearts indicated higher levels of oxidative stress. Additionally, the authors noted changes in mitochondrial structure that were associated with reduced expression of various mitochondria-related molecules and increases in reactive oxygen species [44]. While reactive oxygen species reduction abrogated increases in cardiomyocyte size in $C-Ramp2^{−/−}$ mice, expression of other heart failure genes remained unchanged [44]. Therefore, while mitochondrial dysfunction and reactive oxygen species contributes to disease, loss of RAMP2 affects additional processes that lead to the heart failure and remain to be determined.

These findings are potentially interesting, however the study is confounded by several technical and conceptual inconsistencies. Firstly, several independent publications have indicated that the αMHC-MerCreMer transgenic line spontaneously develop
cardiomyopathy and heart failure with accompanying reduction in cardiac energy and metabolism in the absence of any excised floxed allele [45-47]. Additionally, the cardiomyocyte gene expression levels of Ramp2 are reduced to 60% of controls, a level of expression that is still higher than Ramp2 heterozygotes. So, it is somewhat surprising that the observed phenotype is so dramatic when Ramp2<sup>−/−</sup> animals fail to develop a similar phenotype. Moreover, this study only considered AM as the potential mediator of cardiac dysfunction. While AM likely contributes to these phenotypes, the authors fail to address the fact that RAMP2 associates with numerous other receptors.

This concept becomes apparent in a second paper that investigates an endothelial-specific knockout of Ramp2. Utilizing a vascular endothelial-cadherin promoter, the Shindo lab generated an endothelial cell-specific knockout of RAMP2 (E-Ramp2<sup>−/−</sup> mice) during embryogenesis, which results in perinatal lethality of 95% of the mice [48]. Five percent of E-Ramp2<sup>−/−</sup> mice survive into adulthood, whereas no global Ramp2<sup>−/−</sup> embryos survive past mid-gestation [48]. Interestingly, all mice with endothelial specific knockout of AM (E-Adm<sup>−/−</sup> mice) survive to adulthood. While it is likely that other tissue sources of AM compensate for the loss of AM in the endothelium, it is equally likely that RAMP2 interaction with other GPCRs and peptides contributes to the severity of the phenotype.

Despite this caveat, the study of surviving animals proved to be interesting. Surviving E-Ramp2<sup>−/−</sup> mice express 20% of wildtype Ramp2 levels [48]. This incomplete knockout may facilitate their survival into adulthood. Phenotypic analysis of long-term survivors revealed that adult E-Ramp2<sup>−/−</sup> mice exhibit significant vascular dysfunction, including aortic thinning and increased aortic diameters. Blood pressure was also decreased in E-Ramp2<sup>−/−</sup> mice [48]. This finding is somewhat surprising. As AM is a potent vasodilator, loss of AM signaling would be expected to lead to blood pressure increases. It is possible that the disrupted vascular structure causes the decrease in blood pressure. The authors also note aortic smooth muscle disarray and endothelial cell detachment from the basement membrane [48].
E-Ramp2<sup>−/−</sup> mice also displayed spontaneous vasculitis consistent with endothelial damage. Aged mice exhibited significant organ failure and fibrosis and accelerated vascular senescence, a purported consequence of vascular damage [48]. Two-year-old global Ramp2<sup>−/−</sup> mice similarly exhibited increased vascular senescence compared to wildtype controls, suggesting that even haploinsufficiency for RAMP2 results in vascular damage; further reduction of Ramp2 in the endothelium may increase vascular damage, thereby accelerating senescence.

Loss of endothelial RAMP2 during adulthood also leads to significant vascular dysfunction. After inducing endothelial-specific Ramp2<sup>−/−</sup> in adult mice with tamoxifen (drug-inducible endothelial specific Ramp2<sup>−/−</sup> = DI-E-Ramp2<sup>−/−</sup>), the Shindo group noted significant edema due to enhanced vascular permeability and plasma leakage, with endothelial cell detachment from the basement membrane of aortas. These findings are consistent with the expected role of Ramp2 in vascular permeability. Additionally, aged DI-E-Ramp2<sup>−/−</sup> mice displayed vasculitis similar to that seen in E-Ramp2<sup>−/−</sup> survivors [48]. To examine the postnatal angiogenic potency, the authors utilized a hind-limb ischemia model. Interestingly, blood flow restoration following hindlimb ischemia was also reduced in DI-E-Ramp2<sup>−/−</sup>, indicating decreased angiogenic potential. Endothelial-specific overexpression of Ramp2 in DI-E-Ramp2<sup>−/−</sup> restored blood flow recovery to the hindlimb, suggesting that Ramp2 is indeed responsible for loss of angiogenic potential [48]. Collectively, this extensive study portrays a critical role for RAMP2 in maintenance of vascular integrity throughout development and into adulthood. It is worth mentioning again that GPCR-RAMP2 interactions besides CLR-RAMP2 likely contribute to these phenotypes. Further work is necessary to establish a complete understanding of the role of RAMP2 in vascular dysfunction.

