Study of Lcn2 in inflammation and characterization of its RNA aptamer

Lijie Zhai
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/etd
Part of the Biochemistry Commons, Immunology and Infectious Disease Commons, and the Mechanical Engineering Commons

Recommended Citation
Zhai, Lijie, "Study of Lcn2 in inflammation and characterization of its RNA aptamer" (2012). Graduate Theses and Dissertations. 12541. https://lib.dr.iastate.edu/etd/12541

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
Study of Lcn2 in inflammation and characterization of its RNA aptamer

by

Lijie Zhai

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

Major: Immunobiology

Program of Study Committee:
Marit Nilsen-Hamilton, Major Professor
Mark Ackermann
Douglas Jones
Michael McCloskey
Michael Wannemuehler

Iowa State University
Ames, Iowa
2012

Copyright © Lijie Zhai, 2012. All rights reserved.
TABLE OF CONTENTS

ABSTRACT ........................................................................................................................................ iv

CHAPTER 1 GENERAL INTRODUCTION ........................................................................ 1
  1.1 DISSERTATION ORGANIZATION .................................................................................. 1
  1.2 RESEARCH AIMS AND SIGNIFICANCE ....................................................................... 2
  REFERENCES .......................................................................................................................... 4

CHAPTER 2 LITERATURE REVIEW ................................................................................... 5
  2.1 LIPOCALIN-2 .................................................................................................................. 5
  REFERENCES .......................................................................................................................... 35
  2.2 LIPOPOLYSACCHARIDE AND DEXTRAN SULFATE SODIUM IN ANIMAL INFLAMMATION MODELS .................................................................................................................. 52
  REFERENCES .......................................................................................................................... 55
  2.3 APTAMERS AS BIOSENSORS FOR DISEASE MARKER DETECTION .................. 59
  REFERENCES .......................................................................................................................... 69

CHAPTER 3 LIPOCALIN 2 HAS LITTLE OR NO ROLE IN THE INFAMMATORY RESPONSE .......................................................................................................................... 77
  3.1 ABSTRACT .................................................................................................................. 77
  3.2 INTRODUCTION ......................................................................................................... 78
  3.3 MATERIAL AND METHODS ...................................................................................... 80
  3.4 RESULTS ...................................................................................................................... 83
  3.5 DISCUSSION ............................................................................................................... 85
  3.6 ACKNOWLEDGEMENT ............................................................................................ 88
  REFERENCES ....................................................................................................................... 88
  TABLES ............................................................................................................................... 95
  FIGURES .............................................................................................................................. 97
ABSTRACT

Lipocalin-2 (Lcn2), a member of the lipocalin superfamily, has been implicated in diverse physiological and pathological processes, such as apoptosis, cell differentiation, inflammation, iron metabolism and wound injury healing. However, most of these reports are of correlations and no cause-effect relationship has been established for Lcn2’s role in inflammation and wound healing. Using Lcn2 gene knockout mice (Lcn2−/−), we investigated the role of Lcn2 in both lipopolysaccharide (LPS)-induced acute lung inflammation response and dextran sulfate sodium (DSS)-induced colitis. Under all the treatment conditions, no significant differences were observed in proinflammatory cytokines expression level between the LCN2−/− and wild-type mice for the lung inflammation model. For both of animal models, the histological studies and the observed disease severity also indicated no significant difference between these two types of mice. We conclude from the results of this study that Lcn2 has no causal role in the induction of inflammation nor does it play a protective role against inflammation.

In addition to the functional/mechanism studies, Lcn2 has been re-evaluated as a standard biomarker for many diseases. To develop a new detection tool for Lcn2, in the current study we selected and characterized a mouse Lcn2 (mLcn2) RNA aptamer. Binding affinity analysis demonstrated this RNA aptamer has a dissociation constant for mLcn2 of 0.34 ± 0.07 μM, but does not bind the human and chicken forms of Lcn2. RNA foot printing and mutational assay indicated that the nucleic acids to protein contact regions are mainly located on the loops of the aptamer, which was predicted to fold in a 3-way junction secondary structure. Further analysis from site mutagenesis of mLcn2 revealed that the aptamer-protein interaction involves the amino acids in the pocket of mLcn2 that normally bind its native ligand, iron-siderophore, but does not involve the mLcn2 surface polar amino acids. Application of the mLcn2 aptamer as a detection probe in a homogenous assay was also demonstrated by using micro-cantilever system.
CHAPTER 1 GENERAL INTRODUCTION

1.1 DISSERTATION ORGANIZATION

This dissertation contains five chapters and an appendix.

Chapter 1 is a general introduction to the dissertation organization and the research aims and significance of the studies.

Chapter 2 is a thorough literature review of Lcn2 and application of aptamers. In this chapter, studies covering several aspects of Lcn2 including early identification, gene expression profile, structures, binding ligands and potential mechanisms as well as its relationship to other lipocalins are reviewed and discussed. The second part of Chapter 2 is a literature review of aptamers mainly focusing at its selection, chemical modification and its application as biosensors.

Chapter 3 addresses the first specific aim, role of Lcn2 in inflammation. Experimental results from two mouse inflammation disease models, acute lung inflammation and acute colitis were reported. The fact that no significant difference was observed in both proinflammatory cytokines expression level and histological and disease severity between the LCN2−/− and wild-type mice for both models suggests that Lcn2 has neither a causal role in inflammation nor a protective role. Possible role and mechanism of Lcn2 in inflammation are also discussed in this section. The authors’ contributions are as below: Lijie Zhai designed and performed the experiments and wrote the paper; Yinghua Liu participated in lung inflammation model work; Mark Ackermann, Tania Marchban and Raymond J. Playford did the histological analysis; Thorsten Berger provided the Lcn2 KO mice; Marit Nilsen-Hamilton mentored Lijie Zhai and Yinghua Liu and oversaw the project including data analysis and edit the paper.

Chapter 4 addresses the second specific aim, development of an Lcn2 aptamer. In this chapter, a detailed description of mLcn2 aptamer selection and characterization as well as its preliminary application on micro-cantilever was given. Research presented in Chapter 4 is prepared for submission to *Analytical Biochemistry: Methods in the Biological Sciences*. The contributions of the authors are as follows. Lijie Zhai expressed and purified the recombinant proteins, measured the binding affinity and characterized the protein secondary structures and
RNA secondary structure analysis. Tianjiao Wang selected the mLcn2 aptamer and assessed binding affinity, RNase footprinting, aptamer structure analysis and competition binding assays. Kyungho Kang and Yue Zhao performed the micro-cantilever detection experiments. Pranav Shrotriya mentored Kyungho Kang and Yue Zhao and oversaw the cantilever study. Marit Nilsen-Hamilton mentored Lijie Zhai and Tianjiao Wang and oversaw the project including data analysis. Lijie Zhai wrote the manuscript which was edited by Tianjiao Wang and Marit Nilsen-Hamilton.

Chapter 5 is the general conclusions and perspective towards the future direction. Several important future research directions of Lcn2 are discussed. Further optimization and application of the mLcn2 aptamer are also included in this section.

Supplemental data for mLcn2 aptamer selection and characterization are described in the Appendix followed by the acknowledgement to all the people supporting my Ph.D. study.

1.2 RESEARCH AIMS AND SIGNIFICANCE

Inflammation is the first and the most critical innate immune response to various inflammatory stimuli. Following the initiation of this complicated process, local and systemic reactions are involved. One of the most important systemic reactions is the acute phase response (APR) which can also be triggered by many stress stimuli. During this stress response, the plasma levels of a group of proteins change rapidly. These proteins are called acute phase proteins (APPs) [1]. Lipocalin 2 (Lcn2), a member of the lipocalin super-family, has been shown to be an APP in both animal and humans [2,3]. Many studies indicate the strong correlation between Lcn2 and inflammatory as well as non-inflammatory diseases. Although several putative functions of Lcn2 were reported, such as the induction of myeloid cells apoptosis [4], antibacterial via iron chelating [5], the physiological functions of this protein are still enigmatic as many conflicting experimental results exist [6].

Being an inflammation-associated protein, Lcn2 has been evaluated as a biomarker for many diseases. Among these, acute kidney injury (AKI) is currently the most extensively studied disease in which Lcn2 has been evaluated as a biomarker. A meta-data analysis from studies involving 19 countries and 2538 patients indicated that Lcn2 is a very promising biomarker for AKI diagnosis and prognosis [7]. The role as a valuable biomarker has not only been extended from AKI to other kidney dysfunction associated diseases, but also been
exploited in non-kidney diseases, such as cardiovascular diseases and metabolic disorders [8,9].

Two specific aims are proposed in the current research project with one focusing on the function/mechanism study and the other one centering on the application of Lcn2 as a biomarker.

**Aim 1: Study the role of Lcn2 in different acute inflammation models using Lcn2 \(-/\)- mice.** Although many studies have shown the correlation between Lcn2 and inflammation, *no cause-effect relationship has been established for Lcn2’s role in inflammation.* In other words, it is still unclear whether Lcn2 plays an important regulatory role in inflammation or it is a byproduct of inflammation, perhaps with a later effect in tissue healing. In this regard, a comparative study of Lcn2 knockout and wild-type (Lcn2 \(^{+/+}\)) mice in different inflammation models will address this fundamental question. Because many studies have shown that Lcn2 is highly up-regulated in both respiratory and digestive tissues, two mouse inflammation models, the acute lung inflammation and acute colitis were used in this particular study to elucidate the cause-effect relationship of Lcn2 in inflammation. In order to validate the mouse model and compare the inflammatory responses of the two mice strains, three established parameters (gene expression levels of pro-inflammatory cytokines, histological change and disease severity) were monitored during the experiments. This study was undertaken with the expectation that results from the study could lead to the identification of a new target for anti-inflammatory drug development.

**Aim 2: Select and apply an aptamer as a detection tool for mouse Lcn2.** The significance of Lcn2 as a disease inflammatory marker has promoted the development of more sensitive and specific Lcn2 detection methods with good reproducibility and low cost. Current Lcn2 detection methods for both basic research and clinical centers depend on antigen-antibody based immunoassays. However, the high cost for antibody production becomes a major obstacle for the broad application of these immunoassays. In contrast to the expensive *in vivo* production of antibodies, aptamers are single-stranded nucleic acids isolated from random-sequence nucleic acid pools by cost efficient *in vitro* selection. Meanwhile the greater flexibility of chemical modification makes aptamers compatible with a variety of detection platforms. In this specific aim, *to develop a new detection material for*
mLcn2, we aimed to select and characterize an RNA aptamer specifically recognizing mLcn2 and test its application on a micro-cantilever detection system.

REFERENCES


CHAPTER 2 LITERATURE REVIEW

2.1 LIPOCALIN-2

It has been more than two decades since the discovery of the first two Lcn2 members, the mouse Lcn2 (also known as SIP24) and the chicken Lcn2 (also known as Ch21 or Ex-FABP) [1-3]. During the past twenty years, studies focusing on this protein family have expanded from protein isolation, gene cloning and sequencing to gene expression regulation, structure determination, functional and mechanistic investigation. Meanwhile, the research scope of Lcn2 has extended from the basic studies of cell culture and animal models to more applied investigation with emphasis on the biomarker potential of Lcn2. This is indicated by the fact that, within the last two years there have been 2 to 6 new publications every week in PubMed database (searched by using Lcn2 or lipocalin 2 as the key word) with a majority of these focusing on Lcn2 as a disease biomarker.

As for many other inflammation non-specific biomarkers, although some important features of Lcn2 have been revealed, the explicit cellular function and underlying mechanism of Lcn2 still remain ambiguous and unclear. This review focuses on several key aspects of Lcn2 studies. First, the identification of Lcn2 in different species is introduced to clarify some common nomenclature confusion. Second, the expression profile and expression regulation of Lcn2 from different species are compared. Third, the structural analysis and the ligand binding studies of Lcn2 are summarized. Fourth, based on the first three sections, the functional and mechanistic research work of Lcn2 is discussed with a focus on data interpretation. Fifth, the correlation of Lcn2 with various diseases and its potential as biomarker are briefly reviewed. Finally, Lcn2 is compared to other lipocalins and other inflammatory marker proteins with emphasis on their putative functions in inflammatory response.

The goal of this review is to provide a summary of the most recent Lcn2 research work and to discuss the potential mechanisms underlying the diverse functions of this puzzling protein.
2.1.1 Identification of Lcn2 in different species and its classification and nomenclature

Discovery of Lcn2 in different species was serendipitous as for many other scientific findings. The mouse Lcn2 was first identified as one of the five superinducible proteins with a molecular weight at 24 kDa in the study of growth factor effect on fibroblasts, hence the name of SIP24 [1,2]. Peptide sequencing [4] revealed that SIP24 is the protein product of the mouse 24p3 mRNA, whose cDNA was originally cloned from SV40-infected mouse kidney primary cell cultures [5]. Thus 24p3 was also referred to SIP24. Examination of SIP/24p3 expression profile indicated that this protein was highly up-regulated in mouse uterine luminal fluid upon oestrogen stimulation and uterus epithelium from mice at the parturition and postpartum stages [4,6]. As a result of this observation, SIP24/24p3 was renamed as uterocalin. However, the later use of the same name for a different horse lipocalin made this name less useful for unambiguously identifying the mouse protein [7]. The rat Lcn2 was first named as rat α2-microglobulin-related protein because its cDNA was isolated during the screening of a cDNA library for other purposes and showed sequence similarity to rat α2-microglobulin [8]. By a differential hybridization technique, an identical cDNA was later found to be highly overexpressed in mammary cancers induced by the oncogene neu (HER2/c-erbB-2), which is a potent inducer of mammary cancers in rats. The gene was therefore designated neu-related lipocalin (NRL) [9].

The chicken ortholog of Lcn2 was first identified as a secreted protein with molecular weight of 21 kDa from in vitro differentiating chicken chondrocytes at a late stage of development, hence the name Ch21 [3]. By using native polyacrylamide gel electrophoresis and the Lipidex assay, it was shown that Ch21 can bind fatty acids, and preferentially binds long-chain unsaturated fatty acids with a dissociation constant of 0.2 μM. Therefore the Ch21 protein was proposed to rename as extracellular fatty acid-binding protein (Ex-FABP) [10]. Identification of human Lcn2 originated from the studies of human neutrophil gelatinase. At about the same time, two different groups isolated, purified and sequenced a 25-kDa protein covalently associated with 92-kDa gelatinase/ matrix metalloproteinase 9 (MMP-9) from human neutrophils. It was therefore named as neutrophil gelatinase-associated lipocalin or NGAL [11,12]. The protein
has later been purified by Xu and coworkers, who suggested the name human neutrophil lipocalin or HNL [13].

Sequence analysis of different Lcn2 proteins had Lcn2 classified into the lipocalin family, a large and ever expanding group of proteins exhibiting great structural and functional variation, both within and between species [14]. Lipocalins are typically small (160-180 residues in length), extracellular proteins sharing several common molecular recognition properties: the binding of small, principally hydrophobic molecules; binding to specific cell-surface receptors; and the formation of covalent and non-covalent complexes with other soluble macromolecules. Although proteins in this family share low identity at the sequence level, they all fold into a characteristic tertiary structure, a β barrel consist of 8 to 9 antiparallel β sheets, exhibiting the structural element for ligand binding of lipocalins. In an early study aiming at genomic mapping of lipocalins’ genes, Lcn2 and LCN2 were used to represent the mouse 24p3 gene and human NGAL gene respectively [15]. Therefore Lcn2 or lipocalin 2 was coined as a consensus name for different homologs of this protein in later studies, especially for human and mice. In addition, instead of using a structural property for naming, siderocalin was proposed based on the siderophore binding function of mouse and human Lcn2 [16].

It is not surprising that the Lcn2 literature is difficult to negotiate due to the use of so many different names. As discussed in the following sections, although they share certain common features, Lcn2 proteins from different species also appear to display some fundamental differences that necessitate care in extrapolating the results of experiments on one species to another. A concise and accurate nomenclature of Lcn2 based on function and (or) structure should be established as soon as possible for the research community. In this review, mLcn2 stands for mouse Lcn2 (also 24p3, SIP24, uterocalin and siderocalin), hLcn2 represents human Lcn2 (also NGAL), rLcn2 represents rat Lcn2 (also NRL), cLcn2 represents the chicken Lcn2 (EX-FABP) and Lcn2 refers to all species of Lcn2.
2.1.2 Lcn2 expression and regulation

In this section, several common features of Lcn2 from different species are discussed followed by a comparison of their differences. Signaling pathways involved in Lcn2 regulation are also reviewed.

1) Lcn2 expression under different conditions

The discovery of Lcn2 in several species showed that this protein is highly upregulated under inflammation or stress. However, it is important to know the expression profile of Lcn2 under normal physiological conditions before we delve into a thorough examination of its expression under pathological or physiopathological conditions. Measurements of the mRNA levels of hLcn2 in 50 human normal tissues [17] showed a high level of gene expression in bone marrow but not in peripheral leukocytes, consistent with the observation that biosynthesis of hLcn2 in blood cells occurs only at the myelocyte/metamyelocyte stage of granulocytic maturation [18]. High levels of hLcn2 expression were also observed in the uterus, prostate, salivary gland, stomach, appendix, colon, trachea and lung. These findings were in excellent agreement with the hLcn2 protein distribution data as revealed by immunohistochemical (IHC) staining of tissue samples from every human organ system [19]. In addition, with the technical advantage of specific cell type location using IHC, this study also confirmed the cell type specificity of hLcn2 expression. Among these, hLcn2 was strikingly expressed in normal parietal cells of the gastric fundus and in proximal tubular epithelial cells in the kidney, both of which are involved in active ion transport. While the tissue distribution of hLcn2 in humans and rLcn2 in rats was consistent in most tissues, notable differences were detected in a few organs. rLcn2 was detected in the endocrine portion rather than small ducts of pancreas. rLcn2 also localizes to the kidney distal tubules, contrasting with the presence of hLcn2 in proximal tubules. It is of note that results from these two studies indicate that, although hLcn2 was identified as covalently linked to neutrophil gelatinase, it is not only expressed in differentiating neutrophils but also epithelial cells from a variety of different tissues. Because most studies are focused on pathological or physiopathological conditions or limited to certain specific organ systems, systemic examinations of the mouse and chicken Lcn2 expression profiles under normal conditions are as yet unavailable.
mLcn2 was named as uterocalin because its high expression in mouse uterine luminal fluid upon oestrogen stimulation and uterus epithelium from mice at the parturition and postpartum stages [4,6,20]. Similar to the uterus, the mammary gland, another reproductive tissue undergoing extensive involution during pregnancy also produces high levels of mLcn2 especially during mid-gestation, at birth and throughout lactation [21,22]. It was suggested that a possible function for the high levels of mLcn2 during tissue involution might be to induce local leukocyte apoptosis therefore preventing massive cell death caused by neutrophil infiltration and help tissue remodeling [23]. Consistent with the idea that Lcn2 might play a role in tissue remodeling, the expression of both the rLcn2 and the cLcn2 homologs of mLcn2 were also enhanced in the embryo development in hypertrophic cartilage and muscle fibers (forming myotubes) where active remodeling is taking place [24-26]. However, in contrast to the proposed pro-apoptotic effect of mLcn2 in uterus and mammary gland involution, cLcn2 had been shown an anti-apoptotic role in tissue remodeling [27]. In addition to the female reproductive organs, mLcn2 was also detected in male mice reproductive organ, the early developing epididymal caput with a potential function for iron delivery into spermatozoa [28,29].

Expression of Lcn2 under pathological and physiopathological conditions is diverse and complex as indicated by various stimulators and a large numbers of cell types involved in Lcn2 expression regulation. One of the most common conditions for Lcn2 induction is the inflammatory response. Lcn2 has shown high expression levels in cultured cells from mouse, rat, chicken and human and animal models of these species after treatment with inflammatory agents, including lipopolysaccharide (LPS) and proinflammatory cytokines. For example, mLcn2 is highly expressed in both lung alveolar and hepatic macrophages as well as lung type II epithelial cells after LPS stimulation [30]. It is also strongly induced in hepatocytes and adipocytes both in vitro and in vivo by proinflammatory cytokines, such as interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) [31-33]. Two recent studies using a murine systemic endotoxemia model revealed that, although undetectable under physiological conditions, mLcn2 was highly upregulated in the central nervous system (CNS) with the main cellular source being endothelial cells and microglia and high levels of mLcn2 were found in the choroid plexus/cerebrospinal fluid (CSF) barrier [34,35]. Similar to
mLcn2, rLcn2 and cLcn2 were highly induced in early differentiating chondrocytes and myotubes stimulated by LPS [24,25]. Upregulation of hLcn2 during inflammation is confirmed with data not only from LPS treated cells [36] but also from many inflammatory diseases as discussed in the subsequent sections.

In addition to pathological inflammatory response, the complexity of Lcn2 expression lies in its involvement in more diverse physiopathological conditions or stress responses. For example, mLcn2 is induced in fibroblasts by growth factors such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), prostaglandin F2α [1,2]. Glucocorticoid (dexamethasone) and retinoic acid as well as chemical tumor promoters (turpentine, 12-O-tetradecanoylphorbol-13-acetate (TPA) are also positive inducers for mLcn2 [2,4,37]. Many other biological processes that activate the inflammatory status or stress also contribute to Lcn2’s upregulation. Increased expression of Lcn2 has been reported under many other conditions such as oxidative stress [38], thermal stress [39], circadian rhythm and fed/fasting status [40]. Lcn2 is also abundantly produced by adipocytes [41-43]. The expression and secretion of this protein increases sharply after the conversion of preadipocytes to mature adipocytes. Moreover, considering the involvement in inflammatory response and stress, it is not surprising that a large number of Lcn2 expression data have been accumulated from numerous surveys of almost all known diseases because inflammation and stress are fundamental biological response accompanying many diseases.

In summary, the expression of Lcn2 in different species share some characteristics both spatially and temporally: 1) Although many types of cells can synthesize Lcn2 under various conditions, one of the major types of Lcn2 secreting cells are epithelial cells that are either located in glandular ducts or tissues with externally exposed surfaces that are exposed to microorganisms. This cell type specificity and tissue distribution indicates that Lcn2 might perform cellular substance uptake and (or) anti-microbial infection functions; 2) Induction of Lcn2 usually takes place within a short time period after addition of the inflammatory stimulus, which is consistent with the acute phase protein expression pattern. It was shown that Lcn2 can reach its expression peak at protein level as short as 4 hours after the addition of LPS [30,31]; 3) After induction, the enhancement of Lcn2 expression is drastic, it can reach more than 1000 fold of the control at the mRNA level and hundreds fold at protein
level. Studies of Lcn2 expression in different species suggests that the expression patterns might differ and the genes are differently regulated. For example, although epithelial cells have been shown one of the major cell types for Lcn2 synthesis in human and mouse, cLcn2 has so far only been identified as expressed in chondrocytes and myotubes and granulocytes. Perhaps more relevant to suggesting a possible difference in tissue distribution of Lcn2 expression with species are the studies to show that rLcn2 was detected in the endocrine portion rather than small ducts of pancreas where hLcn2 was detected. rLcn2 was also localized to the renal distal tubules in contrast with hLcn2 being found in proximal renal tubules. These observations may reflect real differences in gene expression between species or changes in gene expression with physiological conditions. However, researchers should be cautious in extrapolating conclusions from studies between species and should always identify the species they used when describing the results of their research work.

