Iron-induced Complement Dysregulation in the Retinal Pigment Epithelium: Implications for Age-Related Macular Degeneration

Yafeng Li
University of Pennsylvania, yafengli@mail.med.upenn.edu

Follow this and additional works at: http://repository.upenn.edu/edissertations
Part of the Biology Commons, Medicine and Health Sciences Commons, and the Molecular Biology Commons

Recommended Citation
http://repository.upenn.edu/edissertations/1088

This paper is posted at ScholarlyCommons. http://repository.upenn.edu/edissertations/1088
For more information, please contact libraryrepository@pobox.upenn.edu.
Iron-induced Complement Dysregulation in the Retinal Pigment Epithelium: Implications for Age-Related Macular Degeneration

Abstract
Age-related macular degeneration (AMD), typically manifesting as a loss of central vision in elderly persons, is a leading cause of blindness in highly developed nations. AMD is a multifactorial disease associated with aging, oxidative stress, complement dysregulation, dsRNA toxicity, among many other possible factors. The formation of extracellular deposits, termed drusen, below the retinal pigment epithelial (RPE) cell layer in the outer retina is a pathognomonic hallmark of AMD. The composition of drusen is complex, but identified elements include iron, complement components, and amyloid protein derivatives. This suggests that iron may be involved in the pathophysiology of AMD. Further support for this hypothesis comes from mice lacking ferroxidases Ceruloplasmin (Cp) and Hephaestin (Heph), which have a primary genetic defect in iron homeostasis. These mice develop some AMD-like morphological features and a telling molecular feature: activated complement component 3 (C3) fragment deposition at the basolateral aspect of the RPE (the location of drusen in AMD). In our studies, we investigated the molecular mechanisms by which C3 is up-regulated by iron in RPE cells. ERK1/2, SMAD3, and CCAAT/enhancer-binding protein-δ (C/EBP-δ) are part of a non-canonical TGF-β signaling pathway that is responsible for iron-induced C3 expression. Pharmacologic inhibition of either ERK1/2 or SMAD3 phosphorylation decreased iron-induced C3 expression levels. Knockdown of SMAD3 blocked the iron-induced up-regulation and nuclear accumulation of C/EBP-δ, a transcription factor known to promote C3 expression by binding to the basic leucine zipper (bZIP1) domain of the gene promoter. We show herein that mutation of this domain reduced iron-induced C3 promoter activity. The molecular events in the iron-C3 pathway represent therapeutic targets for AMD. To better understand the relative contribution of systemic iron and local dysregulation of iron homeostasis to RPE iron accumulation, we used Bmp6 KO mice and WT mice and found that retinal hepcidin levels are not changed, but in fact may be slightly greater in KO compared to WT mice. As such, systemic iron overload by genetic KO or intravenous supplementation in WT mice resulted in increased RPE labile iron and oxidative stress, suggesting that systemic iron overload may lead to retinal iron overload despite the presence of an intact blood retinal barrier. Systemic iron status appears to be a leading determinant of retinal iron status.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Cell & Molecular Biology

First Advisor
Joshua L. Dunaief

Keywords
age-related macular degeneration, aging, complement, iron, ophthalmology, signaling

This dissertation is available at ScholarlyCommons: http://repository.upenn.edu/edissertations/1088
IRON-INDUCED COMPLEMENT DYSREGULATION IN THE RETINAL PIGMENT EPITHELIUM:
IMPLICATIONS FOR AGE-RELATED MACULAR DEGENERATION

Yafeng Li

A DISSERTATION

in

Cellular and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2015

Supervisor of Dissertation

____________________
Joshua L. Dunaief, MD PhD, Professor of Ophthalmology

Graduate Group Chairperson

____________________
Daniel S. Kessler, PhD, Associate Professor of Cell and Developmental Biology

Dissertation Committee

Chair: Jean Bennett, MD PhD, F.M. Kirby Professor of Ophthalmology
Robert B. Wilson, MD PhD, Professor of Pathology and Laboratory Medicine
Andrew B. Dancis, MD, Associate Professor of Medicine
Wenchao Song, PhD, Professor of Pharmacology
IRON-INDUCED COMPLEMENT DYSREGULATION IN THE RETINAL PIGMENT EPITHELIUM:
IMPLICATIONS FOR AGE-RELATED MACULAR DEGENERATION

COPYRIGHT

2015

Yafeng Li
DEDICATION

To my parents and my wife, for their love, endless patience, and unwavering support.
ACKNOWLEDGEMENTS

I must, first, last, and foremost, thank Josh, my mentor. He tirelessly provided great advice and guidance. I learned from him how to really do science and learned that patience is truly a virtue.

To my thesis committee members:
Dr. Bennett, your scientific support and optimistic disposition really helped me through the challenges.
Dr. Wilson, your quote from Francis Bacon stayed with me. Thank you for sharing your scientific acumen.
Dr. Dancis, your brainstorming and trouble-shooting really helped push me over the finish line.
Dr. Song, where would I be without complement? You helped me anchor the scientific basis of my work.
Age-related macular degeneration (AMD), typically manifesting as a loss of central vision in elderly persons, is a leading cause of blindness in highly developed nations. AMD is a multifactorial disease associated with aging, oxidative stress, complement dysregulation, dsRNA toxicity, among many other possible factors. The formation of extracellular deposits, termed drusen, below the retinal pigment epithelial (RPE) cell layer in the outer retina is a pathognomonic hallmark of AMD. The composition of drusen is complex, but identified elements include iron, complement components, and amyloid protein derivatives. This suggests that iron may be involved in the pathophysiology of AMD. Further support for this hypothesis comes from mice lacking ferrooxidases Ceruloplasmin (Cp) and Hephaestin (Heph), which have a primary genetic defect in iron homeostasis. These mice develop some AMD-like morphological features and a telling molecular feature: activated complement component 3 (C3) fragment deposition at the basolateral aspect of the RPE (the location of drusen in AMD). In our studies, we investigated the molecular mechanisms by which C3 is up-regulated by iron in RPE cells. ERK1/2, SMAD3, and CCAAT/enhancer-binding protein-δ (C/EBP-δ) are part of a non-canonical TGF-β signaling pathway that is responsible for iron-induced C3 expression. Pharmacologic inhibition of either ERK1/2 or SMAD3 phosphorylation decreased iron-induced C3 expression levels. Knockdown of SMAD3 blocked the iron-induced up-regulation and nuclear accumulation of C/EBP-δ, a transcription factor known to promote C3 expression by binding to the basic leucine zipper (bZIP1) domain of the gene promoter. We show herein that mutation of this domain reduced iron-induced C3 promoter activity. The molecular events in the iron-C3 pathway represent therapeutic...
targets for AMD. To better understand the relative contribution of systemic iron and local dysregulation of iron homeostasis to RPE iron accumulation, we used Bmp6 KO mice and WT mice and found that retinal hepcidin levels are not changed, but in fact may be slightly greater in KO compared to WT mice. As such, systemic iron overload by genetic KO or intravenous supplementation in WT mice resulted in increased RPE labile iron and oxidative stress, suggesting that systemic iron overload may lead to retinal iron overload despite the presence of an intact blood retinal barrier. Systemic iron status appears to be a leading determinant of retinal iron status.
TABLE OF CONTENTS

DEDICATION ........................................................................................................................................ iii

ACKNOWLEDGEMENTS ................................................................................................................ iv

ABSTRACT ....................................................................................................................................... v

TABLE OF CONTENTS ................................................................................................................ vii

LIST OF TABLES ............................................................................................................................ x

LIST OF ILLUSTRATIONS .............................................................................................................. xi

CHAPTER 1 ...................................................................................................................................... 1

An overview of the mammalian retina .............................................................................................. 1

Classification, epidemiology and pathogenesis of AMD ................................................................. 3

The accumulation of iron in the retinal pigment epithelium in AMD ............................................ 6

The lack of ferroxidase expression leads to retinal degeneration ...................................................... 7

AMD as a genetic disease .................................................................................................................. 8

The association of complement and AMD, with a focus on C3 ...................................................... 9

TGF-β signaling in context and its role in disease ...........................................................................14
CHAPTER 2 ................................................................................................................. 18

Preamble .......................................................................................................................... 18

Abstract .................................................................................................................................. 18

Introduction .......................................................................................................................... 19

Experimental procedures ........................................................................................................ 22

Results ...................................................................................................................................... 28

Iron induced the expression of endogenous C3 in ARPE-19 cell .................................................. 28

Pathway enrichment analysis implicates TGF-β signaling in iron-induced C3 expression ....... 33

Iron-induced C3 up-regulation is independent of TGF-β ligands ............................................. 38

Iron-induced C3 up-regulation involves ERK1/2 and SMAD3 non-canonical TGF-β signaling . 38

Iron-induced changes in SMAD3 phosphorylation are distinct from canonical TGF-β effects ... 49

Iron-induced increases in CEBPD mRNA levels and C/EBP-δ protein levels in both the non-
nuclear and nuclear compartments are dependent on SMAD3 activity .................................. 53

Iron can effect transcriptional up-regulation of C3 via the bZIP1 domain of the C3 promoter ... 59

Iron-induced C3 protein activation and alternative complement pathway activation can be

suppressed by pharmacologic inhibition of SMAD3 and ERK1/2 ............................................. 65

Chronic iron overload in the RPE is associated with increased C3 expression and activation, as
demonstrated by localized C3d deposition in vivo ................................................................. 70

Discussion ............................................................................................................................ 70

Main findings ......................................................................................................................... 70

Discussion ............................................................................................................................. 75

CHAPTER 3 ................................................................................................................. 80
LIST OF TABLES

Table 1.1  
Summary of common AMD associated variants  
Table 2.1  
Taqman® qRT-PCR primers  
Table 2.2  
Sigma MISSION® lentiviral transduction particles for stable knockdown studies  
Table 2.3  
PCR primers used to generate C3 promoter fragments  
Table 2.4  
The most up-regulated and down-regulated annotated transcripts in ARPE-19 cells treated with FAC from the microarray
### LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Schematic of the mammalian retina</td>
<td>2</td>
</tr>
<tr>
<td>1.2</td>
<td>Funduscopic image of dry type age-related macular degeneration (AMD)</td>
<td>4</td>
</tr>
<tr>
<td>1.3</td>
<td>The alternative complement pathway and its regulators</td>
<td>12</td>
</tr>
<tr>
<td>1.4</td>
<td>The Transforming Growth Factor-β (TGF-β) signal transduction pathways</td>
<td>16</td>
</tr>
<tr>
<td>2.1A</td>
<td>Viability of FAC-treated ARPE-19 cells at various doses</td>
<td>29</td>
</tr>
<tr>
<td>2.1B</td>
<td>TFRC mRNA with increasing FAC treatment doses</td>
<td>30</td>
</tr>
<tr>
<td>2.1C</td>
<td>C3 mRNA levels with increasing FAC treatment doses</td>
<td>31</td>
</tr>
<tr>
<td>2.1D</td>
<td>ELISA for C3 protein levels with increasing FAC doses</td>
<td>32</td>
</tr>
<tr>
<td>2.1E</td>
<td>C3 mRNA levels at 2d, 4d, 8d post-FAC treatment</td>
<td>34</td>
</tr>
<tr>
<td>2.1F</td>
<td>C3 mRNA levels of cells treated with Fe³⁺, Mn²⁺, Ni²⁺, or Cu²⁺ at 250µM FAC</td>
<td>35</td>
</tr>
<tr>
<td>2.1G, H</td>
<td>C3 and TFRC mRNA levels of cells treated with 9.75 mg/ml holo-Tf</td>
<td>36</td>
</tr>
<tr>
<td>2.2A</td>
<td>C3 mRNA levels in cells treated with exogenous TGF-β1 and SB431542</td>
<td>39</td>
</tr>
<tr>
<td>2.2B</td>
<td>TGFB1 mRNA and TGF-β1 protein (conditioned medium) levels in FAC-treated cells</td>
<td>40</td>
</tr>
<tr>
<td>2.2C</td>
<td>C3 mRNA levels in cells treated with neutralizing antibody anti-TGF-β1/2/3</td>
<td>41</td>
</tr>
<tr>
<td>2.2D</td>
<td>C3 mRNA levels in cells co-treated with anti-TGF-β1/2/3 antibody and FAC versus FAC only</td>
<td>42</td>
</tr>
<tr>
<td>2.3A</td>
<td>C3 mRNA levels in cells treated with PD98059, SB202190, SP600125</td>
<td>44</td>
</tr>
<tr>
<td>2.3B</td>
<td>C3 mRNA levels in cells treated with SIS3 and FAC</td>
<td>45</td>
</tr>
<tr>
<td>2.3C</td>
<td>C3 protein levels in the conditioned media of cells co-treated with SIS3 or PD98059 and FAC</td>
<td>46</td>
</tr>
<tr>
<td>2.3D</td>
<td>PAI-1 mRNA levels in cells co-treated with SIS3 or PD98059 and FAC</td>
<td>47</td>
</tr>
<tr>
<td>2.3E</td>
<td>Western blot and densitometry for p-ERK1/2 and ERK1/2 in lysates derived from cells at different time points (0, 1, 3, 6h) after FAC treatment</td>
<td>48</td>
</tr>
<tr>
<td>2.4A</td>
<td>Western blot and densitometry of cell lysates for p-SMAD3 (S213), p-SMAD3 (S423/425), SMAD3, and SMAD3</td>
<td>50</td>
</tr>
</tbody>
</table>
TFRC in FAC treatment time course

Figure 2.4B Western blot for cells transfected with FLAG-SMAD3, FLAG-SMAD3 EPSM, and FLAG-SMAD3 EPSM A213S 51

Figure 2.4C Western blot and densitometry of lysates for p-SMAD3 (S213), p-SMAD3 (S423/425), and TFRC in FAC and SIS3 or PD98059 co-treatment 52

Figure 2.5A Western blot and densitometry for p-SMAD3 (S213) and (S423/425) in cells treated anti-TGF-β1/2/3 +/- FAC 54

Figure 2.5B Western blot and densitometry for p-SMAD3 (S213) p-SMAD3 (S423/425) following treatment with TGF-β1 55

Figure 2.6A Western blot and densitometry for SMAD3, p-SMAD3 (S213), and TFRC in ctrl and sh-SMAD3 lines +/- FAC 56

Figure 2.6B C3 mRNA levels in ctrl and sh-SMAD3 lines +/- FAC 57

Figure 2.6C Western blot for p-SMAD3 (S213) in non-nuclear and nuclear lysates of cells, +/- FAC 58

Figure 2.7A CEBPD mRNA levels in cells treated with SIS3 and FAC 60

Figure 2.7B CEBPD mRNA levels in cells treated with neutralizing antibody anti-TGF-β1/2/3 61

Figure 2.7C CEBPD mRNA levels in cells co-treated with anti-TGF-β1/2/3 antibody and FAC versus FAC only 62

Figure 2.7D Western blot and densitometry for C/EBP-δ in non-nuclear and nuclear lysates of cells treated with FAC in a time course 63

Figure 2.7E Western blot and densitometry for C/EBP-δ in ctrl and sh-SMAD3 cell non-nuclear and nuclear lysates 64

Figure 2.8A C3 promoter fragments in use for firefly and renilla luciferase assays 66

Figure 2.8B WT, bZIP1, bZIP2 500 bp C3 promoter fragments in firefly and renilla luciferase assays 67

Figure 2.9A ELISA of C3a from the conditioned media of cells treated with different doses of FAC 68

Figure 2.9B ELISA of C3a from the conditioned media of cells treated with SIS3 and/or PD98059 and FAC versus FAC alone 69

Figure 2.9C ELISA of Ba from the conditioned media of cells 71
treated with SIS3 or PD98059 and FAC versus FAC alone

Figure 2.10A Immunofluorescence for C3d in normal and aceruloplasminemia and normal macula sections (paraffinized)

Figure 2.10B Immunofluorescence for L-ferritin and C3d in OCT-embedded retina sections of 12-month old C3⁻/⁻ and BCRe⁺, Cp⁻/-, HepfF/F mice

Figure 2.11 A model of the molecular mechanism of iron-induced RPE C3 production

Figure 3.1 Quantitative PCR results in liver, neural retina (NR), RPE/choroid (CH), and isolated RPE of Bmp6 KO and WT mice

Figure 3.2 Iron labeling and oxidative stress markers of different retinal layers in i.v. Venofer (iron sucrose)-injected C57BL/6J mice

Figure 3.3 Quantitative PCR results in liver, retina, and RPE of i.v. Venofer (iron sucrose)-injected C57BL/6J mice
CHAPTER 1

An overview of the mammalian retina

The mammalian retina is a highly structured tissue, approximately 0.5mm in thickness, lining the back of eye. This structure is shown schematically in Fig. 1.1. Although the retina is anatomically and functionally complex, we generally consider the retina as having two general components: the neurosensory retina (NR), composed of neuronal and support cells, and the retinal pigment epithelium (RPE).