RAMP2 in the endocrine system
RAMP2 activity in the endocrine system serves as an excellent example of how loss of a RAMP can impact several signaling pathways. Recent work from our lab has demonstrated that haploinsufficiency of Ramp2 leads to a constellation of endocrine-related phenotypes, which are not observed in Calcrl^{−/−} or Adm^{−/−} mice. First, we noticed that the Ramp2^{−/−} colony is difficult to maintain due to small litter sizes and fetal demise [49]. Extensive evaluation of timed-matings throughout pregnancy and the postpartum period revealed peri- and postnatal loss of pups, independent of genotype [50]. Wildtype and mutant pups alike exhibited intrauterine growth restriction, suggesting parental, not fetal, genotype, contributes to fetal demise. Interestingly, fetal loss was only observed when the dam was heterozygous for Ramp2; paternal genotype had no effect on fetal survival, indicating that maternal Ramp2 haploinsufficiency causes fetal loss [50]. The true cause of fetal demise is not well understood but could be due to the loss of signaling through CLR-RAMP2, which is critical for normal development of the reproductive tract. In addition, loss of glucagon signaling, which is critical for fetal development, through the glucagon receptor-RAMP2 complex could also contribute.

We also noticed that pups that died postnataally lacked milk in their stomachs and suspected that prolactin production was disrupted in Ramp2 mutant females. Indeed, Ramp2^{−/−} females exhibited hyperprolactinemia basally and throughout pregnancy. Consistently, pituitary glands, the region of the brain that produces prolactin, of Ramp2^{−/−} females were hyperplastic. Furthermore, examination of the mammary gland of virgin mice revealed accelerated mammary gland development in Ramp2^{−/−} mice, which likely contributes to lactation dysfunction. These symptoms could be attributed to loss of the PTH1R-RAMP2 complex, which fosters signaling of parathyroid hormone-related peptide (PTHrP), a peptide important for fetal and placental development, mammary development, and bone development. Unpublished work from our lab examining placentas of Ramp2-null
mice confirm the PTH1R-RAMP2 interaction *in vivo* and supports the hypothesis that loss of *Ramp2* affects PTH1R function (Kadmiel et al. unpublished).

*Ramp2*<sup>+/-</sup> mice also exhibit skeletal abnormalities including delayed bone development and decreased bone mineral density from the perinatal period through adulthood, suggesting that heterozygosity of *Ramp2* affects bone metabolism independent of hyperprolactinemia. In fact, these skeletal phenotypes are reminiscent of AMY-deficient mice, suggesting that loss of the CTR-AMY receptor complex might cause the bone phenotypes.

Finally, following CRF treatment, adrenocorticotropic hormone (ACTH) response was also suppressed in *Ramp2*<sup>+/−</sup> mice compared to their wildtype littermates, indicating that haploinsufficiency of *Ramp2* also affects the CRF receptor-RAMP2 complex and the physiologic effects of CRF [25]. Ultimately, these data describe an array of endocrine symptoms in *Ramp2* heterozygous mice that cannot be attributed to a single RAMP-GPCR complex. This complex pattern of dysfunction emphasizes that loss of RAMPs can affect many signaling pathways, which often confounds the ability to attribute a given phenotype to one GPCR.

**RAMP3 in the cardiovascular and endocrine systems**

Genetic models of RAMP3 have received the least attention of the three RAMPs. However, exciting data is emerging about the role of RAMP3 *in vivo*. *Ramp3*<sup>−/−</sup> mice are viable, reproduce normally, and display no obvious defects from birth to 8 months [49]. Unlike RAMP1 and RAMP2, loss of RAMP3 had no effect on basal blood pressure or heart rate. The only overt phenotype was observed in aged mice. At nine months, Dackor et al. noticed that *Ramp3*<sup>−/−</sup> mice weighed significantly less than their age-matched controls, though no changes in food or water intake were observed. Despite this leanness, *Ramp3*<sup>−/−</sup>
mice have a similar life expectancy with no obvious decline in health despite their leanness [49].

While no overt phenotypes were observed basally, two recent papers have identified a sex-dependent cardioprotective role for RAMP3. Previous data indicated that Ramp3 is robustly upregulated in the cardiovascular system during hypertension. Additionally, Ramp3 is potently induced by estrogen [51-54]. Therefore, Barrick et al. hypothesized that Ramp3 would be cardioprotective in the setting of hypertension but that the effect would be dependent on sex [51]. To examine this hypothesis, the authors intercrossed Ramp3 and RenTgMK mice, which have high and constant secretion of renin, elevated circulating levels of angiotensin II, and chronic hypertension [51,55]. Interestingly, male but not female RenTgMK;Ramp3\(^{-/-}\) mice exhibit significantly greater renal damage than RenTgMK mice. Similarly, male but not female RenTgMK;Ramp3\(^{-/-}\) mice exhibit significant pathological changes in heart tissue consistent with cardiac hypertrophy. Echocardiography revealed that loss of Ramp3 exacerbates the RenTgMK phenotype of concentric cardiac hypertrophy and caused left ventricular dilation and progression to systolic failure by 5 months of age. This decompensated hypertrophy and transition to heart failure was not evident in RenTgMK or female RenTgMK;Ramp3\(^{-/-}\) mice even when aged 8 months. Collectively, these data make a compelling argument for a sex-dependent role for Ramp3 in the setting of chronic hypertension. While the authors argue that AM signaling through CLR-RAMP3 could be involved, they hypothesize that other GPCRs are also involved.