2) Gene expression regulation of Lcn2

The gene expression regulatory network of Lcn2 is as complex as its expression pattern which is indicated by many intertwined signaling pathways. Genome sequencing data has provided useful information for computational prediction of promoter and transcription activator binding sites (TFBS). Although several common pivotal TFBS have been identified for both hLcn2 and mLcn2, such as nuclear factor-κB (NF-κB) and CCAAT/enhancer-binding protein (C/EBP) binding sites, different signaling pathways involving various TFs have been reported in the Lcn2 gene regulation. In a series of studies focusing on the hLcn2 induction specificity for IL-1β but not TNF-α from human lung epithelial cell line (A549) and human heptoma cells (HepG2), Borregaard and colleagues demonstrated that hLcn2 could be upregulated upon IL-1β stimulation through a IκB-ζ dependent C/EBP independent IL-1:IL-1R:NF-κB pathway in these cells. Based on the requirement for IκB-ζ, they explained why as a similar proinflammatory cytokine, TNF-α had no (or very weak) effect on Lcn2 induction as this cytokine can only stimulate IκB-ζ gene expression through NF-κB activation but does not result in stabilization of the transcript which is required for efficient synthesis of IκB-ζ [31,36,44]. The same mechanism was also revealed in the hLcn2 induction by costimulation of TNF-α and interleukin-17 (IL-17). In this case, the proinflammatory IL-17 provided the signals for IκB-ζ transcript stabilization thereby triggering sustained Lcn2
expression in synergy with TNF-α [45]. Unlike for hLcn2, mLcn2 induction by IL-17 plus TNF-α has been reported to depend on NF-κB and C/EBP but not IκB-ζ in a murine preosteoblast cell line (MC3T3-E1) [46]. However, as these are different cell lines, one cannot conclude that the differences are due to the species.

The essential role of C/EBP for mLcn2 expression has been confirmed by the observation that the C/EBPε deficient mice have severely reduced expression of Lcn2 in the bone marrow [47] and systemic endotoxemia induced mLcn2 via activation of NF-κB and C/EBP [30]. Studies focusing on spermatogenesis indicated that spermatogonial cell (SGC) mediated activation of the NF-κB pathway in Sertoli cells can induce mLcn2 in an IκB-ζ independent way as the expression of mLcn2 was unaffected in the testis of IκB-ζ deficient mice [48]. It is of note, however, spermatogonial cells are a highly specialized cell type that show low responsiveness to inflammatory cytokine stimulation and the inducing factors from SGC were shown to be distinct from inflammatory cytokines.

The dependence of IκB-ζ for mLcn2 induction by inflammatory cytokines in other types of cells can’t be completely excluded. Like many gene regulatory pathways, the independence of C/EBP pathways for hLcn2 induction by IL-1β is cell context dependent. In two recent studies investigating role of hLcn2 in tumorigenesis indicated that C/EBPβ significantly increased hLcn2 promoter activity in 12-O-tetradecanoylphorbol-13-acetate (TPA) treated gastric carcinoma cell lines [49] while C/EBPζ repressed the hLcn2 gene promoter activity in a breast cancer cell line (MDA-MB-231) [50]. In addition to NF-κB and C/EBP, mLcn2 is upregulated by dexamethasone by way of the glucocorticoid responsive core element (GRE) on its gene promoter [37]. Mouse Lcn2 expression is repressed by the Wnt signaling pathway by way of putative TF binding sites for the direct Wnt target MYC [51]. The JAK/STAT pathway had also been reported to participate in the mLcn2 induction. It was shown that, with erythropoietin (EPO) stimulation, knockdown of STAT3 by siRNA caused a significant decrease of mLcn2 gene induction in the mouse myeloid cell line 32D expressing chimeric EPO receptor [52].

Considering its diverse expression profile including many types of cells under various conditions, it is not surprising that Lcn2 expression is cooperatively controlled by a network of transcription factors. This regulation diversity makes Lcn2 more adaptable for rapid
induction/control under different situations. One trend in Lcn2 expression studies is that most research focuses on the induction/upregulation of Lcn2. In comparison, studies focusing on negative control mechanism of Lcn2 at the transcriptional level are scarce. Part of the reason is that Lcn2 is usually highly upregulated upon stimulation and detection of increased response is technically favorable. However, studies of negative control of Lcn2 expression will provide invaluable knowledge for our understanding of the homeostasis of an acute phase protein such as Lcn2.

2.1.3 Structure of Lcn2 and its ligands

1) Structure of Lcn2

Sequence alignment of members from different species indicated Lcn2 belongs to the lipocalin superfamily characterized by a common tertiary structure, a single eight-stranded antiparallel β-sheet closed back on itself to form a continuously hydrogen-bonded β barrel [14]. Lcn2 proteins fall into the kernel lipocalin group as they share the three structurally and sequence conserved regions (SCRs) with other kernel lipocalins which dominate the common core characteristic of the lipocalin fold. More convincing data for Lcn2 3D structure first came from the solution structure of human hLcn2 determined by nuclear magnetic resonance (NMR) spectroscopy [53]. The structure features an eight stranded antiparallel β-barrel, typical of the lipocalin family. One end of the barrel is open, providing access to the binding site within the barrel cavity, while the other is closed by a short N-terminal 3_{10}-helix which is another common structural feature of lipocalins. The free cysteine (Cys87) residue required for association of hLcn2 with pro-MMP-9 lies in an inter-strand (β4- β5) loop at the closed end of the barrel. At about the same time, a crystal structure of hLcn2 was reported which further confirmed the solutions structure [54]. In this study, radically different crystallization conditions including buffer components and pH values yielded essentially identical structures for monomeric and dimeric hLcn2, implying that Lcn2 has a fairly rigid structure. The crystal structure also displays an intramolecular disulfide bond between cysteines 76 and 175 in all determined structures. Formation of the disulfide bond between Cys87 in the dimeric hLcn2 does not occlude or affect the structure of the β-barrel/calyx. The location of Cys87, same as determined in NMR structure, likely allows some interdomain flexibility in the homodimer
or the gelatinase B heterodimer. Other than the disulfide bond there is no other interaction between the two molecules in the homodimer. The crystal structures also showed that the calyx of hLcn2 is unusually large and open and shallower compared to the binding cavities of other lipocalins. The determined volume of hLcn2 calyx is considerably larger even than that of nitrophorin, a lipocalin binds a substituted heme. In addition to a much larger enclosed volume, the hLcn2 calyx is also uncharacteristically lined with many polar and positively charged residues. More recently, the crystal structure of mLcn2 has been solved and compared to hLcn2 structure. Superimposition of the two structures showed that mLcn2 has a very similar protein fold to hLcn2 [55].

2) Lcn2 binds iron-siderophore complex

The solved tertiary structure clearly classified Lcn2 in the lipocalin superfamily, more importantly they identified the binding ligands of hLcn2 and hLcn2, which consequently revealed a likely mechanism for Lcn2 function. Prior to the tertiary structure determination, several hydrophobic and lipophilic inflammation mediators (N-formyl-Met-Leu-Phe, leukotriene B4, platelet activating factor and lipopolysaccharide) had been tested for Lcn2 binding based on the ligand preference for the lipocalin superfamily and a possible anti-inflammatory role for Lcn2 [56,57]. However, the structure of the hLcn2 calyx together with the low binding affinities (millimolar level) for the tested ligands demonstrates that hLcn2 does not specifically bind these ligands. Soon after the first crystal structure of hLcn2 was reported, it was found that hLcn2 copurified with a bacterial chromophore during its expression from an XL-1 Blue strain of *Escherichia coli* (*E. coli*) [16]. Based on the crystallography data and atomic absorption as well as X-ray fluorescence spectroscopy data, this chromophore was predicted to be a bacterial iron chelator, also known as a siderophore. Both protein fluorescence quenching test and bacterial culture proved that the chromophore was a catecholate-type siderophore enterocalin (Ent) that binds to hLcn2 in an iron (ferric status)-siderophore complex (FeEnt) format with a dissociation constant at 0.41 ± 0.11 nM. hLcn2 binds FeEnt by intercalating the positively charged side chains of three protein residues (Arg81, Lys125, and Lys134) between the ligand catecholates through a cyclically permuted, hybrid electrostatic/cation-π interaction. Each of the three catecholate rings fits into a distinct pocket of the trilobate calyx of hLcn2. Because FeEnt rapidly degrades into
dihydroxybenzoyl-serine (DHBS) and dihydroxybenzoic acid (DHBA), interaction of hLcn2: FeDHBA was also determined using a fluorescence quenching assay, which showed a lower but respectable binding affinity ($K_d = 7.9 \pm 1.8 \text{nM}$) compared to FeEnt. It is noteworthy that in this study, the rigidity of hLcn2 structure was once again observed as crystal structures determined in this study were essentially identical to the previous reported structures although the crystallization were performed in remarkably different conditions (from zero to saturating ammonium sulfate, pH from 4.5 to 7.0), suggesting that hLcn2 does not undergo drastic conformational changes due to ligand binding, ionic strength or pH variation. Because the sequence identity between human hLcn2 and mLcn2 or rLcn2 is 62% or 63.5% and the FeEnt interacting amino acids are conserved between these species, it is highly likely that these latter two proteins also bind to FeEnt specifically. Consistent with this expectation, FeDHBA binding by mLcn2 is confirmed in this thesis (chapter 4) and FeDHBA binding assay has become the standard activity assay for commercial recombinant mLcn2 and rLcn2. In contrast to the Lcn2 from human, mouse and rat, the chicken homolog shows no binding to iron-siderophore but specifically binds fatty acid preferentially long-chain unsaturated fatty acids with dissociation constants at 0.2 $\mu\text{M}$ [10]. However, the role (if any) of cLcn2 in fatty acid metabolism has not been elucidated.

3) Lcn2 as a bacteriostatic agent by sequestrating iron-siderophore complex

The identification of bacterial siderophore as the specific ligand for Lcn2 immediately indicated a possible anti-bacterial function of Lcn2 and is consistent with the upregulation of this acute phase protein under infection conditions. Siderophores are bacterial products that sequester iron when the microbes are in an iron-limiting environment. Many siderophores bind iron more tightly than mammalian iron binding proteins, such as lactoferrin and transferrin, therefore competing with the host for iron acquisition. Siderophores are usually classified by the ligands used to chelate the ferric iron. The major groups of siderophores include the catecholates (phenolates), hydroxamates and carboxylates (derivatives of citrate). The primary siderophores of *E. coli* and related enteric bacteria are the catecholate-type siderophore enterobactin (Ent) and the citrate-based, hydroxamate-type siderophore aerobactin. Ent is the strongest soluble Fe$^{3+}$ binding agent known with a $K_d$ of $10^{-49} \text{M}$ [58]. The anti-bacterial function of Lcn2 was validated in a pathogenic *E. coli* infection model
using Lcn2 deficient mice [59]. In this study, intraperitoneal challenge with a sublethal dose of a clinical strain of *E. coli*, H9049, results in an average 1,000-fold greater bacteraemia in Lcn2-deficient mice than in controls, and significant differences in bacterial counts in the liver and spleen were also seen. When infected with a lethal dose of the bacteria, the Lcn2 knockout mice showed substantially accelerated death rates compared with the wild-type mice. The authors also explored the specificity of the protective effect of Lcn2 against Ent-dependent bacterial infection. After being challenged with *E. coli*, H9049, the mice were administered ferrichrome, a hydroxymate-type siderophore to which Lcn2 does not bind [16]. With this treatment the wild-type mice showed similar lethality as that of the Lcn2 knockout mice after the infection. The specificity of Lcn2 protective effect was further confirmed by the observation that Lcn2 had no effect on the survival of mice intraperitoneally infected with *Staphylococcus aureus*, bacteria that acquire iron through siderophores that do not bind Lcn2.

It has been known that bacteria utilize various forms of siderophore as their iron chelating armory. The specificity of the Lcn2 protective effect against the catecholate-type siderophore enterobactin raises the question whether Lcn2 also binds other types of siderophores. Moreover, a close examination of the crystal structure of hLcn2:FeEnt indicated that the electron density quality of the Ent was poor and diffuse and the Ent does not occupy several obvious, underlying pockets in the calyx, implying the possibility of other potential ligands. To further investigate the siderophore binding specificity, Holmes and colleagues tested the binding of hLcn2 with a panel of 18 natural and synthetic siderophores of different types using surface plasmon resonance (SPR), copurification and further confirmed by crystallization [60]. The results showed that hLcn2 binds to a family of catecholate siderophores chemically related to Ent but does not bind to yeast and bacterial siderophores based on hydroxymate or carboxylate iron binding groups. Surprisingly, they found that hLcn2 also binds the carboxymycobactin siderophores of *Mycobacterium smegmatis* (CMB-S) and *M. tuberculosis* (CMB-T), a group of heterogeneous siderophores with iron binding moieties consisting of a hydroxyphenlyoxazoline, a seven-cyclic hydroxamate and a linear hydroxamate with a fatty acid tail. The binding pattern of hLcn2:FeCMB is very similar with that of hLcn2:FeEnt as Lys125 and Lys134 participate in cation-π bond formation and the hydroxybenzoyl of CMB superimposes almost identically onto the DHBA moiety of Ent in
the same sub-pocket. This study also showed that the mLcn2 displays an equivalent binding specificity for all the 18 siderophores. Taken together, these findings strongly support that Lcn2 binds to siderophores mainly through their highly polar ferric hydroxybenzoyl moiety, implying a degenerate recognition pattern for its antibacterial role. Meanwhile, observation that Lcn2<sup>−/−</sup> mice, intratracheally challenged with <i>M. tuberculosis</i>, showed increased number of bacteria in the lung alveolar epithelia than that of wild-type mice further supported the <i>in vitro</i> ligand binding data [61].

4) Role of Lcn2 in iron transportation

The finding that Lcn2 binds to iron-siderophores influenced the thinking regarding the cellular mechanism of this protein. If Lcn2 binds siderophores of bacterial origin does it also bind an endogenous eukaryotic siderophore? In this regard, some intriguing questions need to be addressed, such as does Lcn2 interact with iron-siderophore within the cells and what are the downstream events after iron-siderophore is sequestered by Lcn2. A sketch of Lcn2 mediated iron cellular traffic has been drawn from several lines of evidence although the exact molecules involved need to be investigated. The first thorough study of Lcn2 mediated iron cellular trafficking was reported at the same time as the hLcn2:FeEnt crystal structure was published[62]. mLcn2 was reported to be an epithelial inducer from ureteric bud cell line (UB) for renal mesenchymal cells and that UB cell expressed mLcn2 could co-precipitate with iron when the cells were pulsed with <sup>59</sup>Fe. During partial purification of mLcn2 from the UB cells, a chromatic cofactor of similar size to Ent was found. Moreover, the purified and iron-depleted native mLcn2 from UB cells bound <sup>59</sup>Fe as determined by filter assay. These data indicated there might be mammalian siderophores that mLcn2 binds and this mammalian siderophore-mLcn2 complex can mediate iron trafficking under physiological conditions. This hypothesis is consistent with the observation that Lcn2 is not only upregulated upon bacterial infection but also induced under many physiological inflammatory or stress conditions during which bacterial siderophores are very limited. Further characterization revealed that both the mLcn2 purified from UB cells and hLcn2 purified from <i>E. coli</i> (XL-Blue strain) can deliver iron to cells through receptor mediated endocytosis that could be blocked by lowering temperature or competed with by unlabeled Fe<sup>3+</sup>. Using fluorescently labeled mLcn2/hLcn2 and markers of different organelles, it was
demonstrated that Lcn2 could be taken up by kidney cell lines using a different pathway from other iron transfer protein such as transferrin and that the intracellular trafficking of Lcn2 involves late recycling endosomes (rab11+, [63]) and late endosomes/prelysosomes but not early endosomes (rab5+) and lysosomes (dextran positive). The steady-state distribution of lipocalin-2 was apparently different from that of transferrin as the latter only occupied a small perinuclear compartment while Lcn2 occupied larger vesicles located at the periphery of the transferrin compartment and throughout the cytoplasm. The localization of Lcn2 in acidic late endosomes indicated one potential mechanism for iron dissociation from hLcn2:FeEnt complex similar as that of transferrin. It was also confirmed in this study that hLcn2 released iron at low pH (n=5) and the protein was not significantly degraded after entering the endocytic pathway. In addition to cellular trafficking pathway, this particular study also showed that mLcn2/hLcn2 could regulate iron-responsive genes including the genes encoding transferrin receptor1 and ferritin and that both forms of Lcn2 were involved in primordial renal cell epithelialization in contrast to the importance of transferrin function in late kidney developmental stages.

Both the intracellular trafficking pathway and iron dissociation under low pH environment had been further confirmed by several other studies. Using human heptoma cell line HepG2 with ectopic expression of a putative mLcn2 receptor (24p3R), Devireddy et al. [64] concluded that mLcn2 was able to transfer iron into cells by 24p3R mediated endocytosis. Colocalization with a fluorescently tagged Rab11 supported the conclusion that mLcn2 is recycled through the endosomal pathway. It was also demonstrated that mLcn2 could associate with siderophore intracellularly as previously reported. In contrast to the endocytic pathway in “normal” cell lines, a study of a mouse Mycobacterium avium infection model showed that mLcn2 is mainly localized in the lysosomes of M. avium-infected macrophages[65]. The pH effect on iron release from the hLcn2-iron-siderophore complex was extensively tested in vitro by fluorescence quenching, UV-visible spectroscopy and EXAFS (extended X-ray absorption fine structure measurement) and crystallography [66]. It was found that both Ent and FeEnt dissociated from Lcn2 at pH lower than 4 although the protein retained its native structure. Acidification also caused siderophore hydrolysis leading to ligand destabilization. This ligand hydrolysis increases reduction potential of the ferric
complex, within range of physiological reductants. Thus, the reduction of ferric iron in acidic intracellular compartment such as late endosome and lysosome, may contribute to iron release.

Exciting results simultaneously reported by three different research groups provide strong support and for the hypothesis that Lcn2 plays a role in iron trafficking via endogenous siderophore-like iron binding molecules. In one of these studies [67], which was based on a previous finding that Lcn2 can mediate iron cellular uptake when secreted from FL5.12 cells, a murine interleukin-3 (IL-3)-dependent pro-B lymphocytic cell line [23], the endogenous iron binding ligand(s) for Lcn2 was investigated by sequential chromatography and mass spectrometry. A putative mammalian siderophore iron binding moiety (2,5-DHBA) was isolated, characterized and found similar to the iron-binding component of enterobactin (2,3-DHBA). These investigators also identified the genes encoding the enzymes responsible for 2,5-DHBA synthesis from both mammals and zebrafish, implying the evolutionary conservation of iron metabolism through siderophores. In the other study [68], urinary organic compounds were screened for their ability to bind with iron alone or iron plus Lcn2. It was revealed that catechol is the major binding ligand among all the tested compounds. In contrast to the Ent which shows high affinity with Lcn2 even without Fe$^{3+}$, catechol only tightly binds to Lcn2 in the presence of Fe$^{3+}$ with nanomolar $K_d$. Structural analysis showed that the Lcn2-Fe$^{3+}$-catechol complex also depends on electrostatic/cation-π bonds involving Lys125 and Lys134 within the binding pocket. Furthermore, similar to acid-dependent iron release in hLcn2:FeEnt complex, both catechol and catechol-iron dissociate from the Lcn2-Fe$^{3+}$-catechol complex at pH 6.0. The biological relevance of the Lcn2-Fe$^{3+}$-catechol complex was also investigated by observing the tissue distribution in mice injected with $^{55}$Fe$^{3+}$ labeled Lcn2-Fe$^{3+}$-catechol complex. Another possible Lcn2 ligand, L-norepinephrine (NE), a neuroendocrine catecholamine that can be utilized by many bacteria as an iron chelator from the host, bind hLcn2 in the presence of Fe$^{3+}$ with a dissociation constant of 50 nM. By contrast, there is no detectable binding between hLcn2 and NE in the absence of Fe$^{3+}$. The specific binding to NE was further confirmed by the fact that addition of hLcn2 could effectively inhibit the growth of NE dependent *E. coli* and *Bacillus subtilis* [69].
It is now clear that iron-siderophore (bacterial/mammalian) is a specific ligand for mammalian Lcn2. Considering the fundamental role of iron metabolism in almost every organism, it is not surprising that this property confers important functions to Lcn2 with respect to various biological processes. Although the siderophore is not the only virulence factor for many bacteria, infection models from mLcn2 deficient mice demonstrated the antibacterial role of mLcn2. However, the importance of Lcn2’s role in iron metabolism and its related functions could not be validated in the Lcn2 deficient (Lcn2^{-/-}) mice [59,70], which could be explained by the existence of redundancy. Because only single copy of Lcn2 gene is identified in the genome, this proposed redundancy is not through genetic duplication. If the hypothesis is true then redundancy must derive from other functionally related proteins. The cell signaling pathways triggered by Lcn2-iron-siderophore complex (if they exist) have not been identified. Research focusing on this area will provide insights into our understanding the cellular mechanisms of Lcn2 and will help us to design/discover efficient drug targets for infectious diseases and other disorders.

2.1.4 Other cellular functions of Lcn2 and the potential mechanisms

The involvement of mammalian Lcn2 in iron trafficking partially explains why this protein has important cellular functions in a broad range of biological processes, such as cell proliferation/apoptosis, differentiation and migration, cellular oxidative stress. Corresponding to these fundamental biological processes, the role of Lcn2 has been extensively studied in cell apoptosis, tissue injury and repair, tumorigenesis, metastasis, metabolism syndrome and many other inflammation associated diseases. However, the molecular links between Lcn2-iron-siderophore complex and the immediate cellular response(s) have yet to be elucidated.

1) Lcn2 and cell apoptosis/proliferation/differentiation

The cellular functions of Lcn2 are not yet well-defined and may differ with cell type or environment. This is especially true for the apoptotic effect. The role of Lcn2 in cell apoptosis was first reported from a study of the effect of IL-3 deprivation on the FL5.12 murine IL-3-dependent pro-B lymphocytic cell line [23]. It was shown that withdrawal of IL-3 induced expression of mLcn2 from FL5.12 cells and the conditioned media from IL-3 depleted FL5.12 cells could induce apoptosis of naïve FL5.12 even in the presence of IL-3.
This apoptotic effect of mLcn2 was demonstrated with many other cytokine-dependent and -independent cell lines as well as primary hematopoietic cells from both human and mice. By contrast, nonhematopoietic cells and monocyte-derived macrophages as well as IL-7 dependent leukocytic cell line (mouse D1-F4) were resistant to the apoptotic effect, indicating a cell and cytokine specificity of Lcn2’s pro-apoptotic effect. It was also demonstrated that addition of mLcn2 to FL5.12 cells could activate Bad, a pro-apoptotic member of the Bcl-2 family by its dephosphorylation. Another anti-apoptotic member of Bcl-2 family, Bcl-XL, was shown to antagonize the apoptotic effect of Lcn2 as FL5.12 cells overexpressing Bcl-XL showed no death upon IL-3 withdrawal and mLcn2 addition. In their subsequent studies, Devireddy and colleagues showed that Bim, a BH3-only pro-apoptotic member of Bcl-2 family also plays crucial role in Lcn2 induced apoptosis. First, Bim is highly expressed upon IL-3 withdrawal (Lcn2 induction) from FL5.12. Second, knockdown of Bim using RNA interference (RNAi) substantially reduced the apoptosis [64]. In addition to the Bcl-2 family proteins, ATFx, a mouse transcriptional factor of the ATF/CREB family also participates in mLcn2 induced apoptosis as reported by the same group [71]. In this study, the investigators reported that mLcn2 causes apoptosis of FL5.12 cells by repressing ATFx expression while constitutive expression of ATFx renders FL5.12 resistant to mLcn2-mediated apoptosis. These results indicated that intrinsic apoptosis mediators are essential for the cell type specific apoptosis induced by Lcn2. The cell apoptotic effect of Lcn2 was also correlated with its iron trafficking function [64]. The addition of holo-mLcn2 (mLcn2-siderophore-iron complex purified from XL-Blue E. coli strain) to cells which are susceptible to Lcn2 induced apoptosis significantly increased the cell viability with a remarkable suppression of Bim expression and an increased intracellular iron concentration. By contrast, depletion of intracellular iron level by the addition of apo-mLcn2 (mLcn2 purified from the BL-21 E. coli strain) or membrane-permeable iron chelator, desferrioxamine (DFO) induced apoptosis with increased Bim expression at a comparable level to that induced by IL-3 deprivation. Together, these data were taken to suggest that mLcn2 exerts its apoptotic function through an iron and apoptotic protein family-dependent way. Consistent with these results, studies of primary hematopoietic cells isolated from mice or humans also showed that purified mLcn2 and hLcn2 have effect on apoptosis of erythroid progenitor cells and
monocyte/macrophage lineage cells, but not of other hematopoietic progenitor cell lineages [72,73]. Treatment with Lcn2 also inhibited further differentiation of both human and mouse erythroid progenitor cells and administration of mLcn2 to anemic mice resulted in significantly reduced red blood cells compared to no mLcn2 injection. This negative regulatory role in hematopoiesis of mLcn2 has been confirmed using Lcn2 deficient mice in a recent study [74]. A recent study also showed that addition of purified mLcn2 to primary neonatal rat cardiomyocytes and a cardiomyocyte cell line induced a strong apoptosis response via a mechanism involving elevated intracellular iron and translocation of the Bcl-2 family member Bax to the mitochondrial membrane[75].