The RPE, as the name suggests, is a pigmented monolayer of cells that makes contact with the photoreceptors to phagocytose their outer segments on a daily basis, regenerates the visual chromosphere 11-cis retinal, and maintains the blood-outer retina barrier while regulating epithelial transport, among its various critical functions.

The NR has at least five types of neurons: photoreceptors, bipolar cells, ganglion cells, horizontal cells, and amacrine cells. The support, or glial cells are the astrocytes and the Müller cells. All of these cells can be located within the three layers of neuronal cell bodies and two layers of synapses that compose the NSR.

The outer nuclear layer (ONL) contains the cell bodies of the rod and cone photoreceptors, which are sensitive to different wavelengths of lights. In the photoreceptor outer segments, light energy in the form of photons are converted into graded electrochemical potentials. The structures that allow this to occur within the photoreceptors are densely packed lipid-bilayer disc structures and photo-sensitive proteins, such as opsins and transducins. The inner segments of the photoreceptors contain the cell nuclei and are rich in mitochondria. The synaptic connections between rod/cones, vertically running bipolar cells, and laterally-oriented horizontal cells occur in the outer plexiform layer (OPL).
Figure 1.1 Schematic of the mammalian retina. Proceeding from the top to bottom, we begin with the outermost layer (top), the retinal pigment epithelium (RPE, white with gray nuclei). Photoreceptors, as either rods (blue) or cones (red, green, aqua blue), have nuclei in the outer nuclear layer (ONL). Between the RPE and the ONL lies the outer segments (OS) and inner segments (IS) of the photoreceptors. The synaptic layer between the photoreceptors and the bipolar cells is called the outer plexiform layer (OPL). The cell bodies of Müller glia (gray outline), bipolar cells (taupe), horizontal cells (yellow), and amacrine cells (orange) are located in the inner nuclear layer (INL). But horizontal cells synapse in the OPL and amacrine cells synapse in the IPL, the layer between bipolar cells and ganglion cells. The ganglion cell layer (GCL) contains the cell bodies of ganglion cells (purple). Müller glia span from the inner limiting membrane (ILM) adjacent to the vitreous to the outer limiting membrane (OLM), where photoreceptor cell OS takes form. Figure adapted from http://webvision.med.utah.edu.
The inner nuclear layer (INL) contains the cell bodies of the bipolar, horizontal, and amacrine cells. The Müller glia cell bodies can also be found in the INL. These unique cells project their processes all the way from the inner limiting membrane, near the ganglion cell layer (GCL), in one direction, all the way to the outer limiting membrane, at the outer edge of the ONL, in the opposite direction. Generally, bipolar cells receive input from photoreceptors and relay the signals to ganglion cells. The horizontal cells serve as interneurons that modulate the lateral transmission of signals at the synaptic interface of photoreceptor and bipolar cells. Amacrine cells are a different type of interneuron, in that they modulate the lateral interactions of the synapses of bipolar and ganglion cells, otherwise known as the inner plexiform layer (IPL).

The ganglion cell layer (GCL), the innermost retinal structure, contains the cell bodies of ganglion cells. Ganglion cells receive the integrated signals from the neural processing networks in the IPL, and transmit, to the brain, information regarding the visual image as action potentials along its own axons, which constitute the optic nerve.

**Classification, epidemiology and pathogenesis of AMD**

AMD is a degenerative disease of the central retina (macula) that results primarily in the loss of central vision in elderly persons. Loss of central vision impairs most activities of daily living and presents a great health burden on the individuals affected and their families. AMD is clinically classified into dry (atrophic) or wet (neovascular) types. In dry AMD, clinical findings may include subretinal drusen deposits, focal or more widespread geographic atrophy of the RPE, and RPE detachments/clumping. Drusen are localized deposits of extracellular material, usually found in the macular region, and appear as small, yellowish-white spots on ophthalmoscopy (Fig. 1.2). In many individuals over the age of 50, a few small, hard drusen are detected as a normal consequence of aging (Jager et al., 2008). On the other hand, larger, soft drusen are more strongly associated with disease progression and vision loss. These larger, soft drusen, possibly accompanied by RPE clumping, increases the risk for developing the wet form of AMD (Jager et al., 2008). Wet AMD is characterized by the abnormal growth of blood vessels
Figure 1.2 Funduscopic image of dry type age-related macular degeneration (AMD) with findings of pervasive, small, yellowish-white drusen and a region of atrophy in the macula. Reproduced with permission from the Massachusetts Eye & Ear Infirmary, Photographer, David Walsh. Graphic 58340 Version 2.0; Accessed from Up-To-Date: Age-related macular degeneration.
from the choroidal circulation, and less frequently from the retinal circulation, into the subretinal space. These leaky new vessels form pockets of subretinal fluid and/or blood, leading eventually to fibrous scarring. Although the wet form of disease only accounts for 10 to 15% of AMD cases, it accounts for more than 80% of all AMD cases with severe vision loss or legal blindness (Jager et al., 2008).

The prevalence and forecast data for AMD are available from numerous studies. To date, one of the most comprehensive studies was published in 2004 by The Eye Diseases Prevalence Research Group (EDPRG), which estimated that in the year 2000, 1.47% of adults over the age of 40 in the United States, or approximately 1.75 million people, are affected by AMD, wet and/or dry (Friedman et al., 2004). By 2020, only two decades later, AMD would affect nearly 3 million people. With the aging of the population, this figure would surely shift upwards in the coming decades. In a more recent publication, the overall prevalence of AMD may be slightly lower, given several modifiable factors such as smoking and the effects of changes in therapy for people at higher risk (genetic or modifiable) of developing advanced AMD (Klein et al., 2011).

We will review aspects of AMD pathogenesis, though we note that the disease mechanism for dry AMD remains incompletely understood. Generally, it is thought that abnormalities in Bruch’s membrane components (Johnson and Anderson, 2004), along with inflammation and chronic infection, may play important roles (Kalayoglu et al., 2005; Seddon et al., 2005a; Vine et al., 2005). Several more specific mechanisms of disease, including ischemia of the photoreceptor and RPE due to decreased Bruch’s membrane/choroidal perfusion selectively damaging the macular region (Grunwald et al., 2005), primary RPE senescence, RPE cell apoptosis due to activation of components of the inflammatory and immune system, i.e., Toll-like receptor 3 activation (Yang et al., 2008), and genetic polymorphisms in numerous disease susceptibility genes (Gorin, 2012) have been proposed. Nevertheless, a combination of genetic and environmental factors related to one or more of these abnormalities may lead to the development of drusen, the pathognomonic feature of AMD.
The molecular basis of angiogenesis and wet AMD is better understood than that of dry AMD. Since establishing the conceptual framework that choroidal neovascularization is tightly controlled by a substance that either promote or inhibit blood vessel formation/development, many groups have shown that a key angiogenic “Factor X,” later to be identified as vascular endothelial growth factor (VEGF), is responsible for retinal neovascularization associated with various forms of retinopathies (Aiello et al., 1994; Miller et al., 1997; Tolentino et al., 2002). Several VEGF isoforms have been identified, and VEGF-A, the one most associated with angiogenesis (Bhisitkul, 2006), has been the target of current, mainstay anti-VEGF therapies used in the treatment of wet AMD. In this regard, there has been considerable success as the loss of vision due to poorly-controlled wet AMD has decreased substantially where treatment has become available and prevalent.

AMD remains a complex, multi-factorial disease with many possible and likely genetic and environmental contributory factors. This introduction does not attempt to cover the depth and scope of work done with the aim of better understanding AMD pathogenesis, but we hope it can provide a basis for understanding the main work that is to be presented in this thesis.

The accumulation of iron in the retinal pigment epithelium in AMD

Work in the Dunaief lab using Perls’ staining identified increased iron deposition in the RPE/Bruch’s membrane underlying the macula region of AMD retinas compared to that of normal, age-matched controls (Hahn et al., 2003). In a case report of a 72-year old patient with advanced geographic atrophy, the cells in the photoreceptor layer also exhibited strong labeling for iron as well as ferroportin, the only known exporter of iron, and ferritin, an iron storage protein (Dentchev et al., 2005). Both ferroportin and ferritin have been found to be up-regulated in response to elevated iron levels in the mouse retina (Hahn et al., 2004a, 2004b). Taken together, these findings in patients and mice suggest a pathogenic role for iron not only in the RPE/Bruch’s membrane, but also in the neurosensory retina. One logical possibility for iron-induced damage to retinal tissues is oxidative stress.
Ferrous iron (Fe\(^{2+}\)) participates in Fenton chemistry with peroxide, H\(_2\)O\(_2\) to produce the ferric form of iron (Fe\(^{3+}\)), and the highly reactive/damaging hydroxyl radical (OH•). This implies that an increase in non-bound, labile iron concentration would lead to an increase in reactive oxygen species (ROS) that would impair RPE cell function. In fact, increased iron in the retina can lead to lipid peroxidation of photoreceptors. In turn, phagocytosis of such peroxidized outer segments by the RPE can damage the RPE membranes (Guajardo et al., 2003). Furthermore, in \textit{vitro} studies using iron-loaded ARPE-19 cells exhibited much decreased phagocytosis activity, interrupted cathepsin D processing, and reduced cathepsin D activity (Chen et al., 2009).

**The lack of ferroxidase expression leads to retinal degeneration**

The conversion of ferrous to ferric iron requires the activity of proteins known as ferroxidases. Four proteins with ferroxidase activity have been identified in mammals, which are ceruloplasmin, hephaestin, zyklopen, and amyloid precursor protein. All four of these proteins have been localized in the mammalian retina, and the RPE seems to express all of them (Chen et al., 2010, 2003, 1997; Hahn et al., 2004a).

Aceruloplasminemia (CP\(^{-/-}\)) is a rare, adult-onset autosomal recessive condition caused by mutations in the ceruloplasmin gene on chromosome 3q (Harris et al., 1995). It was first reported that the patient had late-onset retinal and basal ganglia degeneration, the latter of which was associated with iron deposition as seen on magnetic resonance imaging. Studies in the Dunaief lab have shown that a Caucasian patient with aceruloplasminemia had early-onset maculopathy as demonstrated by yellowish-white subretinal drusen-like lesions and RPE cell atrophy, which resemble some of the morphological features of AMD (Dunaief et al., 2005; Wolkow et al., 2011). The presentation of retinal and RPE degeneration in human patients with this disease, a primary disorder of iron metabolism, indicates that dysregulation of iron homeostasis may contribute to the retinal degeneration seen in the clinical presentation of AMD.
The ceruloplasmin gene-deficient mouse (Cp⁻/⁻) has been previously developed to better understand the role of the ferroxidase ceruloplasmin in CNS iron homeostasis. On histology, the retinas of these mice show neurodegenerative changes in the inner nuclear layer, the main site of ceruloplasmin expression. Many cells of this layer had condensed chromatin and darkened cytoplasm (Patel et al., 2002). Since these changes are not drastic, a mouse with a combined Cp mutation and a sex-linked anemia (sla) mutation in the hephaestin gene, herein referred to as the double knockout (DKO), was developed by our laboratory. These mice have an progressive, severe retinal degeneration accompanied by retinal iron accumulation (Hadziahmetovic et al., 2008a; Hahn et al., 2004a). In these studies, increased ferritin staining by immune-labeling was evident at 6 months of age, and Perls’ positive RPE cells were detected by 9 months. Similar to AMD, ultra-structural analysis of the RPE shows undigested photoreceptor outer segments, indicating RPE dysfunction. Bruch’s membrane integrity is also compromised, with the appearance of widely-spaced college in the sub-RPE space. Most pertinent to our current work, increased RPE iron content in DKO mice leads to cellular autofluorescence and accumulation of complement component 3 (C3) in the RPE/Bruch’s membrane compartment.

AMD as a genetic disease

Although many non-genetic and/or environmental factors have been implicated in AMD development, such as smoking, nutritional antioxidant levels, and omega-3 fatty acid intake, accumulating evidence suggests that genetic predisposition plays an important role in conferring AMD risk. The heritability of AMD, or the relative contribution of genetic to non-genetic factors, is estimated at a relatively high value of between 45% and 70% (Seddon et al., 2005b). It is said that the genetics of AMD is unique among multi-factorial diseases in that several genetic loci confer strong effect sizes with reported odds ratios of >2.0 per risk allele. For examples, strong risk variants in the CFH gene (Edwards et al., 2005; Haines et al., 2005; Klein et al., 2005) on chromosome 1q32 and the ARMS2/HTRA1 genes (Jakobsdottir et al., 2005; Rivera et al., 2005) on chromosome region 10q24 were identified. These were followed by numerous other risk
variants in complement genes and genes related to immunity, lipid metabolism, and extracellular matrix homeostasis (Grassmann et al., 2014).

In recent years, there has been an impressive output of genome-wide association studies (GWASs) involving a large number of subjects in the field of AMD genetics. An international multicenter meta-analysis involving more than 18 individual GWAS datasets, which combined included more than 17,000 cases of AMD and 60,000 control patients (Fritsche, 2013), contributed to defining an association between AMD and genetic variants in a total of 19 chromosomal regions (Table 1.1). The genes that lie in these regions belong to different categories of biological processes or signaling pathways. Some of the major categories, listed in Table 1.1, include extracellular matrix organization, angiogenesis, regulation of inflammation, cholesterol metabolism, and above all, complement activation (Weber et al., 2014). Cumulatively, these genetic findings may enable, through extensive downstream studies, the establishment of possible causal relationships between the development of AMD and a host of biological processes or signaling pathways. This may be particularly relevant for complement system of innate immunity, which is the focus of the thesis work herein. As can be inferred, a better understanding how aberrant complement activation contributes to the disease phenotype and progression of AMD may prove to be useful for both genetic risk prediction/stratification and the development of novel targeted therapies.

The association of complement and AMD, with a focus on C3

Complement, or sometimes referred to as the “complement cascade,” is an arm of the innate immune system consisting of a complex network of plasma proteins that interact and cooperate to provide defenses against microbial invasion and maintain healthy tissue integrity. The major steps involved in complement activation are initiation, formation of the C3 convertase protein complex, cleavage of the C3 molecule, formation of a C5 convertase complex, cleavage of the C5 molecule, and formation of the membrane attack complex (MAC) as part of the terminal...
Table 1.1 Summary of common AMD associated variants (adapted from Fritsche, 2013).