Indeed, a follow-up study identified a novel interaction of GPR30, now known as GPER, with RAMP3 that confers sex-dependent cardioprotection in vivo [27]. Studies have shown that GPR30 is involved in mediating the effects of estrogen in the heart [56]. Synthetic activation of GPR30 with the specific GPR30 agonist G-1 protects cardiac tissue from injury in several models of heart disease [57-59]. Despite the substantial functional data, controversy regarding the trafficking, subcellular location, and ligand binding of GPR30
confounded the field. Based on these questions, the functional role of RAMP3, and the cardioprotective nature of both GPR30 and RAMP3, Lenhart et al. hypothesized that GPR30 and RAMP3 could form a complex that mediates cardioprotection during cardiovascular stress [27].

After confirming a protein-protein interaction between GPR30 and RAMP3 in vitro, investigation of mouse cardiac tissue revealed that GPR30 was mislocalized in the absence of RAMP3. In both male and female Ramp3−/− mice, the proportion of GPR30 at the plasma membrane was decreased with a concomitant increase in cystolic GPR30 protein relative to wild-type controls. Sex differences in GPR30 localization were also observed, as the proportion of GPR30 protein at the plasma membrane was increased in females compared to males across all genotypes. However, the difference between males and females was most significant when Ramp3, an estrogen regulated gene, was present.

More importantly, absence of Ramp3 and subsequent mislocalization of GPR30 has functional consequences. As expected, G1 treatment, which signals through GPR30 agonist and confers cardioprotection, causes a statistically significant decrease in cardiac disease pathology in RenTgMK;Ramp3+/+ male mice. Cardiac fibrosis and markers of hypertrophy, left ventricle weight to body weight measurements and cardiomyocyte area, were all decreased in G1- vs. placebo-treated RenTgMK;Ramp3+/+ male mice. However, G1 had no effect RenTgMK;Ramp3−/− mice, suggesting that G1 cardioprotection is due directly to the GPR30-RAMP3 function in the heart. G1 treatment of female mice had no effect regardless of Ramp3 genotype status, suggesting that because they exhibit higher GPR30 levels at the cell membrane basally, female mice remain cardioprotected even in the absence of Ramp3 [27]. Conversely, male mice only respond to G1 treatment if Ramp3 is present to help maintain cell surface localization of GPR30.

These data are exciting for several reasons. First, it provides a novel, physiologically significant GPCR-RAMP pair. Secondly, the sex-dependent nature of the
GPR30-RAMP3 may elucidate important pathways that contribute to the gender differences seen in cardiovascular disease and may allow for gender-specific treatment. Finally, this is the first identification of a RAMP interacting with a Family A GPCR. With the exception of CaSR, the RAMP field has focused exclusively on Family B, the secretin family of receptors. These data suggest that RAMPs could have a much broader impact than originally suspected.

Concluding remarks and future questions

The discovery of RAMPs elucidated a novel mechanism for the regulation of GPCRs and transformed our understanding of GPCR pharmacology. Future studies will aim to identify additional RAMP-GPCR pairs with a particular focus on their functional consequences. If these pairs are found to have roles in normal or disease physiology, targeting at the RAMP-GPCR interface could prove to be a very powerful tool.

Parsing out the exact physiological effect of each RAMP-GPCR pair will be the focus of future studies. The ability of RAMPs to bind many receptors confounds the study of genetic mouse models. Targeting the GPCR-RAMP interface, therefore, will not only prove useful for the treatment of disease, but also in the determination of the functionality of specific GPCR-RAMP pairs.

Additionally, there is much more to understand about the biochemistry of RAMPs. For example, the stoichiometry of RAMPs to their GPCR partner has been studied but is still debated. Evidence exists for a 1:1, 2:2, and 2:1 ratio of CLR:RAMP; however, whether the stoichiometry differs by cellular environment or between GPCRs is not known [15,60,61]. It is possible that these questions will become even more convoluted with the identification of GPCR heterodimers that interact with RAMPs. So far, studies have only focused on the interaction of one GPCR with one RAMP, but it is possible that two different GPCRs could
complex with a RAMP, or two different RAMPs could bind to a single GPCR. It is possible that we have just begun to discover the breadth of RAMP biology.