In contrast to the pro-apoptotic function of mLcn2, hLcn2 seems to play a protective role in cell survival. In one study of the effect of hLcn2 on cell toxicity [76], it was shown that hLcn2 was induced in human epithelial type tumor cell lines upon stimulation by apoptosis-inducing xenobiotics (5-lipoxygenase-activating-protein inhibitor MK886). Knockdown of hLcn2 expression by siRNAs further increased the inhibitory effect of a PDK1 (phosphoinositide-dependent kinase 1) inhibitor on cell proliferation, while overexpression of hLcn2 reduced cell death induced by this compound. Moreover, recombinant hLcn2 protein showed no cytotoxic effects, whereas an antiserum against full-length hLcn2 was toxic. A similar observation that purified recombinant hLcn2 has no apoptotic effect on primary human bone marrow cells was also reported [31].

Evidence for a cell protective effect of mLcn2 came from in vivo studies of a mouse model of renal ischemia-reperfusion injury [77,78]. Intravenous injection of purified mLcn2 into mice both in the absence and presence of ischemia-reperfusion injury resulted in its rapid uptake by tubule epithelial cells and the observation that exogenously administered mLcn2 can ameliorate histopathologic damage to injured tubules in this animal model. It was also shown that administration of mLcn2 can reduce the apoptotic tubule cell death induced by ischemia-reperfusion injury as determined by TUNEL (transferase-mediated dUTP nick-end labeling) staining and can enhance tubule cell proliferation after ischemic injury as shown by IHC staining of proliferating cell nuclear antigens (PCNA). The cell protective effect of mLcn2 was demonstrated iron dependent as both the apo-mLcn2 (purified from BL-21 strain of E. coli which does not produce siderophore) and replacement of Fe$^{3+}$ with a similar
trivalent metal ion gallium in the mLcn2-siderophore-iron complex showed no protective
effect in the ischemic injury model. Heme oxygenase 1 (HO-1), a key player for cell survival
through augmentation of intracellular iron efflux [79], was also shown to participate the cell
protection function of mLcn2 as administration of mLcn2 in the ischemia-reperfusion injured
mice caused much higher expression of HO-1 compared to the controls and injection of HO-
1 inhibitor abolished the protective effect. As an indirect evidence for its cell protection role,
cLcn2 was shown as a constitutive survival protein because down-regulation of cLcn2 by
antisense technique in chondrocytes and myoblasts led to dramatic loss of cell viability and a
strong inhibition of cell proliferation and differentiation [80].

The contradictory observations of apoptosis versus proliferation and differentiation in
response to Lcn2, make it difficult to define its cellular functions. After close examination,
however, there are several possible explanations that may reconcile the seemingly opposite
functions of Lcn2. First, similar to its gene regulation, the cellular response to Lcn2 may be
cell-type dependent. For example, Lcn2 mainly increases apoptosis in certain hematopoietic
cell lineages and this effect is also cytokine dependent. This might explain why there was no
apoptotic effect for hLcn2 on epithelial type cancer cell lines [76]. Although there had been
several reports of Lcn2 induced apoptosis on epithelial type cell lines [81,82], our lab data
has tested the apoptotic effect of Lcn2 on at least one of these cell lines and found no
significant difference from the controls (unpublished data). The apoptotic effect may also be
a function of the differentiation state of the cell because only immature erythroid cells were
shown to be susceptible to Lcn2 induced apoptosis while mature erythroid cells were
resistant. Therefore, under physiological conditions, this tight cell-dependent response would
result in specific repression of hematopoietic cells by Lcn2 while most cells are resistant.

Compared to the extensive studies of apoptotic induction of cultured cells by Lcn2, there
have been fewer reported studies showing an increase in cultured cells of proliferation,
differentiation or protection from apoptosis due to Lcn2 treatment. Thus the protective effect
of Lcn2 observed in in vivo animal models needs to be carefully examined as it is difficult to
exclude the possibility that this effect might be due to inhibition of inflammatory cells by
Lcn2-induced apoptosis.
The contradictory reports on Lcn2 function may also arise from the fact that many intrinsic factors crucial for cell survival or apoptosis, such as the Bcl-2 family proteins, ATF/CREB family proteins and HO-1 are very likely to be regulated by many other signals in addition to Lcn2. Also, the intracellular iron level which is of importance to cell survival and growth, is also influenced by iron transfer proteins other than Lcn2.

In summary, it is not surprising that Lcn2 may exert different effects in different contexts because of the complex in vivo coordinated regulatory network in which it appears to be involved.

2) Cell surface receptor(s) of Lcn2

To fully understand the precise mechanisms underlying various functions of Lcn2, it is essential to explore the involved signaling pathways. As it is the initial point of such pathways, the identification of an Lcn2 cell surface receptor is of great importance. A putative cell surface receptor of mLcn2 (24p3R) was first reported from studies using a cell panning technique with FL5.12 cells [64]. Sequence alignment indicated that 24p3R gene matched a murine protein named brain type organic cation transporter (BOCT). A highly conserved human homolog was also found through a database search. Immunoblot detection of murine tissues demonstrated wide expression of 24p3R under normal conditions. In contrast, cell lines that were resistant to Lcn2-induced apoptosis do not express 24p3R. Conversely, the ectopic expression of 24p3R converted these cell lines to become susceptible to mLcn2-induced apoptosis and enabled them to take up mLcn2-siderophore-iron complex, further confirmed that this cell surface protein is required for the iron transfer and cell apoptosis mediated by mLcn2. In this study, the receptor dependence was also investigated in Lcn2-apoptosis resistant cells, such as oncogene BCR-ABL overexpressing cell lines. Consistent with another report [83], the BCR-ABL⁺ myeloid cell lines showed persistently high level of mLcn2, but significantly reduced level of 24p3R. This reciprocal regulation was proposed to depend on the BCR-ABL kinase activity. More recently, a detailed analysis of the transcription regulation of mLcn2 and 24p3R revealed that BCR-ABL up-regulates the JAK/STAT pathway and elevates mLcn2 expression but also activates the Ras signaling pathway to repress the expression of 24p3R [84]. The down-regulation of 24p3R on BCR-ABL⁺ marrow cells provided a potential explanation for how blood cancer cell invasion is
controlled in these cells. In this explanation, mLcn2 proteins secreted from leukemia cells cause apoptosis on normal hematopoietic cells through interacting with 24p3R, while the cancer cells are more resistant due to the low expression of 24p3R. This local suppression of normal hematopoiesis would allow the leukemic clone to preferentially survive, expand and invade the normal marrow and the spleen. This hypothesis was tested using a murine leukemia model in which transplantation of marrow cells expressing BCR-ABL but lacking mLcn2 (from mLcn2-/- mice) did not cause leukemia or any disease after 75 days, whereas all mice receiving wild type BCR-ABL donor cells died with chronic myeloid leukemia (CML)-like disease [85].

Although the 24p3R has been characterized, especially for its role in Lcn2 mediated iron trafficking and apoptosis, several key questions remain unaddressed. First, so far there has been no evidence showing direct binding between 24p3R and mLcn2, raising the question of whether 24p3R is an mLcn2 specific cell surface receptor. Second, compared to numerous studies describing the expression of Lcn2 under diverse physiological or pathological conditions, research data for 24p3R expression profile under corresponding conditions are very rare. Therefore, a good correlation between 24p3R and mLcn2 has yet to be validated. Third, the biochemical properties of 24p3R have not been fully characterized, nor are its downstream signaling components known. Answers for these questions will eventually reveal the missing links between Lcn2-mediated iron trafficking and its pro-apoptotic and (or) anti-apoptotic effect.

Human megalin, also known as low-density lipoprotein receptor-related protein 2 (LRP2), was shown by surface plasmon resonance (SPR) to bind both apo-hLcn2 and holo-hLcn2 tightly with affinity of \( K_d = \sim 60 \) nM. Megalin also mediates the endocytosis of hLcn2 as demonstrated by the uptake of fluorescently tagged hLcn2 into rat yolk sac epithelial cell line and the internalization could also be blocked by megalin specific antibody. It is well known that megalin binds and endocytoses a variety of ligands including other iron binding proteins, such as transferrin and lactoferrin [86]. In a recent study overexpression of 24p3R on both non-renal and renal tubule derived cell lines was shown to mediate the uptake of various metal ion binding proteins with high affinity, including mettallomethionein, transferrin, and serum albumin [87]. The results of these studies suggest that 24p3R is also
not specific for mLcn2. Therefore it is of importance to ask the question whether these two proteins (megalin and 24p3R) are authenticate signaling receptors for Lcn2 or there are some other unidentified receptors truly responsible for this function. Regardless the receptor specificity, complex regulation of multiple receptors also well explains the “contradictory” cellular functions of Lcn2 as discussed previously.

3) Role of Lcn2 in tumorigenesis and metastasis

Because of its role in cell survival and proliferation/differentiation, numerous reports have described the involvement of Lcn2 in various tumor diseases. In contrast to research on hematopoietic cancers, which mainly focus on the 24p3R mediated apoptosis/survival balance, investigation of the role of Lcn2 in solid tumors center on its association with gelatinase B/MMP-9 which is ambiguous and controversial. Matrix metalloproteinases (MMPs) are a family of endopeptidases whose activities depend on metal ions, such as Zn$^{2+}$ and Ca$^{2+}$. Collectively, MMPs are capable of degrading all the molecular components of extracellular matrix, the barrier separating the tumor cells from their normal surrounding tissues, which is disassembled as part of the metastatic process [88]. MMP-9 is a zinc-dependent enzyme involved in extracellular matrix remodeling through digesting specific substrates including gelatin, elastin, and types V and X collagens [89]. Many studies have demonstrated the role of MMPs, in particular, MMP-9 in tumorigenesis and metastasis [90]. The covalent association of hLcn2 with MMP-9 suggests that hLcn2 may play a role in tumor invasion and metastasis in addition to its iron-siderophore binding functions. In consistent to this postulation, Yan et al. [91] first detected the MMP-9-hLcn2 complex in the urine sample from patients with breast cancer. In addition, the complex of MMP-9-hLcn2 could be reconstituted in vitro by mixing MMP-9 and hLcn2 in gelatinase buffers with pH values in the range of urine and in normal urine as well. This study also showed that hLcn2 could protect MMP-9 from autodegradation as demonstrated by increased MMP-9 enzyme activity resulting from either incubation of MMP-9 with hLcn2 in vitro or overexpression of hLcn2 in breast cancer cell line. However, this study indicated the protective interaction between hLcn2 and MMP-9 is transient as the reconstituted MMP-9-hLcn2 complex underwent the same degradation rate as free MMP-9. In addition, overexpression of hLcn2 in the tumor cell line has no effect on the mRNA levels of both MMP-9 and TIMP-1 (tissue
inhibitor of matrix metalloproteinase-1), suggesting that the increased MMP-9 activity did not result from transcriptional regulation by hLcn2. Another study also provided evidence for a positive role of hLcn2 in activation of MMP-9 [92]. In light of these in vitro findings, different research groups have tested the biological consequence of the MMP-9-hLcn2 complex in vivo. Studies including xenograft of hLcn2 overexpressing or knockdown tumor cells and spontaneous cancer models from Lcn2-/- mice have supported the finding that this complex formation leads to increased tumor growth accompanied by an increase in MMP-9 activity, tumor angiogenesis, and tumor cell proliferation[93-97].  

Although it is more reasonable to deduce that hLcn2 can form heterodimer with MMP-9 through its free cysteine (Cys87), the chemical basis for mLcn2 and rLcn2 to covalently associate with MMP-9 is elusive as both of these two proteins lack the free cysteine. Previous data indicated even the interaction between hLcn2 and MMP-9 is transient [91] and the activation function was demonstrated through a nonphysiological pathway [92], suggesting the association may be serendipitous. Furthermore, study from a mouse spontaneous breast cancer model indicated that the slight decrease in MMP-9 activity could not explain the markedly decreased tumor burden found in the mLcn2 knockout [98]. Consequently, Lcn2’s oncogenic role is at best only partly dependent on its ability to stabilize MMP-9. In addition to the MMP-9 stabilization theory, possible mechanism involved in Lcn2’s role in tumor biology has been attributed to the epithelial to mesenchymal transition (EMT), an important process for tumor cells to become more aggressive and metastatic [99]. It was found that when ectopically expressed in a human breast cancer cell line (MCF-7), hLcn2 induces a typical EMT change in the cell morphology, accompanied by the loss of epithelial marker (E-cadherin), increased expression of the mesenchymal markers (vimentin and fibronectin) and increased invasiveness [100]. This observation is contradictory to the reported mesenchymal to epithelial transition (MET) function of both hLcn2 and mLcn2 on renal tubule cell development [62,78]. Not only does the previously reported MET function under physiological condition apparently conflicts with the putative EMT effect which is crucial for tumor metastasis, studies from several mice tumor models also showed opposite results and strongly suggested that Lcn2 might play an anti-tumor role for specific cell types. Hanai et al. first reported that mLcn2 overexpression suppressed Ras-transformed murine breast cancer
4T1 cell invasion and lung metastasis in vivo by reactivation of E-cadherin expression through antagonizing the ras mitogen activated protein kinase (Ras-MAPK) pathway [101]. In a subsequent study, the same group revealed that mLcn2 can suppress the Ras-induced expression of vascular endothelial growth factor (VEGF) via down-regulation of Ras-MAPK and Ras phosphatidylinositol-3-kinase (Ras-PI3K) pathways [102]. Consistent to these findings, another two independent studies further demonstrated that ectopic expression of hLcn2 in Ras transformed human colon cancer cells and pancreas cancer cells markedly reduced tumorigenesis and metastasis in mice tumor models [103,104]. Taken together, the role of Lcn2 in cancer biology is extremely complicated as indicated by the involvement of a variety of oncogenic regulators and signaling pathways as well as the diverse outcomes from different type of tumors at different developmental stages. Considering that iron metabolism is closely associated with tumorigenesis and 24p3R has been identified as an important player in hematopoietic cancers, it is very surprising that there have been very few studies looking into the correlation between iron trafficking and 24p3R with Lcn2’s role in solid tumor biogenesis and metastasis. Thus it is imperative for the Lcn2 researchers to focus their future work on this area.

4) Role of Lcn2 in cell migration

Several studies have tested the effect of Lcn2 on cell migration by directly adding the proteins onto cells [100,105,106]. In a recent study focusing on the role of Lcn2 in central nerve system (CNS), it was shown that Lcn2 promotes migration of multiple cell types in the CNS by a mechanism that involves up-regulation of chemokine CXCL10 via activation of JAK2/STAT3 and IKK/NF-κB signaling pathways. [107]. It is very likely that these signaling pathways are context dependent considering the complexity of Lcn2 biological functions. A fully elucidation of the involved signaling network will be helpful for future application of Lcn2 in tissue-injure or wound healing therapy.

5) Role of Lcn2 in metabolic syndrome

It was not until recently that Lcn2 has been closely related with metabolic syndrome. Since the first report showing that Lcn2 is an inflammatory marker closely associated with obesity, insulin resistance, and hyperglycemia in humans [108], numerous clinical studies have confirmed the association of serum Lcn2 concentrations with various metabolic
parameters and inflammatory markers [109-115]. However, in contrast to the more consistent clinical research data, the basic research data regarding the mechanism and function of mLcn2 in metabolic syndrome remain controversial with very limited agreement. So far mouse Lcn2 has only shown high expression in perigonadal (epididymal and periuterinal) white adipose tissue (WAT) but no expression in inguinal and brown adipose tissue (BAT) [43,116,117], suggesting adipose deposit specificity. The results of expression profiles of mLcn2 in mouse obesity models including spontaneous obesity mice (ob/ob) and diabetic mice (db/db) as well as high-fat diet (HFD) induced obesity are ambiguous as some studies showed elevated mLcn2 in epididymal WAT [116,117] while other indicated decreased expression of mLcn2 in periuterinal adipose tissue [43]. More conflicting results came from studies using Lcn2 deficient mice obesity models. Among these, a profound difference in body weight was reported between Lcn2−/− compared with wild-type (WT) mice, with Lcn2−/− mice significantly leaner than WT mice under HFD conditions [118]. Lcn2−/− mice on both chow and HFD in the study also displayed a striking improvement in whole body glucose metabolism. This study showed that Lcn2−/− mice on a standard chow diet had a minimal body weight phenotype but displayed significantly lower fasting glucose as well as fasting insulin levels. A subsequent study reported very different results that Lcn2−/− mice were significantly heavier than WT controls on both chow and HFD. Lcn2−/− mice showed markedly impaired glucose tolerance and insulin sensitivity on both chow and HFD [119]. Both HFD- and standard chow-fed Lcn2−/− mice had higher fasting glucose levels. Quite opposite to the previous two studies, a third study using Lcn2−/− mice showed no effect of Lcn2 on body mass or body composition regardless of age or diet. Consistent with this observation, the authors reported almost no effect of Lcn2 on glucose tolerance or insulin sensitivity [120]. In conclusion, Lcn2 has been shown an independent risk factor for insulin resistance, diabetes as confirmed by large number of clinical surveys, but its precise functions and underlying mechanisms have to be elucidated yet.

In summary, over the past three decades, Lcn2 has been shown an important player exhibiting a great variety of biological functions. One can tentative speculate that iron transportation may be the authenticate function of Lcn2, because iron metabolism is associated either directly or indirectly with all the potential functions of Lcn2. As another
corroboration to this speculation, recent studies have indicated Lcn2 may serve an anti-
oxidant agent in either heme oxygenase 1 (HO-1) dependent [121] or independent way [122].
Considering the essential role of iron in oxidative stress, this observation is not unexpected.
Currently, it is of extreme importance to find the missing links between iron trafficking and
the observed Lcn2 functions.

2.1.5 Lcn2 as a disease biomarker

Ever since the identification of Lcn2 as an acute phase protein closely associated with the
inflammatory response, a large body of evidence has accumulated and indicated that Lcn2
could be a potential biomarker for many inflammatory diseases.

1) Lcn2 as a biomarker in kidney diseases

In addition to many types of cancers and metabolic syndrome, another major group of
diseases in which Lcn2 has been substantially evaluated as a biomarker are kidney
dysfunction-associated diseases with special focus on acute kidney injury (AKI). AKI
represents a common and serious problem in clinical medicine with mortality at 60.3% [123].
Renal ischemia-reperfusion injury (IRI) is the major cause of AKI in the native and
transplanted kidney. Although researches based on animal models have provided successful
therapeutic approaches, these approaches have been unsuccessful on human AKI due to lack
of early specific markers for AKI and therefore a delay in initiating the therapy that is vital
for stopping the progress of the disease. Currently the AKI diagnosis entirely depends on
increases of serum creatinine (Scr) or decreases of urine output [124]. However, Scr has been
shown insensitive and imprecise for AKI [124,125]. Thus there is an urgent need for an
accurate early AKI diagnostic marker. mLcn2 was identified as an early AKI biomarker from
a cDNA microarray analysis of global gene expression changes during the early reperfusion
periods following ischemic injury in an established mouse model [126]. Further experiments
showed that induction of mLcn2 mRNA was detected in ischemic mouse kidney tissue after
as little as 3 h of reperfusion (4.1 ± 0.5-fold, mean ± SD), with expression peak at 12 h (9 ±
0.6-fold, mean ± SD). Consistent with the mRNA expression level, elevation of mLcn2
protein level (3-fold) could be detected after 3 h of reperfusion and reached the maximal
level at 24 h (> 12-fold). Remarkably, the mLcn2 protein could also be easily detected by
using only 1 µl unprocessed urine sample within 2 h of ischemia and persisted throughout the
Furthermore, this sensitivity of early detection is not species specific as the same pattern of expression file was also observed in rat ischemia-reperfusion model. In light of this initial finding, numerous experimental and clinical studies have been conducted to evaluate Lcn2 as an early biomarker in AKI across a range of different clinical settings. It also has been studied in both adult and pediatric populations. The ever expanding interest in this research field can be reflected by the fact that there have been more than three original research papers every week in PubMed over the last three years on this topic. In 2009 a systematic review and meta-analysis was performed to validate the accuracy of hLcn2 in diagnosis and prognosis in AKI [127]. Using a hierarchical bivariate generalized linear model to calculate the diagnostic odds ratio (DOR) and sample size–weighted area under the curve for the receiver-operating characteristic (AUC-ROC), the authors analyzed data from 19 studies and 8 countries involving 2,538 patients, of whom 487 (19.2%) developed AKI. It was found that the diagnostic accuracy of plasma/serum hLcn2 was similar to that of urine hLcn2 and that the hLcn2 value had a better predictive ability for children (25.4 [95% CI, 8.9-72.2]/0.930 [95% CI, 0.883-0.968]) compared with for adults (10.6 [95% CI, 4.8-23.4]/0.782 [95% CI, 0.689-0.872]). The hLcn2 level was a useful prognostic tool with regard to the prediction of renal replacement therapy initiation (12.9 [95% CI,4.9-33.9]/0.782 [95% CI, 0.648-0.917]) and in-hospital mortality (8.8 [95% CI, 1.9-40.8]/0.706 [95% CI, 0.530-0.747]). Therefore it was concluded that hLcn2 level appears to be of diagnostic and prognostic value for AKI. As the authors pointed out, however, it needs broader investigation (more geographic areas) with more standardized detection methods to further validate the robustness of hLcn2 as an early biomarker for AKI.

2) Lcn2 as a biomarker in other diseases

In addition to AKI, Lcn2 has been reported as a potential biomarker for a variety of renal and non-renal diseases. For instance, in many chronic kidney diseases (CKD) including polycystic kidney disease [128], glomerular diseases (systemic lupus erythematosus (SLE) and IgA nephropathy as well as glomerulonephritis) [129-131], the assessment of hLcn2 is of significant prognostic value; it can, in particular, predict the risk of disease progressing to more advanced CKD stages in the short to medium term. In addition to being an excellent biomarker for renal physiopathology, hLcn2 might also be a promising biomarker for
cardiovascular diseases as suggested by studies of two different animal models in which mLcn2 is highly induced upon cardiovascular injury [132,133]. Based on its upregulation under inflammatory conditions, clinical researchers have also investigated the correlation between hLcn2 and atherosclerosis. In one study of 156 middle-aged patients with asymptomatic atherosclerosis, hLcn2 levels in plasma were found significantly higher in patients compared to healthy controls and correlated to the diastolic pressure and age [134]. This study also reported significantly higher level of hLcn2 in female patients with hypertension than in healthy controls, suggesting that hypertension itself, independent of atherosclerosis, can cause an upregulation in systemic hLcn2. Consistent with this observation, one recent study [135] detected higher serum hLcn2 in patients with normal serum creatinine but with systemic hypertension and stable coronary artery disease (CAD) than healthy controls. Although the serum creatinine was normal, the estimated glomerular filtration rate was significantly lower in these patients, suggesting the elevated systemic hLcn2 is a marker for early kidney injury which could cause the hypertension. In addition to the above correlation studies, one recent study focusing on neutrophils effect on local inflammatory sites such as atherosclerotic plaques showed higher systemic IL-18 and hLcn2-MMP-9 in CAD patients than normal controls[136]. Moreover, using a skin blister model, this study also demonstrated that the in vivo transmigrated neutrophils in the CAD patients had increased tendency to express hLcn2-MMP-9 compared to the healthy controls, suggesting a potential role of activated neutrophils in CAD pathogenesis.