<table>
<thead>
<tr>
<th>SNP</th>
<th>Candidate genes in region</th>
<th>Biological process</th>
<th>Frequency of risk variant in AMD patients</th>
<th>Odds ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs10737680/A</td>
<td>CFH</td>
<td>Complement activation</td>
<td>64%</td>
<td>2.43</td>
<td>(2.39 to 2.47)</td>
</tr>
<tr>
<td>rs13081855/T</td>
<td>COL8A1</td>
<td>Extracellular matrix organization</td>
<td>10%</td>
<td>1.23</td>
<td>(1.17 to 1.29)</td>
</tr>
<tr>
<td>rs6795735/T</td>
<td>ADAMTS9</td>
<td>Extracellular matrix organization</td>
<td>46%</td>
<td>1.10</td>
<td>(1.07 to 1.14)</td>
</tr>
<tr>
<td>rs4698775/G</td>
<td>CFI</td>
<td>Complement activation</td>
<td>31%</td>
<td>1.14</td>
<td>(1.10 to 1.17)</td>
</tr>
<tr>
<td>rs429608/G</td>
<td>C2/CFB</td>
<td>Complement activation</td>
<td>86%</td>
<td>1.74</td>
<td>(1.68 to 1.79)</td>
</tr>
<tr>
<td>rs943080/T</td>
<td>VEGFA</td>
<td>Angiogenesis</td>
<td>51%</td>
<td>1.15</td>
<td>(1.12 to 1.18)</td>
</tr>
<tr>
<td>rs3812111/T</td>
<td>COL10A1</td>
<td>Extracellular matrix organization</td>
<td>64%</td>
<td>1.10</td>
<td>(1.07 to 1.14)</td>
</tr>
<tr>
<td>rs3130783/A</td>
<td>DDR1</td>
<td>Extracellular matrix organization</td>
<td>79%</td>
<td>1.16</td>
<td>(1.11 to 1.20)</td>
</tr>
<tr>
<td>rs13278062/T</td>
<td>IER3</td>
<td>Regulation of inflammatory processes/apoptosis</td>
<td>79%</td>
<td>1.16</td>
<td>(1.11 to 1.20)</td>
</tr>
<tr>
<td>rs334353/T</td>
<td>TGFBR1</td>
<td>Extracellular matrix organization/angiogenesis</td>
<td>73%</td>
<td>1.13</td>
<td>(1.10 to 1.17)</td>
</tr>
<tr>
<td>rs10490924/T</td>
<td>HTRA1</td>
<td>Extracellular matrix organization</td>
<td>30%</td>
<td>2.76</td>
<td>(2.72 to 2.80)</td>
</tr>
<tr>
<td>rs9542236/C</td>
<td>B3GALTL</td>
<td>Protein glycosylation</td>
<td>44%</td>
<td>1.10</td>
<td>(1.07 to 1.14)</td>
</tr>
<tr>
<td>rs8017304/A</td>
<td>RAD51B</td>
<td>DNA repair/apoptosis</td>
<td>61%</td>
<td>1.11</td>
<td>(1.08 to 1.14)</td>
</tr>
<tr>
<td>rs920915/C</td>
<td>LIPC</td>
<td>Cholesterol/LDL/HDL metabolism</td>
<td>48%</td>
<td>1.13</td>
<td>(1.09 to 1.17)</td>
</tr>
<tr>
<td>rs1864163/G</td>
<td>CETP</td>
<td>Cholesterol/LDL/HDL metabolism</td>
<td>76%</td>
<td>1.22</td>
<td>(1.17 to 1.27)</td>
</tr>
<tr>
<td>rs2230199/C</td>
<td>C3</td>
<td>Complement activation</td>
<td>20%</td>
<td>1.42</td>
<td>(1.37 to 1.47)</td>
</tr>
<tr>
<td>rs4420638/A</td>
<td>APOE</td>
<td>Cholesterol/LDL/HDL metabolism</td>
<td>83%</td>
<td>1.30</td>
<td>(1.24 to 1.36)</td>
</tr>
<tr>
<td>rs5749482/G</td>
<td>TIMP3</td>
<td>Extracellular matrix</td>
<td>74%</td>
<td>1.31</td>
<td>(1.26 to 1.36)</td>
</tr>
<tr>
<td>rs8135665/T</td>
<td>SLC16A8</td>
<td>Membrane transport</td>
<td>21%</td>
<td>1.15</td>
<td>(1.11 to 1.19)</td>
</tr>
</tbody>
</table>
complement pathway. On the most basic level, three complement pathways (classical, lectin, and alternative) can each be uniquely activated by specific inciting factors all with the end result of C3 activation, which promotes pro-inflammatory reactions and activation the terminal complement pathway leading to MAC formation (Sahu and Lambris, 2001).

The importance of complement biology in AMD is, at this point, well established. Without undertaking a review of complement-related diseases, we note that inherited complement deficiencies, such as mutations in components of the cell lytic, terminal pathway increases a patient’s susceptibility to Gram-negative bacterial infections; on the other hand, mutations that impair the function of complement cascade regulatory proteins have been linked to many autoimmune diseases, such as systemic lupus erythematosus (SLE). Returning to the eye and as described above in the AMD genetics section of this Chapter, variants in genes encoding complement components and regulatory proteins (Table 1.1) confer increased disease risk. It is thus reasonable to hypothesize that the decreasing ability of complement to maintain healthy tissue as a result of its dysregulation likely contributes to the pathogenesis of AMD. It is telling that some well-studied iron-related loci have not been implicated in AMD (i.e. HFE for high iron and TMPRSS6 loss of function for low iron). Perhaps this is because these loci by themselves have low disease penetrance compared to complement gene polymorphisms, but that as this thesis will show, iron dysregulation may serve as a crucial component that drives complement dysregulation in AMD.

Among the three complement pathways, it is widely accepted that the alternative complement pathway is the most important in the AMD disease context. The alternative pathway, interestingly, is continuously activated by a spontaneous hydrolysis of the internal thioester bond in C3 to form C3(H2O). Because this species can fulfill the same role as C3b in participating in the formation of the C3 convertase, there is a low-level of baseline pathway activity, a phenomenon also known as “tick-over” (Pangburn, 1981). C3(H2O) binds to the Bb portion of complement factor B (CFB), which itself is cleaved by the complement serine protease factor D
Figure 1.3 The alternative complement pathway and its regulators (fluid phase and membrane-bound). The alternative pathway is central to the pathogenesis of AMD. Genetic variants in complement component and regulator genes result in altered protein function and thus altered dynamics of the system. The ▽ symbol (red) indicates decay-accelerating function (of convertase) or inactivation (of C3b to iC3b), + (green) indicates enzymatic action, and – (maroon) indicates inhibitory action. Figure is adapted from Bradley et al., 2011.
(CFD). The Ba fragment that results from CFD-mediated splitting of CFB is most likely retained in the fluid phase, and provides a suitable marker for alternative pathway activation (Kolb et al., 1989). The C3 convertase, C3(H2O)Bb, cleaves C3 into C3a and C3b and activates the molecule. To amplify this reaction, C3b feeds into the positive feedback loop by binding to Bb and forms more C3 convertase enzyme complexes. A diagram illustrating these fundamental aspects of the alternative complement pathway is provided in Fig. 1.3 (Bradley et al., 2011). C3b is also deposited on cell/tissue surfaces and can be further processed to C3d. Given the continuous manner by which the alternative pathway is activated, the key role of inhibitory regulators is to prevent inappropriate over-activation and damage to self-tissues.

In AMD drusen deposits, almost all alternative complement pathway proteins have been identified, including CFH, C3, and the derivatives of its activation and processing. Furthermore, the fragment C3d and the Ba fragment of CFB, collectively considered as chronic complement activation markers, as well as and CFD, are significantly increased the blood plasma in AMD patients (Scholl et al., 2008). However, the interaction or correlation between systemic complement activation and ocular pathology is not well characterized. Although CFH is the first and arguably the most important of the complement cascade members to be genetically implicated in AMD, we now turn our focus to C3, the main subject of our study. It must be noted that C3 is the central component of the entire complement cascade. One of the most strongly associated genetic variants in C3, rs2230199 (R80G), results in a change in protein electrical charge as evidenced by the differentiation of fast and slow electrophoretic variants. Although the variant was mapped to the C3b portion of the precursor protein, which affects renal graft survival, the functional implications of the polymorphism on AMD pathogenesis remains, as yet, unclear (Yates et al., 2007). More recently, two reports have identified a rare variant in C3 that is associated with advanced AMD. This variant, rs147859257 (K155Q), is rarer than the aforementioned rs2230199. The rare variant is associated with ~2.91-fold increased risk for macular degeneration, and the resulting C3b variant is functionally hypothesized to have reduced binding to CFH (Zhan et al., 2013). In the companion paper that also characterized this rare variant, the co-incubation of CFH, CFI, and K155 (wild-type) C3 protein displayed much more
efficient cleavage of the C3 protein alpha-chain compared to the parallel experiment containing the Q155 (variant) protein (Seddon et al., 2013).

Given the wealth of genetic evidence implicating complement gene polymorphisms in AMD, it is prudent to also consider the effect of environmental exposures to the activities of the complement cascade. One particularly promising and fruitful line of research in this area has been smoking, a factor that has been found to increase AMD risk consistently. On the epidemiology front, the Age-Related Eye Disease Study (AREDS) reported an odds ratio of 1.6 for wet AMD and 1.8 for geographic atrophy when comparing people who had smoked >10 pack-years to those who had no smoking history (2005); The Blue Mountains Eye Study found that current smokers had a relative risk of 3.9 for advanced AMD compared with non-smokers (Tan, 2007). On the functional front, cigarette smoke has been shown to modify the C3 protein in vitro such that it is less susceptible to inactivation by CFH and CFI (Kew et al., 1985); More recently, cigarette smoke has been shown to increase expression and activation of C3 in cultured ARPE-19 cells (Kunchithapautham et al., 2014). A prevailing hypothesis for some of these findings is that smoke-associated oxidative stress may lead to the aberrant over-activation of complement, as seen with modifiable proteins and increased gene expression. Which other environmental factors can affect oxidative stress? Iron, the subject of our longstanding interest, logically may be one answer to this question.

**TGF-β signaling in context and its role in disease**

The Transforming Growth Factor-β (TGF-β) superfamily is a group of highly conserved growth and differentiation factors that play pleiotropic roles in cell proliferation, differentiation, morphogenesis, tissue homeostasis, and tumorigenesis (Massague, 2012). The classical category of factors, for which the superfamily is named, is the TGF-β ligand. These are secreted molecules that contain 7 conserved Cys residues in the primary sequence. Two TGF-β polypeptides form a dimer, forming inter- and intra-subunit disulfide bonds in a tight structure called the "cysteine knot" (Shi and Massagué, 2003). The TGF-β ligand is initially synthesized as
an approximately 400 aa precursor that is cleaved to form the mature protein containing a pro-
peptide. Once this pro-peptide is removed, the TGF-β is rendered biologically active (Massagué,
1998).

TGF-β signaling commences with ligand binding to the TGF-β receptor complex, which is
composed of the type II and type I transmembrane serine/threonine kinase receptors. After
ligand-induced conformational changes, the constitutively active type II receptor phosphorylates
the type I receptor, which in turn phosphorylates the receptor-regulated Smad proteins, or R-
Smads known as Smad2 or Smad3 (Feng and Derynck, 2005). In canonical TGF-β signaling,
phosphorylated R-Smads interacts with the common mediator Smad (co-Smad) such as Smad4,
co-translocate to the nucleus, where they recruit other co-activators and/or co-repressors to
modulate target gene expression (Fig. 1.4). To end the signaling effect and Smad-mediated
transcription, cells employ a number of different means, including but not limited to phosphatase
action to remove the phosphorylation of Smad C-terminal residues (Bruce and Sapkota, 2012)
and ubiquitylation for targeted turnover of the R-Smad molecule itself (Gao et al., 2009). As such,
Smad proteins are a central pillar of TGF-β superfamily signaling, transducing the signals
conveyed by ligand binding to specific receptors.

Smad proteins may not only act as an effector of canonical TGF-β ligand-induced
signaling inputs. More and more interesting facets of Smad biology have come to light in recent
years, resulting in a new understanding Smad protein biology. The R-Smad protein has two
conserved domains, one N-terminal and the other C-terminal, designated as MH1 and MH2
domains, respectively. Between the MH1 and MH2 domains lie a proline-rich linker, a region that
is divergent in sequence and length (Shi and Massagué, 2003; Wang et al., 2009). This linker
region may be a hub for integrating varied regulatory inputs (Massague, 2012), and studies have
demonstrated the Smad3 protein harbors four putative phosphorylation sites in the linker region:
Thr 179 (T179), Ser 204 (S204), Ser 208 (S208), and Ser 213 (S213). Quite importantly, the
phosphorylation of these linker region residues appears to be highly context dependent, with
Figure 1.4 The Transforming Growth Factor-β (TGF-β) signal transduction pathways. TGF-β is secreted in an inactive latent form that can be activated by proteases. In canonical signaling, dimerized TGF-β ligand induces a heterodimerization of TGF-β types I and II receptors. Active TGF-beta type I receptors act on downstream effectors, including the receptor SMADs (R-Smads) that are phosphorylated on the C-terminus to permit binding to the co-Smad, Smad4, and nuclear localization. Nuclear Smad complexes bind with low affinity to specific SMAD binding elements (SBEs) in the promoter sequences of regulated genes. Transcription complexes are formed with transcriptional binding partners and transcriptional activators/inhibitors to specifically induce or repress gene expression. TGF-β can also activate other pathways: Akt, JNK, p38, Erk, etc. as part non-canonical signaling. Figure and legend adapted from Rich, 2003.
different kinases and/or different conditions yielding varied patterns of phosphorylation and with those, downstream effects. For examples, cyclin-dependent kinase (CDK) 2/4 phosphorylates Smad3 at the T179 and S213 in the linker region (Matsuura et al., 2004) and epidermal growth factor (EGF)-induced ERK1/2 activation results in the phosphorylation of T179, S204, and S208, with S208 as the purported best ERK1/2 site of action (Matsuura et al., 2005). Furthermore, besides phosphorylating the C-terminal residues of Smad3, as defined in canonical signaling, TGF-β can induce a rapid phosphorylation of the linker region residues, adding a layer of complexity to the paradigm of canonical signaling. This may be called non-canonical TGF-β signaling. However, in this case the ligand receptor does not itself phosphorylate the Smad3 linker, but instead recruits other kinases, such as those in the CDK family, to mediate C-terminal-dependent linker phosphorylation (Wang et al., 2009). Interestingly, the Smad3 linker region may also physically interact with co-activators or co-repressors (i.e. p300), thereby modulating Smad3 C-terminal phosphorylation-dependent gene expression (Wang et al., 2009). This level of cross-talk most likely allows Smad3 to generate a much larger range of transcriptional activities, which translates to a large set of distinct biological responses.

The role that iron may play in modulating Smad3 action in the process of up-regulating C3 expression will be examined in the next Chapter. In these studies, iron, not TGF-β ligand, stimulates Smad3 activity via a non-canonical pathway; meanwhile, iron suppresses Smad3-mediated canonical signaling activity. To our knowledge, this is the first detailed study on how iron modulates TGF-β signaling, which sets it apart from the majority of the literature in the field, which focuses, rightly, on ligand-induced signaling.
CHAPTER 2

Preamble

This chapter contains the manuscript entitled “iron-induced complement component 3 (C3) up-regulation via non-canonical TGF-β signaling in the retinal pigment epithelium,” which represents the main work of my thesis. Some information in the Abstract and Introduction may be slightly redundant from those presented in Chapter 1, but it is our opinion that these concepts are worth re-visiting prior to the presentation of experimental data.

The manuscript herein (written entirely by me) was submitted to the Journal of Biological Chemistry on 17 February 2015; it was revised, re-submitted, and accepted for publication on 23 March 2015. The abstract and all subsequent sections have been preserved from the manuscript.

Abstract

Dysregulation of iron homeostasis may be a pathogenic factor in age-related macular degeneration (AMD). Meanwhile, the formation of complement-containing deposits under the retinal pigment epithelial (RPE) cell layer is a pathognomonic feature of AMD. In this study, we investigated the molecular mechanisms by which C3, a central protein in the complement cascade, is up-regulated by iron in RPE cells. Modulation of TGF-β signaling, involving ERK1/2, SMAD3, and CCAAT/enhancer-binding protein-δ (C/EBP-δ), is responsible for iron-induced C3 expression. The differential effects of spatially distinct SMAD3 phosphorylation sites at the linker region and at the C-terminus determined the up-regulation of C3. Pharmacologic inhibition of either ERK1/2 or SMAD3 phosphorylation decreased iron-induced C3 expression levels. Knockdown of SMAD3 blocked the iron-induced up-regulation and nuclear accumulation of C/EBP-δ, a transcription factor that has been shown previously to bind the basic Leucine Zipper 1 (bZIP1) domain in the C3 promoter. We show herein that mutation of this domain reduced iron-
induced C3 promoter activity. *In vivo* studies support our *in vitro* finding of iron-induced C3 upregulation. Mice with a mosaic pattern of RPE-specific iron overload demonstrated co-localization of iron-induced ferritin and C3d deposits. Humans with aceruloplasminemia causing RPE iron overload had increased RPE C3d deposition. The molecular events in the iron-C3 pathway represent therapeutic targets for AMD or other diseases exacerbated by iron-induced local complement dysregulation.