RAMPs provide a beautiful example of the complexity of GPCR pharmacology. Though a finite number of GPCRs exist, their ability to heterodimerize with additional proteins allows for exponential combinations and diverse cellular signaling. While this makes comprehensive understanding of their activity challenging, it may allow for highly specific drug targeting. Consequently, further biochemical and genetic studies are warranted in order to harness the therapeutic potential of the RAMP-GPCR interface in human disease.
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Figure 4-1. Effects of RAMP association on CLR and CTR. (A) For cell surface expression, calcitonin receptor-like receptor requires the presence of one of the RAMP family members. CLR associates with RAMPs in the ER, which facilitates plasma membrane expression. Association with RAMP1 yields a high affinity calcitonin gene related peptide (CGRP) receptor, whereas CLR association with RAMP2 or -3 results in a potent adrenomedullin (AM) receptor. (B) Conversely, calcitonin receptor (CTR) is trafficked to the plasma membrane independent of RAMPs. In the absence of a RAMP, CTR binds calcitonin. In association with RAMP1, -2, -3, the CTR-RAMP complex binds amylin (AMY).
Figure 4-2. The effects of RAMP association on GPCR pharmacology.
REFERENCES


Chapter 5: The Novel Interaction of Receptor Activity Modifying Proteins with the Family A Seven Transmembrane Chemokine Receptors

Introduction

Receptor activity modifying proteins (RAMPs) are single pass transmembrane proteins that complex with certain G protein-coupled receptors (GPCRs) to alter ligand binding, receptor trafficking, and downstream signaling [1,2]. First discovered in 1998, RAMP modulation of GPCRs added an additional layer of complexity to GPCR pharmacology [3]. However, the RAMP-GPCR complex resolved significant debate over the ligand specificity and cellular trafficking of certain receptors [4]. For example, the interaction of calcitonin receptor-like receptor (CLR) with RAMP2 or RAMP3 constitutes a functional receptor for the peptide hormone, adrenomedullin (AM), while CLR association with RAMP1 changes the ligand affinity to a different peptide, CGRP [4,5]. Additionally, plasma membrane expression of CLR is dependent on a RAMP chaperone [4]. While not all GPCRs require them, RAMPs offer a unique opportunity for drug discovery, as the RAMP-receptor interface could yield specific, high affinity drugs [2,6]. Consequently, there has been great interest in discovering alternate RAMP-GPCR pairs.

Chemokine receptors (CKRs) are seven transmembrane receptors that belong to the largest family of GPCRs, Rhodopsin-like or Family A receptors. CKRs and their ligands have critical roles in immune defense – mediating attraction, migration, proliferation, and activation of immune cells [7]. Roles also exist for these receptors outside of the immune system during normal and disease physiology [8-12]. While RAMP research has largely
focused on the Family B G protein-coupled secretin receptors, the association of RAMPs with the Family A receptor G protein-coupled estrogen receptor 1 (GPER), formerly known as GPR30, has recently been appreciated and broadened the scope of RAMP-GPCR interactions [13].

Increasing evidence suggests a potential interaction of RAMPs with the CKRs. Firstly, many CKRs have promiscuous ligand binding, displaying the ability to bind a broad spectrum of molecules. Interestingly, their promiscuity extends beyond typical chemokines, as two recently published papers establish that peptide hormones bind the chemokine receptor CXCR7 [10,14]. This diverse binding profile is reminiscent of RAMP modulation of CLR ligand specificity.

Furthermore, several CKRs belong to a small family of atypical chemokine receptors (ACKR) or non-signaling decoy receptors, which act as molecular sinks for ligands, and whose pharmacology resonates with RAMP modulation of GPCRs. In order to promote the rapid sequestration of ligand, a decoy receptor must be quickly recycled. Indeed, several studies have noted rapid shuttling of these receptors to and from the cell surface and the endosome [15,16]. RAMP3 is unique among the RAMP family as it contains a PDZ domain in the C-terminus that allows for the interaction of RAMP3 with N-ethylmaleimide-sensitive factor (NSF), a trafficking protein that facilitates the rapid recycling of receptors [17]. Association of the RAMP3-GPCR complex with NSF converts the receptor lifecycle from a degradative to a recycling pathway. Whether RAMP3 could participate in CKR recycling has not yet been investigated.

Finally, the intracellular environment of a particular ACKR, CXCR7, dramatically alters the downstream signaling of the receptor. Consistent with its function as a ligand sink, initial studies demonstrated that CXCR7 fails to elicit downstream signaling upon ligand binding [18]. However, more recent studies indicate that CXCR7 can induce beta-arrestin mediated signal [19]. Moreover, while CXCR7 does not typically couple to a G protein, data
suggests that, in astrocytes, CXCR7 is capable of coupling to G_\alpha_i [19-22]. No determinant of these functional differences has been identified, however, RAMPs offer a potential explanation for these inconsistencies. We thus hypothesized that chemokine receptors interact with RAMPs.

Specifically, based on the Caron laboratory’s interest in CXCR7 (Chapter 2), we aimed to determine whether three related members of the chemokine receptor family, CXCR7, CXCR4, and CCR5 interact with the RAMP family. These CKRs are capable of heterodimerizing with each other – CXCR4 forms stable heterodimers with both CXCR7 and CCR5 [21,23,24] – and they share biological activity. CXCR7 and CXCR4, which both bind CXCL12, have been extensively studied for their roles in cancer progression [25]. Additionally, CCR5 and CXCR4, and to a smaller degree CXCR7, act as co-receptors for human immunodeficiency virus (HIV). RAMP interaction with CKRs, therefore, offers exciting potential for drug discovery. Here, we utilize bioluminescence resonance energy transfer (BRET), confocal imaging, and a fluorogen activating protein (FAP) assay to present preliminary data demonstrating novel protein-protein interactions between these three CKRs and the RAMP family.