Although many preliminary studies have shown the potential of hLcn2 as a biomarker in cardiovascular diseases as mentioned above, people should be cautious about this promising marker for several reasons. First, as for AKI, large scale clinical studies are still necessary to thoroughly evaluate the accuracy of Lcn2 in diagnosis and prognosis of particular types of cardiovascular diseases. Second, as discussed in the hypertension case study [135], the elevated systemic Lcn2 may be the output of organs/tissues other than the heart as part of more extensive syndrome that includes cardiovascular disease. Therefore, the specificity of Lcn2 for reflecting cardiovascular function needs further elucidation.

In conclusion, Lcn2 is an emerging biomarker in diagnosis and prognosis of many inflammatory- and injury-associated diseases. However, large scale clinical surveys using
standardized detection methods are still necessary to validate the robustness of Lcn2 as a valid biomarker.

2.1.6 Lcn2 and other lipocalins

The failure to replicate in knockout mice many of reported functions of Lcn2, such as apoptosis and kidney development, suggests that there might be redundant mechanisms for Lcn2 function. In addition, although Lcn2 can recognize phenolate/catecholate type siderophores and mycobacterial CMBs, it fails to bind many other bacterial siderophores and all types of fungal siderophores [60]. As the innate immune system always utilizes different armories for host defense, it would be unusual that anti-siderophore effect is only mediated by Lcn2. Furthermore, if the iron transportation via mammalian siderophore is the authentic function of Lcn2 under physiological conditions, functional redundancy is highly likely because iron metabolism is essential for living organisms. Finally, the highly conserved β barrel binding calyx provides the structural basis for the possibility that certain lipocalins might share functional similarity.

Several studies focusing on other lipocalin family members have suggested some lipocalins that might provide functional redundancy for Lcn2. After hLcn2 was reported as an iron-siderophore chelator, it was reported that Lcn1 (tear lipocalin, von Ebner’s Gland protein), a lipocalin very distant in sequence from Lcn2, also binds iron-siderophores [137]. Lnc1 inhibits the growth of a broad range of bacteria and fungi through ferric siderophore (both catecholate type and hydroxymate type) sequestration. However, the binding affinity of Lcn1 for siderophore is relatively low ($K_d = 0.5 \, \mu M$ for fungi siderophore, triacetylfusarinine C) and the nature of the recognition mechanism has yet to be fully investigated. More recently studies the Q83 lipocalin has been added to the list of iron-siderophore binding lipocalins [138,139]. The Q83 lipocalin was originally identified based on its overexpression in quail embryo fibroblasts transformed by the $v$-myc oncogene and is 23% and 87% identical in sequence to hLcn2 and cLcn2 respectively. Using a fluorescence quenching assay, the Q83 was shown to bind ferric enterobactin with almost the same affinity ($K_d = 0.54 \pm 0.05 \, nM$, mean $\pm$ SD) as that of hLcn2. More importantly, the NMR structure of the Q83:Fe:Ent revealed that this siderophore binding lipocalin shares the same binding mode as hLcn2:Fe:Ent, which is the characteristic electrostatic/cation-π interaction
between a triad of positively-charged amino acids and the three catechol rings embedded in the three sub-pockets within the calyx. cLcn2 also shows a triad of positively-charged amino acids in the calyx as does a more distantly related lipocalin, C8γ, a well-studied member of the complement cascade for which there is a high-resolution crystallographic structure [140]. However, neither protein has been reported to bind bacterial siderophores.

In addition to iron-siderophore binding, other lipocalins may also share similarities with Lcn2 in other features. One example is α-1-acid glycoprotein (AGP) (also known as oroscomucoid) [141]. As is Lcn2, AGP is one of the major positive acute phase proteins in human, rats, mice and other species. Besides the increased hepatic synthesis in response to systemic tissue injury, inflammation or infection, AGP has also been found widely expressed in many other extra-hepatic organ systems and tissues including, but not exclusively, urogenital system (kidney, testes, uterus), the respiratory system (lung, alveolar macrophages), digestive system (colon, ileum, stomach), CNS (brain), thymus, leukocytes, endothelial cells. Expression of the AGP gene is controlled by a combination of the major regulatory mediators, i.e. glucocorticoids and a proinflammatory cytokine network involving mainly IL-1β, TNFα, IL-6 and IL-6 related cytokines. Sequence analysis indicates that the AGP promoter contains similar TF binding sites as Lcn2, such as C/EBP binding sites and GRE. AGP is highly glycosylated (45% carbohydrate) and has very low pI of 2.8-3.8 [142]. This unique property confers on AGP the ability to bind and to carry numerous basic and neutral lipophilic drugs from endogenous (steroid hormones) and exogenous origin [143].

Other than drug binding, the exact biological function of AGP remains unknown although various immunomodulating effects have been described, such as inhibition of neutrophil activation [144], modulation of LPS-induced cytokine secretion [145], maintaining capillary permeability [146] and protection of mice from hepatitis and lethal shock [147]. Although it is imprudent to draw a conclusion that AGP functions are partially overlapping with those of Lcn2, the similarities in regulation of these genes suggest that it may be worth testing whether AGP has a role in iron binding.
2.1.7 Conclusion and perspective

After almost three decades of extensive studies, a large body of knowledge of Lcn2 has accumulated, including expression regulation, tissue distribution, ligand identity and its value as disease biomarker. However, as for most other lipocalins, the precise molecular mechanism of this protein remains enigmatic and challenging. To fully solve these puzzles, several aspects of this protein should be top priority for future studies.

1) Identification of the authentic cell receptor of Lcn2. As discuss in section 2.1.4, it is likely that Lcn2 exerts its function through other cell surface receptors other than 24p3R and megalin. In addition, expression profile of 24p3R needs to be thoroughly investigated under more pathophysiological conditions to validate its correlation with various Lcn2 functions;

2) Biochemical and biophysical characterization of 24p3R. So far, there has been no data showing the structure or adaptor/signaling proteins associated with 24p3R upon its activation. Lack of this important data makes it difficult to link the Lcn2/24p3R to downstream signaling pathways;

3) Characterization of iron metabolism after its uptake through an iron-siderophore-Lcn2 complex. The detailed molecular events after ferric iron is taken up by cells as a result of Lcn2 are still unclear even though in vitro data showing that the iron dissociates from the siderophore-Lcn2 complex in the acidic environment of the late endosomes. Delineation of this iron metabolism pathway will eventually help us understanding the fundamental mechanism(s) involved in various Lcn2 functions.

REFERENCES


[50] L. Wang, H. Li, J. Wang, W. Gao, Y. Lin, W. Jin, G. Chang, R. Wang, Q. Li, L. Ma, and T. Pang, C/EBP ζ targets to neutrophil gelatinase-associated lipocalin (NGAL) as a


[139] M. Hartl, T. Matt, W. Schüler, G. Siemeister, G. Kontaxis, K. Kloiber, R. Konrat, and K. Bister, Cell transformation by the v-myc oncogene abrogates c-Myc/Max-mediated


2.2 LIPOPOLYSACCHARIDE AND DEXTRAN SULFIDE SODIUM IN ANIMAL INFLAMMATION MODELS

2.2.1 Lipopolysaccharide induced inflammation

Lipopolysaccharides (LPSs) are amphiphilic molecules produced by Gram-negative bacteria. Although there are a variety of LPSs from different bacteria, all LPSs consist of a hydrophilic polysaccharide component covalently linked to a hydrophobic lipid component termed lipid A via the acidic deoxysugar 3-deoxy-2-octulosonic acid (KDO). The polysaccharide is composed a core oligosaccharide and the O-specific chain that defines the serologic specificity of the LPS. Lipid A is the bioactive core of LPS, as demonstrated by the observation that free natural or chemically synthesized lipid A can reproduce the biological effects of LPS [1].

LPS is one of the most potent inducers of inflammation and can lead to toxic shock and death [2]. LPS activates many types of cells to produce proinflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8, and IL-12 [3]. In addition, LPS activated macrophages also secrete a wide variety of other inflammation mediators including platelet-activating factor, prostaglandins, enzymes, and free radicals [3]. Endothelial cells, LPS targeted cell-type, also produce adhesion molecules that are essential for inflammatory cell recruitment [4]. Consequently, these inflammatory cytokines and mediators efficiently control growth and dissemination of invading pathogens. However, excessive and uncontrolled production of these inflammatory cytokines and mediators may lead to serious systemic complications such as microcirculatory dysfunction, tissue damage, and septic shock [5]. On the other side, host is permanently exposed to low amounts of LPS via the airways or by translocation from gut bacteria, which proves to be beneficial for the host by stimulating its resistance to infection and malignancy [6,7]. Prior exposure to low doses of LPS can also result in a transient state of low responsiveness to subsequent LPS stimulation, known as endotoxin tolerance [8].

Signaling cascades triggered by LPS have been extensively studied within past decades to fully elucidate the molecular mechanisms underlying the profound functions of LPS. The first host protein involved in the recognition of LPS is LPS-binding protein (LBP) [9]. LBP binds with high affinity to the amphipathic lipid A moiety of LPS. It facilitates the monomerization of LPS and transfers LPS to the membrane-bound (m)CD14 [9], which is a
component of the cellular LPS receptor complex. In addition, LBP transfers LPS to soluble (s)CD14, resulting in activation of mCD14-negative endothelial and epithelial cells [10]. The LPS/LBP/CD14 complex further interact with the cell surface toll like receptor 4 (TLR4), one of TLR family members that play essential roles as pattern recognition receptors [11]. Myeloid differentiation factor 2 (MD-2), a secreted glycoprotein lacking a transmembrane domain, is also an indispensable extracellular adaptor molecule for LPS-initiated signaling pathway by associating with TLR4 extracellular domain [12]. It is of note that, although LBP plays an essential role in the formation of the LPS receptor complex, CD14-independent activation by LPS has been reported. The major substitutes for CD14 include heat shock proteins, Hsp70 and Hsp90, chemokine receptor 4 (CXCR4) and growth differentiation factor 5 (GDF5) [13].

The TLR4 signaling cascade initiated by LPS binding is enhanced by homodimerization of the receptor and subsequent recruitment of the TIR domain-containing adaptor molecules (TIRAP) to the cytoplasmic domain of the receptor [14]. These adaptors include myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like protein (Mal), also called TIRAP, TIR-containing adaptor inducing IFNβ (TRIF), also known as TIRAP-1 (TICAM-1), and TRIF-related adaptor molecule (TRAM), also called TIRAP-2 (TICAM-2). These players are activated through the NF-κB, MAPK and PI3K/Akt pathways [15]. In the MyD88-dependent pathway, LPS-bound TLR4 recruits MyD88 and TIRAP. IL-1 receptor-associated kinase 1 (IRAK1) and 4 (IRAK4) are then recruited to the receptor complex via interactions between the death domains (DD) of MyD88 and IRAKs. IRAK1 recruits and activates TRAF6, leading to the downstream activation of the IKK complex and the MAPKs. Activation of the IKK complex results in phosphorylation and degradation of IκB, which activates NF-κB and subsequent expression of proinflammatory molecules. Moreover, activation of the MAPKs, mainly JNK, can also result in activation of the transcription factor AP-1. MyD88 or TRAF6 can also associate with PI3K, resulting the activation of NF-κB through an Akt-dependent signaling pathway. In addition to the above mentioned MyD88-dependent early-phase activation of NF-κB pathways, the late-phase activation of NF-κB is through the MyD88-independent pathway which relies on the adaptor molecules, TRIF and TRAM. TRIF interacts with TRAF6 and receptor-interacting protein 1(RIP1), as well as NF-κB activating
kinase associating protein (NAP1) and TRAF family member-associated NF-κB activator (TANK) binding kinase 1 (TBK1), further activating the transcription factors NF-κB and IFN regulatory factor 3 (IRF3), respectively.

LPS has been long used as an inflammation inducer in various animal inflammation models involving every organ system. A simple search using lipopolysaccharide and inflammation as key words will retrieve more than ten thousands research papers in PubMed. Among a variety of inflammation models, LPS-induced acute lung injury (ALI) has been established and accepted as a model system to study the pathophysiology and molecular mechanisms of ALI. It also provides invaluable information for diagnosis/prognosis and new therapy methods of ALI [16].

2.2.2 Dextran sulfate sodium (DSS) induced colitis

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn’s disease (CD), is a chronic, relapsing and remitting inflammatory condition that affects individuals throughout life [17]. Several experimental colitis models have been reported within last decades and some characteristic pathological features of IBD can be investigated using these models, depending on the agents used to induce the experimental colitis [18].

The dextran sulfate sodium (DSS) induced colitis model, in which oral administration of 3%-10% DSS in drinking water can induce acute or chronic colitis was originally used to investigate the role of leukocytes in the development of colitis[19]. Mice administered 3%-10% DSS for 5-7 d followed by regular water, develop acute and extensive colon injury with complete crypt depletion and relatively slow regeneration of the colonic epithelium. The clinical signs of disease include weight loss, loose stools/diarrhea, and rectal bleeding. Histopathological analysis of colon sections typically reveals extensive crypt and epithelial cell damage, infiltration of granulocytes and mononuclear immune cells, and tissue edema, often accompanied with severe epithelial ulceration. Pathological scoring is generally performed on the distal segment of the colon, which is the most severely affected portion [20]. Histopathological evaluation based on hematoxylin and eosin staining of the tissue sections is based on three parameters: severity of inflammation, extent of inflammation, and crypt damage. Moreover, mice received 5 administration cycles of DSS (5% DSS for 7 days
followed by distilled water for 10 days of each cycle) can develop chronic colitis [19]. It is noteworthy that long-term DSS administration produces colorectal carcinoma, which is similar to the colonic dysplasia that develops as consequence of human ulcerative colitis [21].

The mechanism of induction and pathogenesis in DSS-induced colitis has not been fully elucidated. DSS is a heparin-like polysaccharide containing up to three sulfate groups per glucose molecule [22]. A tissue distribution study found DSS mainly in the Kupffer cells of the liver, the macrophages of the mesenteric lymph node (MLN) and the lamina propria of the large intestine [23]. Acute mucosal damage was also observed in DSS-treated SCID (severely combined immunodeficiency) mice indicating that acquired immune responses are not involved in the induction of DSS-induced colitis [24]. In contrast, macrophages and CD4+ T cells are more frequent in the area of wound healing during the chronic stage and these CD4+ T cells secrete increased levels of IFN-γ and IL-4, suggesting Th1 and Th2 cells may play a pathogenic role in chronic DSS-induced colitis [25]. While the role of gut bacteria in the pathogenesis of DSS-induced colitis is unclear, this form of colitis can be ameliorated by treatment with antibiotics, underlining the importance of commensal bacteria in the development of colitis [17,26]. Many other factors have also been associated with DSS-induced colitis, such as cytokines (IL-18)[27], chemokines (CCR5) [28], growth factors (TGF-α) [29], hormones (leptin) [30], lipid mediators (lipoxin A4) [31] and pivotal transcription factors (STAT3) [32].

REFERENCES


2.3 APTAMERS AS BIOSENSORS FOR DISEASE MARKER DETECTION

2.3.1 Introduction

Nucleic acid aptamers are single-stranded DNA or RNA molecules between 30 and 70 nucleotides in length that bind to their ligands with high affinity and specificity. To date, many aptamers have been selected against a broad range of targets with binding affinities comparable to monoclonal antibodies [1]. In some cases, the use of aptamers might be more suitable than antibodies as scientific and biotechnological tools because aptamers have several unique advantages: 1) they are produced chemically in a readily scalable process; 2) they are non-immunogenic, which makes them favorable as potential drugs; 3) they are smaller in size, which allows more efficient entry into biological compartments; 4) they can be chemically modified with various functional groups during synthesis; 5) they can be selected against some toxic substance for which antibody production is not possible[1].

2.3.2 Selection of aptamers

The word aptamer is derived from the Latin ‘aptus,’ meaning ‘to fit.’ Because of their elaborate three-dimensional structures and shapes, aptamers can bind to a wide variety of target molecules with high specificity and affinity [2]. So far the most effective way to select aptamers is by systematic evolution of ligands by exponential enrichment (SELEX) technology (also known as in vitro selection) [3,4]. SELEX comprises an iterative process of in vitro selections of target-bound nucleic acids and partition of unbound nucleic acids from large pools (usually more than $10^{15}$) of randomized nucleic acid sequences. The SELEX process contains five main steps: binding, partition, elution, amplification, and conditioning (Figure 1). However, so far there has been no standardized aptamer selection protocol for any target. The design and specific selection conditions of SELEX vary according to the target itself, the randomized oligonucleotide pool or the features and application of the aptamers. It is therefore important to pay close attention to several key steps in the SELEX process in order to increase the chances of successfully selecting desired aptamers.
First, there are some general requirements for the targets used for SELEX. Pure and abundant single target molecules help to minimize the enrichment of nonspecific binders. Positively charged groups (e.g. primary amino groups), hydrogen bond donors and acceptors and planarity (e.g. aromatic compounds) are certain target features that facilitate an aptamer selection [5,6]. Aptamer selection is more difficult for hydrophobic and negatively charged molecules (e.g. containing phosphate groups). These target requirements are caused by the basic principles of the intermolecular interactions between aptamers and their targets, including the complementarity in shape, stacking interactions between aromatic compounds and the aptamer nucleobases, electrostatic interactions or hydrogen bonds [2]. So far, numerous aptamers have been selected against many different types of targets, including peptides, proteins, carbohydrates, and antibiotics as well as cells or whole organisms [6]. Interestingly, in an exception to the positive charged target rule, Winkler et al. reported that a

Figure 1 In vitro selection of target specific aptamers using SELEX technology (Reprint from ref [1], copyright (2005) with permission from Elsevier.)
“naturally selected” aptamer (riboswitch), found in the mRNAs encoding enzymes involved in thiamine synthesis in *E. coli*. specifically recognize thiamine pyrophosphate with particular binding to the phosphate groups [7]. This result demonstrates the diversity of aptamer targets.

Another important aspect of SELEX technology is the design of the random oligonucleotide library. Most SELEX experiments start the aptamer selection with an oligonucleotide library containing a randomized internal region flanked by two fixed primer regions. Researchers have developed different strategies to modify the randomized region to improve the aptamer selection efficiency and facilitate subsequent application of the selected aptamers, such as the insertion of a short stable stem-loop forming sequence into the randomized region as a structural anchor for the GTP aptamer selection [8]. Similarly, a short sequence complementary to a biotinylated capture oligonucleotide was inserted into the randomized region so that the library could be immobilized on an avidin coated surface [9]. Once bound to the target, the oligonucleotides were released from the surface due to their altered structures, therefore eliminating the need for target immobilization. Because fixed primer regions are sometimes difficult to remove as they may participate target binding, a primerless random sequence library was also designed to eliminate the need for these regions [10,11].

The third critical aspect of the SELEX process is to efficiently partition target-bound and unbound oligonucleotides. One common method for partitioning is to utilize affinity chromatography with immobilization of the target on sepharose or agarose, but it requires larger amounts of target material than other methods [12]. For example, magnetic beads require fewer targets and provide simpler handling [13]. The other commonly used method of partitioning that does not require target immobilization is filtration through nitrocellulose [3]. However, high non-specific binding is occasionally a problem for this method. Within the past years, more new technologies have been applied to the SELEX partition step, such as capillary electrophoresis (CE) [14], flow cytometry (FC) [15], and surface plasmon resonance, SPR [16]. In parallel to the application of new partitioning methods, several modifications of SELEX procedure have also been developed to minimize the background
binding, which include the negative SELEX [17,18], counter-SELEX or subtractive SELEX [19,20].

The last step before continuing to the next cycle of SELEX is conditioning, during which the dsDNAs amplified by PCR are either transcribed into RNA or separated into ssDNA for the next round selection. Several methods for efficient dsDNA separation have been developed, including linking biotin to one of the primers for PCR followed by strepavidin-binding separation [21], using asymmetric PCR to obtain ssDNA [22], and separation based on size difference of the two strands of PCR amplicons produced by modified primers [23,24].

Although SELEX design and specific selection conditions are target dependent, SELEX can be automated to reduce the work load and accelerate the selection speed for highthroughput aptamer array design. The first automated SELEX station was built for the selection of nucleic acid ligands [25]. Subsequently, this workstation was modified by substituting magnetic separation with vacuum-filter separation to select aptamers for protein target [26]. This platform can conduct eight selections in parallel and will complete approximately 12 rounds of selection in 2 days. Eulberg et al. have developed an automated SELEX system with high flexibility and versatility in terms of choice of buffers and reagents as well as stringency of selection conditions, which mimics the manual operation well and may increase the probability of successful selection [27]. More recently, a microfluidic, microline-based assembly of automatic SELEX platform was designed and applied for the selection and synthesis of an anti-lysozyme aptamer [28].

2.3.3 Chemical modifications of aptamers

Both DNA and RNA aptamers are susceptible to nuclease-mediated degradation and, to a lesser extent chemical and physical degradation, which are very common conditions for body fluids. It was reported that DNA aptamers composed of unmodified nucleotides have half-lives in the blood as short as 2 minutes [29]. It is therefore necessary to chemically modify the selected aptamers to increase their nuclease resistance and physicochemical stability for future application. Chemical modifications also introduce new features like functional groups that provide new possibilities for interaction of the aptamer with target molecules.
The chemical modification can be introduced into the aptamer selection at two different steps: 1) using modified nucleotides during the SELEX process; 2) post-SELEX modification of the already selected aptamers. For the first type of modification, there are a large number of available functionally modified nucleotides but a crucial aspect is their compatibility with enzymes used in the SELEX process, such as the DNA and RNA polymerases which are essential for PCR amplification and *in vitro* transcription. Typical modifications focus on the 2'-position of the sugar in RNA libraries. The ribose 2'-OH group of pyrimidine nucleotides is replaced with a 2'-NH$_2$ or 2'-F or 2'-O-methyl group, which protects the RNA from degradation by nucleases [30-32]. Replacement of the 4'-oxygen atom in the nucleotide ribose ring with a thio group has also been shown to increase aptamer nuclease resistance 50 times more [33]. Another common modification is replacement of the unlinked oxygen in the phosphodiester linkage by sulfur. This phosphorothioate linkage is more resistant against nuclease digestion than the phosphodiester bond [34]. Besides the sugar and phosphate backbone modification, a very innovative design based on stereochemistry has been applied in aptamer selection. Spiegelmers are aptamers in which the sugars are the enantiomers (mirror images) of natural nucleic acid sugars. Because nucleases are highly stereo-selective these molecules are several orders of magnitude more nuclease-resistant than the corresponding natural nucleic acids [35]. In addition to modifications that make aptamers more resistant to nucleases, other types of chemical modifications introduce a great variety features. Modifications of the bases at the C-5 position of pyrimidines or at the C-8 position of purines have been reported to increase the chances of selecting aptamers against anionic target molecules [36]. Furthermore, aptamers containing 5-bromouracil and 5-iodouracil have been selected and can be activated by UV irradiation to form a covalent linkage with the bound target [37].

The second approach for aptamer modification is post-SELEX modification. Replacement of the ribose 2'-OH group as aforementioned can also be done at this step. It is noted that, however, this post-SELEX modification may cause unwanted structural alteration of the aptamers and a subsequent affinity decrease. In this situation, a reselection with the modified aptamer pool is necessary to guarantee binding to the desired target. A successful example is the 2'-fluoropyrimidine modified aptamer against Rous sarcoma virus [38].
Moreover, modification at the 3' and 5' ends also protects aptamers from exonuclease digestion. A 3'-end capping with streptavidin-biotin or inverted thymidine (3'-idT, creates a 3'-3' linkage) or several 5'-caps (amine, phosphate, polyethylene glycol (PEG), cholesterol, fatty acids, proteins, etc.) protect aptamers from exonucleases [1,39]. 5' capping with large molecular mass polymers, such as PEG and cholesterol also significantly reduce the renal filtration rates of aptamers, which helps to overcome another shortcoming of aptamers as in vivo therapeutics due to their small size [32]. In addition, locked nucleic acids (LNAs), a type of natural nucleic acid analogue which have a bicyclic sugar ring due to the covalent linkage between 2'-oxygen and the 4'-carbon with a methylene [40], have shown great promise for stabilizing aptamers because of their substantially increased helical thermostability, excellent mismatch discrimination and nuclease resistance [41].