**Introduction**

Dysregulation of iron homeostasis may be an etiologic factor in several neurodegenerative disorders such as age-related macular degeneration (AMD) (Dunaief, 2006), although the detailed mechanisms by which iron acts as a driver of AMD-like pathology have not been reported. AMD is considered a chronic, localized inflammatory disease (Johnson et al., 2001; Zipfel et al., 2010) with a genetic component (Grassmann et al., 2014). Several genetic factors that confer major risk for AMD are polymorphisms within complement component genes (Edwards et al., 2005; Gold et al., 2006; Haines et al., 2005; Klein et al., 2005; Seddon et al., 2013; Yates et al., 2007). Since complement dysregulation can lead to inflammatory reactions (Markiewski and Lambris, 2007; Morgan et al., 2005), both genetic and environmental factors may work concurrently to determine the activity of the complement cascade in the outer-retinal milieu. AMD is characterized by primary pathology in the retinal pigment epithelium (RPE) monolayer and the formation of sub-RPE deposits called drusen. In human drusen samples, both C3 and C3 activation fragments were identified (Anderson et al., 2002). C3, the central molecule of the complement cascade that includes the classical, alternative, and lectin pathways, is activated by numerous inciting factors that require a rapid inflammatory reaction (Markiewski and Lambris, 2007; Sahu and Lambris, 2001). In AMD, the aberrant activation of the alternative pathway and the resultant release of inflammatory mediators are involved in drusen formation (Anderson et al., 2002). In the alternative pathway, C3 activation is under the control of Factor B,
a key pathway component, and Factor H, a potent negative regulator, among others (Chen et al., 2008; Pickering et al., 2002). Both Factor B and Factor H are expressed by the RPE cell (Chen et al., 2007, 2008; Coffey et al., 2007). Increased Factor B levels are accompanied by increased C3 expression and activation in aged mouse RPE (Chen et al., 2008). The interaction between C3 and Factor H is supported by the finding that C3b is a major physiological ligand of Factor H (Perkins et al., 2012). Furthermore, both C3 and Factor H are genetically implicated as contributory factors to AMD etiology (Klein et al., 2005; Yates et al., 2007).

Previous studies have shown elevated total iron levels in the RPE/Bruch’s membrane complex as well as photoreceptors in the human macula of post-mortem dry and wet AMD eyes compared to those of healthy controls (Hahn P et al., 2003). The iron-containing species in these samples identified by histochemical and chelation tests comprise both loosely bound, chelatable iron and non-chelatable iron components. The non-chelatable iron components may be iron derivatives that are incorporated into, and are essential for, the function of reactive oxygen species (ROS)-producing enzymes (Dixon and Stockwell, 2014). The chelatable iron is capable of directly generating ROS via Fenton chemistry. Evidence exists that ROS can in turn damage iron-containing proteins to produce more labile iron (Dixon and Stockwell, 2014; Flint et al., 1993), suggesting the interchangeability of non-chelatable and chelatable iron. Thus, it is reasonable to study the biological impact of an increased intracellular labile iron pool (LIP) on complement regulation in the RPE.

Our group has demonstrated previously increased RPE iron levels in mice with knockout of the \( \text{Cp} \) gene (\( \text{Cp}^{-/-} \)) and a naturally occurring \( \text{Sla} \) mutation in the \( \text{Heph} \) gene (\( \text{Heph}^{\text{Sla/Sla}} \) or \( \text{Heph}^{\text{Sla/Y}} \)), referred to as the double knockout (DKO) mouse model (Hadjiahmetovic et al., 2008b; Hahn et al., 2004a). When compared to the wild-type mice, these DKO mice exhibited some pathological
features of AMD, and the generalized high iron level in retina and RPE/choroid were associated with an accumulation of sub-RPE activated C3 fragments (Hadziahmetovic et al., 2008b). Our group has also shown that systemic administration of the iron chelator deferiprone decreased murine retinal C3 mRNA levels in a light-induced degeneration model (Song et al., 2012a) and RPE C3 mRNA levels in a sodium iodate-induced degeneration model (Hadziahmetovic et al., 2012a). These findings suggest that diminishing iron levels may reduce retinal complement expression. However, whether and how an increase in LIP within RPE cells is capable of triggering endogenous C3 activation are, as yet, unknown.

In cultured RPE cells, we found that iron exposure increases both C3 mRNA and protein levels. To understand the underlying molecular events, we first pursued a bioinformatics approach using a gene expression microarray, which identified the TGF-β signaling superfamily as the pathway(s) most strongly affected by iron. Interestingly, the TGF-β Receptor Type I gene, TGFBR1, is genetically associated with advanced AMD (Fritsche, 2013) and elevated urinary TGF-β1 protein levels are associated with early AMD (Guymer et al., 2011). In canonical TGF-β signaling, TGF-β ligands bind to receptor serine/threonine kinases that phosphorylate transcription factors SMAD2 and SMAD3 at the C-terminal SSXS motif (Feng and Derynck, 2005). While non-phosphorylated SMAD proteins shuttle in and out of the nucleus dynamically, upon phosphorylation, SMAD2/3 docks with SMAD4 and undergoes nuclear translocation. Once in the nucleus, this complex recruits co-factors to form a larger complex and differentially regulates target gene expression. In this study, we identified in cultured human RPE cells an iron-induced, non-canonical TGF-β pathway leading to C3 up-regulation. Corresponding in vivo studies showed that deposition of the C3 activation product C3d is spatially associated with iron overloaded RPE cells.
Experimental procedures

**Cell Culture and Cell Treatment Reagents**—ARPE-19 cells from the American Type Culture Collection (ATCC, Manassas, VA) were cultured in 1:1 DMEM/F12 (Invitrogen, Grand Island, NY) supplemented with 10% FBS (HyClone, Logan, UT). Once confluent, cells were maintained in medium with 1% FBS for 4 weeks prior to experiments to obtain mature monolayers (Kunchithapautham et al., 2014). One day prior to experiments, cells were placed in serum-free medium to deplete residual serum complement components. Iron in the form of Ferric Ammonium Citrate (FAC; MP Biomedicals, Santa Ana, CA), dissolved in serum-free medium, was used to treat cells for the indicated times. Alamar Blue reagent for cell viability was from Invitrogen. Transition metals suitable for cell culture were from Sigma (St. Louis, MO). Purified apo- and holo-Transferrin (Tf) were from Millipore (Billerica, MA). Expression plasmids pCS2 FLAG-SMAD3 (Kretzschmar et al., 1999), pCS2 FLAG-SMAD3 (EPSM) (Kretzschmar et al., 1999), and pCS2 FLAG SMAD3 EPSMA213S (Gao et al., 2009) were gifts from Joan Massagué (Addgene plasmids #14052, 14963, 27113). Pharmacologic inhibitors, recombinant proteins, and neutralizing antibodies were obtained as follows: PD98059, U0126, SB202190, SP600125, and human recombinant TGF-β1, β2, β3 (Cell Signaling, Danvers, MA); SIS3 (Millipore); SB431542 (Tocris, Minneaples, MN); anti-TGF-β1/2/3 antibody and isotype control (R&D Systems, Minneapolis, MN).

**RNA Extraction, Quantitative RT-PCR, Microarray Processing and Data Analysis**—Total RNA was isolated using QIAzol® reagent and the miRNeasy Mini Kit from Qiagen (Valencia, CA). Quantitative reverse transcriptase-PCR (qRT-PCR) using the standard ΔΔCt method was performed using Taqman primers (Applied Biosystems, Waltham, MA) listed in Table 2.1, with 18S rRNA as the internal control. Microarray processing and data analysis services were provided by the Penn Molecular Profiling Facility using the Affymetrix GeneChip® Human Gene 2.0 ST Array (Affymetrix, Santa Clara, CA). For each group, untreated and FAC-treated, three
Table 2.1 Taqman® qRT-PCR primers

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Symbol</th>
<th>Catalog number</th>
</tr>
</thead>
<tbody>
<tr>
<td>complement component 3 (human)</td>
<td>C3</td>
<td>Hs01100879_m1</td>
</tr>
<tr>
<td>transferrin receptor (human)</td>
<td>TFRC</td>
<td>Hs00174609_m1</td>
</tr>
<tr>
<td>plasminogen activator inhibitor type 1 (PAI-1; human)</td>
<td>SERPINE1 (PAI-1)</td>
<td>Hs01126607_g1</td>
</tr>
<tr>
<td>transforming growth factor, beta 1 (human)</td>
<td>TGFB1</td>
<td>Hs00998133_m1</td>
</tr>
<tr>
<td>CCAAT/enhancer binding protein (C/EBP), delta (human)</td>
<td>CEBPD</td>
<td>Hs00270931_s1</td>
</tr>
</tbody>
</table>
independent arrays were performed, probing >40,000 Transcript IDs from more than 24,800 genes. Processing steps were conducted as described in the Ambion WT Expression Manual and the Affymetrix GeneChip Expression Analysis Technical Manual. For data analysis, probe intensity (.cel) files were imported into Partek Genomics Suite (v6.6, Partek Inc., St. Louis, MO), where RMA normalization was applied yielding log2-transformed intensities. These values were tested for differential expression using Significance Analysis for Microarrays (SAM; samr v2.0, Stanford University) (Tusher et al., 2001), yielding a fold-change and q-value (false discovery rate) for each transcript. For a transcript to be considered for input into DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov; accessed February 2015) for pathway analysis, the thresholds of fold-change ≥1.5 (up or down) and q-value ≤10% were applied. The KEGG and BioCarta pathway mapping databases as well as GOTERM_BP_FAT biological processes database were included within the analysis. The top pathways/processes were determined by setting a corrected false discovery rate (Benjamini-Hochberg) <10%. The complete array dataset can be accessed in the Supplemental Table at the following URL:
http://www.jbc.org/content/early/2015/03/23/jbc.M115.645903/suppl/DC1.

Whole Cell Protein Extraction, Cell Compartment Protein Extraction and Western Blotting — Whole cell protein lysates were extracted using Laemmli SDS Lysis Buffer supplemented with Protease/Phosphatase inhibitor cocktail and PMSF (Cell Signaling) according to standard methods. Lysates were sonicated for 10s prior to quantification with the 660nm protein assay (Pierce, Rockford, IL). For each sample, 30 µg of protein were loaded onto a 4-12% Bis-Tris gel for Western blotting. Non-nuclear (cytoplasmic, membranous, cytoskeletal) and nuclear protein lysates were extracted using the Qproteome® cell compartment kit (Qiagen) according to manufacturer’s instructions. Primary antibodies used are as follows: anti-p-ERK1/2, anti-ERK1/2, anti-p-SMAD2 (S245/250/255), anti-GAPDH, and anti-α-tubulin clone 11H10 (Cell Signaling); anti-SMAD3, anti-FLAG M2, and anti-Actin AC-40 (Sigma); anti-p-SMAD3 (S213) (Assay Biotech,
Sunnyvale, CA); anti-Transferrin Receptor (TFRC; Invitrogen); anti-p-SMAD3 (S423/425) and anti-Lamin B1 (Abcam, Cambridge, MA); anti-C/EBP-δ (clone L46–743.92.69) was provided by BD Biosciences Pharmingen (San Jose, CA) as an outcome of an Antibody Co-development Collaboration with the NCI. Secondary antibodies IRDye® 680RD Donkey anti-Rabbit and 800CW Donkey anti-Mouse (LI-COR Biosciences, Lincoln, NE) were used in combination when possible for two-channel infrared detection with an Odyssey imager. ImageJ Software 1.46r was used for band densitometry.

**Knockdown of SMAD3 using Lentiviral Vectors**—This was performed per MISSION shRNA’s instructions (Sigma). Briefly, ARPE-19 cells were seeded overnight to 70% confluence prior to treatment with lentiviral transduction particles (Sigma) that express shRNA targeting SMAD3 transcripts (sh-SMAD3). All particles used, including controls are listed in Table 2.2. Cells were used for qRT-PCR 72h post-transduction, without selection or puromycin was used to select for stably transduced cells for 2-3 passages prior to qRT-PCR and Western blotting.

**Plasmid Construction, Transfection and Dual Luciferase Assays**—DNA fragments consisting of four different sized, overlapping sequences of the C3 gene promoter, flanked by 5’ and 3’ MluI and BglII sites, respectively, were amplified by the Q5® High-Fidelity DNA Polymerase from New England BioLabs (NEB, Ipswich, MA). The fragments, which are approximately 500 bp (-481 to +52), 1.0 kb (-1078 to +52), 1.5 kb (-1555 to +52), and 2.0 kb (-2047 to +52), were cloned into the pCR-Blunt II TOPO vector using the Zero Blunt TOPO kit (Life Technologies, Grand Island, NY) according to the product instructions. The bZIP1 and bZIP2 mutant fragments, with the same base substitutions as previously reported (Juan et al., 1993), were generated using the Q5® Site-Directed Mutagenesis Kit (NEB). All primers are listed in Table 2.3. Once sequence-verified, the fragments were sub-cloned into the pGL3-Basic Vector (Promega, Madison, WI) for luciferase assays. ARPE-19 cells at 80% confluence, grown in complete medium with no antibiotics, were transfected with either empty or promoter-inserted pGL3 vector using the reagent Lipofectamine.
### Table 2.2 Sigma MISSION® lentiviral transduction particles

<table>
<thead>
<tr>
<th>Product name</th>
<th>Abbreviation used</th>
<th>Catalog number</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLKO.1-puro empty vector control</td>
<td>Vector</td>
<td>SHC001V</td>
</tr>
<tr>
<td>pLKO.1-puro non-mammalian shRNA control</td>
<td>sh-Null</td>
<td>SHC002V</td>
</tr>
<tr>
<td>pLKO.1-puro-CMV-TurboGFP™ Positive Control</td>
<td>t-GFP (not shown)</td>
<td>SHC004V</td>
</tr>
<tr>
<td>pLKO.1-puro SMAD3 shRNA</td>
<td>sh-SMAD3</td>
<td>SHCLNV-NM_005902 TRCN0000330127</td>
</tr>
</tbody>
</table>

### Table 2.3 PCR primers used to generate C3 promoter fragments

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 bp/WT</td>
<td>ACGTACCGCCTAGTTTCTGTGTGGGCTC</td>
<td>ACGTAGATCTGACAGTGAGGGGCAGAG</td>
</tr>
<tr>
<td>1 kb</td>
<td>ACGTACCGGTCTACTTTGGGTGTTGAGC</td>
<td>ACGTAGATCTGACAGTGAGGGGCAGAG</td>
</tr>
<tr>
<td>1.5 kb</td>
<td>ACGTAGCGTGAGGGCGAGAATTGTTT</td>
<td>ACGTAGATCTGACAGTGAGGGGCAGAG</td>
</tr>
<tr>
<td>2 kb</td>
<td>ACGTACCGGTGCAGGGCTCATAAGTGTG</td>
<td>ACGTAGATCTGACAGTGAGGGGCAGAG</td>
</tr>
<tr>
<td>500 bp/bZIP1</td>
<td>AATGGTTATTTGTCTGACTGCGGAGCC</td>
<td>TCCTAAGCTTTTCAGTCC</td>
</tr>
<tr>
<td>500 bp/bZIP2</td>
<td>GAAAAGCTTATCGCGAGGTATTGAGAAATCTGGGGGCAGCCCCAAAAGG</td>
<td>AGTCCCTGGGGGCAACAT</td>
</tr>
</tbody>
</table>
LTX (Life Technologies) at 2:1 LTX:DNA ratio for 4h before initiation of FAC treatment.

Luciferase activity was then determined with the Dual-Luciferase reporter assay system kit (Promega) according to manufacturer’s protocols. Luminescence was read for 1.5s by an Infinite M200 Pro plate reader (Tecan Systems, Morrisville, NC).

**ELISA**—Detections of secreted C3 precursor protein (Abcam), C3a (BD Biosciences) and Factor B fragment Ba (Quidel, San Diego, CA) in ARPE-19 cell culture conditioned media were performed per each manufacturer’s instructions. The conditioned media used for C3 assay did not require concentration, but those for C3a and Ba required an approximately 70-fold concentration for detection in the standard range. Amicon Ultra-15 centrifugal filters with a 3 kDa molecular weight cutoff membrane (Millipore) were used for media concentration.