**Experimental procedures**

*Bioluminescent Resonance Energy Transfer*

To test the association of the chemokine receptors with the RAMPs, BRET titration studies were performed as described previously [13,26]. Briefly, HEK293T cells were transfected with the positive control (CLR-Rluc), the negative control (B_2ADR-Rluc), or experimental plasmids (CXCR7-Rluc, CXCR4-Rluc, CCR5-Rluc), and increasing amounts of RAMP1-YFP, RAMP2-YFP, or RAMP3-YFP. Cells were treated with a luciferase substrate, coelanterazine H, for 10 minutes, and plates were read on a Berthold Technologies Mithras LB940 plate reader. BRET signal was calculated as the ratio of light emitted from YFP (530
nm) to the light emitted from Rluc (485 nm) ratio. Total YFP (measured in untreated wells) and total luminescence were used to control for the transfection efficiency. BRET data were analyzed and evaluated using a non-linear regression equation assuming a single binding site (GraphPad Prism).

Confocal Imaging

HEK293T cells were plated on 35 mm MatTek glass bottom culture dishes and co-transfected with RAMP3-YFP and untagged positive control (CLR), negative control (B₂ADR or V2R) or experimental plasmids (CXCR7, CXCR4, and CCR5). 24 hours following transfection, live cells were imaged. Optical sections were acquired on the confocal microscope using the Zeiss LSM Image Browser v4.2.

FAP assay

Plasmids. FAP-RAMP3 was generated using multisite gateway cloning following manufacturer’s instruction (Life Technologies). Briefly, a RAMP3 entry clone (attL5-RAMP3-attL2) was constructed by inserting an att-flanked RAMP3 geneblock (element 2, attB5-RAMP3-attB2; Integrated DNA Technologies) into a P5-P2 pDONR vector using BP clonase II. Recombination of the RAMP3 entry clone with a FAP entry clone (element 1, attL1-RAMP3-attR5) into a destination vector yielded the final FAP-RAMP3 expression clone.

FAP assay. HEK293T cells were transfected with 0.75 µg FAP-RAMP3 and 0.75 µg CLR, V2R, B₂ADR, or CCR5. Cells were washed with PBS and treated with a cell impermeant malachite green (MG) fluorogen (1:4000) ~5 minutes prior to imaging. Cells were imaged on an Odyssey scanner (Li-COR).
Results

In order to investigate whether the CKRs interact with RAMPs, we first performed BRET studies, which have been previously used to demonstrate protein-protein interactions between RAMPs and GPCRs [13,27]. Here, HEK293T cells were transfected with constant amounts of a Renilla luciferase (Rluc)-GPCRs and increasing amounts of YFP-tagged RAMP. The well-established interaction of CLR with RAMPs were utilized as a positive control, while beta-2 adrenergic receptor (B2ADR) provided a negative control. In cells expressing CLR, we observed a robust change in BRET signal with increasing RAMP2-YFP (Figure 5-1A, green). Conversely, B2ADR-expressing cells demonstrated a linear relationship to increasing concentration of RAMP2-YFP, which is characteristic of a non-specific interaction (Figure 5-1A, red) [28].

We then examined the interaction of the CKRs with the RAMP family. Similar to the positive control control, we observed an increase in BRET signal in cells cotransfected with CXCR7 and increasing amounts of RAMP2-YFP or RAMP3-YFP (Figure 5-1B), indicating a potential interaction between CXCR7 and two members of the RAMP family. Conversely, we detected a linear, non-specific interaction between CXCR7 and RAMP1-YFP (Figure 5-1B), suggesting that CXCR7 and RAMP1 fail to associate. It is possible that the donor (Rluc) and acceptor (YFP) molecules are not in the correct orientation for interaction. Therefore, these data are considered inconclusive [28]. In cells expressing CXCR4-Rluc, we detected an increase in BRET signal with increasing amounts of RAMP2-YFP and RAMP3-YFP (Figure 5-1C). Cells expressing CCR5-Rluc also demonstrated robust changes in BRET signal with increasing doses of RAMP2-YFP and RAMP3-YFP (Figure 5-1D). CCR5-expressing cells demonstrated an inconclusive, linear relationship to increasing doses of RAMP1-YFP.