In summary, the large range of possible chemical modifications has made aptamers adaptable for a large variety of applications under diverse conditions. One excellent example for the successful modification of an aptamer is the aptameric drug for age-related macular regeneration, Pegaptanib (trade name Macugen). This aptamer is fully modified, composed of 2'-O-methyl purine/2'-fluoro pyrimidine with two 2'-ribo purines conjugated to 40 kDa PEG and 3'-idT. It has half-lives as long as one day in rodents and 10 days in humans [1]. It is very likely that, in the near future, more nucleotide analogues and engineered DNA/RNA polymerases will be created and our ability to manipulate nucleic acid aptamer development will expand.

2.3.4 Application of aptamers as biosensor and other tools

By virtue of their ligand diversity, large number of possible chemical modifications as well as the intrinsic hybridization ability with extra nucleotide fragment, the use of aptamers has been explored in a broad range of research and application fields. In general, applications of aptamers can be divided into three main categories: 1) as therapeutic reagents; 2) as biosensors for target detection; 3) application in biological process to investigate molecular function and mechanism. Studies in the first category mainly focus on selection against known molecules involved in various diseases and modification of aptamers [1,6,42,43]. Because the current thesis is reporting research data about aptamer application as a biosensor, an in-depth literature review is given in this section for recent advances in the second
category. Meanwhile, because many aptamer-based biosensors have been developed and applied for investigation of biological systems, important progress in the third category is also briefly described.

1) Application of aptamers as biosensors

The essential role of a biosensor is to capture analytes and produce a detectable signal. Depending on the mechanism of signal production, aptamer-based biosensors can be generally divided into three types: 1) signal production without the aid of additional molecular reporters; 2) signal production with the aid of molecular reporters; 3) signal production through an amplification mechanism.

For the first type of aptameric biosensor the physical properties (e.g., mass or conductivity) on the surface of a biosensor will change during the process of analyte capture. Based on these principles, aptamers have been used to develop mass responsive or electrochemical biosensors. One example is the SPR sensor that uses aptamer-conjugated biochips. Aptamer-based SPR analysis has been used to detect the C-reactive protein [44], the HIV-1 Tat protein [45], human IgE [46], and retinol binding protein 4 (RBP4) [47]. Aptamers have also been coated on the surface of quartz crystals to develop quartz crystal microbalances (QCM) [48], which showed limits of detection (LODs) for HIV-1 Tat protein [45] and thrombin [49] at 0.25 ppm and ~1 nmol/L, respectively. To achieve the goal of simultaneously detection of multiple targets, microfabrication techniques have been studied to improve the scalability of aptamer-based biosensors. A typical example is the microfabricated cantilever-based biosensor [50]. The binding of different target proteins is detected by measuring the differential cantilever bending between the sensor cantilever (the aptamer-modified surface) and the reference cantilever (the single-stranded control DNA-modified surface). The differential cantilever bending can range from 3 to 32 nm, which is detectable by use of an interferometer. In addition to the mass-dependent measurement devices, several label-free electrochemical biosensors have been developed based on changes in conductivity due to aptamer–target interactions [51], such as the reusable thrombin biosensor [52] and aptamer coupled carbon nanotube field effect transistor (CNT-FET) [53].

To achieve more sensitive detection, signal production with the aid of molecular reporters has been studied in the development of aptamer-based biosensors. The inherent flexibility of
single stranded DNA or RNA allows aptamers to fold into specific tertiary structures upon binding to corresponding targets. The structures of aptamers can also be further modulated by intermolecular hybridization once aptamers interact with their complementary sequences. Molecular reporters have been incorporated into aptameric biosensors based on these features. One example is the molecular beacon probe, a single DNA or RNA strand with a linked fluorophore and quencher attached to opposite ends of the aptamer. A structural change caused by analyte binding results in a change in distance between the fluorophore and quencher, thereby creating a fluorescent on/off switch controlled by the analyte based on this fluorescence resonance energy transfer (FRET) interaction. This technique has been applied for detection of a variety of molecules, such as cocaine [54] and thrombin [55]. Based on the principle that the structural change in the separation distance of the 5' and 3' ends, fluorescence FRET probes have also been applied in aptamer-based biosensor detection of angiogenin in which the fluorophore interaction results in emission at a longer wavelength due to FRET [56]. Furthermore, analyte-induced structural changes have been combined with electrodes for biosensor development. In a delicate experimental design, redox-active methylene blue (MB) was linked to a thrombin aptamer to detect thrombin level in serum. Upon binding to thrombin, the aptamer forms a G-quadruplex conformation and prevents the MB molecules from communicating with the electrode, creating an electronic “on” and “off” switch [57]. Similarly, a MB-modified cocaine aptamer was recently reported for real-time monitoring of cocaine in blood serum [58].

In addition to the biosensors that depend on an analyte-promoted aptamer structural change, designs that utilize the intrinsic complementary hybridization between aptamer and short DNA/RNA sequences have also been extensively studied. An allosteric aptamer-based biosensor, also known as targeted reversibly attenuated probes (TRAP) [59] consists of three functional parts: an aptamer at one end with its cis-complementary sequence (attenuator) at the other end, the central portion (intervening sequence) between the aptamer and the attenuator is complementary to a target nucleic acid. In the absence of the target nucleic acids, the ATP aptamer hybridizes with its attenuator and shows no binding to ATP. When the target nucleic acid is present, such as an expressed mRNA, the aptamer is dissociated from the attenuator and can bind its ligand, ATP. Data from this study also showed that
activation of the aptamer in the TRAP by the complementary nucleic acid at physiological temperatures is sensitive to single-base mismatches, further proving the fine tuning ability of the intermolecular hybridization on aptamer-based biosensors.

For analytes with very low concentrations, it is crucial to develop ultrasensitive aptameric biosensors. One of the sensitive aptameric biosensors is virtually identical to the conventional ELISA in which the aptamer-functionalized plate was applied in a sandwich aptamer-linked immobilized sorbent assay, also known as the enzyme linked oligonucleotide assay (ELONA) [60]. Another system utilizes aptamer-functionalized nanoparticles as a support for reporter molecules. For instance, gold nanoparticles (AuNPs) have been used to carry a large number of aptamers with a 25-adenine tail. After the aptamer binds its target, the poly-adenine tails are hydrolyzed by a nuclease to release a large number of adenines as the detection signal [61]. Similarly, aptamer-functionalized AuNPs can be used to carry smaller CdS nanoparticles which can release many Cd²⁺ ion under acidic conditions. This ultrasensitive biosensor can detect as low as 0.55 fmol/L thrombin [62]. Aptamers have also been combined with PCR technique to amplify the signal for detection [63]. After forming complexes with their target, the aptamers can be amplified through PCR to provide stronger signals. This method can be further improved by using real-time rolling circle amplification (RCA), which is different from regular PCR, as an isothermal amplification method [64].

In summary, significant achievement has been accomplished for aptamer-based biosensor over the past years. However, several aspects still remain challenging. First, although aptamers are selected for high affinity and specificity, the biological sample conditions are more complicated than the defined aptamer selection buffers. The aptamer-based biosensor may show decreased affinity or increased non-specific binding in these altered environments. Second, the understanding of molecular recognition between aptamers and their targets lags behind the development of new biosensing technology. For example, the rational design for the sequence and length of intermolecular hybridization are still trial-and-error based for many aptameric biosensors. Third, as discussed in section 2.3.2, there has been no standardized SELEX procedure established. Therefore, the selection of new aptamers is relatively slow compared to the procedure of obtaining antibodies. Consequently, there are
fewer new aptamers available for biosensor development than antibodies. Finally, many aptameric biosensors are still not as highly sensitive as the conventional ELISA.

2) Additional applications of aptamers

Besides their use in the biosensor field, aptamers have also been explored as research tools in many other areas. Based on their high specificity, aptamers are used as specific ligands in affinity chromatography, affinity capillary electrophoresis, capillary electrochromatography and flow cytometry [65]. Aptamers have also been applied in a ligand/aptamer-controlled gene expression system to study cellular regulatory circuits. In this case, ligand-specific aptamer sequences were inserted into the 5'-untranslated regions (UTR) of the genes of interest, which hindered the transcription of gene of interest when ligand binds the aptamer. When the ligands were not present, gene expression and the following regulatory processes occurred. This method has been applied to control gene expression in prokaryotic and eukaryotic cells systems [66,67].

Aptamers have also been utilized as an important tool for antibiotics research. Certain antibiotics such as aminoglycosides exert their antibacterial function through binding to bacterial RNA. Knowledge of antibiotic-RNA interactions, obtained by studying the structural interaction of such antibiotics with antibiotic-specific RNA aptamers, might shed light on the rational design of new and highly active antibiotic compounds [2,68].

As discussed in previous section, aptamer-based biosensor can be coupled with PCR technique to amplify the detection signal. In one particular example, this signal amplification approach improves the detection sensitivity of aptameric biosensor to zeptomol (10^{-20} mol) amounts in combination with proximity ligation [69]. In this study, the binding of a target protein by two aptamers brings the aptamers into close proximity and promotes ligation of oligonucleotides linked to each aptamer probe. Subsequently, the two aptamer-linked oligonucleotides are ligated via a specific connector oligonucleotide. The proximity ligation forms a DNA sequence for PCR amplification which further produces detection signals.

Aptamers have been explored for in vivo imaging for various physiological processes with special focus on cancers. For instance, the in vivo administration of an anti-elastase aptamer developed for inflammation imaging achieved a target-to-background ratio of 4.3±0.6 at 2 h [70]. For IgG-based detection system, a ratio of 3.1±0.1 was achieved at 3 h.
Some aptamers capable of binding cell surface proteins have been studied for \textit{in vivo} tumor cell imaging. These include an aptamer against MUC 1, a large transmembrane glycoprotein [71] and an aptamer against prostate-specific membrane antigen (PSMA) that was functionalized on quantum dots and superparamagnetic iron oxide nanoparticles for prostate cancer imaging and therapy [72,73].

\textbf{2.3.5 Conclusion and perspective}

Within less than 30 years of their discovery, tremendous advances have been made for aptamer development and application, largely due to the combined application of biological, physicochemical and engineering research methods. In contrast to the chemical property and engineering design strategy, our understanding of intrinsic characteristics of aptamer (e.g. folding, hybridization) still falls behind. This limitation raises several challenges for current aptamer research. First, although \textit{in vitro} selection is advantageous for aptamer development, the selected aptamers do not always perform well under \textit{in vivo} conditions in which they can display lower affinities or higher non-specific binding. Second, \textit{in vitro} selection, which is performed manually, requires a lot of personnel time and costly reagents. Third, aptamer development is limited to trial and error approaches because the mechanism of DNA/RNA folding is not well understood. The latter capability would allow rational design of aptamers.

A partial solution for the above challenges may be the broader application of automated SELEX workstations with greater flexibility and versatility for selection conditions. It is noteworthy that many current aptamer studies only focus on several well-characterized aptamers, such as the thrombin, theophylline, ATP, cocaine and aminoglycoside aptamers. Considering the effect of target complexity on aptamer development and application, aptamer selection and characterization against new targets may broaden our knowledge for aptamer biology.

\textbf{REFERENCES}


CHAPTER 3  LIPOCALIN 2 HAS LITTLE OR NO ROLE IN THE INFAMMATORY RESPONSE

Lijie Zhai, Yinghua Liu, Mark Ackermann, Tania Marchban, Thorsten Berger, Raymond J. Playford, Marit Nilsen-Hamilton

3.1 ABSTRACT

Inflammation is a fundamental biological process that requires tight regulation to maintain homeostasis. Many factors are involved in this profound regulatory circuit. Identification and characterization of the crucial regulatory factors is important for our understanding and ability to prevent inflammation. Lipocalin-2 (Lcn2), a secreted protein belonging to the lipocalin superfamily, is highly up-regulated under a large number of inflammatory conditions. However, there are no data indicating whether this protein is a pivotal regulator for inflammation or merely a byproduct of inflammation. To address this important question we compared the inflammation response level between lipocalin-2 knockout (KO, Lcn2−/−) and wild-type (WT, Lcn2+/+) mice in lipopolysaccharide (LPS) induced acute lung inflammation and dextran sulfate sodium (DSS) induced acute colitis models. By measuring the gene expression level of proinflammatory cytokines, tissue histological changes and disease activity index, we have confirmed that administration of bacterial LPS and DSS induces inflammation in Lcn2 KO and WT mice. However, no significant differences were observed between these two mouse strains in any of the three parameters. Moreover, macrophage surface makers also showed similar expression levels between these two strains upon induction of acute lung inflammation. Based on these results, we conclude that Lcn2 plays little or no role in development of the inflammatory response.

---

a Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA 50011
b Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University, Ames, IA 50011-1250
c Centre for Gastroenterology, Barts and The London, Queen Mary’s School of Medicine and Dentistry, London, UK E1 2AD
d Ontario Cancer Institute, University of Toronto, ON, Canada M5G 2C1
3.2 INTRODUCTION

Inflammation is the response of host tissues to challenges by microbial infections or tissue injury. It is a coordinated process that involves the release of exogenous and endogenous biochemical mediators, recruitment of leucocytes, and damage and destruction of the host tissues. Although inflammation helps to constrain and eliminate infection and promote tissue repair, in extremely serious cases, multiple organ failure or even death can occur due to uncontrolled inflammatory response [1]. Thus, it is essential for the host to evolve a means to regulate this process. To date, numerous inflammatory mediators have been identified and characterized [2]. Knowledge based on these findings has greatly aided the design of more efficient anti-inflammatory treatments and provided broader range of candidate targets for development of anti-inflammatory drugs [3,4].

Lipocalin-2, a secreted protein, which belongs to the lipocalin superfamily [5], has been identified in both human and animals as an inflammation-induced protein under a variety of pathological and physiological circumstances. For instance, many inflammation inducers, such as LPS, turpentine, 12-O-tetradecanoylphorbol-13-acetate (TPA) can significantly up-regulate the expression of Lcn2 both in vitro and in vivo [6-9]. Furthermore, a large body of clinical evidence shows that Lcn2 is highly induced in both infectious and non-infectious inflammatory diseases [10-16]. More intriguingly, Lcn2 has been shown to be up-regulated during physiological “sterile inflammation”, such as tissue involution [17,18], oxidative and thermol stress [19,20], adipogenesis [21], and embryo development in hypertrophic cartilage and muscle fibers [22]. Lcn2 is universally expressed in response to various stressors by a range of cell/tissue types and organ systems, including epithelial cells [18], endothelial cells [23], hepatocytes, leukocytes [7], astrocytes [24], adipocytes [21], and spermatozoa [25].

Considering its apparent pervasive involvement in inflammation, it seems reasonable to better understand and clarify the precise function and underlying mechanism of lipocalin-2. Reported functions of Lcn2 have been related with cell apoptosis/survival [26,27], cell proliferation and migration [28,29], tumor metastasis [30,31], insulin resistance [32-34], ischemia-reperfusion injury repair [28], wound healing [35], and anti-oxidation [20]. This apparent functional diversity may result from a fundamental function of this protein that is common to all cellular activities affected.
One identified fundamental function of Lcn2 is its tight binding to bacterial siderophores [36,37]. Siderophores are bacterial products with extremely high affinities for ferric iron that provide a means for pathogens of sequestrating iron in the mammalian host’s iron-limited environment [38]. Thus, Lcn2 has been proposed to be an innate immune factor that blocks bacterial acquisition of iron-siderophore [39]. Lcn2 can also regulate intracellular iron level \textit{via} Lcn2-iron-siderophore complex trafficking and this iron trafficking may contribute to the apoptotic effect of Lcn2 observed with certain cells [40,41]. Interestingly, several mammalian counterparts of bacterial siderophores have been found that bind Lcn2 in the same way as bacterial siderophores. These results suggest that Lcn2 may mediate cellular iron uptake in addition to its anti-bacteria effect [42,43]. It is of note that the exact molecular events connecting Lcn2-iron-siderophore cellular uptake and various functions of Lcn2 are still unknown. Two molecules, megalin and 24p3R have been proposed as the cell surface receptors for Lcn2 [41,44], but the precise signaling pathways after the engagement of ligand and receptor have not been delineated.

Regardless of the precise molecular mechanisms, it would be of significant interest to determine if Lcn2 plays an essential role in the development of inflammation. The current literature on this subject is insufficient to answer this question. For example, some studies using Lcn2 knockout mice only addressed the role of Lcn2 as a bacteriostatic agent by preventing bacterial utilization of iron-siderophore [45-48], while others focused on the role of Lcn2 in tumorigenesis and metastasis [30,31]. One previous study showed no difference with wild-type (WT) and Lcn2 KO mice in LPS induced septic shock and ischemia-reperfusion injury [49]. Another study, focusing on Lcn2 expression in central nervous system, reported that no significant difference between Lcn2 KO and WT mice in LPS-induced endotoxemia [50]. Contrary to these two studies, another study reported that Lcn2 KO mice showed enhanced airway resistance and increased airway inflammation induced by ovalbumin compared with WT controls [51]. Three independent investigations of role of Lcn2 in insulin resistance and obesity reported inconsistent conclusions [32-34].

Given the complexity of Lcn2’s function, it is necessary to further explore and validate the role of Lcn2 in other organ systems in response to different inflammatory stimuli. In the current study, we investigated the necessity of Lcn2 in inflammation occurring in respiratory
system and gastrointestinal system respectively. A bacterial inflammatory agent (lipopolysaccharide, LPS) and chemical inflammation inducer (dextran sulfate sodium, DSS) was investigated to assess if Lcn2 might function differently during different inflammatory responses. By comparing the inflammatory response severity of Lcn2 KO and WT mice, we confirmed that Lcn2 is highly expressed in both inflammation models, but is not essential for the progress of inflammation. We conclude that Lcn2 does not likely play a role in development of the inflammatory response but might play some important role in a related process such as post-inflammatory healing.

3.3 MATERIAL AND METHODS

Animals

Specific-pathogen-free BALB/c (College of Veterinary Medical School, Iowa State University, Ames, IA) and Lcn2 KO mice [49] were backcrossed for six generations on a BALB/c background, all mice were genotyped via tail PCR and WT littermates were used as controls. Animals were housed in microisolator cages with a 12/12 h light–dark cycle and maintained on sterile food and pyrogen-free water ad libitum before being used in the experiment. Procedures involving mice and their care were conducted in compliance with the institution's guidelines, as outlined in the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health.

Induction of inflammation

Both male and female Lcn2−/− and WT littermate mice with ages of 7~15 weeks (average 11 weeks) were used in the acute lung inflammation model. Lung injury was induced as previously reported [52] with the slight modifications described here. Mice were anesthetized by inhalation of carbon dioxide (CO2). LPS (from Escherichia coli O55:B5; Sigma-Aldrich, St Louis, MO), diluted in 10~30 μl endotoxin free PBS (2.68 mM KCl, 1.47 mM KH₂PO₄, 136.89 mM NaCl, 8.1mM Na₂HPO₄, pH 7.4), was instilled intranasally (i.n.). Control mice received sterile PBS only. Mice were treated with LPS at different doses per body weight (2 mg/kg, 4 mg/kg or 8 mg/kg) for 6 or 8 h to determine the optimal conditions for the inflammation induction.
For the acute colitis model, female Lcn2\(^{-/-}\) and WT littermate mice of ages 9 to 21 weeks (average 14 w) were fed with various concentrations of DSS (molecular mass 36 to 50 kDa; MP Biomedical, Solon, OH) dissolved in sterile, distilled water \textit{ad libitum} throughout the experiment (5 or 7 days). The DSS solutions were changed on days 3 and 5. Control mice had access to water without DSS.

All mice were sacrificed by CO\(_2\) inhalation at the end of the experiment.

**Determination of the clinical score, colon length and histological scoring**

For the acute lung inflammation model, after sacrificing the mice, lung and liver were removed, excised and some samples were immediately frozen in liquid nitrogen and one piece with volume at 0.5 cm\(^3\) were fixed in 10\% buffered formalin for paraffin embedding. Tissue sections from paraffin blocks were processed and stained with hematoxylin & eosin (H&E). H&E stained samples were analyzed by a board certified pathologist who was blinded for the groups (M. Ackermann). To quantify inflammation and damage, the lung and liver tissue slides were semi-quantitatively scored as shown in Table 1.

To determine the inflammation severity in DSS induced colitis, samples were assessed using the disease activity index (DAI; Table 2). DAI is a clinical score that includes the factors of weight loss, changes in stool consistency, and the presence of blood in the stool [53]. Postmortem the entire colon was removed from the caecum to the anus, and the colon length was measured as marker of inflammation. The colon was cut into 3 equally long segments: the proximal/cecum end (segment A), the mid-segment (segment B), and the distal/colon end (segment C). Each segment was further cut into two halves longitudinally. One half was fixed, processed and stained in the same way as for the lung tissue for histological analysis. The other half was immediately frozen in liquid nitrogen for later use. Histological scoring was performed in a blinded fashion by a board-certified pathologist (T. Marchbank). Briefly, the proximal colon, mid-colon and distal colon were assessed by the severity of inflammation (0–3), extent of inflammation (0-3), crypt damage (0–4), and percentage involvement (0–4) (Table 3). The scores from both individual segments and the sum of all three segments were used to evaluate the severity of inflammation.
RNA isolation and RT-PCR

Tissues were frozen immediately in liquid nitrogen upon removal from the animal and stored at -80 °C. Total RNA was isolated from whole tissue using the Trizol Reagent (Life Technologies, Grand Island, NY) following the manufacturer's instructions. Reverse transcription was done as described previously [54]. Briefly, 1μg total RNA was treated with 1 unit DNase I (Life Technologies, Grand Island, NY) for 15 min at room temperature followed by 15 min incubation at 65 °C for deactivation. The treated RNA samples were reverse transcribed in a 20 μl final volume containing 250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂, 100 mM DTT, 10 mM dNTP, 200 μM 18mer oligo dT and 200 U Superscript II RNase H⁻ reverse transcriptase (Life Technologies, Grand Island, NY). The real-time PCR was performed in an Opticon (MJ research, Waltham, MA) or a Mini Opticon (Bio-Rad Laboratories, Hercules, CA) with FullVelocity™ QPCR Master Mix (Stratagene, La Jolla, CA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as the reference gene to normalize gene expression in each sample. Primers used in the experiments were: mouse Lcn2, sense: 5’-CAATGTCACCTCCATCCTGGTCA, anti-sense: 5’-GCGAACTGGTTGTAGTCCGTGGT-3’; GAPDH, sense: 5’-TGACATCAAGAGGTGGTGAAGCA-3’, anti-sense: 5’-GGTCCACCACCCTGTTGCTGT-3’; interleukin 6 (IL-6): sense: 5’-GAGGATACCACTCCCAACAGACC-3’, anti-sense: 5’-AAGTGCATCATC-TGTTGTTTCATACA-3’; tumor necrosis factor-alpha (TNF-α), sense: 5’-CATCTTCTCCTCATCGGATCAGCAACAGACC-3’, anti-sense: 5’-AAGTGCATCATC-TGTTGTTTCATACA-3’; CD14, sense: 5’-CAGGGTGACACCCCAGGATTACAT-3’, anti-sense: 5’-CACAGCTGCATGCTGGTA-3’; F4/80, sense: 5’-TTTCTCGCTGCTTCTTTC-3’, anti-sense: 5’-CCCCGTCTCTGTATTCAACC-3’.