**Immunofluorescence of Human Aceruloplasminemia Retina Paraffin Sections and Mouse Retinal Cryosections**—Ocular tissue from a 60 year-old male donor with aceruloplasminemia was prepared as paraffin sections, as described in a previous publication, in adherence to the tenets of the Declaration of Helsinki (Wolkow et al., 2011). The paraffin sections were stained with anti-human C3d antibody (1:100, Abcam). Generation of the systemic ceruloplasmin null, bestrophin promoter-driven cre recombinase, hephaestin floxed/floxed mouse on a C57BL/6 background (Bcre+, Cp−, HephF/F) has also been described in a previous publication (Wolkow et al., 2012). Background-matched C3 null (C3−) mice were provided by Wenchao Song (University of Pennsylvania). All mice were handled in accordance with the Institutional Animal Care and Use Committee of the University of Pennsylvania. Retina cryo-sections of 10 μm thickness derived from 12 month old-mice were used for immuno-labeling studies. The primary antibodies used were rabbit anti-L-ferritin clone F-17 at a 1:100 dilution (generous gift from Paolo Arosio, Università Degli Studi di Brescia, Brescia, Italy) and goat anti-mouse C3d at a 1:100 dilution.
(R&D Systems). Slides were examined on a Nikon Eclipse 80i microscope, and images were acquired using NIS-BR Elements v4.1 software (Nikon, Melville, NY).

**Statistics**—All experiments were performed in at least triplicate, with mean ± SEM reported for each comparison group. The means were analyzed using either a two-tailed Student’s t-test or a one-way ANOVA, followed by a Bonferroni post hoc test (Prism 5.0; GraphPad Software, San Diego, CA).

**Results**

**Iron induced the expression of endogenous C3 in ARPE-19 cell**

In a previous publication we have shown that photo-oxidative stress in mouse RPE cells can lead to elevated C3 mRNA levels (Hadziahmetovic et al., 2012b). These changes are accompanied by altered expression of iron regulatory proteins. More directly, a mouse model of retinal iron overload, *Cp*−/− *Heph* Δa, exhibited deposition of activated C3 fragments in the basolateral RPE and Bruch’s membrane (Hadziahmetovic et al., 2008b). Since C3 is a central player in the complement cascade, here we investigated if iron can specifically induce C3 transcription and translation in the RPE cell. Using the human ARPE-19 cell line differentiated in low serum for one month, then cultured in serum-free medium, we determined that the viability of these cells decreased by 30% but remained stable at all iron (ferric ammonium citrate; FAC) doses in the 25 µM to 1 mM range (**Fig. 2.1A**). In this same analysis, transferrin receptor (TFRC) mRNA levels decreased by at least 50% in the same dose range (**Fig. 2.1B**), indicating an increase of the LIP and an intact iron regulatory axis. Concurrently, the C3 mRNA levels increased by 3 to 4-fold relative to untreated cells (**Fig. 2.1C**). In a corresponding experiment, the C3 protein levels in the conditioned medium were found to increase 2 to 3-fold within the range of FAC used (**Fig. 2.1D**). Renewal of culture medium and continued treatment with FAC beyond 2d (i.e. 4d and 8d),
Figure 2.1A Viability of cells grown as one-month differentiated monolayers and treated with FAC at increasing doses for 2d. Viability is decreased with 25μM iron treatment compared to untreated but remains stable for higher doses examined. Data are expressed as mean ± SEM (error bars), with n ≥3 using the following statistical notations: * $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$; these notations are consistent in all subsequent figures within Chapter 2.
**Figure 2.1B** TFRC mRNA with increasing FAC treatment doses for 2d. The mRNA levels are decreased with 25µM iron treatment compared to untreated but remain stable for higher doses examined.
Figure 2.1C C3 mRNA levels with increasing FAC treatment doses for 2d. The C3 mRNA levels are increased at the lowest iron dose relative to untreated but remain stable at the higher doses.
Figure 2.1D C3 protein levels increase, relative to untreated, in the conditioned medium of cells treated with increasing doses of FAC for 2d.
resulted in further increases in C3 mRNA levels up to 20-fold (Fig. 2.1E). Yet, it was determined that only treatment with FAC, but not other transition metals (Mn$^{2+}$, Ni$^{2+}$, and Cu$^{2+}$), induced increases in C3 mRNA levels relative to untreated (Fig. 2.1F). C3 mRNA up-regulation was also observed in cells treated with holo-transferrin (holo-Tf), the extracellular iron carrier protein. The levels increased by ~2.5-fold (Fig. 2.1G), while TFRC mRNA levels decreased by ~40% (Fig. 2.1H). Overall, these data delineate a specific, iron-induced C3 gene response within the RPE cell.

Pathway enrichment analysis implicates TGF-β signaling in iron-induced C3 expression

To characterize the potential molecular pathway(s) affected by iron treatment and identify the one(s) responsible for C3 induction, we performed a whole genome microarray on untreated ARPE-19 cells and cells treated with 250 µM FAC for 48h/2d (Supplemental Table). Iron loading resulted in 95 transcripts with up-regulated expression and 122 transcripts with down-regulated expression of magnitude ≥1.5-fold and q <10%. After eliminating non-annotated entries, we derived a list of 70 up-regulated and 87 down-regulated transcripts (total 157), of which 9 up-regulated ones and 7 down-regulated ones are of magnitude ≥2.0-fold (Table 2.4). Both C3 and TFRC are among these “top hits” (Table 2.4), validating our results above in Fig. 2.1A and 2.1B. Using the list of 157 transcripts with expression change magnitude ≥1.5-fold as input into the program DAVID, we generated another list of “pathways” and “biological processes” that best represent these transcripts. The entry “TGF-β signaling” topped this list, fitting within the constraint of false-discovery rate (Benjamini-Hochberg) <10%. Subsequent entries do not meet this statistical significance cutoff.
Figure 2.1E C3 mRNA levels are increased relative to control cells at each of the 2d, 4d, and 8d time points after the initial 250µM FAC treatment.
Figure 2.1F C3 mRNA levels of cells treated with Fe$^{3+}$, Mn$^{2+}$, Ni$^{2+}$, or Cu$^{2+}$ at 250µM concentration for 2d, with only Fe$^{3+}$/FAC showing an increase in C3 mRNA relative to untreated. Cell viabilities for cells treated with the other transition metals were similar to those for Fe$^{3+}$. 
Figure 2.1G and H C3 mRNA levels of cells treated with 9.75 mg/ml holo-Tf are increased relative to those of apo-Tf-treated cells, with a corresponding decrease in TFRC mRNA.
Table 2.4. The most up-regulated and down-regulated annotated transcripts in ARPE-19 cells treated with FAC

<table>
<thead>
<tr>
<th>Transcript ID</th>
<th>RefSeq</th>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Fold change (log2)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>16955939</td>
<td>NM_015541</td>
<td>leucine-rich repeats and immunoglobulin-like domains 1</td>
<td>LRIG1</td>
<td>2.69089</td>
</tr>
<tr>
<td>16911261</td>
<td>NM_001200</td>
<td>bone morphogenetic protein 2</td>
<td>BMP2</td>
<td>2.44466</td>
</tr>
<tr>
<td>16996813</td>
<td>NM_005582</td>
<td>CD180 molecule</td>
<td>CD180</td>
<td>2.30087</td>
</tr>
<tr>
<td>17089003</td>
<td>NM_182487</td>
<td>olfactomedin-like 2A</td>
<td>OLFML2A</td>
<td>2.24716</td>
</tr>
<tr>
<td>16750996</td>
<td>NM_020039</td>
<td>amiloride-sensitive cation channel 2, neuronal</td>
<td>ACCN2</td>
<td>2.18511</td>
</tr>
<tr>
<td>16867784</td>
<td>NM_000064</td>
<td>complement component 3</td>
<td>C3</td>
<td>2.18391</td>
</tr>
<tr>
<td>16829801</td>
<td>NM_001124758</td>
<td>spinster homolog 2 (Drosophila)</td>
<td>SPNS2</td>
<td>2.18123</td>
</tr>
<tr>
<td>17096285</td>
<td>NM_001333</td>
<td>cathepsin L2</td>
<td>CTSL2</td>
<td>2.16549</td>
</tr>
<tr>
<td>16785938</td>
<td>NM_015351</td>
<td>tetratricopeptide repeat domain 9</td>
<td>TTC9</td>
<td>2.09855</td>
</tr>
</tbody>
</table>

Transcripts with down-regulated expression

<table>
<thead>
<tr>
<th>Transcript ID</th>
<th>RefSeq</th>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Fold change (log2)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>17056984</td>
<td>NM_002192</td>
<td>inhibin, beta A</td>
<td>INHBA</td>
<td>-2.02469</td>
</tr>
<tr>
<td>16907639</td>
<td>NR_036227</td>
<td>microRNA 2355</td>
<td>MIR2355</td>
<td>-2.07836</td>
</tr>
<tr>
<td>16976599</td>
<td>NM_014465</td>
<td>sulfotransferase family, cytosolic, 1B, member 1</td>
<td>SULT1B1</td>
<td>-2.11595</td>
</tr>
<tr>
<td>16912362</td>
<td>NM_181353</td>
<td>inhibitor of DNA binding 1, dominant negative helix-loop-helix protein</td>
<td>ID1</td>
<td>-2.96384</td>
</tr>
<tr>
<td>16963241</td>
<td>NM_003234</td>
<td>transferrin receptor (p90, CD71)</td>
<td>TFRC</td>
<td>-3.07206</td>
</tr>
<tr>
<td>16683377</td>
<td>NM_002167</td>
<td>inhibitor of DNA binding 3, dominant negative helix-loop-helix protein</td>
<td>ID3</td>
<td>-4.0267</td>
</tr>
<tr>
<td>17084723</td>
<td>NM_001216</td>
<td>carbonic anhydrase IX</td>
<td>CA9</td>
<td>-4.35892</td>
</tr>
</tbody>
</table>

²Data are relative to control, untreated cells.
Iron-induced C3 up-regulation is independent of TGF-β ligands

To determine whether TGF-β ligands drive C3 up-regulation following iron treatment, we incubated ARPE-19 cells with human recombinant TGF-β1, TGF-β2, or TGF-β3. By qRT-PCR analysis, TGF-β1 (Fig. 2.2A), and to a lesser degree TGF-β3 (data not shown) showed significant down-regulation of C3 mRNA levels, whereas TGF-β2 showed no significant difference when compared to untreated control (data not shown). These results suggest that the ligands, via canonical TGF-β pathway activation, do not account for the increase in C3 mRNA levels observed with iron loading. The significant up-regulation of specific downstream target genes of these ligands, such as PAI-1 in the case of TGF-β1 (Fig. 2.2A), demonstrated that the canonical TGF-β pathway is intact and that TGF-β receptor blockade using SB431542 is efficacious. We then investigated the levels of TGFB1 mRNA and TGF-β1 protein under untreated and FAC-treated conditions and found that mRNA levels were increased slightly by ~1.3-fold, but the secreted protein levels were moderately but significantly decreased, by ~20% (Fig. 2.2B). This led to the hypothesis that basal TGF-β levels, though suppressed by FAC, are important for regulating C3 mRNA levels. To test this hypothesis, we treated cells with anti-TGF-β1/2/3 neutralizing antibody and found that C3 mRNA levels were increased by ~1.7-fold relative to control conditions and PAI-1 mRNA levels were decreased by ~60% in the same comparison (Fig. 2.2C). We further asked whether basal TGF-β activity affects the degree of FAC-induced C3 up-regulation. To determine this, we co-treated anti-TGF-β1/2/3 antibody and FAC, which showed no significant difference in C3 mRNA levels compared to FAC only (~2.5-fold over untreated) (Fig. 2.2D). PAI-1 mRNA levels were repressed by FAC though not further lowered by neutralizing antibody (Fig. 2.2D).

Iron-induced C3 up-regulation involves ERK1/2 and SMAD3 non-canonical TGF-β signaling

Iron has been shown previously to induce ERK1/2 phosphorylation in rat hippocampal neurons and mouse neurosensory retina (Hadziahmetovic et al., 2011a; Muñoz et al., 2011). To examine
Figure 2.2A C3 mRNA levels in ARPE-19 cells are repressed by exogenous TGF-β1 (10 ng/ml; 2d) and this inhibition is partially relieved by TGF-β receptor inhibitor SB431542 (100 nM; 2d). In contrast, PAI-1 mRNA levels are significantly increased by TGF-β1 and this increase is partially inhibited by SB431542.
Figure 2.2B TGFB1 mRNA levels show an increase with FAC treatment; TGF-β1 protein levels in the conditioned medium of FAC-treated cells show a significant decrease relative to untreated.
Figure 2.2C C3 mRNA levels are increased with addition of neutralizing antibody anti-TGF-β1/2/3 (10µg/ml; 2d) when compared to control or IgG-treatment conditions. PAI-1 mRNA levels are decreased in the same comparison.
**Figure 2.2D** C3 mRNA levels are not significantly changed with co-treatment of anti-TGF-β1/2/3 antibody and FAC when compared to FAC only. PAI-1 mRNA levels are decreased to similar levels with co-treatment of anti-TGF-β1/2/3 antibody and FAC compared to FAC only.
if ERK1/2 plays a role in iron induction of C3, we pre-treated ARPE-19 cells with PD98059, a pharmacologic inhibitor of MEK1/2, the kinase upstream of ERK1/2, followed by FAC treatment. This inhibitor was able to block FAC-induced C3 up-regulation (**Fig. 2.3A**). Another inhibitor that functions by a similar mechanism, U0126, achieved the same effect (data not shown). In contrast, inhibitors to the two other MAPK classes, SB202190 against p38 and SP600125 against JNK1/2/3, did not suppress FAC-induced C3 up-regulation (**Fig. 2.3A**).

ERK1/2 can phosphorylate the linker region residues of SMAD2/3, central components in the TGF-β signaling pathway (Bae et al., 2012; Hough et al., 2012; Kamato et al., 2014; Matsuura et al., 2005; Wang et al., 2009). In the present study, FAC did not induce linker region phosphorylation of SMAD2 (data not shown). Thus, to assess whether SMAD3 plays a direct role in C3 induction, we co-administered a specific inhibitor of SMAD3 function, SIS3 (Jinnin et al., 2006), with FAC, and found that this compound suppressed the up-regulation (**Fig. 2.3B**). SIS3 alone also decreased C3 mRNA levels relative to the untreated baseline (**Fig. 2.3B**), suggesting that the inhibitor modulates basal C3 expression. Furthermore, ARPE-19 cells co-treated with SIS3 or PD98059 and FAC showed diminished levels of C3 protein in the conditioned media compared to cells treated with FAC alone (**Fig. 2.3C**). Expression of TGF-β1-responsive gene PAI-1 was inhibited by SIS3, but not by PD98059. The FAC-induced repression of PAI-1 mRNA levels was relieved by PD98059 (**Fig. 2.3D**). To determine the temporal sequence of ERK1/2 phosphorylation and SMAD3 activity, we treated cells with FAC for different lengths of time (0, 1, 3, 6h) in the absence and presence of SIS3 and found no difference in p-ERK1/2 levels by Western blotting at each of the time points examined (**Fig. 2.3E**). In contrast, PD98059 inhibited FAC-induced SMAD3 linker phosphorylation at S213 (see below, **Fig. 2.4C**). These findings place ERK1/2 upstream of SMAD3 in an FAC-induced signaling pathway.
Figure 2.3A C3 mRNA levels in ARPE-19 cells treated with MEK1/2 inhibitor PD98059 (5 µM; 2d), p38 inhibitor SB202190 (20 µM; 2d), or JNK1/2/3 inhibitor SP600125 (50 µM; 2d) alone and plus FAC. Control contains vehicle only (DMSO). Relative to FAC alone, only PD98059 showed a significant decrease in C3 mRNA levels when co-treated with FAC.
Figure 2.3B The specific inhibitor of SMAD3, SIS3 (2 µM; 2d), inhibited FAC-induced C3 up-regulation. SIS3 decreased basal C3 mRNA levels.
Figure 2.3C Iron-induced increases in C3 protein levels in the conditioned media are diminished by SIS3 and PD98059.
Figure 2.3D SIS3 inhibited PAI-1 mRNA levels to the same extent as FAC, while PD98059 had no significant effect. Co-treatment with SIS3 and FAC showed no additional suppression of PAI-1 mRNA levels, but co-treatment with PD98059 and FAC restored them to baseline.
Figure 2.3E Western blot and densitometry analysis for p-ERK1/2 and ERK1/2 in lysates derived from cells at different time points (0, 1, 3, 6h) post-FAC treatment, with or without SIS3 co-treatment. SIS3 showed no effect on the FAC-induced increase of p-ERK1/2 at 1h.
Iron-induced changes in SMAD3 phosphorylation are distinct from canonical TGF-β effects

Since C3 up-regulation involves SMAD3 activity, we probed the phosphorylation status of SMAD3 following iron treatment. Using phospho-specific antibodies directed against a linker region residue (S213) and the C-terminal residues (S423/425), we found two directly opposite effects: p-SMAD3 (S213), shown as a 150 kDa band, gradually increased at time points up to 48h/2d, while p-SMAD3 (S423/425) decreased in the same time course (Fig. 2.4A). The p-SMAD3 (S213) antibody-detectable 52 kDa band remained at baseline levels. TFRC protein levels diminished as a reflection of increasing LIP (Fig. 2.4A). To test the specificity of the anti-p-SMAD3 (S213) antibody in Western blotting (Fig. 2.4B), we transfected cells with a construct expressing FLAG-SMAD3 and detected a band at 54 kDa. We also transfected a construct expressing FLAG-SMAD3 EPSM (with mutated linker region phosphorylation sites) and detected virtually no band. Finally, we transfected a construct expressing FLAG-SMAD3 EPSM A213S (with residue 213 reverted back to Serine) and detected the band once again. This, together with SMAD3 shRNA data to be shown below (Fig. 2.6A), suggest that the 150 kDa band detected in un-transfected cells contains p-SMAD3 (S213). Western blots also showed that the SMAD3 inhibitor SIS3 and MEK1/2 inhibitor PD98059 can suppress iron-induced increases in p-SMAD3 (S213) levels, supporting further the model that ERK1/2 and SMAD3 are in the same signaling pathway (Fig. 2.4C). Of note, p-SMAD3 (S213) levels decreased with SIS3 treatment compared to the untreated baseline. This finding correlates with decreased C3 mRNA levels observed in the same comparison in Fig. 2.3B. In addition, SIS3 can suppress basal p-SMAD3 (S423/425) levels to the same extent as FAC alone while PD98059 showed no such effect on the phosphorylation of these C-terminal residues (Fig. 2.4C).