Based on our interest in the trafficking potential of RAMP3 and the positive BRET signal observed with CXCR4 and CCR5, we sought to confirm the interaction of these CKRs with RAMP3 using the classical assay for RAMP association. As RAMPs do not traffic to the
plasma membrane without a receptor, the ability of a GPCR to translocate a RAMP to the cell surface is the standard in the field for determining RAMP-receptor interactions [13,27]. We therefore investigated whether the CKRs were able to transport RAMP3-YFP to the plasma membrane. As shown in Figure 5-2A,B, in HEK293T cells transiently expressing B₂ADR or vasopressin 2 receptor (V2R), RAMP3-YFP does not localize to the plasma membrane. Conversely, in CLR-expressing cells RAMP3-YFP is robustly expressed at the plasma membrane (Figure 5-2C, white arrows). Interestingly, we observed similar plasma membrane expression with all three CKR receptors (Figure 5-2D-F, white arrows). Plasma membrane localization of RAMP3-YFP was more obvious in cells expressing CXCR4 and CCR5 than with CXCR7. However, plasma membrane localization was particularly well visualized at cell-cell contacts in CXCR7-expressing cells, where presence of two plasma membranes allowed for clear linear fluorescence (Figure 5-2D, white arrows). Importantly, this linear fluorescence is not observed in V2R and RAMP3 expressing cells (Figure 5-2B), where multiple cells are imaged.

Finally, we utilized a FAP assay to quantitatively determine plasma membrane expression of RAMP3. Developed by the Waggoner group in 2007, fluorogen activated proteins are protein reporters that generate fluorescence only when bound to a specific chemical label (fluorogen) [29]. Exposing N-terminally FAP-tagged proteins to cell-impermeant fluorogen allows for visualization of plasma membrane localized proteins, while intracellular proteins that have not been activated by the fluorogen remain dark [30]. Conveniently, the fluorogen has absorption and emission wavelengths such that it can be directly imaged on the Odyssey Imaging System (Li-COR). We thus generated and expressed an N-terminally tagged RAMP3 in HEK293T cells. While minimal FAP-RAMP3 was visualized when co-expressed with V2R or B₂ADR (Figure 5-3A, second and third columns), we observed a statistically significant increase in plasma membrane localization of FAP-RAMP3 when cells expressing CLR or CCR5 were treated with cell impermeant
Discussion

In the current study, we present preliminary evidence suggesting an interaction between RAMPs and CXCR7, CXCR4, and CCR5. First, utilizing a well-characterized BRET assay for determining protein-protein interactions, we observed robust interactions of CXCR7, CXCR4, and CCR5 with members of the RAMP family. Co-expression of CXCR7 and CCR5 with the RAMP1-YFP constructs resulted in BRET curves similar to those of the negative control (B2ADR). Because the placement of the donor and acceptor molecules on the proteins is critical to facilitate the interaction, we could not conclude that CXCR7 and CCR5 do not interact with RAMP1. However, numerous receptors have been shown to complex with some but not all of the members of the RAMP family (Table 4-1); the association of CXCR7 and CCR5 with RAMP2 and -3, but not RAMP1, would therefore be consistent with previous reports.

For the remainder of our studies, we focused on CKR association with RAMP3. RAMP3 is a particularly attractive candidate for association with CKRs, as the PDZ domain of RAMP3 regulates receptor trafficking [17,31]. ACKRs, like CXCR7 (also known as ACKR3), must continuously cycle from the plasma membrane and endosomal compartments, thus enabling efficient internalization and delivery of ligand for lysosomal degradation [32]. ACKR interaction with RAMP3 may facilitate this rapid recycling via NSF [17]. Furthermore, typical CKRs may need to remain at the cell surface to perpetuate signaling. The interaction of the PDZ domain of RAMP3 with Na+/H+ exchanger regulatory factor-1 (NHERF-1), an adaptor protein that prevents receptor internalization, could stabilize receptor expression at the plasma membrane [31]. Therefore, RAMP3 association with CKRs may result in functional consequences that are both interesting and easily assessed.
As RAMPs do not traffic to the cell surface independently, visualization of RAMP expression at the plasma membrane by confocal microscopy is considered the standard in the field for demonstrating RAMP-GPCR interactions. Consistent with our BRET data, we found that co-expression of CXCR4 or CCR5 with RAMP3-YFP allowed for robust visualization of RAMP3-YFP at the cell surface, providing further evidence that these CKRs associate with RAMPs. We also noted some plasma membrane localization of RAMP3 in cells expressing CXCR7, suggesting that CXCR7 does indeed interact with RAMP3.

In addition to these well-characterized assays, we utilized a newly described FAP assay to quantitatively determine RAMP expression at the cell surface [29]. While RAMP translocation by GPCRs has been the gold standard for determining RAMP associations, it is inherently biased by which cells are imaged. In contrast, the FAP assay allows for visualization of plasma membrane expression of entire populations of cells. Again, consistent with our previous findings, we found that similar to CLR, FAP-RAMP3 successfully localizes to the plasma membrane when co-expressed with CCR5, whereas minimal plasma membrane expression of FAP-RAMP3 was observed in cells expressing B2ADR and V2R. Collectively, these data suggest that CKRs are able to form protein complexes with RAMPs.