Statistical analysis

For histological score and gene expression data the statistical significance of any difference was determined by the unpaired, one-tailed t-test using Microsoft Office Excel2007 (Microsoft, Seattle, WA). A difference was considered as statistically significant with a p < 0.05.
3.4 RESULTS

**Effect of Lcn2 on LPS-induced acute lung inflammation**

To investigate the effect of Lcn2 on lung inflammatory injury, an acute lung inflammation model induced by intranasal instillation of LPS was utilized in this study. Different doses of LPS and treatment time periods were first tested with WT mice to establish the inflammation model. In our preliminary experiments, mice treated with lower doses of LPS (0.4 mg/kg, 4 mg/kg) for 4 or 6 h showed no or very mild microscopic signs of inflammation. The mRNA levels of proinflammatory cytokines also showed no difference between LPS and PBS groups (data not shown). We therefore increased the dose of LPS to 8 mg/kg and treated the mice for 8 h. Histological analysis indicated that, even under this high-dose condition, only a few of WT mice (2 out of 8 for both lung and vascular systems) showed significant inflammation changes while most mice had a score at zero in lung and liver as well as vascular systems (Fig 1, WT). A similar pattern was observed for mLcn2 KO mice. Only one out of eight KO mice had an evident histological change in the lung and vascular system with most mice showing no inflammation response (Fig 1, KO). Because liver dysfunction has also been related with systemic inflammation caused by acute lung injury [55], the histopathology of liver was also examined. With the exception of one mouse (Fig 1, KO, liver in PBS treatment), all the mice including both WT and KO showed no sign of inflammatory response in liver tissue regardless of the presence or absence of LPS treatment.

Statistical analysis showed no significant difference for the histology scores between any two groups in the study (LPS vs. PBS for both KO and WT, WT vs. KO for LPS treatment). The one PBS-treated KO mouse with a high liver histological score of (Fig 1, KO, liver, PBS) did not show evidence of inflammation in its lung and vascular system. One WT mouse in PBS group showed mild lung inflammation change (Fig 1, WT, lung, PBS).

Taken together, the above data indicate that current experimental treatment only causes very mild inflammation in this LPS induced acute lung injury model. Disruption of mouse Lcn2 (mLcn2) gene did not increase murine susceptibility to lung inflammation stimulation.

Although the microscopic observation did not reveal significant gross inflammatory changes between LPS treated and untreated animals, gene expression analyzed with real-time
RT-PCR provided more sensitive results. The mRNA levels of two pivotal proinflammatory cytokines, IL-6 and TNF-α showed statistically significant (p<0.05) difference between LPS and PBS groups in both WT and KO mice from lung and liver (Fig 2, 3). One exception to this is the comparison of lung IL-6 levels in KO mice between PBS and LPS, which shows no significant difference due to high variation in LPS-treated KO mice (Fig 2, IL-6). Statistical analysis indicates there is no significant difference for IL-6 and TNF-α between KO and WT mice in both presence and absence of LPS.

To confirm mLcn2 as an acute phase protein (APP) [9], gene expression of mLcn2 was also analyzed by real-time RT-PCR for WT mice. In consistence to its APP role, the mLcn2 mRNA from liver samples showed significant increases (p<0.05) upon LPS stimulation (Fig 3, mLcn2). In contrast, no statistically significant difference was observed for the lung samples due to high variation in both LPS and PBS groups (Fig 2, mLcn2). This variation may originate from differences in individual response to the stress intranasal instillation, which can also partially explain the mild aforementioned inflammation in the PBS treated lung.

Previous in vivo and in vitro studies have shown that mLcn2 is highly expressed in LPS-stimulated macrophages [6,7] and macrophage maturation has been reported as inhibited by mLcn2 [56]. The tissue resident macrophage level was determined by quantifying the level of F4/80 mRNA, a major murine mature macrophage surface marker, to test if mLcn2 knockout has any effect on macrophage maturation. For mLcn2 WT mice, the mRNA level of F4/80 showed no difference between PBS and LPS in lung and liver sample sets. The same result was observed for KO mice. Furthermore, comparison between KO and WT mice revealed no significant differences under any treatment regime (Fig 2, F4/80). These data show that, the current experimental treatment with LPS showed no statistically significant effect on Lcn2 and had no obvious effect on the tissue resident macrophage level.Expression of CD14, another common surface marker for both monocytes and granulocytes was also examined. Although it is obvious that both LPS-treated WT and KO mice had higher levels of CD14 expression compared to PBS-treated mice, not all the differences were statistically significant due to the large observed variation (Fig 2, CD14). The large
variation may be explained by the histological observation that only a small number of the mice showed any extent of neutrophil infiltration.

**Effect of Lcn2 on DSS induced acute colitis**

Two conditions were used in the DSS induced acute colitis, which were 3% DSS for 5 days and 2% DSS for 7 days. Histology analysis indicates that both treatment conditions induced the intestinal inflammation response (Fig 4). Because previous studies have shown that in DSS-induced colitis the distal segment of the colon is the most severely affected portion [53], the histopathology for each of three individual segments of colon were evaluated in the 3%-7d treatment condition. Both the proximal colon (segment A) and mid-colon (segment B) as well as the sum of three segments’ scores showed significant differences in histological score between DSS treated and untreated groups for both WT and KO mice (Fig 4). For the distal colon (segment C), only the KO mice showed a significant difference between DSS and control group, but not for WT mice. For mice treated with 2% DSS for 7 days, the histological scores based on segment C showed clear difference between treated and untreated mice for both genotypes. For all the comparisons, there was no significant difference between the responses of the KO and WT animals.

The disease activity index was also monitored throughout the experiment period as another parameter for evaluating inflammatory severity. For both 3%-5d and 2%-7d treatments, no stool consistency changes were observed during the experiment. The DAI scores are the average value of weight change and rectal bleeding. Only the DAI scores of the longer treatment period (2%-7d) are significantly different between treated and untreated mice for both genotypes (Fig 5B). There is no difference in DAI scores between KO and WT mice. Colon length, another inflammation severity parameter, also showed the same difference (Fig 5B). Although there is a significant difference in colon length between KO and WT animals treated with 3%-5d DSS treatment (Fig 5A), this may not reflect a real biological significance considering that there were no differences in any other parameters.

**3.5 DISCUSSION**

It has been clearly shown that Lcn2 is up-regulated in both animal lung inflammation model [7,47,48,51]and human pulmonary inflammatory diseases [57,58]. In addition, high
expression of Lcn2 has also been reported from patients of inflammatory bowel diseases [16] and in mice after chemically induced gastrointestinal mucosa injury [35]. These data show a strong correlation between Lcn2 induction and the inflammatory response, but do not address the question of the role (if any) of Lcn2 in the induction, progression or response to inflammation. In the present study, we investigated the involvement of Lcn2 in the induction of the inflammatory response by assessing inflammation severity, disease activity and proinflammatory cytokine expression level in WT and KO animals. We found no effect of Lcn2 on the induction of both LPS induced acute lung inflammation and DSS triggered acute colitis, implying no causal role of Lcn2 for the initiation inflammation.

An intranasal instillation of LPS was utilized to induce lung inflammation, which has been fully characterized in previous study [52] in which administration of LPS at 10 μg per mouse could induce significant lung inflammation as early as 2h. After 8 h treatment, the percentage of neutrophils in alveolar wall increased to 31.3 ± 11.7% with an average histopathology score at 2.6 ± 0.5, in contrast to 0% neutrophil infiltration and 0 histopathology score in PBS group. With a higher dose and longer treatment (100 μg per mouse, 24 h), the percentage of alveolar neutrophils reached to 31 ± 13% with an average histopathology score at 1.8 ± 0.6, in contrast to 0 % and 0 score in PBS group. However, using similar and higher doses of LPS (0.4 mg/kg, 4 mg/kg, 8 mg/kg, average mice body weight 24 ± 3 g) and the same treatment time (8 h), we did not observe the same degree of inflammatory response (Fig 1). This discrepancy is not due to genetic background of the mice and the LPS used, as both our study and the previous research used BALB/c mice and LPS from *E. coli* serotype 055:B5. Our study differed from the previous work in using CO₂ for anesthesia instead of using a chemical anesthetic. Although CO₂ inhalation has been reported to induce acute inflammatory response [59] we saw little evidence of lung inflammation under our experimental conditions even with high LPS doses. We also tested isoflurane as the anesthetic for intranasal instillation with the same results. Histopathological analysis revealed no inflammation change after treatment with 8 mg/kg LPS for 8 h (data not shown). These results indicate that some factors other than the anesthesia caused the inconsistency between our results and those of the previous report.
mLcn2 was recently reported to be up-regulated in mouse hippocampus following psychological stress. Furthermore, disruption of the mLcn2 gene is reported to promote a stress-induced increase in spine density and caused an increase in the proportion of mushroom spines, which also correlated with higher neuron excitability and elevated stress-induced anxiety [60]. However, it is not understood whether this altered neuron excitability has any effect on the acute inflammatory response. In the present work, in addition to the LPS stimulation, the procedure for anesthesia and intranasal instillation is another potential stress which is very likely to perturb the central nervous system. For both LPS and PBS treated mice, we did not observe any significant difference between Lcn2 KO and WT mice in proinflammatory cytokine expression (Fig 2, Fig 3, IL-6, TNF-α), nor was any behavioral difference was noticed between these two strains throughout the experimental period (data not shown). This suggests that the enhanced neuron excitability caused by Lcn2 gene knockout might not contribute to the acute inflammation induction.

Our finding that Lcn2 has no effect on acute lung inflammation induction differs from the results of another reported study in which Lcn2 deficient mice showed enhanced airway resistance and increased airway inflammation as shown by increased lymphocyte and eosinophil presence in the bronchoaveolar lavage fluid (BALF) compared with in WT mice [51]. However, a number of important differences between the two studies may explain the discrepancy. First, ovalbumin (OVA) mixed in aluminium hydroxide was used as an airway allergen in the previous study to investigate mLcn2’s role in airway inflammation as opposed to LPS in the current study. Also, prior to inducing the allergic response, the mice in the previous study were first sensitized by multiple intraperitoneal injections of OVA then the OVA was delivered to them in a form of an aerosol for three consecutive days with 20 min per day. This allergic inflammation model involves much more handling of the animals with the accompanying stress and is very different from the single intranasal instillation of LPS used in this study. The previous study also did not collect data to test for lung neutrophil infiltration or levels of proinflammatory cytokine levels, which limits the extent to which the studies can be directly compared.

In a previous study, we showed that recombinant mLcn2 could ameliorate indomethacin-induced gastric injury through a subcutaneous route but not through oral administration,
which suggested that the role of mLcn2 might be post-inflammatory, perhaps in wound healing. In the current study, deletion of mLcn2 gene did not result in exacerbated colitis induced by DSS. However, this is a short term study and these results do not distinguish between the possibility that mLcn2 has special healing function in the stomach but not in the large intestine or if there is functional redundancy for mLcn2, which results in no observed difference between Lcn2 KO and WT mice.

3.6 ACKNOWLEDGEMENT

We thank Lee Bendickson for his great help with animal maintenance.

REFERENCES


### Table 1 H&E stain scoring criteria for LPS-induced inflammation

<table>
<thead>
<tr>
<th>Liver (^a) (Inflammation)</th>
<th>Liver (vacuolar change)</th>
<th>Lung</th>
<th>Vascular</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 = no remarkable change</td>
<td>0 = no remarkable lesions</td>
<td>0 = no remarkable lesion</td>
<td>0 = no remarkable lesion</td>
</tr>
<tr>
<td>1 = earliest detectable increase in neutrophils in blood vessels</td>
<td>1 = earliest detectable vacuolar change</td>
<td>1 = earliest detectable increase in neutrophils in capillaries/septa/vessels</td>
<td>1 = earliest detectable increase in eosinophilia (redness) of the vascular</td>
</tr>
<tr>
<td>2 = neutrophils in aggregates or marginating in vessels</td>
<td>2 = diffuse vacuolar change, mild</td>
<td>2 = neutrophils in aggregates in the airways</td>
<td></td>
</tr>
<tr>
<td>3 = neutrophil infiltration into the liver parenchyma</td>
<td>3 = diffuse vacuolar change, moderate</td>
<td>3 = neutrophils filling alveolar lumens/airways</td>
<td></td>
</tr>
<tr>
<td>4 = diffuse vacuolar change, severe</td>
<td>4 = extensive neutrophils involving &gt;30% of the section</td>
<td>E = edema around vessels</td>
<td></td>
</tr>
</tbody>
</table>

\(a\): Histopathology of liver was scored based on two categories: inflammation and vacuolar change. Each category is rank from 0 to 3/4 (no inflammation to severe). The score from each category were added for a final liver histological score for each mouse with a maximal possible value as 7.

### Table 2 DAI scoring criteria for DSS-induced colitis \(^a\)

<table>
<thead>
<tr>
<th>Score</th>
<th>Weight Loss (%)</th>
<th>Stool Consistency</th>
<th>Rectal Bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>Well formed pellet</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>1-5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6-10</td>
<td>Pasty and semiformed stools that did not adhere to the anus</td>
<td>Positive occult blood test</td>
</tr>
<tr>
<td>3</td>
<td>11-20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>&gt;20</td>
<td>Liquid stools that did adhere to the anus</td>
<td>Gross bleeding</td>
</tr>
</tbody>
</table>

\(a\): Ref [53]. Each mouse was scored based on three categories: weight loss, stool consistency and rectal bleeding. Each category is rank from 0 to 4 (no inflammation to severe). The score from each category were added and divided by 3 for a final DAI score for each mouse. In our experiment, no stool consistency change was observed for any treatment, therefore only weight loss and rectal bleeding were scored and the sum was divided by 2. The maximal possible DAI score for each mouse is 4.
<table>
<thead>
<tr>
<th>Score</th>
<th>Inflammation Severity</th>
<th>Inflammation Extent</th>
<th>Crypt Damage</th>
<th>Percentage Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>0%</td>
</tr>
<tr>
<td>1</td>
<td>Mild</td>
<td>Mucosa</td>
<td>Basal 1/3 damaged</td>
<td>1%-25%</td>
</tr>
<tr>
<td>2</td>
<td>Moderate</td>
<td>Mucosa and submucosa</td>
<td>Basal 2/3 damaged</td>
<td>26%-50%</td>
</tr>
<tr>
<td>3</td>
<td>Severe</td>
<td>Transmucosa</td>
<td>Crypts lost surface epithelium present</td>
<td>51%-75%</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td>Crypts and surface epithelium lost</td>
<td>76%-100%</td>
</tr>
</tbody>
</table>

Table 3 H&E stain scoring criteria for DSS-induced colitis

- The colon tissue was divided into 3 equally long segments (A, B, C). Each segment was scored based on four categories: inflammation severity, inflammation extent, crypt damage, percentage involvement. Each category is rank from 0 to 4 (no inflammation to severe). The score from each category were added for a final score for each segment. The maximal possible score for each segment is 16. For 3%DSS-5d treated mice, the sum of the three segments scores were also used to compare histopathology between WT and KO mice.
FIGURES

Figure 1 Histopathology score of LPS-induced lung inflammatory response. H&E staining of liver and lung tissue samples was performed as described in Material & Method. Histopathology scores were evaluated according to the criteria in Table 3. The sample size for each group was given at the x-axis. Each open (WT mice) or filled (KO mice) circle represents the score of an individual mouse. The group mean score larger than zero was shown as the cross (+) symbol.

Figure 2 The level of mLcn2, cytokine and cell surface marker mRNA transcripts in the lung of WT versus Lcn2 KO mice following intranasal instillation of LPS. Mice were i.n. instilled with either 8 mg/kg LPS in sterile PBS or PBS alone for 8 h, total RNA was extracted and real-time RT-PCR was performed to determine the mRNA level of various genes of interest. All the mRNA values are normalized to GAPDH. For significant difference between LPS and PBS groups, * represents p<0.05 and ** is for p<0.01.

Figure 3 The level of mLcn2, cytokine and cell surface marker mRNA transcripts in the liver of WT versus Lcn2 KO mice following intranasal instillation of LPS. Mice were i.n. instilled with either 8 mg/kg LPS in sterile PBS or PBS alone for 8 h, total RNA was extracted and real-time RT-PCR was performed to determine the mRNA level of various genes of interest. All the mRNA values are normalized to GAPDH. For significant difference between LPS and PBS groups, * represents p<0.05 and ** is for p<0.01.

Figure 4 Histopathology score of DSS-induced acute colitis. H&E staining of colon tissue was performed as described in Material & Method. Histopathology scores were evaluated according to the criteria in Table 2. The sample size for each group was given at the x-axis. Each open (WT mice) or filled (KO mice) circle represents the score of an individual mouse. The group mean score larger than zero was shown as the cross (+) symbol. For significant difference between DSS treatment and no DSS treatment groups, * represents p<0.05 and ** is for p<0.01.

Figure 5 Assessment of DAI and colon Length in DSS-induced colitis. Disease activity index (DAI) was monitored and scored as described in Table 1. length were measured at the
end of experiment. The sample size for each group was given at the x-axis. For significant difference between DSS treatment and no DSS treatment groups, * represents p<0.05 and ** is for p<0.01. In addition, a statistical significant difference between WT and KO mice at 3% DSS treatment is also marked with **.
Figure 1
Figure 2
Figure 3

Graphs showing the mRNA levels of mLcn2, IL-6, TNF-α, F4/80, and CD14 under PBS and LPS treatment in WT and KO mice.
CHAPTER 4 AN RNA APTAMER-BASED MICROCANTILEVER SENSOR TO DETECT THE INFLAMMATORY MARKER, MOUSE LIPOCALIN-2

A paper to be submitted to *Analytical Biochemistry: Methods in the Biological Sciences*

Lijie Zhai*a,*, Tianjiao Wang*a,*, Kyungho Kangb, Yue Zhao b, Pranav Shrotriya b, Marit Nilsen-Hamilton*a,†

4.1 ABSTRACT

Lipocalin-2 (Lcn2) has been identified as a biomarker for many inflammatory-based diseases including acute kidney injury, cardiovascular stress, diabetes and various cancers. Inflammatory transitions occur rapidly in kidney and cardiovascular disease, for which an inline monitor could be beneficial. Micro-cantilever devices with aptamers as recognition elements can be effective and rapidly responsive sensors. Here, we have selected and characterized an RNA aptamer that specifically binds mouse Lcn2 (mLcn2) with a dissociation constant of 340 ± 70 nM. The aptamer competes with a catechol iron-siderophore, the natural ligand of mLcn2. This and the results of studies with mLcn2 mutants demonstrate that the aptamer binds to the siderophore binding pocket of the protein. A microcantilever sensor was developed with the aptamer as the recognition element. The system showed a detection limit of 4 nM and dissociation constant for mLcn2 of 38 ± 22 nM. The higher apparent affinity of the immobilized aptamer compared with the aptamer in solution may result from its close surface packing, which makes it effectively multivalent with a lower apparent off-rate. This aptamer-coated micro-cantilever sensor has potential for

---

*a* Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA, 50011  
b* Department of Mechanical Engineering, Iowa State University, Ames, Iowa 50011  
*Equally contributing author  
† Corresponding author: marit@iastate.edu
development as an in-line monitoring system for mLcn2 in studies of acute diseases such as kidney and cardiac failure.

4.2 INTRODUCTION

Lipocalin-2 (Lcn2) is a member of the lipocalin superfamily. It was first described as a growth-factor regulated protein [1,2] and then identified as an acute phase protein [3]. Since the discovery of this small secreted protein, a variety of biological functions have been related to Lcn2 such as induction of cell apoptosis and cell differentiation, cancer metastasis, tissue wound healing, insulin resistance in adipocytes and an antibacterial effect [4-8]. Many of these events involve growth factors and the inflammatory response. The human form of this protein (NGAL) was first described as a protein expressed by neutrophils [9], which are also critical players in inflammation. With its expression being associated with inflammatory responses, it is not surprising that increased Lcn2 levels have been reported for a large number of diseases in clinical analyses and animal disease models. Among these, Lcn2 has been identified as a reliable and early indicator of acute kidney injury (AKI) and proposed as a promising marker for AKI diagnosis and prognosis based on a meta-data analysis from studies involving 19 countries and 2538 patients [10].

As do other lipocalins, Lcn2 forms a beta-barrel-type structure known as the lipocalin fold that surrounds an internal pocket or calyx for ligand binding [11]. The ligand for Lcn2 is one of a number of catechol siderophores [11]. The ability to bind siderophores produced by pathogenic prokaryotes provides Lcn2 with a role in bacteriostasis in vivo [8], which may also be fundamental to its involvement in iron transport [12,13].

Being an acute phase protein produced by the liver, the epithelium, and dendritic cells of the innate immune response and secreted by activated neutrophils, Lcn2 levels are expected to increase in most inflammatory conditions. This is consistent with increased levels of Lcn2 associated with a variety of kidney dysfunction diseases, other diseases involving inflammation, including cardiovascular disease, atherosclerosis, acute myocardial infarction and metabolic disorders including diabetes and obesity. Because it is an early marker of inflammation and tissue stress, this protein is most useful as a biomarker for acute inflammatory responses such as acute kidney injury that can occur during medical intervention. For this application, in-line monitors are most effective.
Currently Lcn2 is detected by antibody based immunoassays, which are not readily incorporated into in-line detection systems, in part due to the susceptibility of antibodies to irreversible denaturation caused by repeated use. Nucleic acids are more stable to temperature fluctuations and aptamers have been demonstrated as good sensors that can be used repeatedly for real-time detection. To date, many aptamers have been selected against a broad range of targets with binding affinities comparable to monoclonal antibodies and have been incorporated into many sensor platforms [14].

Here, we describe the selection of an RNA aptamer that specifically recognizes mouse lipocalin-2 (mLcn2) but not to its human and chicken orthologues. The aptamer and protein were independently evaluated to determine the regions involved in their interaction with the conclusion that the binding site includes the conserved iron-siderophore binding pocket of mLcn2. The mLcn2 aptamer was incorporated into a micro-cantilever system and demonstrated capable of selectively detecting mLcn2 in a homogeneous assay. The results of these studies suggest that a microcantilever-aptamer detection system might be developed for in-line detection of mLcn2 in studies of animal models of critical human diseases.

4.3 MATERIALS AND METHODS

Reagents

The reagents used in these studies were: T4 polynucleotide kinase and RNase I (Promega, Madison, WI), RNase T1 and DNase I (Epicentre, Madison, WI), RNase V1 (Life Technologies, Grand Island, NY), Taq DNA polymerase (GenScript, Piscataway, NJ), recombinant T7 RNA polymerase (prepared by us), dNTP and NTP (Thermo Fisher Scientific Inc., Glen Burnie, MD), $^{\gamma}$-$^{32}$P-ATP $^{\alpha}$-$^{32}$P-ATP (MP Biomedicals, Solon, OH), $^{55}$FeCl$_3$ (PerkinElmer, Waltham, MA), 2,3-DHBA (Sigma-Aldrich, St. Louis, MO), ThermoScript™ reverse transcriptase and TOPO XL PCR cloning kit (Life Technologies, Grand Island, NY), TALON metal affinity resin (Clontech, Mountain View, CA), superflow Ni-NTA resin (Qiagen, Valencia, CA) and other chemicals (Sigma-Aldrich, St. Louis, MO).

All oligonucleotides were chemically synthesized with standard desalting by either the DNA Facility (Iowa State University) or Integrated DNA Technology (IDT, Coralville, IA).
The 5’ thiolated oligonucleotides were synthesized with HPLC purification by IDT. All oligonucleotide sequences are given in Table S1 (supplementary data).

**Recombinant Protein**

The 6xHis-mLcn2 protein, expressed in the murine myeloma cell line NS0, was purchased from R&D Systems Inc. Expression vectors for 6xHis-tagged wild-type mLcn2 and its mutants, human Lcn2 (hLcn2) and chicken Lcn2 (cLcn2) were prepared by us by subcloning these sequences into TrcHis expression vector (Invitrogen, Carlsbad, CA) [15]. The proteins were expressed and purified by Nickel-NTA affinity chromatography as previously described [15]. To remove the 6xHis tag and unwanted amino acids encoded by the plasmid sequence, the recombinant fusion proteins were incubated with recombinant enterokinase (EMD Chemicals, Inc.) in the buffer containing 20 mM Tris, 50 mM NaCl, 2 mM CaCl₂, pH 7.4 at room temperature for 24 hours. The uncut proteins and the His tag were removed by capturing them on a Nickel-NTA resin. The successful cleavage was confirmed by running SDS-PAGE.