To gain insight into the role of endogenous TGF-β on the regulation of p-SMAD3 (S213) and p-SMAD3 (S423/425), we treated cells with BSA, IgG or anti-TGF-β1/2/3 antibody with or without FAC. Anti-TGF-β1/2/3 antibody alone dramatically decreased basal, TGF-β-sustained p-SMAD3
Figure 2.4A Western blot and densitometry analysis of ARPE-19 cell lysates for p-SMAD3 (S213), p-SMAD3 (S423/425), SMAD3, and TFRC in a FAC treatment time course experiment. The p-SMAD3 (S213) 150 kDa band increased while the p-SMAD3 (S423/425) and TFRC bands decreased in intensity in a time-dependent manner. The p-SMAD3 (S213) antibody-detectable 52 kDa band showed no significant change in the same time course. The SMAD3 protein levels also remain unchanged.
Figure 2.4B Cells transfected with constructs expressing FLAG-tagged SMAD3, SMAD3 EPSM (linker mutations), and SMAD3 EPSM A213S (mutation reverted at residue 213) showed specificity of the antibody used to detect p-SMAD3 (S213) in Western blots.
Figure 2.4C Western blot and densitometry analysis of p-SMAD3 (S213) showed that co-treatment with SIS3 or PD98059 and FAC reduced the intensity of this band relative to FAC alone. SIS3 decreased the basal p-SMAD3 (S213) levels. Blot and analysis of p-SMAD3 (S423/425) demonstrated that SIS3 reduced band intensity to the same extent as FAC alone, while PD98059 had no significant effect. Co-treatment with either inhibitor with FAC showed no significant difference with FAC alone.
In the presence of this antibody, iron retained its ability, albeit less, to induce formation of the p-SMAD3 (S213) complex. Anti-TGF-β1/2/3 antibody alone also decreased p-SMAD3 (S423/425) levels, as expected, while iron treatment potentiated this suppressive effect. In contrast, cells treated with exogenous TGF-β1 for 1h demonstrated no significant difference in p-SMAD3 (S213) levels but up-regulated p-SMAD3 (S423/425), consistent with canonical signaling effects (Fig. 2.5B). This increase returned to basal level at 2d (data not shown).

To verify that the 150 kDa band does contain SMAD3 and more specifically, p-SMAD3 (S213), we transduced ARPE-19 cells with lentiviral constructs expressing shRNA to stably knockdown SMAD3 protein (Fig. 2.6A). With SMAD3 knockdown (sh-SMAD3), the iron-induced increase in intensity of the 150 kDa band as detected by anti-p-SMAD3 (S213) is diminished, unlike in the control cell lines un-transduced, vector, and sh-null, which are shown in the same panel (Fig. 2.6A). In addition, qRT-PCR measurement of C3 mRNA levels in sh-SMAD3 cells revealed a lack of FAC-induced up-regulation, which was present in the three control cell lines (Fig. 2.6B).

To determine whether the p-SMAD3 (S213)-containing protein complex (150 kDa) undergoes nuclear translocation, we performed cellular fractionation on un-transduced cells with or without FAC treatment. This band, regardless of iron treatment, localized only to the non-nuclear fraction, suggesting that the complex does not translocate to the nucleus (Fig. 2.6C). In addition, the p-SMAD3 (S213) antibody-detectable 52 kDa protein is not induced by FAC in either the non-nuclear or the nuclear compartment (Fig. 2.6C).

Iron-induced increases in CEBPD mRNA levels and C/EBP-δ protein levels in both the non-nuclear and nuclear compartments are dependent on SMAD3 activity

It has been reported that SMAD3 physically interacts with C/EBP-δ and inhibits the transcription of C/EBP target genes important for adipocyte differentiation (Choy and Derynck, 2003). To
**Figure 2.5A** Western blot and densitometry analysis of p-SMAD3 (S213) and (S423/425) in cells treated with BSA, IgG, or anti-TGF-β1/2/3 antibody in the presence or absence of FAC showed the combined effect of iron and neutralization of canonical TGF-β signaling. SMAD3 protein levels increased after anti-TGF-β1/2/3 antibody treatment relative to control condition, BSA with no FAC. Only the antibody plus FAC condition is statistically significant.
Figure 2.5B Western blot and densitometry analysis following treatment with exogenous TGF-β1 (10 ng/ml; 1h) showed no significant change in p-SMAD3 (S213) and increased p-SMAD3 (S423/425).
Figure 2.6A Western blot and densitometry analysis for SMAD3, p-SMAD3 (S213), and TFRC of ARPE-19 cell lysates derived from un-transduced (control), vector, sh-null, and sh-SMAD3 lines, each in the absence or presence of FAC. Efficient knockdown of SMAD3 decreased the formation of p-SMAD3 (S213)-containing complexes at 150 kDa.
Figure 2.6B The FAC-induced increase in C3 mRNA levels is observed in the un-transduced, vector, and sh-null cell lines but is absent in the sh-SMAD3 cell line.
Figure 2.6C Western blot for non-nuclear and nuclear lysates of untreated and FAC-treated cells. The p-SMAD3 (S213)-containing complex at 150 kDa localize only to the non-nuclear fractions. The p-SMAD3 (S213) antibody-detectable band at 52 kDa is essentially unchanged by FAC treatment in both the non-nuclear and nuclear fractions.
probe the regulatory relationship between iron, SMAD3, and C/EBP-δ, we measured CEBPD mRNA levels and found that FAC up-regulated CEBPD. Co-treatment with SIS3 and FAC significantly decreased these levels (Fig. 2.7A). This finding posits a crucial role for SMAD3 in FAC-induced CEBPD gene regulation. In testing TGF-β-mediated basal CEBPD regulation, we found that anti-TGF-β1/2/3 antibody alone increased CEBPD mRNA levels by a modest ~1.3-fold relative to control (Fig. 2.7B). FAC increased CEBPD mRNA levels by ~2-fold, with co-treatment of FAC and anti-TGF-β1/2/3 antibody not significantly different from FAC alone (Fig. 2.7C). The trends in these neutralizing antibody experiments correlate with those found when measuring C3 mRNA levels (Fig. 2C, 2D).

FAC also increased non-nuclear and nuclear C/EBP-δ protein levels in a time-dependent manner (Fig. 2.7D). Next, we aimed to understand SMAD3’s role in iron-induced C/EBP-δ nuclear accumulation by comparing nuclear C/EBP-δ levels in the absence or presence of FAC for the sh-SMAD3 and control cell lines. We showed that SMAD3 knockdown blocked the iron-induced increase within the nuclear as well as the non-nuclear compartments (Fig. 2.7E). Interestingly, knockdown of SMAD3 elevated basal C/EBP-δ protein levels in both compartments in the absence of iron stimulus. In agreement with protein quantitation, CEBPD mRNA levels were increased in both the puromycin-selected (Fig. 2.7E) and unselected sh-SMAD3 cells relative to controls (data not shown). Taken together, these results suggest that SMAD3 mediates FAC-induced increases in both CEBPD mRNA and C/EBP-δ protein in the non-nuclear and nuclear compartments.

Iron can effect transcriptional up-regulation of C3 via the bZIP1 domain of the C3 promoter
It has been reported that C/EBP-δ binds to the bZIP1 domain of the C3 promoter following stimulation by IL-1 (Juan et al., 1993; Maranto et al., 2008; Wilson et al., 1990). To determine if
Figure 2.7A CEBPD mRNA levels in ARPE-19 cells are decreased by co-treatment with SIS3 and FAC compared to FAC alone, which is increased relative to control.
Figure 2.7B CEBPD mRNA levels are increased with addition of neutralizing antibody anti-TGF-β1/2/3 (10µg/ml; 2d) when compared to control or IgG-treatment conditions.
Figure 2.7C CEBPD mRNA levels are elevated with FAC treatment, but not significantly changed with co-treatment of anti-TGF-β1/2/3 antibody and FAC.
**Figure 2.7D** Western blot and densitometry analysis for C/EBP-δ in non-nuclear and nuclear lysates of cells treated with FAC in a time course. C/EBP-δ protein levels in the non-nuclear fraction show a time-dependent increase. Nuclear accumulation of C/EBP-δ occurs with the duration of FAC treatment.
Figure 2.7E Knockdown of SMAD3 in sh-SMAD3 cells impaired FAC-induced increases in nuclear C/EBP-δ when compared to sh-null and un-transduced cells. Basal C/EBP-δ levels increased in the sh-SMAD3 cell line relative to the controls.
Iron can similarly stimulate the C3 promoter, we generated a set of luciferase constructs harboring 4 overlapping but discretely sized C3 promoter fragments and transfected them into ARPE-19 cells (Fig. 2.8A). Cells transfected with each of the four fragments, approximately 500 bp, 1 kb, 1.5 kb, or 2.0 kb in length relative to the C3 transcription start site, responded to iron treatment with increased Firefly luciferase activity, while Renilla luciferase, serving as transfection control, showed no significant difference between with or without iron conditions (Fig. 2.8A). In a follow-up luciferase assay, cells transfected with a vector harboring the wild-type proximal 500 bp promoter fragment were compared with cells transfected to contain the same fragment with either the bZIP1 or bZIP2 domain mutations described previously (Juan et al., 1993). As shown, bZIP1, but not bZIP2, lost its iron-inducible luciferase activity (Fig. 2.8B). Therefore, bZIP1, the putative C/EBP-δ binding site, is likely to be important for iron-mediated C3 transcriptional up-regulation.

Iron-induced C3 protein activation and alternative complement pathway activation can be suppressed by pharmacologic inhibition of SMAD3 and ERK1/2

To examine iron’s effect on C3 protein activation, we used ELISA to measure the levels of the cleavage byproduct of C3 activation, C3a, in ARPE-19 cell conditioned media. There was an iron dose-dependent increase in C3a levels (Fig. 2.9A). We also found a significant decrease in C3a levels when comparing cells that are co-treated with SIS3 or PD98059 inhibitor and FAC to cells treated with FAC alone (Fig. 2.9B). Evidently, pharmacologic suppression of C3 mRNA (Fig. 2.3A, 2.3B) and protein (Fig. 2.3C) levels also translated into decreased C3 protein activation. By approximation, iron induced a comparable 2-fold change in both C3 and C3a levels relative to untreated (Fig. 2.3C, 2.9B). Analogously, co-treatment with SIS3 or PD98059 and FAC compared to FAC alone resulted in ~25% decrease in both C3 and C3a levels (Fig. 2.3C, 2.9B). In addition, co-treatment with both inhibitors and FAC achieved commensurate levels of C3a protein as co-treatment of a single inhibitor and FAC (Fig. 2.9B). To study the alternative complement pathway activation status, we measured the levels of the Factor B activation product,
Figure 2.8A ARPE-19 cells transfected with luciferase vectors containing a set of nested but different-sized C3 promoter fragments, as schematically indicated, uniformly displayed responsiveness to FAC treatment. The results were corrected for transfection efficiency as measured by Renilla luciferase activity, which was not significantly changed by FAC.
Cells transfected with the smallest fragment (500 bp) containing a wild-type (WT) sequence, the same fragment with a mutant bZIP1 domain, or the same fragment with a mutant bZIP2 domain are treated with or without FAC. The bZIP1 mutant suppressed baseline and FAC-inducible luciferase activities. The results were corrected for transfection efficiency as measured by Renilla luciferase activity, which was not significantly changed by FAC.
Figure 2.9A ELISA of C3a from the conditioned media of untreated ARPE-19 cells and those treated with different doses of FAC for 2d. Compared to either untreated or 25 µM FAC-treated cells, the 250 µM FAC-treated cells showed a significant increase in C3a production.
Figure 2.9B C3a production is significantly decreased by co-treatment with SIS3 or PD98059 and FAC compared to FAC alone at 4d; co-treatment with both inhibitors and FAC did not further reduce C3a levels compared to co-treatment with a single inhibitor and FAC.
Factor Ba, and determined that relative to untreated, FAC induced Factor Ba production. This effect is inhibited by co-treatment with SIS3 or PD98059 inhibitor and FAC (Fig. 2.9C). Although this finding points to a possible co-regulation of Factor B and C3 expression, it is not further explored herein.

**Chronic iron overload in the RPE is associated with increased C3 expression and activation, as demonstrated by localized C3d deposition in vivo**

Our group has published a case report of post-mortem retinal findings in a human patient with aceruloplasminemia showing iron overload in the RPE cells (Wolkow et al., 2011). In the present study, we used paraffin-embedded sections containing RPE cells of the macular region, where iron deposition is the most concentrated, and detected stronger signals for C3d, a C3 activation fragment, than in the same region of an eye from an age-matched patient without retinal disease (Fig. 2.10A). The fluorescence pattern is punctate and distributed throughout the cell, a pattern that is absent in the sections from the normal eye. Further, a ferroxidase conditional knockout in the RPE serves as a good ocular disease model for the human disease aceruloplasminemia. As such, these BCre+, Cp+/-, HephF/F mice have a mosaic pattern of gene ablation, with select RPE cells exhibiting iron overload detected by L-ferritin antibody staining. Cells showing strong L-ferritin signal are highly correlated with cells showing increased C3d deposition (Fig. 2.10B).