Whether these interactions have functional consequences has yet to be determined. However, RAMP association could explain some of CKR pharmacology, including chemokine promiscuity, receptor recycling, and inconsistencies in downstream signaling. Endogenous expression of RAMPs may affect the biological activity of CKRs. The presence of a particular CKR-RAMP complex may alter ligand specificity or affinity. Likewise, as it has been shown to do for other GPCRs, RAMP association with CKR may affect G protein coupling [33,34]. For example, the presence of a RAMP may account for CXCR7 coupling to Gαi in astrocytes, whereas in other cell types, where endogenous RAMP expression is low, CXCR7 fails to G protein couple [20].
Moreover, although these results are preliminary, the CKR-RAMP interaction may have pathophysiological roles. CKRs and their ligands play pivotal roles in normal physiology. As such, dysregulation of their signaling can result in severe pathologies, including autoimmune disorders, pulmonary disease, transplant rejection, cancer, and vascular disease [35]. Moreover, viral exploitation of CKRs can facilitate their entry into host cells [35]. For example, HIV primarily uses CCR5 and CXCR4 for entry into human CD4+ T cells [36]. Consequently, direct antagonism of chemokine receptors offers potential for treatment of a wide variety of disease. CXCR4 and CXCR7 have already received significant attention from pharmaceutical companies for their roles in cancer metastasis [37]. If their association with RAMPs has functional consequences, the RAMP-CKR interface could be exploited for anticancer therapies.

More interestingly, several naturally occurring mutations in the coding region of CCR5 have been identified to affect the transport, binding, and signaling of CCR5 [38]. CCR5-893(−) is a single-nucleotide deletion observed exclusively in Asians, which results in premature termination of translation, a 54 amino acid deletion at the C-terminus, and poor cell surface expression of CCR5 [39,40]. While this mutation has not been studied extensively, it is possible that this truncation interferes with the association of CCR5 with a RAMP, thus preventing cell surface expression. Further research is necessary to evaluate whether the CCR5-RAMP association has biological consequences. Nevertheless, drugs designed to target the CCR5-RAMP interface could prove to be an exciting prospect for HIV therapy.

In summary, our results demonstrate evidence for the novel interaction of CXCR7, CXCR4, and CCR5 with the RAMP family. The RAMP-CKR interaction adds complexity to an already complex CKR system. GPCR homo- and heterodimerization is a well-known phenomenon. It will be interesting to determine whether CKR heterodimers, like CXCR7-CXCR4 or CCR5-CXCR4, form complexes with the RAMPs, and if RAMP-heterodimer
interactions yield different biological activities. Future studies will aim to address these questions. Furthermore, these data emphasize the breadth of RAMP interaction with GPCRs. While they are still mostly considered modifiers of Family B GPCR, we may find that RAMPs touch every branch of the GPCR tree.
Figure 5-1. BRET analysis of CKR-RAMP protein-protein interaction

(A) BRET analysis of CLR (green) and B₂ADR (red) with RAMP2. CLR+RAMP2 shows a robust increase in BRET signal with increasing doses of RAMP2-YFP. B₂ADR+RAMP2 displays a linear, non-specific interaction. (B) BRET analysis of CXCR7 with RAMP1 (blue), -2 (periwinkle), -3 (purple). CXCR7+RAMP1 displays a linear, nonspecific interaction. However, both CXCR7+RAMP2 and CXCR7+RAMP3 show positive changes in BRET signal with increasing RAMP-YFP. (C) BRET analysis of CXCR4 with RAMP2 (periwinkle) and -3 (purple). Both CXCR4+RAMP2 and CXCR4+RAMP3 show positive changes in BRET signal with increasing RAMP-YFP (D) BRET analysis of CCR5 with RAMP1, -2, and -3. CCR5+RAMP1 demonstrates a linear, non-specific interaction with increasing doses of RAMP1-YFP. CCR5-Rluc-expressing cells, however, show robust changes in BRET signal with increasing doses of RAMP2-YFP or RAMP3-YFP.
Figure 5-2. Localization of the GPCR-RAMP3 complex in vitro

(A,B) In order to determine RAMP3 localization in the absence of an interacting receptor, B₂ADR and V2R were co-transfected with RAMP3-YFP and live cells were visualized by confocal microscopy. While significant fluorescence was visible in the golgi apparatus, no plasma membrane localization of RAMP3 was observed. (C) CLR, a well-established RAMP3 partner, clearly enables RAMP3-YFP localization to the plasma membrane (white arrows). Plasma membrane localization was also observed when CXCR7 (D), CXCR4 (E), or CCR5 (F) were co-transfected with RAMP3-YFP. Plasma membrane localization was particularly well visualized at cell-cell contacts, where presence of two plasma membranes allowed for clear linear fluorescence (D, white arrows). Note that these are not seen in V2R+RAMP3-YFP (B), where multiple cells are imaged.
Figure 5-3. FAP assay of CCR5 interaction with RAMP3
(A) HEK293T cells were transfected with positive (CLR) or negative (V2R and B\textsubscript{2}ADR) controls, or CCR5 and FAP-RAMP3 (FAP-R3). Cells were treated with a membrane impermeant fluorogen for 5 minutes and then imaged on an Odyssey scanner (LI-COR). Robust fluorogen labeling of cell surface proteins was observed in the positive control (FAP-R3+CLR, first column) and with FAP-R3+CCR5 (fourth column). The negative controls had negligible cell surface labeling following treatment. (B) Quantitation of FAP assay. All wells were normalized to the average of the four nontransfected (NT) wells (column five in A). While no significant difference was observed between FAP-R3+CLR or FAP-R3+CCR5, a significant difference was observed in both conditions over FAP-R3+V2R and FAP-R3+B\textsubscript{2}ADR (*p<0.05, **p<0.01, ***p<0.001)
REFERENCES