**Circular Dichroism (CD)**

CD measurements were performed with a Jasco J-710 spectropolarimeter. The samples were scanned in the far-UV wavelength range from 190–250 nm in a quartz cell with a path-length of 0.1 cm at 22 °C. All experiments were performed in 10 mM Na₂HPO₄, pH 7.4. CD values after buffer background subtraction were used as the final reading for each protein sample. The raw CD readings were input into CDFit (http://www.ruppweb.org/cd/cdtutorial.htm#Program%20CDFIT) to calculate the mean molar ellipticity per residue and to predict the secondary structure.

**In Vitro Selection of Aptamers**

*In vitro* selection of an aptamer for mLcn2 was carried out by SELEX (Systematic Evolution of Ligands by EXponential enrichment) [16]. The detailed procedure of aptamer selection is described in the Supplemental Data. Briefly, a random RNA oligo pool was generated from the ssDNA library (oligo 487) by primer extension and *in vitro* transcription. Radioisotope labeled ATP was incorporated into the transcripts during certain rounds of SELEX to monitor the binding percentage of the aptamers. For selection, the transcribed RNAs were mixed with Talon resin, which was coupled with mLcn2 (for mLcn2-Resin
preparation, see Supplemental Data). For negative selections, the RNAs were first incubated with resin then the wash-off was used to mix with mLcn2-resin. RNAs incapable of binding mLcn2 were washed off from the resin and the mLcn2-binding RNAs were eluted from the resin using imidazole. The protein-bound RNAs were reverse transcribed into cDNAs, which were PCR amplified to generate the enriched pool for the next round selection. SELEX rounds of in vitro transcription, RNA-protein binding and partition and RT-PCR were carried out until significant enrichment of RNA binders was observed. Finally the enriched pool was cloned into the TOPO XL PCR cloning plasmid (Invitrogen, Carlsbad, CA) for sequencing.

**Binding Affinity Measurement**

A standard filter binding assay was used to determine the dissociation constant $K_d$ and inhibition constant $K_i$ [17]. The detailed experimental procedure is given in the Supplemental Data. Equation of $B = B_{min} + (B_{max}-B_{min})*[M]/(K_d+[M])$ was used to derive $K_d$. To derive inhibitor constant $K_i$, the competition data were first fit to the equation of $B = B_{min} + (B_{max}-B_{min})/(1+10^{(\log[M]-\log[IC_{50}])})$ to obtain $IC_{50}$. Then $K_i$ was calculated from the equation of $K_i=IC_{50}/(1+[M]/K_d)$. In the equations, $B$ is percent bound of a ligand; $B_{min}$, minimal percent bound of a ligand; $B_{max}$, maximal percent bound of a ligand; $[M]$, ligand concentration and $IC_{50}$, 50% inhibitory concentration. Isothermal titration calorimetry (ITC) was also used to measure the $K_d$ and thermodynamics of aptamer-mLcn2 interaction at room temperature and in the presence of 50 mM Tris, 150 mM NaCl, 5 mM MgCl2, pH 7.4

**RNase Footprinting**

5’-end labeling of RNAs with $^{32}$P was carried out at 37 °C for 1.5 h in a 20 μl mixture of 70 mM Tris (pH 7.6 at 25 °C), 10 mM MgCl2, 5 mM DTT, 1U/μl T4 polynucleotide kinase and 2 μCi/μl $\gamma$-$^{32}$P-ATP (6000 Ci/mmol). The labeled RNAs were purified by PAGE through an 8% polyacrylamide gel containing 7M urea. The $^{32}$P-labeled aptamer with mLcn2 or hLcn2 at different concentrations were mixed in 10 μl of reaction buffer (2.5 mM, 50 mM KH$_2$PO$_4$, 150 mM NaCl, 5 mM MgCl2, pH 7.4). RNase I (5 x 10$^{-4}$ U/μl) and RNase V1 (1 x 10$^{-5}$ U/μl ) were added and incubated at 23 °C for 10 min. Partial alkaline hydrolysis was done in 50 μM Na$_2$CO$_3$, pH 9.0 at 95 °C for 5 min. RNase T1 digestion of the labeled aptamer was carried out in 10 μl reaction mixture containing 0.5 to 1 U/μl RNase T1, 5 M
urea, 350 mM sodium citrate, 0.7 mM EDTA, pH 5.0, at 50 ºC for 4 min. The reaction was stopped by addition of gel loading dye containing 47.5% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol FF. Samples were analyzed, after separation through a 10% denaturing polyacrylamide gel by PAGE, with Typhoon 8600 Variable Model Imager (GE Healthcare, Piscataway, NJ).

**Micro-cantilever Detection Assay**

The micro-cantilever detection assay was performed as previously described [18]. In the current study, the sample cantilever was coated with 5’ thiolated oligo 569, while the reference cantilever was coated with an irrelevant thiolated DNA oligo (Table S1). The differential surface stress changes were plotted against the protein concentrations, and the $K_d$ was calculated by the nonlinear fitting curve with the same equation as used for $K_d$ determination in the filter binding assay with the $B_{max}$ and $B_{min}$ representing maximal and minimal surface stress changes respectively. The limit of detection (LOD) is derived from the lowest measured signal ($X_L$) using the nonlinear fitting equation, with $X_L = X_{bl} + 3S_{bl}$ for which $X_{bl}$ is the mean of the blank measurement, and $S_{bl}$ is the standard deviation of the blank measurement [19].

### 4.4 RESULTS

**Selection of mLcn2 Aptamers**

The SELEX procedure was performed for 10 rounds, after which the mLcn2 bound RNA was found enriched to 18%, from the starting 0.4%, bound to the protein (Fig. 1A). By round 10, more than 90% of the RNA bound to the mLcn2-linked resin, but only 1.7% bound to the Talon-resin alone (Fig. 1A). Among the 56 cloned RNAs from the 10th round, 21 aptamer candidates were identified by sequence alignment (Fig. 1B). Although no single consensus sequence could be identified for all 21 candidates, two sequences (A1 and A4) dominated, being about 50% of the population (Fig 1B). Both A1 and A4 had a 3-way junction motif as predicted by an RNA secondary structure prediction program S-fold (Fig 1C) and the truncated 3-way junction of A1 had the best affinity for mLcn2 compared to full length A1, A4 and truncated A4 (Fig S1). Thus the truncated A1, designated as oligo569, was chosen for further characterization.
Binding Affinity and Stoichiometry of the mLcn2 Aptamer

The binding of oligo569 to mLcn2 was evaluated by filter binding and isothermal titration calorimetry (ITC) from which dissociations constants of $0.34 \pm 0.08 \, \mu M$ and $0.21 \pm 0.004 \, \mu M$ (mean ± S.D.) were determined respectively (Fig. 2). The stoichiometry of binding determined by ITC was $0.87 \pm 0.04$ (Fig 2B).

The recombinant protein that was used for SELEX differs from the endogenous protein in two ways. First, it has a His tag (6 histidines with additional associated peptide). Second, the endogenous protein is glycosylated. To ensure that neither the His tag nor the glycosylation influenced the binding constants for the aptamer, we determined the $K_d$ for oligo569 to recombinant mLcn2 without the His tag and to His-tagged glycosylated recombinant mLcn2 expressed by mammalian cells and obtained values of $0.43 \pm 0.09 \, \mu M$ and $0.38 \pm 0.22 \, \mu M$ respectively (Fig 2C). These results show that oligo569 (subsequently referred to as the mLcn2 aptamer) binds mLcn2 with submicromolar affinity in a manner that is affected by neither glycosylation nor the His tag.

Binding Sites and Secondary Structure of the mLcn2 Aptamer

RNAse footprinting was used to define the binding sites for mLcn2 on the aptamer using the human lipocalin-2 (hLcn2) as a negative control. From this analysis, we identified nucleotides including A11-A17, A25-A28 and U32 as protected from the RNase I digestion and nucleotides including C20-G21 and U32-U33 as protected from RNase VI digestion in the presence of mLcn2 but not hLcn2 (Fig 3A). Based on the RNA footprinting results and secondary structures predicted by S-fold and MC-FOLD (Fig S2) the most probably secondary structure of oligo 569 is proposed to be a three-way junction with the two loops and stem 2 involved in binding to mLcn2, which is also the most stable structure predicted by S-fold (Fig 3B). Stem 1 probably has no direct contact with mLcn2 but may serve as a clamp to stabilize the 3-way junction structure.

To further confirm the proposed secondary structure and binding interactions, sequence variants of the aptamers were synthesized (Table S1) and tested for their affinities to mLcn2 (Fig 3C). A summary of the results of filter binding assays showed that deletion or substitution of the nucleosides on the two loops (oligos 572, 573, 577, 579 and 580) either abolished or dramatically decreased the aptamer binding affinity to mLcn2 (Fig 3C).
contrast, shuffling of stem 2 (oligo 578) did not change the binding affinity and addition of G·C pairs to the stem 1 (oligo 571, oligo 576) slightly increased the binding affinity. These affinity changes are not likely due to changes in overall secondary structure of the variants as the secondary structures predicted by S-fold and MC-FOLD for these aptamer variants retained the 3-way junction of the parent sequence except that the short G·C stem near loop 1 was lost in the predicted secondary structure of oligo 573 due to deletion of the CCG residues (Fig S3). Taken together, these results identify the key residues on the mLcn2 aptamer contributing to high affinity binding with mLcn2 as being in loop 1 (A11-U17) and loop 2 (A25-G29). The effects of sequence variations in the junction regions (U16-U18, C36-A37) on affinity could be because the junction is a region of direct interaction with mLcn2, but could also be because changes in these interloop regions change the distance between loop 1 and loop 2, which could be critical for the 3D structure of the aptamer. The stems may also be important for aptamer structure even if they do not themselves contact the protein. For example, stem 2 may serve as a structural scaffold for loop 2 to promote its interaction with mLcn2 while stem 1 may stabilize the overall 3-way junction.

**Identification of Binding Sites of the Aptamer on Mouse Lipocalin-2**

To identify the mLcn2 protein side chains that interact with the aptamer, recombinant mutant proteins were produced with alanine substitutions for charged and polar surface and calyx-exposed amino acid side chains. These substitutions included ten regions of the protein surface and the amino acids in the protein calyx that are responsible for iron-siderophore binding (Arg103, Lys 147, Lys156). Homology modeling of the mLcn2 surface was performed after alignment with the hLcn2 (NGAL) sequence for which pdb parameters were available from the crystal structure (Fig S5). Since this work was performed the crystal structure of mLcn2 has been deposited in the database and found to match the homology-based simulation. Mutations of the surface amino acids had no significant effect on the binding of mLcn2 to the aptamer, with $K_d$ changes of less than 4-fold for any of these mutants compared with the wild-type protein (Table I). Larger effects on binding were observed with mutations in the calyx. Binding to a single site mutant (mLcn2_R103A) decreased the affinity by 5-fold, the double mutant (mLcn2_R103A_K147A) and triple mutant (mLcn2_R103A_K147A_K156A) did not bind the aptamer (Fig 4A). To verify that
the three calyx alanine substitutions did not grossly alter the overall mLcn2 structure, we performed circular dichroism in which we observed similar percentages of the major secondary structural elements for these mutants as for the wild-type mLcn2 (Fig S4). A crystallography study of hLcn2 has also demonstrated no global or local structural changes due to double-site mutation that included Arg103 in human Lcn2 [20]. Consequently, we concluded that the three basic calyx amino acid residues constitute part, or all, of the binding site for the aptamer on mLcn2.

The three basic calyx residues bound by the aptamer are also essential for the iron-siderophore binding to hLcn2 [21]. To confirm that a similar relation exists for mLcn2, we tested the binding affinity of the three calyx mutants for the siderophore, Fe(DHBA)₃. In line with the aptamer binding results, the single alanine substitution for Arg103 did not have a large effect on the affinity of the protein with the mutant mLcn2 having a $K_d$ 1.48 ± 0.05 fold of wild-type mLcn2. However, mLcn2 mutants with double- and triple-substitutions did not bind Fe(DHBA)₃ sufficiently to establish a $K_d$. (Fig 4B). Thus the three basic amino acids in the calyx are important for both iron-siderophore and aptamer binding to mLcn2.

One explanation for a similar amino acid residue requirement for the aptamer and the siderophore is that the aptamer and siderophore bind to the protein by way of the same amino acid residues. To test this hypotheses, we examined whether the aptamer and Fe(DHBA)₃ compete or cooperate for mLcn2 binding and found that Fe(DHBA)₃ and the aptamer compete for mLcn2 binding (Fig 4C, D). The aptamer variant sequences were tested for competition with Fe(DHBA)₃. The resulting $IC_{50}$s were 12.4 ± 0.2 μM ($K_i$ at 3.6 ± 0.1 μM) and 2.8 ± 1 μM ($K_i$ at 1.1 ± 0.5 μM) for oligo 569 and oligo 576 respectively (Fig 4C, D). The lower $K_i$ for oligo576 may result from the increased stability afforded the aptamer structure by virtue of the G·C pairs at stem 1. Under the same conditions in which oligos 569 and 576 were competitive for Fe(DHBA)₃ binding to mLcn2, the negative controls, either $Fe^{3+}$ or an irrelevant RNA oligonucleotide, showed no competition. From these results we conclude that the aptamer binds to the same three basic residues in the mLcn2 calyx as those of the hLcn2 siderophore binding sites and that the aptamer recognizes properly folded and functional mLcn2.
Detection of mLcn2 Using Aptamer Coated Micro-Cantilever

For in-line measurement of mLcn2 levels by the aptamer a sensor platform is required that responds rapidly to the presence of analyte. For this, we have chosen a micro-cantilever detection system that we have previously demonstrated capable of detecting cocaine in a configuration in which the cocaine aptamer was linked to the cantilever [18]. Thiol-modified mLcn2 aptamer and an irrelevant oligonucleotide (Table S1) were coated on sample and reference microcantilevers, respectively. Upon addition of analytes (mLcn2 or the R103A_K147A double mutant mLcn2) the differential surface stress between these two cantilevers was recorded to measure the binding force. The binding affinity was estimated by nonlinear curve fitting of the surface stress vs. concentration. In this context, the aptamer affinity for mLcn2 was determined as 38 ± 22 nM. This value is significantly lower than that measured for the aptamer in solution and may be due to aptamer crowding on the cantilever surface with a resulting decrease in apparent k_{off}. By fitting the experimental data using a non-linear fitting equation the value of 7 mN/m was determined as the sensitivity of the surface stress measurements, the limit of detection for the mLcn2 concentration was estimated to be 4 nM. The microcantilever gave no significant response to the R103A_K147A mutant of mLcn2, which we have previously shown does not bind the aptamer (Fig 5).

4.5 DISCUSSION

In the current study, we describe the successful selection of an RNA aptamer that specifically recognizes mouse lipocalin-2. The binding affinity of this aptamer was determined to be 200-300 nM using filter binding assay and ITC for the analysis. The micro-cantilever system, however, showed a 10-fold higher affinity of this aptamer. The increased apparent affinity could be explained by the fact that the apparent dissociation rate of the protein from the cantilever surface on which the aptamers are highly concentrated is likely to be lower than in solution due to the high probability of protein rebinding after its initial dissociation from the aptamer at the cantilever surface. This argument applies to the effect of duplicating binding sites on the same molecule, such as for antibodies. In addition, the tight molecular packing on the cantilever surface may stabilize the aptamer-ligand complex and thereby increase the binding affinity by decreasing the percent of time the aptamer adopts a
non-binding structure. Similarly, the slightly increased affinity of the two variants of oligo 569 (oligo 571 and oligo 576, Fig 3C) may also be attributed to structural stabilization due to the addition of more G·C pairs to stem 1. The latter speculation is supported by the lower free energies predicted for these variants by the MC-Fold and S-fold programs (Fig S3). Increased structural stability might also explain why oligo 576 is more competitive with Fe(DHBA)$_3$ than oligo 569 binding for binding to mLcn2 (Fig 4D).

With the current aptamer, the micro-cantilever can detect as low as 4 nM (96 ng/ml) mLcn2 and is much less sensitive than the current ELISA assay, which has a detection limit of 0.2 nM (R& D, Minneapolis, MN). However, the reported basal concentration of mLcn2 in blood is 100 ng/ml and can increase to 3000 ng/ml [8], which is within our detection range. The micro-cantilever is also compatible with in-line sensing that cannot be achieved with an ELISA.

A common feature of aptamers is their exceedingly high specificity for the analyte against which they were selected. In line with this general observation, the aptamer is highly specific for mouse lipocalin calyx even though the same trilogy of amino acids is present in the same positions in the human lipocalin calyx, which is not bound by the aptamer. Sequence alignment showed that, in addition to the three iron-siderophore binding amino acids, other amino acids forming the iron-siderophore binding pockets are conserved for both human and mouse lipocalin-2 (Fig S5 and ref [Fig S5 and’ 21]). The fact that mutation of the three conserved amino acids completely eliminates binding of the aptamer to mLcn2 suggests that these three amino acids form the major interaction sites of the aptamer on mLcn2 and that the difference in interaction of the aptamer with the human and mouse proteins may be due to a dynamic aspect of their structures that is not evident in the X-ray crystallographic data or that the aptamer may also bind to another, as yet unidentified, region on mLcn2 that is different in the human Lcn2.

In conclusion, we have selected an RNA aptamer that specifically bind to mLcn2 with an affinity $210 \pm 4$ nM in solution and of $38 \pm 22$ nM when immobilized. The RNA-protein interaction involves bases on the aptamer loop regions and the iron-siderophore binding amino acid trilogy in the calyx of the protein molecule. The aptamer is specific for the mouse protein over the human protein, despite the identity of amino acid trilogy to which the
aptamer binds in the calyx. We have shown the potential of this aptamer as a detection probe on a micro-cantilever platform.

4.6 ACKNOWLEDGEMENT

We thank F. Descalzi (University of Genoa, Italy) for the pDR5 plasmid containing the exFABP (cLcn2) sequence [22], J. Ryon for preparing the hLcn2 expression vector, and E. Will for preparing the cLcn2 expression vector. This work was funded in part by the Institute for Physical Research and Technology.

REFERENCES:


<table>
<thead>
<tr>
<th>Mutated Amino Acids</th>
<th>$K_d$ (mean ± SD, μM)</th>
<th>Mutation Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>mLcn2</td>
<td>0.34 ± 0.07</td>
<td>wild-type</td>
</tr>
<tr>
<td>S23A_N26A</td>
<td>0.65 ± 0.28</td>
<td>surface</td>
</tr>
<tr>
<td>D41A</td>
<td>1.08 ± 0.16</td>
<td>surface</td>
</tr>
<tr>
<td>D45A_Q46A</td>
<td>1.08 ± 0.24</td>
<td>surface</td>
</tr>
<tr>
<td>T65A_E66A</td>
<td>0.76 ± 0.10</td>
<td>surface</td>
</tr>
<tr>
<td>H120A</td>
<td>0.72 ± 0.15</td>
<td>surface</td>
</tr>
<tr>
<td>R176A_K179A</td>
<td>0.67 ± 0.10</td>
<td>surface</td>
</tr>
<tr>
<td>E80A_N81A_N82A</td>
<td>1.46 ± 0.46</td>
<td>surface</td>
</tr>
<tr>
<td>E150A_N151A</td>
<td>0.92 ± 0.61</td>
<td>surface</td>
</tr>
<tr>
<td>E169A_E172A</td>
<td>0.82 ± 0.26</td>
<td>surface</td>
</tr>
<tr>
<td>D195A_Q196A_D199A</td>
<td>1.69 ± 0.39</td>
<td>surface</td>
</tr>
<tr>
<td>R103A</td>
<td>1.97 ± 0.75</td>
<td>iron-siderophore binding sites</td>
</tr>
<tr>
<td>R103A_K147A</td>
<td>NB^a</td>
<td>iron-siderophore binding sites</td>
</tr>
<tr>
<td>R103A_K147A_K156A</td>
<td>NB</td>
<td>iron-siderophore binding sites</td>
</tr>
</tbody>
</table>

^a: NB, No binding
FIGURES

Figure 1 Selection of mLcn2 aptamers. A. The enrichment of the mLcn2 binding RNAs during the progress of in vitro selection was monitored as the binding percentage of the RNA pool in the presence (+) and absence (-) of mLcn2 using the filter binding assay; B. Dendrogram of 21 aptamer candidate sequences. After the 10th round of SELEX the RNAs were cloned and sequenced. Twenty one different sequences were obtained from 56 clones. Sequence alignment using AlignX (VectorNTI, Invitrogen) showed that 2 of the 21 sequences (A1, A4) account for 25% and 21% of the population respectively. Each sequence is denoted by a capital letter followed by Arabic numbers. The frequency of each sequence is shown on the right side of the dendrogram; C. The secondary structures of the two dominant sequences, A1 and A4, were predicted by S-fold. The common structural element between these two candidates was identified as a 3-way junction (in rectangle). Truncations of the original full length candidates were made based on this 3-way junction.

Figure 2 Determination of mLcn2 Aptamer Binding Affinity and Specificity. A. Filter binding assay. Lcn2 from mouse (mLcn2) were titrated against Oligo 569 to determine the $K_d$ in more than 10 independent filter binding assays using different batches of 6xHis-mLcn2 fusion protein and oligo 569. One representative data with nonlinear fitting curve is shown. The binding of oligo 569 to human (hLcn2) and chicken (cLcn2) Lcn2 orthologs were determined with 3 independent filter binding assays. No detectable binding was observed for these two orthologs even the protein concentration reached more than 10 μM. For each filter binding assay, duplicates were performed to obtain the standard deviation within each assay; B. Isothermal Titration Calorimetry. The mLcn2 aptamer (oligo 569) was titrated into 5 μM recombinant 6xHis-mLcn2. The integrated area under each heat release peak (●) from each injection was plotted against the molar ratio of aptamer to mLcn2 and the curve was fitted with a one-site independent binding model (solid line). The first titration data point was omitted from the analysis. One representative result from two independent experiments is shown; C. The mLcn2 aptamer (oligo 569) binds to recombinant bacterial nonglycosylated mLcn2 lacking a His- tag (●) and glycosylated His-tagged mLcn2 expressed from mammalian cells (○). Solid line: nonlinear fitting curve for EK cut mLcn2; dotted line:
nonlinear fitting curve for the mammalian form mLcn2. One representative result from two independent experiments is shown for each protein.

**Figure 3 Identification of the binding sites and secondary structure of mLcn2 aptamer.**

A. RNAse Footprinting of the mLcn2 aptamer. 2.5 nM 5' 32P -labeled oligo 569 was incubated with or without 4 μM hLcn2 or mLcn2 at (0.3 and 3 μM) and subjected to digestion by RNase I (left panel) and RNase V1 (right panel). Control channels include: OH, partial alkaline hydrolysis of the aptamer; G: G ladder of the aptamer generated by partial RNase T1 digestion; -: aptamer alone with no treatment. The figure shows the results of one of at least four independent experiments; B. Predicted secondary structure of the mLcn2 aptamer (oligo 569). A consensus structure from M-Fold and S-Fold predictions is shown with a summary of the results for protection from cleavage by RNase I (่า) and RNase V1 (▼). Lines between the residues indicate base-pairing predicted as dynamic (dotted lines) or stable (solid lines). Possible secondary structural elements were shown in rectangles with labels; C. Effect of variations in aptamer sequence on binding activity. A schematic representation of the mLcn2 aptamer shows the effect of varying its sequence (Table S1) on the affinity for mLcn2. The box with dash-line border indicates deletion; the boxes with solid line border indicate substitutions. Oligo numbers are shown followed by $K_d$ change (mean ± standard deviation) in comparison to oligo569 (average from 2 to 3 independent experiments). nb: no binding ($K_d$ was unable to be estimated as no saturation binding even at 20 μM protein concentration).