**Discussion**

**Main findings**

The main findings of our study suggest a model, schematically diagrammed in Fig. 2.11, as follows: First, increased iron in RPE cells stimulates the phosphorylation of ERK1/2, followed by SMAD3 (S213) phosphorylation in a pathway leading to C3 up-regulation. Second, iron induces
Figure 2.9C ELISA using the same comparison as in B showed that production of Factor Ba, a marker of alternative complement pathway activation, is decreased by co-treatment with SIS3 or PD98059 and FAC compared to FAC alone.
Figure 2.10A Paraffin-embedded sections of normal and aceruloplasminemia human macular retina, with retina pigment epithelium (RPE) and Bruch’s membrane (BrM) labeled, are stained with antibodies against C3d (red) and DAPI for nuclei (blue). Scale bars are equivalent to 50 µm.
Figure 2.10B Representative images of RPE cells (arrow) and BrM (arrowhead) in OCT-embedded retina sections of 12-month old $C3^{-/-}$ and $BCre^+\!,\! Cp^{-/-},\! Heph^{F/F}$ mice. Cells with increased L-ferritin levels (green, left panels) have an associated increase in C3d deposition (red, middle panels). The merged images, demonstrating co-localization, are shown in the right panels. Scale bars are equivalent to 50 µm.
Figure 2.11 The molecular mechanism of iron-induced RPE C3 production involves ERK1/2, SMAD3, and C/EBP-δ signaling. Increased intracellular iron (Fe$^{3+}$) in RPE cells from FAC treatment stimulates the phosphorylation of ERK1/2, followed by SMAD3 linker (S213) phosphorylation (orange up arrow), in a non-canonical TGF-β pathway leading to up-regulation of C3, the central molecule of the complement cascade (The FAC-induced pathway is delineated by orange arrows). MEK1/2 inhibitor PD98059 and SMAD3 inhibitor SIS3 can block FAC-induced C3 up-regulation. FAC also results in decreased phosphorylation of SMAD3 C-terminal residues (S423/425) at the SSVS motif (orange down arrow). Through SMAD3, FAC increases CEBPD mRNA and C/EBP-δ protein levels, possibly by inducing CEBPD expression and relieving SMAD3-mediated inhibition at baseline (orange X over blue inhibitory line). The p-SMAD3 (S213) complex remains extra-nuclear. In addition, FAC promotes the nuclear accumulation of C/EBP-δ protein. C/EBP-δ likely binds to the bZIP1 domain of the C3 promoter, inducing C3 expression. Once translated into protein, C3 is secreted and cleaved into C3a and C3b. Concurrently, Factor D catalyzes the activation of Factor B to form Bb and the cleavage product Factor Ba. Factors C3b and Bb together form the alternative pathway (AP) C3 convertase to amplify the activation of C3 by forming more C3b and C3a. The iron-induced non-canonical TGF-β pathway (orange) may cross-talk with the basal, canonical TGF-β pathway (blue; see discussion), which is initiated by the binding of TGF-β ligand to the TGF-β Receptor complex (Type I and II) at the plasma membrane. Endogenous TGF-β binding maintains the phosphorylation levels of SMAD3 at both S213 and S423/425, ultimately acting as a negative regulator of basal C3 expression. Stimulatory arrows and inhibitory lines represent a functional link between the subsequent entities, not necessarily a direct interaction.
increases in linker region phosphorylation, i.e. p-SMAD3 (S213), and decreases in C-terminal phosphorylation, i.e. p-SMAD3 (S423/425). Together these changes represent the effects of cross-talk between an iron-induced non-canonical TGF-β pathway and an intrinsic, canonical TGF-β signaling pathway. Third, SMAD3 mediates the iron-induced up-regulation and nuclear accumulation of C/EBP-δ, a transcriptional inducer of C3. Fourth, the bZIP1 domain, previously identified as a C/EBP-δ-binding region within the C3 promoter, is responsive to iron stimulation. Fifth, activation of C3 protein and Factor B, an activator in the alternative pathway, are induced by increased iron. Sixth, in vivo studies support the molecular association between RPE intracellular iron overload and increased deposition of activated C3 fragments.

Discussion
The mechanistic findings in the present study are relevant to AMD, as the disease is associated with elevated RPE iron levels (Hahn P et al., 2003). The increased iron leads to oxidative stress by Fenton chemistry in RPE cells, likely overwhelming their anti-oxidant defenses. As a general concept, oxidative stress is considered as an inciting factor of complement over-activation in the RPE (Handa, 2012; Kunchithapautham et al., 2014; Thurman et al., 2009). To determine whether iron can up-regulate RPE complement immune-mediated defenses, we treated ARPE-19 cells with a standard range of FAC doses (Salvador and Oteiza, 2011) and measured C3 mRNA and protein levels. C3 activation indicates a mobilized arm of innate immunity and a possible buildup of an adaptive immune response (Markiewski and Lambris, 2007; Morgan et al., 2005). We have, for the first time, provided evidence that iron triggers the transcriptional up-regulation, protein secretion, and proteolytic activation of C3. Several other transition metals did not lead to C3 up-regulation in the same experimental model. We have also identified components of the TGF-β signaling superfamily that mediate iron-induced C3 expression.
Exogenous TGF-β1 ligand stimulates the canonical pathway (Massague, 2012; Wang et al., 2009), resulting in repression of C3 and induction of PAI-1 mRNA levels. These findings demonstrate that in ARPE-19 cells the machinery of the canonical TGF-β pathway is intact. When endogenous TGF-β ligands are neutralized in the absence of FAC, the resultant up-regulation of C3 and down-regulation of PAI-1 mRNA levels reveal the regulatory role of the canonical TGF-β pathway at baseline. Since addition of anti-TGF-β1/2/3 antibody alone up-regulated C3 expression, one could hypothesize that signaling events of the iron-induced non-canonical TGF-β pathway could cross-talk with those of the canonical pathway, leading to inhibition of the latter’s inhibitory effect on C3 expression. Consistent with this hypothesis, we found that iron treatment results in a modest but significant decrease in endogenous TGF-β1 levels, thereby attenuating canonical signaling. Apparently, iron-mediated effects on the non-canonical and canonical TGF-β pathways act together to up-regulate C3. We further explored the relative contributions of these two pathways to C3 up-regulation. The up-regulation of C3 as an outcome of activated ERK1/2 and SMAD3 in non-canonical signaling is approximately 1.5-fold more robust than the effect of neutralizing endogenous TGF-β signaling (Fig. 2.2C, 2.2D). Furthermore, co-treatment with anti-TGF-β1/2/3 antibody and iron did not show any additive effect on C3 up-regulation when compared to iron alone. Taken together, it is plausible that inhibition of the inhibitory canonical TGF-β signaling plays an adjunctive role in iron-induced C3 up-regulation.

In the context of iron stimulation, the regulatory cross-talk between these two pathways may be mediated, in part, by ERK1/2. ERK1/2 is thought to have a pathogenic role in both atrophic and neovascular AMD because p-ERK1/2 is increased in human geographic atrophy retinas (Dridi et al., 2012) and RAS/ERK signaling is implicated in oxidative stress-mediated VEGF secretion by ARPE-19 cells (Kunchithapautham and Rohrer, 2011). ERK1/2 has been shown previously to phosphorylate several SMAD3 linker region residues in a variety of cellular contexts (Kretzschmar
et al., 1999; Matsuura et al., 2005; Tarasewicz and Jeruss, 2012; Wang et al., 2009; Wang, Guannan, 2008). In the present study, we observed that iron-induced activation of ERK1/2, but not p38 or JNK1/2/3, up-regulates C3 in a non-canonical TGF-β pathway. Further, the MEK1/2 inhibitor PD98059 blocks iron-induced increases in p-SMAD3 (S213). Taken together, this suggests that iron activates ERK1/2 to phosphorylate SMAD3 at S213, which likely contributes to C3 up-regulation. Meanwhile, phosphorylation of C-terminal residues (S423/425) is not affected by ERK1/2 inhibition in the absence or presence of iron (Fig. 2.4C). Although induction of the TGF-β1 target gene PAI-1 is SMAD3 C-terminal phosphorylation-dependent (Hua et al., 1998; Liu et al., 1997), phosphorylation of the linker region by activated ERK1/2 may be important for iron’s inhibitory effect on PAI-1 expression. We have shown that inhibition of ERK1/2 activity restores iron-suppressed PAI-1 mRNA levels back to baseline (Fig. 2.3D). This finding implies that phosphorylation of the SMAD3 linker region plays a regulatory role in canonical, C-terminal phosphorylation-dependent gene expression. Nevertheless, iron appears to utilize spatially distinct SMAD3 phospho-isoforms as a platform to convey different signals to effector genes, as exemplified by the transcriptional up-regulation of C3 versus the down-regulation of PAI-1.

In ARPE-19 cells, the 150 kDa band detected by p-SMAD3 (S213) antibody, and up-regulated by iron, contains SMAD3. Results from antibody specificity tests, SIS3 inhibition, and targeted knockdown of SMAD3 support this finding. Previously, it was determined that SMAD3-containing complexes are distributed over a wide range of molecular weights (Jayaraman and Massagué, 2000), and 150 kDa is within this range. However, this 150 kDa complex should have dissociated under SDS-PAGE conditions. The lack of dissociation then raises the possibility that either the complex is extraordinarily stable or it represents a covalently modified form of SMAD3 that cannot be effectively disrupted by SDS. In post-mortem human RPE/choroid tissues, the most significant band detected by the same antibody against p-SMAD3 (S213) was higher than 52 kDa (approximately 70 kDa; data not shown). Taken together, these results suggest that the apparent
molecular weights of p-SMAD3 (S213)-containing species are context-dependent. The 150 kDa, p-SMAD3 (S213)-containing species remains in the non-nuclear compartment after iron treatment. Phosphorylation at S213 may affect SMAD3’s interaction with other signaling mediators, one of which could be transcription factor C/EBP-δ, to effect the latter’s nuclear accumulation. SMAD3 has been shown to directly interact with C/EBP-δ in the context of adipocyte differentiation (Choy and Derynck, 2003), though the details of how p-SMAD3 (S213) in ARPE-19 cells may regulate this interaction remain unknown.

C/EBP-δ acts as the major protein responsible for the acute-phase C3 gene expression by binding to the bZIP1 domain in the promoter sequence (Juan et al., 1993; Maranto et al., 2008; Wilson et al., 1990). The bZIP1 domain is required for basal and cytokine-inducible C3 expression. Similarly, we find this domain to be important for iron-induced C3 transcription in ARPE-19 cells. Also, we showed that iron-induced C3 up-regulation is accompanied by increased CEBPD mRNA and C/EBP-δ protein levels. SMAD3 activity appears to mediate these increases, since SIS3 blocks iron-induced increases in CEBPD mRNA, and there was no significant difference in nuclear C/EBP-δ levels between untreated and iron-treated sh-SMAD3 cells. It should be noted that C/EBP-δ protein levels are elevated in sh-SMAD3 cells compared to the control cell lines, suggesting that SMAD3 inhibits basal C/EBP-δ expression. In other words, the observed iron-induced increases in C/EBP-δ levels may result, at least in part, from the relief of SMAD3 inhibition of basal C/EBP-δ expression. This mechanistic model is consistent with that of vascular smooth muscle cells in an anti-inflammatory state, in which TGF-β1-stimulated SMAD3 represses C/EBP-δ expression (Feinberg et al., 2004). In a further study of basal repression of CEBPD, neutralization of endogenous TGF-β ligands in the absence of FAC resulted in a modest up-regulation of CEBPD, which is comparable in magnitude to the iron-induced up-regulation of CEBPD (Fig. 2.7B, 2.7C). Thus, similar to the regulation of C3 mRNA
levels, CEBPD mRNA levels are likely induced by iron through a non-canonical TGF-β pathway and through the relief of SMAD3-mediated basal inhibition.

In AMD pathogenesis, it is thought that over-activation of the complement cascade, especially in the alternative pathway, contributes to the chronic inflammatory state. Our findings support the concept of a locally produced complement-mediated disease process, as iron insult of the RPE cell results not only in increased C3 transcription, but also in protein secretion and activation. With more activated C3 in the RPE milieu, the complement cascade is mobilized to initiate immune-mediated inflammation. Within the cascade, formation of the alternative pathway C3 convertase requires Factor B and generates byproduct Factor Ba so that more C3 can be activated (Kolb et al., 1989; Sahu and Lambris, 2001). Although C3 activation and its downstream effects on RPE pathology are complex, our findings of increased C3a and Factor Ba levels are still suggestive of C3 and alternative pathway activation, respectively. Befitting this complexity, while pharmacologic blockade of ERK1/2 or SMAD3 incompletely suppressed iron-induced C3a formation, blockade of both targets provided no synergistic suppression (Fig. 2.9B). This supports our model of ERK1/2 and SMAD3 functioning in a single iron-mediated pathway. The incomplete suppression of C3a by the inhibitors, singly or in combination, points to the likely involvement of other iron-induced factors or processes in C3 activation. Additionally, we provide strong evidence that excess intra-RPE iron can lead to local complement dysregulation, as reflected by C3d accumulation, in the individual RPE cells of an aceruloplasminemia patient and an animal model of RPE-specific iron overload. In AMD, localized complement production and activation within the RPE layer may be more active in individuals with disease-associated complement gene polymorphisms. Since ERK1/2, SMAD3, C/EBP-δ, and endogenous TGF-β are now implicated in RPE iron-C3 dysregulation, these signaling molecules may serve as useful therapeutic targets for ameliorating RPE pathology in AMD.
CHAPTER 3

Preamble
In the previous chapter, we examined the mechanism(s) by which iron may lead to complement dysregulation in the RPE cell. An important follow-up question is whether increase in iron systemically or locally contributes more to iron overload in the RPE. To determine how the retina, and more specifically the RPE may be loaded with iron in the disease context of AMD, we performed studies detailed below. The work in this Chapter, entitled “A high serum iron level causes more retinal iron accumulation despite an intact blood-retinal barrier,” has been published as a Short Communication article in a November 2014 issue of *American Journal of Pathology* with Liangliang Zhao and myself, Yafeng Li, as the co-first authors, respectively.

Abstract
The retina can be shielded by the blood-retinal barrier. Since photoreceptors are damaged by excess iron, it is important to understand whether the blood-retinal barrier protects against high serum iron levels. Bone morphogenetic protein 6 (*Bmp6*) knockout mice have serum iron overload. Herein, we tested whether the previously documented retinal iron accumulation in *Bmp6* KO mice might result from the high serum iron levels or, alternatively, low levels of retinal hepcidin, an iron regulatory hormone whose transcription can be upregulated by Bmp6. Further, to determine whether increases in serum iron can elevate retinal iron levels, we injected iron intravenously into wild-type (WT) mice. Retinas were analyzed by qPCR and immunofluorescence to assess the levels of iron-regulated genes/proteins and oxidative stress. Retinal hepcidin mRNA levels in *Bmp6* KO retinas were the same as, or greater than, that of age-matched WT retinas, indicating that *Bmp6* KO does not cause retinal hepcidin deficiency. Changes in mRNA levels of L-ferritin and transferrin receptor indicated increased retinal iron in intravenous iron-injected WT mice. Oxidative stress markers were elevated in photoreceptors of mice receiving intravenous iron. These findings suggest that elevated serum iron levels can
overwhelm local retinal iron regulatory mechanisms.

**Introduction**

Iron is necessary for life, but in excess it can be toxic. Therefore, iron is tightly regulated. Many of the genes that regulate iron on the systemic level are expressed in the retina and may play a role in local iron regulation (Gnana-Prakasam et al., 2010). We previously studied double knockout (DKO) mice deficient for the iron exporting ferrooxidases ceruloplasmin (Cp) and hephaestin (Heph) (Song and Dunaief, 2013). These DKO mice have an age-dependent retinal iron accumulation and degeneration (Hadziahmetovic et al., 2008b; Hahn et al., 2004c). DKO mice have retinal iron overload despite systemic iron deficiency, so it is likely that the retina accumulates iron as a result of impaired retinal iron export. We also studied mice with systemic knockout of the iron-regulatory hormone hepcidin (Hamp). These mice also have age-dependent retinal iron accumulation with degeneration (Hadziahmetovic et al., 2011b). Bmp6 is known to upregulate Hamp in the liver and retina (Meynard et al., 2009), and Bmp6 KO mice have retinal iron accumulation similar to Hamp KO mice (Hadziahmetovic et al., 2011c). Since Hamp and Bmp6 KO mice have elevated serum iron levels, it is unclear whether the retinal iron accumulation in these mice results from elevated serum iron caused by diminished liver Hamp production or from low retinal Hamp levels impairing local iron regulation in the retina.

There are several routes for iron influx yet only one for cellular iron efflux (Donovan et al., 2000; Gunshin et al., 1997; Soe-Lin et al., 2009); the transmembrane protein ferroportin can export iron out of cells. Ferroportin can be regulated by Hamp, which triggers its internalization and degradation, leading to decreased export of cellular iron (Nemeth et al., 2004). Hamp expression in the liver is increased by iron overload, and, following secretion into the bloodstream, limits intestinal iron absorption, macrophage iron recycling and hepatocyte iron release. In comparison,
iron deficiency anemia and tissue hypoxia can inhibit Hamp expression to increase iron supply for the body (Liu et al., 2005; Pak et al., 2006; Ramos et al., 2011).