Chapter 6: Conclusions and Future Directions

Summary of Results

We have presented preliminary data indicating that the chemokine receptors, CXCR7, CXCR4, and CCR5 form protein-protein interactions with members of the RAMP family. BRET studies suggest that CXCR4 and CCR5 interact with RAMP2 and RAMP3 in vitro. While BRET studies for CXCR7 were inconclusive, confocal microscopy investigating plasma membrane localization of RAMP3 demonstrated that all three chemokine receptors are capable of translocating RAMP3 to the cell surface. Finally, a quantitative FAP assay showed that CCR5 facilitates cell surface expression of RAMP3 at an equivalent level to the well-established RAMP-interacting GPCR, CLR. Plasma membrane expression of RAMP3 in CCR5-expressing cells was significantly increased above all negative controls.

Collectively, these data identify a novel group of Family A Rhodopsin-like GPCRs that interact with RAMPs. While the functional consequences of these interactions have not been characterized, the potential for modulating receptor pharmacology during inflammation or to alter viral receptivity could prove interesting. Future studies will characterize CKR-RAMP interactions and aim to delineate their biological roles.

Future Studies

As the current state of the RAMP field is comprehensively addressed in Chapter 4, this section will focus on the current and future studies associated with the data presented in Part II, where we identify the novel interaction between CKR and RAMPs.
Our first aim will focus on completing the trifecta of experiments for CXCR7, CXCR4, and CCR5 and all three RAMPs: BRET studies, confocal imaging, and FAP assays. We are confident that CCR5 forms a true protein-protein interaction with RAMP3, as we noted positive results for all three experiments. While CXCR4 and CXCR7 exhibited promising interactions with RAMPs, we must confirm these data with further assays. Thus, we have generated stable cell lines expressing 3xHA-RAMP1, -2, or -3. Additionally, we have acquired Flag epitope tagged CKR constructs. If these CKRs truly associate with RAMPs, live cell staining of HEK293T cells co-expressing a 3xHA-RAMP and a Flag epitope tagged CKRs will allow for visualization of CKR and RAMP co-localization at the plasma membrane.

Furthermore, though the live cell imaging of RAMP3-YFP was encouraging, the high intercellular fluorescence due to RAMP3-YFP presence in the golgi apparatus made discerning the plasma membrane challenging. With HA-tagged constructs, we can perform live cell staining on permeabilized and non-permeabilized cells. This experiment will allow for robust visualization of RAMP expression at the plasma membrane in non-permeabilized cells without the interference of intracellular fluorescence. HA-staining in permeabilized cells will ensure RAMP-HA expression. We have also generated FAP-tagged RAMP1 and -2 and plan to perform FAP assays with all three CKRs. This assay is both quantitative and exquisitely sensitive, and together with live cell imaging will provide convincing evidence as to which CKRs are capable of RAMP translocation to the cell surface.

Additionally, the ease of the FAP assay facilitates a much larger experiment. We have acquired a library of nearly 350 known druggable GPCRs (also known as the druggable “GPCR-ome”) with which we will perform a semi-high throughput screen for GPCR interactions with RAMPs [1]. We will test this system first with a pilot study, utilizing 19 CKRs. Here, we will transfect cells stably expressing FAP-RAMP1, -2, and -3 with CKRs, treat with membrane impermeant fluorogen and image the cells to determine which CKRs traffic RAMPs to the plasma membrane.
If this screen proves reliable and yields interesting results, we can perform this assay on the entire druggable GPCR-ome. Remarkably, four percent of the GPCRs in the GPCR-ome library are not efficiently surface expressed [1]. It will be interesting to determine whether co-transfection with a RAMP family member allows for cell surface expression of these GPCRs. As all of the receptors are Flag epitope tagged, we can monitor cell-surface expression of both the FAP-RAMP and the Flag-GPCR.

**Concluding Remarks**

Collectively, the studies presented in Part II support the interaction of CKRs with the RAMP family and contribute additional Family A GPCRs to the growing number of receptors that interact with RAMPs. Over the past decade and a half, there has been great interest in understanding how RAMPs affect GPCR pharmacology, but determining which receptors interact with RAMPs has been a slow process. As discussed in Chapter 4, RAMPs have historically been thought to only interact with Family B G protein-coupled secretin receptors. However, since their discovery, RAMPs have also been shown to modulate at least one member of both family A and family C GPCRs [2,3]. Our preliminary data suggest a much wider group of GPCRs interact with RAMPs. The proposed semi-high throughput screen will allow us to characterize the breadth of RAMP interactions, facilitating a shift in the focus of RAMP research to the design of functional assays to determine whether a given GPCR-RAMP association affects pharmacology or biological activity. A thorough understanding of RAMP effects on biology has the potential to have significant clinical impact, as targeting of the RAMP-GPCR interface could yield highly specific, high affinity drugs.
REFERENCES