**Figure 4 Identification of binding sites on mLcn2.**

A. The effects of mutations in the calyx on the affinity of mLcn2 to its RNA aptamer. The nonlinear fitting curves are shown by the solid and dotted lines for mLcn2 (●) and R103A (○) respectively, while the double- (△) and triple-mutants (▲) showed no binding. One representative result is shown from at least 4 independent experiments for each protein; B. The effects of alanine substitutions in the mLcn2 calyx on iron-siderophore binding. Recombinant mLcn2 proteins with combinations of alanine substitutions of the amino acids Arg 103, Lys147, Lys156 were titrated against $^{55}$Fe(DHBA)$_3$. ●, mLcn2; ○, R103A; △, R103A_K147A; ▲, R103A_K147A_K156A. The figure shows one representative data from at least four repeats; C. Aptamer binding to
mLcn2 in the presence of varying concentrations of ferric iron or Fe(DHBA)₃. The relative amount of 2 nM ³²P-aptamers (oligo 569, oligo 576) bound to 4 μM mLcn2 in the presence of various concentrations of ferric iron (right panel) or Fe(DHBA)₃ (left panel) with the binding in the absence of Fe³⁺ or Fe(DHBA)₃ set at 1; D. Fe(DHBA)₃ binding to mLcn2 in the presence of varying concentrations of aptamers or control oligonucleotide. The relative amount of 100 nM ⁵⁵Fe- Fe(DHBA)₃ bound to 200 nM mLcn2 in the presence of various concentrations of oligonucleotides with the binding in the absence of oligonucleotide set at 1.

**Figure 5 The mLcn2 aptamer can be applied on micro-cantilever system for lipocalin-2 detection.** A. Schematic representation of the sensing strategy for mLcn2 detection (left panel) and optical circuit of differential surface stress sensor (right panel). Laser wavelength is 635 nm. A pair of microlens arrays with lens of 240 and 900 μm diameters; and pitches of 250 μm and 1 mm, respectively were used to direct the beams toward the sensing/reference pair; B. Surface stress values as a function of protein concentrations. Non-linear fitting curve (solid line) was calculated from the averaged values of six independent assays for mLcn2 (●) or two independent assays for mLcn2-K125A (○). The two dotted lines below and above the fitting curve for mLcn2 represent the 95% confidential interval. The estimated Kₐ for mLcn2 is at 0.038 ± 0.022 μM.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

A

Sensing surface coated with aptamer molecules

Aptamer molecules

Exposure to analyte leads to affinity complex formation on sensing surface and surface stress change

Analyte molecules

Affinity complexes

B

Surface Stress Change (mN/m)

Protein Concentration (µM)

-20

0

20

40

60

80

100

120

mLCn2

R103A_K147A
CHAPTER 5  GENERAL CONCLUSIONS

5.1 ACUTE PHASE PROTEINS (APPs) AND INFLAMMATION

The acute phase response (APR) is a prominent systemic reaction of the organism to local or systemic disturbances in its homeostasis caused by infection, tissue injury, trauma or surgery, neoplastic growth or immunological disorders [1]. The acute phase response may be divided into changes in the concentrations of many plasma proteins, known as the acute phase proteins (APPs), and a large number of behavioral, physiologic, biochemical, and nutritional changes. An acute phase protein has been defined as one whose plasma concentration increases (positive APPs) or decreases (negative APPs) by at least 25 percent during inflammatory disorders [2]. The expression characteristic of Lcn2 has clearly classified it as a positive acute phase protein [3].

Although it has been more than eighty years since the description of the first APP, C-reactive protein (CRP) [4], the functions and mechanisms of most APPs have not been fully elucidated. The assumption that the changes in plasma concentrations of acute-phase proteins are beneficial is based largely on the known functional capabilities of the proteins and on logical speculation as to how these might serve useful purposes in inflammation, healing, or adaptation to a noxious stimulus. The positive APPs are regarded as having general functions in opsonization and trapping of micro-organisms and their products, in activating complement, in binding cellular remnants like nuclear fractions, in neutralizing enzymes, scavenging free haemoglobin and radicals, and in modulating the host’s immune response. In this regard, the bacteriostatic effect of Lcn2 via iron-siderophore sequestration fit the pattern. However, the findings that Lcn2 binds to mammalian like siderophore and its up-regulation under “sterile inflammation” suggest that Lcn2 may exert much more general functions than against bacterial infection. Unfortunately, although many studies have indicated that Lcn2 actively participates in other functions, experiments using Lcn2 deficient mice have not supported a role of Lcn2 as an essential factor for functions, such as cell apoptosis, kidney embryonic development.

In the third chapter of this thesis, I present research work that supports previous findings of no effect of Lnc2 on the inflammatory response. In two different inflammation models
including LPS-induced acute lung injury and DSS-induced colitis, mLcn2 deficient mice showed no significant differences in inflammation initiation and progression. Together with previous research data, it is very clear that Lcn2 does not influence certain types of inflammation response.

The simplest explanation for no observed effect of Lcn2 on inflammation is that the role of this protein is other than in development of progression of inflammation and that it might have a role in recovery from inflammation. Another explanation for no observed effect of deleting Lcn2 on the inflammatory response could be genetic redundancy. A similar finding was made for CRP, another APP. Highly sensitive measurements have revealed that even modest elevations in stable baseline CRP plasma levels can be correlated with a significantly increased risk of future cardiovascular disease, suggesting potential effects of CRP on atherogenesis. However, a study of CRP deficient mice did not provide a clear conclusion because there was no significant difference between the CRP and WT mice [5].

5.2 ASSESSMENT OF APPs AS DISEASE BIOMARKER

Upon stimulation, the plasma concentration change of certain positive APPs such as CRP can reach as high as 1000-fold of the starting point [6]. Thus, measurement of APPs, despite the lack of diagnostic specificity for some of them, is useful to clinicians because such changes reflect the presence and intensity of an inflammatory process and are useful in managing the patient’s disease because the concentration often reflects the response to and need for therapeutic intervention. Furthermore, in some diseases, serial measurements APPs are of prognostic value [6].

Lipocalin-2 has long been confirmed as a robust biomarker in many diseases because of its high stability and expression level in both serum and urine samples (described in Chapter 2.1.5). More importantly, Lcn2 as a novel marker for AKI and CKD has been extensively studied and shown to be very promising for both diagnosis and prognosis. Therefore, in addition to its diverse biological functions, Lcn2 is also an excellent target for biomarker detection probe development. To this end, the fourth chapter of my thesis mainly describes the experimental results of selection, characterization and application of mLcn2 nucleic acid aptamers. Through collaboration with other colleagues, we have selected an mLcn2 specific RNA aptamer with affinity at 340 nM ± 70 nM. Interestingly, the aptamer was found to bind
to the sites on the protein molecule that includes the conserved iron-siderophore binding amino acids. Consistent with this observation, the aptamer can compete with iron-siderophore binding to mLcn2. Finally, using an mLcn2 aptamer functionalized micro-cantilever, we are able to detect mLcn2. These results suggest a potential application of this aptamer for mLcn2 detection. The results of this study have identified a new detection tool and for mLcn2 that can be used to track the levels of this protein in future studies of its function and/or mechanism.

5.3 FUTURE WORK

For practical application of the mLcn2 aptamer it requires additional development. Because our aptamer was selected using unmodified dNTPs, it is ribonuclease sensitive and cannot be readily applied to detect proteins in biological samples. This problem can be solved by synthesizing chemically modified mLcn2 RNA aptamer. Because modification can alter aptamer structure, affinity and specificity, the modified aptamer will need to first be optimized to ensure that the chemical modification does not adversely affect the aptamer-protein binding. It will then be tested in various conditions mimicking the physiological environments.

Although our aptamer showed certain degree of competition with iron-siderophore for its binding to mLcn2, its lower affinity for Lcn2 compared with the siderophore makes it incapable of totally blocking the binding of siderophore to mLcn2. It is possible that one of the 19 remaining aptamer candidates might have a higher affinity and these can be screened. If none are found to bind mLcn2 with higher affinity then a reselection might be done based on current aptamer sequence to enhance the affinity. Increasing the binding affinity of the mLcn2 aptamer to a similar or higher level as that of siderophore will create a potential antagonist for mLcn2 of the aptamer, which would provide it with more potential applications, such as opposing the mLcn2 mediated apoptotic effect on normal hematopoietic cells in leukemia (Chapter 2, 2.1.4).

In addition to the above proposed future work, more applications of the mLcn2 aptamer could be explored. For example, in parallel with micro-cantilever system, the mLcn2 aptamer can be applied in combination with the mLcn2 antibody in the ELONA system [7] (described
in Chapter 2.3.4) that combines antibody and aptamer for mLcn2 detection. Establishment of this detection platform will provide a more cost-efficient detection method for mLcn2.

REFERENCES

APPENDIX SUPPLEMENTAL DATA FOR CHAPTER 4

MATERIAL AND METHODS

**Preparation of mLcn2-Resin**

His-tagged proteins were captured on Talon cobalt affinity resin (Clontech) in the buffer containing 50 mM KH$_2$PO$_4$, 150 mM NaCl, 0.02% NaN$_3$, 0.05% Tween-20, pH 7.4. The unbound proteins were removed by washing the resin with the buffer 3 times. The protein-resin coupling efficiency was calculated by determining the protein concentrations in the original protein prep and the flow-through and washes. Protein-resin was stored in 50% slurry in the above buffer at 4 °C with a concentration at 0.70 mg mLcn2 per ml beads. 50% slurry of the resin without addition of the proteins was prepared in the same procedure and used as the negative control for the in vitro aptamer selection.

**In Vitro Selection of Aptamers**

In vitro selection of an aptamer for mLcn2 was carried out by SELEX (Systematic Evolution of Ligands by EXponential enrichment). A ds-DNA library was generated from the ss-DNA library (oligo 487) by Taq polymerase extension using oligo 484 as a primer. The reaction was carried out at 72 °C in 2 ml mixture of 1.67 μM oligo487, 3.33 μM oligo484, 0.5 mM dNTP, 50 mM KCl, 10 mM Tris (pH 9.0 at 25 °C), 1.5 mM MgCl$_2$, 0.1% Triton X-100 and 0.05 U/μl Taq polymerase . After purification with the QIAquick PCR purification kit (Qiagen), PCR products (360 pmol for the first round and 1/3 of the PCR products for the rest rounds) were in vitro transcribed in 40-80 μl mixture of 30 mM Tris, 10 mM DTT, 2 mM spermidine, 20 mM Triton X-100, 20 mM MgCl$_2$, pH 8.5, 5 mM each of ATP, GTP, UTP, CTP, 0.7 μM T7 RNA polymerase, 0.2 U/ml inorganic pyrophosphatase (New England Biolabs) at 42 °C for 1 hour. At 1$^{st}$, 6$^{th}$, 9$^{th}$ and 10$^{th}$ round of the selection, 10 μCi α-$^{32}$P-ATP (6000 Ci/mmol) was added to the reaction mixture to prepare radioisotope labeled transcripts for monitoring RNA binding to mLcn2 on talon-resin. The reactions were stopped by the addition of 20-40 U of RNase free DNase I and incubation at 37 °C for 30 min. The transcribed RNA was precipitated by ethanol and subsequently dissolved in 20-50 μl of 7M urea prior to being fractionated by 8% (w/v) denaturing (7 M urea) PAGE
(acrylamide/bisacrylamide, 19:1, w/v) in buffer containing 45 mM Tris/borate (pH 7.5) and 1 mM EDTA. RNA products were then visualized by UV-shadowing, and the bands corresponding to the full-length RNAs were excised. The transcripts were eluted by a solution of 0.3 M sodium acetate and 1 mM EDTA from the gel slices overnight at 23 °C. The elution was extracted twice with phenol/chloroform followed by ethanol precipitation. The final RNA was dissolved in 20-30 μl deionized water and the concentration was determined by NanoDrop at 260 nm.

The transcribed RNAs were diluted in selection buffer of 50 mM KH2PO4, 150 mM NaCl, 5 mM MgCl2, pH 7.4 and loaded onto the column containing 0.2 ml of mLcn2-resin equilibrated with the same buffer. For 3rd, 6th and 9th round of selection, the RNAs were first loaded onto column containing only resin without mLcn2 as a negative selection. The columns were rinsed with 300 μl of selection buffer and the eluates (unbound RNAs) were then loaded on to a second column containing 0.2 ml mLcn2-resin. In positive selection, the protein and RNA concentration started with 7.75 μM and 16 μM respectively in a 200 μl reaction volume and gradually decreased to 0.9 μM and 1.2 μM respectively in a 500 μl volume at round 10. Following 1 h incubation at room temperature, the columns were washed with 5 to 10 ml of selection buffer and the mLcn2-RNAs complex was eluted by 150 mM imidazole (pH 7.4). Eluted RNAs were extracted twice with phenol/chloroform followed by ethanol precipitation.

Aliquots of the above purified RNAs (1/3 volume) were then reversed transcribed by ThermoScript reverse transcriptase using oligo 485 as the primer. The reverse transcription (RT) was carried out in 20 μl mixture of dNTPs (1mM each), 2.5 μM oligo 485, 0.75 U/μl reverse transcriptase, 50 mM Tris acetate (pH 8.4 at 25 °C), 75 mM potassium acetate, 8 mM magnesium acetate. Half of the reverse transcribed cDNAs were then amplified by PCR in 100 μl mixture of 50 mM KCl, 10 mM Tris (pH 9.0 at 25 °C), 1.5 mM MgCl2, 0.1% Triton X-100 and 0.025 U/μl Taq polymerase, dNTPs (0.2 mM each), 2 μM reverse primer oligo 484 and 2 μM forward primer oligo 485 with the temperature control of 1 cycle of 93 °C, 3 min; 6-12 cycles of 93 °C, 30 sec, 65 °C, 1 min, 72 °C, 1min and 1 cycle of 72 °C, 7 min. The rounds of in vitro transcription, RNA-protein binding and partition and RT-PCR were
carried out until significant enrichment of RNA binders was observed. At round 10, the enriched pool was cloned into pCR-XL-TOPO vector for sequencing and further characterization.

**Binding Affinity Measurement**

Standard filter binding assay was used to either determine $K_d$ for aptamers / Fe(DHBA)$_3$ – mLcn2 interactions or study the competition between the aptamer and Fe(DHBA)$_3$ for mLcn2 binding at 23 $^\circ$C. The $^{55}$Fe(DHBA)$_3$ was prepared by mixing 0.5 mM $^{55}$FeCl$_3$, 1.5 mM DHBA, 0.1 mM Tris (pH 8.0 at 25 $^\circ$C) for 30’ at 23 $^\circ$C. For competition assay and Kd measurement of Fe(DHBA)$_3$ – mLcn2 interaction, the buffer contained 50 mM Tris, 150 mM NaCl, 10 mM CaCl$_2$, 5 mM MgCl$_2$, pH 7.4. For Kd measurement of aptamer – mLcn2 interaction, the buffer was 50 mM KH$_2$PO$_4$, 150 mM NaCl, 5 mM MgCl$_2$, pH 7.4. Briefly, 50 μl samples for binding or competition assays were filtered through a 96-well filtration apparatus (Bio-Rad) assembled with buffer soaked nitrocellulose membrane (GE Water & Process Technologies) on top of buffer soaked positively charged nylon membrane (Millipore) by 55 cm Hg vacuum. The membranes were then washed with 0.5 ml buffer and air-dried. The radioisotope signals on the membranes were recorded by phosphor screen, scanned by Typhoon 8600 Variable Model Imager (GE Health Care Life Sciences) and quantified by ImageQuant (GE Health Care Life Sciences). The percent bound was calculated as the ratio of the intensity on nitrocellulose membrane to that of the sum on nitrocellulose membrane and nylon membrane. For every filter binding assay, each sample had duplicates or triplicates.

ITC experiments were performed using a Nano-ITC isothermal titration calorimeter (TA Instrument). In each experiment, 50 μM aptamer dissolved in the binding buffer was titrated using the computer-controlled syringe into the sample cell (0.95 mL) containing either binding buffer or 5 μM mLcn2 dissolved in the same buffer at 25 $^\circ$C. The syringe was set at a stirring speed of 150 rpm. After a 60 s initial delay, each titration involved an initial 1 μL injection followed by 15 serial injections of 15 μL each at intervals of 300 s. The raw data obtained in each experiment were corrected for the effect of titrating aptamer from the syringe to the sample cell containing the buffer only but no protein. The thermodynamic
parameters were calculated using a one-site binding model in the analysis software (NanoAnalyze) provided by TA Instrument.

**Bioinformatics**

The 3-D structure of mLcn2 was predicted based on homologous modeling using SWISS-MODEL following program instructions. The surface amino acids were identified as amino acids with more than 30% solvent accessible as analyzed by the Swiss Pdb-Viewer. The secondary structures of RNA aptamers were predicted by both Sfold (Sma module) and MC-FOLD using the aforementioned experimental conditions as prediction parameters.
### Supplemental Table 1

<table>
<thead>
<tr>
<th>Oligo Number</th>
<th>Use</th>
<th>Oligo Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligo 487</td>
<td>Random ssDNA pool</td>
<td>GCCTGTTGTGAGCCTCCTCTGTCGAA(N53)TTGACGTTTTATATTGTCTCCCC</td>
</tr>
<tr>
<td>Oligo 484</td>
<td>reverse primer for SELEX</td>
<td>TAATACGACTCACTATAGGGAGACAAGAATAAAACGCTCAA</td>
</tr>
<tr>
<td>Oligo 485</td>
<td>forward primer for SELEX</td>
<td>GCCTGTTGTGAGCCTCCTGTCGAA</td>
</tr>
<tr>
<td>Oligo 258</td>
<td>negative control for Fe(DHBA)_3 competition binding</td>
<td>GGAUCCCGACUGGCGAGAGCCAGGUACGAAUGGAUCC</td>
</tr>
<tr>
<td>Oligo 569</td>
<td>wild-type mLcn2 aptamer</td>
<td>CCUCCGGCUCAUACCUCUUCGAAGACAAGCUUCGACAGGAGG</td>
</tr>
<tr>
<td>Oligo 571</td>
<td>mutated mLcn2 aptamer</td>
<td>CCUCCGGCUCAUACCUCUUCGAAGACAAGCUUCGACAGGCGGG</td>
</tr>
<tr>
<td>Oligo 572</td>
<td></td>
<td>CCUCCGGCUCAUACCUCUUCGAAGACAAGCUUCGACAGGAGG</td>
</tr>
<tr>
<td>Oligo 573</td>
<td></td>
<td>CCUCCGGCUCAUACCUCUUCGAAGACAAGCUUCGACAGGAGG</td>
</tr>
<tr>
<td>Oligo 576</td>
<td></td>
<td>GGCGCGCGCUCAUACCUCUUCGAAGACAAGCUUCGACAGGCCGCC</td>
</tr>
<tr>
<td>Oligo 577</td>
<td></td>
<td>CCUCCGGCUCAUACCUCUUCGAAGCUUCGACAGGAGGAG</td>
</tr>
<tr>
<td>Oligo 578</td>
<td></td>
<td>CCUCCGGCUCAUACCUCUUCGAAGACAAGCUUCGACAGGAGG</td>
</tr>
<tr>
<td>Oligo 579</td>
<td></td>
<td>CCUCCGGCUCAUACCUCUUCGAAGACAAGCUUCGACAGGAGG</td>
</tr>
<tr>
<td>Oligo 580</td>
<td></td>
<td>CCUCCGGCUCAUACCUCUUCGAAGACAAGCUUCGACAGGAGG</td>
</tr>
</tbody>
</table>

a: Underlined residues are substitution for original nucleosides

b: Hyphen represents residue deletion

c: Residues in bold represent addition to original sequence
FIGURES

Figure S1 Selection of a mLcn2 specific aptamer. The full length of A1 and A4 and their truncated sequence (oligo 569 from A1, oligo 570 from A4) based on the 3-way junction structures were tested for the binding to mLcn2.

Figure S2 Predicted secondary structure of oligo 569. A. Ten secondary structures of oligo 569 with the best ranking scores predicted by MC-Fold; B. Ten secondary structures of oligo 569 with best ranking scores predicted by S-fold.

Figure S3 Predicted secondary structure of oligo 569 variants. A. Ensemble secondary structures of 8 mutated mLcn2 aptamers predicted by S-fold; B. Ensemble secondary structures of 8 mutated mLcn2 aptamers predicted by MC-Fold.

Figure S4 CD analysis of mLcn2 and its calyx core mutants. Decreased binding of the iron-siderophore binding sites mutants to both Fe(DHBA)$_3$ and aptamer is not likely due to global conformation change upon mutation. A. Circular dichroism scan of the wild-type mLcn2 and the iron-siderophore binding sites mutants. Result shown is the averaged mean molar ellipticity per residue from 4-7 independent scans; B. Percentage of secondary structures by fitting CD data from 4-7 independent scans. Rfac%: R value as quality indicator for the fit which is calculated by the linear least squares minimization.
Figure S1
Figure S2A
Figure S2B
Figure S3A

Oligo 571

\[ \Delta G_{298}^\circ = -14.30 \]

Oligo 572

\[ \Delta G_{298}^\circ = -17.10 \]

Oligo 573

\[ \Delta G_{298}^\circ = -11.70 \]

Oligo 576

\[ \Delta G_{298}^\circ = -21.0 \]

Oligo 577

\[ \Delta G_{298}^\circ = -12.4 \]

Oligo 578

\[ \Delta G_{298}^\circ = -13.7 \]

Oligo 579

\[ \Delta G_{298}^\circ = -13.5 \]

Oligo 580

\[ \Delta G_{298}^\circ = -13.10 \]
Figure S4

<table>
<thead>
<tr>
<th>protein</th>
<th>$\text{R}_{\text{fac}}$</th>
<th>helix</th>
<th>sheet</th>
<th>coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>mLcn2</td>
<td>22 ± 5.5</td>
<td>1.4 ± 2.0</td>
<td>47 ± 3.1</td>
<td>51 ± 2.8</td>
</tr>
<tr>
<td>R103A_K147A</td>
<td>24 ± 4.6</td>
<td>3.3 ± 2.7</td>
<td>44 ± 5.7</td>
<td>52 ± 4.4</td>
</tr>
<tr>
<td>R103A_K147A_K156A</td>
<td>25 ± 2.7</td>
<td>3.4 ± 3.4</td>
<td>45 ± 5.0</td>
<td>52 ± 3.0</td>
</tr>
</tbody>
</table>

Figure S5
ACKNOWLEDGEMENT

Big grace obviously thanks. There is no words can express my appreciation for all the people who have helped me throughout my Ph.D. study. Without the guidance, advice, help and encouragement from my mentors, families, and friends, it would be impossible for me to reach this far on my research career.

I want to express the most sincere appreciation to my advisor, Dr. Marit Nilsen-Hamilton, for her meticulous and patient mentoring throughout my eight years of Ph.D. training. From the very first peer-review publication of my Master degree studies (Hepatitis E virus genotyping based on full-length genome and partial genomic regions. J. Virus Res. 2006 Sep; 120(1-2):57-69) to the current thesis, Marit has helped and mentored me to learn every aspect of how to do the research in a scientific way. I also want to thank the members on my POS committee: Drs. Mark Ackermann, Douglas Jones, Michael McCloskey, and Michael Wannemuehler. Their advices and help are invaluable contributions to both my basic knowledge and research work.

Many colleagues have helped me lot during my Ph.D. study. Yinghua Liu, Wei Zhao, Tianjiao Wang and our lab manager Lee Bendickson have had many discussions and advices regarding both my research projects and other aspect of my research career. I really appreciate their kind help.

Finally I want to express the most gratefulness and love to my family for their long-lasting and selfless support all the way through my Ph.D. study. Although they are separated from me in long distance, my parents have always accompanied me through the up and down along the road. Their encouragement helps me overcoming countless difficulties in both my study and life. My dearest wife, Yueyue Yu, has been there with me since the start of my Ph.D. study. Her persistency and patience are the strength and motivation for me to complete the long journey.