Hamp is expressed in many tissues, including the retina within photoreceptors, Müller cells, and RPE (Gnana-Prakasam et al., 2008). Ferroportin, the target of Hamp, is localized to RPE, photoreceptor inner segments, the outer plexiform layers, and inner limiting membrane (Hahn et al., 2004b). Locally synthesized Hamp may regulate ferroportin in the NR. To determine whether retinal Hamp deficiency contributes to the retinal iron accumulation in Bmp6 KO mice, we compared Hamp mRNA in Bmp6 KO versus WT mice. We also tested whether WT mice given intravenous (I.V.) injections of iron sucrose would develop retinal iron accumulation despite the presence of Hamp produced locally in the retina.

**Materials and methods**

**Animals**—Bmp6 KO mice on the CD1 background were generated as described previously (Solloway et al., 1998). Bmp6 knockout male mice aged 2.5, 3.5, 5 and 10 months and age matched wild-type (WT) male CD1 mice were used for experiments. Wild-type C57BL/6J mice at 2.5 months were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The C57BL/6J mice were treated with or without 1.2mg iron-sucrose (Venofer, American Regent, Shirley, NY) in 200ml 0.9% saline solution via tail vein injection 3 times (once per week) until time of sacrifice. Experimental procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmology and vision research. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. The eyes were enucleated immediately after sacrifice and were fixed overnight in 4% paraformaldehyde for immunofluorescence.
**Immunolabeling**—Mouse globes fixed in 4% paraformaldehyde were rinsed in PBS and the eyecups were dissected. The eyecups were cryoprotected in 30% sucrose solution overnight then embedded in optimal cutting temperature compound (Tissue-Tek; Sakura Finetek, Torrance, CA) and slowly frozen in 2-methylbutane on dry ice. Immunofluorescence was performed on sections of 10 µm thickness, as described previously (Dunaief et al., 2002). Primary antibodies were rabbit anti-L-ferritin (E17) at 1:200 dilution (kind gift from Paolo Arosio, University of Brescia), rabbit anti-MDA at 1:100 dilution (Alpha Diagnostic, San Antonio, TX) and rabbit anti-HNE at 1:100 dilution (Alpha Diagnostic, San Antonio, TX). Primary antibody was detected using fluorophore-labeled secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Control sections were treated identically, except that primary antibodies were omitted. Sections were analyzed by fluorescence microscopy with identical exposure parameters using a Nikon TE300 microscope equipped with ImagePro Plus version 6.1 (Media Cybernetics INC., Bethesda, MD) software.

**RPE Isolation**—We used a recently developed method for the rapid isolation of RPE cells for RNA extraction and analysis (Xin-Zhao Wang et al., 2012) This employs simultaneous RPE cell isolation and RNA stabilization. Briefly, eyes were enucleated and placed on an ice-cold PBS-soaked sponge. The peri-ocular tissues were removed and the eye was opened posterior to the limbus using Vannas scissors, then the anterior segment and the retina were removed. The resulting posterior eye cups were quickly dipped into a PBS-containing microcentrifuge tube to remove any loosely adherent debris. Afterwards, the eye cup was immediately transferred into a microcentrifuge tube containing 200 µl of ice-cold RNA-protect cell reagent (Qiagen, Cat. No. 76526) with gentle manual tapping of the tube every 1-2 min to accelerate the release of the RPE cells. After 10 min, the eye cup was removed from the solution with forceps. RPE cells were then centrifuged (5 min at 2750 rpm). The RNA isolation was performed with an RNeasy plus micro kit (Qiagen, Cat. No. 74034) according to the manufacturer’s protocol.
Quantitative real-time PCR—Liver, NR and RPE samples obtained from Bmp6 KO and WT mice were analyzed using quantitative real-time PCR for gene expression, as described previously (Song et al., 2012b). RNA isolation was performed with the RNeasy mini kit (Qiagen, Cat. No. 74106) according to the manufacturer’s protocol. The RNA was quantified with a spectrophotometer and stored at -80°C. cDNA was synthesized using TaqMan reverse transcription reagents (Applied Biosystems, Cat. No. 808-0234) according to the manufacturer’s protocol. TaqMan gene expression assays were obtained from Applied Biosystems and used for PCR analysis. Probes included hepcidin antimicrobial peptide (Hepc1, Mm04231240_s1), transferrin receptor (Tfrc, Mm 00441941_m1), L-ferritin (Ftl1, Mm03030144_g1), Von Willebrand factor homolog (Vwf, Mm00550376_m1), retinal pigment epithelium 65 (Rpe65, Mm00504133_m1), Collagen, type VI, alpha 1 (Col6a1, Mm00487160_m1). Eukaryotic 18S rRNA (Hs99999901_s1) served as an internal control due to its constant expression level among the studied sample sets. Real-time TaqMan RT-PCR (Applied Biosystems, Foster City, CA) was performed on an ABI Prism 7500 detection system using the ΔΔC_T method, which provided normalized expression values. The amount of target mRNA was compared among groups of interest. All reactions were performed in biological (three mice) and technical (three real-time PCR replicates per mouse) triplicates.

Statistical Analysis—The mean and the standard error were calculated for each comparison group. Statistical analysis was performed using the Student's two group, two sided t-test. For multiple comparisons, we used one way ANOVA with post hoc pairwise comparisons using Bonferroni correction for multiple comparisons. P<0.05 was considered statistically significant. All statistical analysis was performed with GraphPad Prism version 5 (GraphPad Software, San Diego, CA).
Results

Regulation of hepcidin in NR in response to iron status

Expression of Hamp in the liver, a key regulator of systemic iron metabolism, can be up regulated by Bmp6. Consistent with a previous report (Meynard et al., 2009), Hamp mRNA levels in the livers of male Bmp6 KO mice at several ages were diminished compared to WT males (Fig. 3.1A, B and C).

Because the Bmp6 pathway can increase Hamp expression at the systemic level, to determine whether it can also regulate retinal Hamp, we measured Hamp mRNA levels in NR and RPE. First, to test the level of RPE RNA enrichment in isolated RPE cells, we measured mRNA levels of RPE65, an RPE-specific marker, which was almost 6-fold higher in purified RPE samples compared with the RPE/choroid samples. In addition, the vWF and Collagen VI mRNAs, which are expressed in choroid and sclera, were decreased significantly in purified RPE samples compared with the RPE/choroid samples (Fig. 3.1D, E and F).

The Hamp mRNA levels in NR showed no significant difference between Bmp6 KO and WT mice at 2.5 months and 3.5 months, and increased in Bmp6 KO compared with WT mice aged from 5 months to 10 months (Fig. 3.1G). The Tfrc mRNA levels in Bmp6 KO NR showed no significant difference at 2.5 months, were decreased at 3.5 months, unchanged at 5 months and decreased at 10 months (Fig. 3.1H) in Bmp6 KO mice compared with age and sex matched WT mice. Decreased Tfrc mRNA levels indicate elevated iron levels, since increased intracellular iron prevents IRP1 and 2 from stabilizing the Tfrc mRNA (Rouault, 2006).

Hamp mRNA levels in RPE showed no significant differences between Bmp6 KO mice and WT mice at both 5 and 10 months (Fig. 3.1I, K). The Tfrc mRNA level in RPE showed a significant decrease in Bmp6 KO mice compared with WT mice (Fig. 3.1J, L).
Figure 3.1 Quantitative PCR results in liver, neural retina (NR), RPE/choroid (CH), and isolated RPE of Bmp6 KO and WT mice. Bmp6 KO mice have lower Hamp mRNA levels in liver than age- and sex-matched WT controls at 2.5mo (A), 3.5mo (B) and 10mo (C) of age. Relative mRNA levels of RPE65 (D), vWF (E) and collagen VI (F) in RPE/CH versus isolated RPE. RPE samples had enriched RPE65, limited vWF and barely detectable Col6a1 mRNA compared to RPE/CH. Relative mRNA levels of Hamp (G, I, and K) and Tfrc (H, J, and L) in NR of Bmp6 KO and WT at different ages. G: Hamp mRNA levels in 2.5mo and 3.5mo Bmp6 KO were not different from those in WT controls. However, Hamp mRNA levels are significantly higher in 5mo and 10mo old Bmp6 KO compared to WT controls. H: Tfrc mRNA levels in Bmp6 KO NR show no significant difference at 2.5mo and 5mo, and was decreased at 3.5mo and 10mo compared with age and sex matched WT mice. I and K: Hamp mRNA levels in RPE show no significant difference between Bmp6 KO mice and WT mice at both 5mo and 10mo. J and L: However, Tfrc mRNA levels show a significant decrease in Bmp6 KO mice compared to WT controls at both 5mo and 10mo (J and L). (Values shown are means of ΔΔCT ±SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. N=3 biological and technical replicates for all qPCR experiments)
Eyes from C57BL/6J mice treated with I.V. iron have increased ferritin-L, and oxidative stress markers

The levels of the cytosolic iron storage protein ferritin are regulated by labile iron levels through iron regulatory proteins IRP1 and IRP2 (Rouault, 2006). Accordingly, when labile iron levels rise, ferritin translation increases, leading to increasing levels of both L-ferritin and H-ferritin proteins (Muckenthaler et al., 2008). RPE cells showed L-ferritin immunoreactivity that was stronger in the mice treated with I.V. iron than age-matched controls (Fig. 3.2A, B, C, and D). Iron accumulation-induced lipid peroxidation was assessed by immunofluorescent detection of malondialdehyde (MDA) and 4-hydroxy-nonenal (HNE). These are reactive intermediates in the formation of advanced lipoxidation end products (ALEs). Thus, they are frequently measured as indicators of lipid peroxidation and oxidative stress. We detected an increase of MDA and HNE in the inner segment and outer plexiform layers of mice that were injected with iron compared to the controls (Fig. 3.2E, F, G, and H); accompanying quantification is presented (Fig. 3.2I, J, K, and L).

Hepcidin, L-ferritin and transferrin receptor mRNA levels in C57BL/6J mice treated with I.V. iron

In C57BL/6J mice treated with three weekly I.V. iron sucrose injections, Hamp and L-ferritin mRNA levels were significantly higher in the liver compared to control mice (Fig. 3.3A, C), whereas Tfrc mRNA levels were unchanged (Fig. 3.3B). Hamp mRNA levels were not significantly different in NR and RPE of the mice treated with or without iron (Fig. 3.3D, G). Tfrc mRNA levels were significantly lower in NR of iron-treated mice compared to control mice (Fig. 3.3E), whereas L-ferritin mRNA levels (a less sensitive measure of iron) were unchanged (Fig. 3.3F). L-ferritin mRNA levels were significantly higher in the RPE of iron sucrose-injected mice compared with controls, and Tfrc mRNA levels were significantly lower (Fig. 3.3H, I).
Figure 3.2. Iron labeling in RPE of i.v. Venofer (iron sucrose)-injected C57BL/6J mice. Iron level as determined by immunolabeling for L-ferritin (red), are greater in the RPE of Venofer-injected mice (B) compared to controls (A). C and D show the high magnification (60x objective) of the RPE layer (arrows) in A and B, respectively. Oxidative stress levels determined by immunolabeling for MDA and HNE, are greater in inner segments (arrows) and the outer plexiform layer (arrowheads) with Venofer injection (F and H) compared to controls (E and G). I-L: Pixel density quantification of MDA and HNE immunostaining in the inner segments and outer plexiform layer of three mice with Venofer injection or controls. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. The scale bar in A and B represents 100µm (A, B, E-H); 33.3µm (C and D). Statistical analysis was performed using the Student’s two group, two sided t-test. *P < 0.05, **P < 0.01.
Figure 3.3 Quantitative PCR results in liver, retina, and RPE of intravenous Venofer (iron sucrose)-injected C57BL/6J mice. Relative mRNA levels of Hamp, Tfrc and L ferritin (Ftl) in liver (A-C), NR (D-F) and RPE (G-I) of mice with or without Venofer injection. Hamp (A) and Ftl (C) mRNA levels in liver were significantly higher in mice with Venofer injection compared to the controls. B: Tfrc mRNA levels in liver of mice with or without Venofer injection were not different. Hamp (D) and Flt (F) mRNA levels in NR of mice with or without Venofer injection are not different. E: Tfrc mRNA levels were significantly lower in mice with Venofer injection compared to the controls. G: Hamp mRNA levels in RPE of mice with or without Venofer injection were not different. H: Tfrc mRNA levels were significantly lower in RPE Venofer injected mice compared to the mice without Venofer injection. I: Ftl mRNA levels were significantly higher in the RPE of mice with Venofer injection compared to the controls. Values shown are means of ΔΔCT ±SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. N=3 biological and technical replicates for all qPCR experiments.
Discussion

Previous studies showed elevated serum and retinal iron levels in systemic *Hamp* and *Bmp6* KO mice (Hadziahmetovic et al., 2011b, 2011c). To determine whether the retinal iron accumulation was due to loss of Bmp6-stimulated retinal production of Hamp, we measured Hamp mRNA in the NR and RPE by qPCR in *Bmp6* KO mice. Our results indicate an important influence of systemic Hamp (and, consequently, serum iron) levels on retinal iron. In *Bmp6* KO mice, liver but not retinal Hamp levels were diminished.

*Bmp6* can upregulate Hamp expression systemically, and we hypothesized that there was a similar local regulatory mechanism in the retina. Despite retinal expression of Bmp6 and its receptors, retinal Hamp expression was not diminished in *Bmp6* KO mice; it increased significantly. These results provide evidence that basal retinal Hamp expression is independent of the Bmp6 pathway, though it is difficult to speculate precisely what that regulatory mechanism could be. As the retina takes in more iron from the iron-rich blood in *Bmp6* KO mice, this iron appears to upregulate Hamp expression. As a measure of retinal iron levels, Tfrc mRNA at 2.5 months was similar between KO and WT. The Tfrc level was decreased at age 3.5 months, indicating elevated retinal iron levels. At 5 months, Hamp expression in NR was increased in *Bmp6* KO mice, possibly diminishing the iron influx, as the Tfrc levels were normal. However, at 10 months, Hamp levels were still higher in *Bmp6* KO than age-matched WT, but the Tfrc levels were lower in the KO suggesting that the increased Hamp levels were insufficient to prevent further iron influx.

In the RPE, 5 and 10 month old *Bmp6* KO mice had decreased Tfrc mRNA compared to age and sex matched WT mice. This reflects increased RPE iron caused by high systemic iron levels; a reduction in local retinal Hamp levels was not responsible, since there was no difference in RPE Hamp mRNA levels between *Bmp6* KO mice and WT.

To further assess the effects of elevated systemic iron levels on retinal iron, we injected mice with I.V. iron sucrose, which is used in patients to treat iron-deficiency anemia. Following three weekly
I.V. injections, we confirmed that liver Hamp mRNA levels increased, reflecting elevated hepatocyte iron levels. Tfrc levels did not change in the liver, suggesting that sub-populations of liver cells did not experience enough of an iron load to down-regulate Tfrc mRNA. In both the RPE and NR of iron sucrose treated mice, Tfrc mRNA decreased compared with the control. The L-ferritin mRNA levels increased in the RPE of iron sucrose injected mice. These results show that in WT mice, high systemic iron levels caused increased iron levels in the RPE, and, to a lesser extent, NR (since only Tfrc mRNA but not the less sensitive measure L-ferritin mRNA changed in the NR). Therefore, Hamp produced locally in the retina is insufficient to prevent retinal iron uptake in the context of increased blood iron levels. While elevated iron levels in the liver are a strong stimulus for Hamp expression, following iron sucrose injection, Hamp mRNA levels in the NR were unaltered; the acute systemic iron challenge did not cause retinal Hamp up-regulation.

After I.V. iron injection, oxidative stress markers MDA and HNE increase in the photoreceptor inner segments. The RPE accumulates more iron more than the NR, but oxidative stress markers were not elevated in the RPE. It is likely that the RPE has a more robust anti-oxidant defense system than the photoreceptors, resulting in the appearance of spatial separation between foci of increased ferritin staining and foci of increased oxidative stress marker accumulation. Currently, we do not have complement data to accompany these findings.

These studies emphasize the importance of blood iron levels on retinal iron levels; elevated blood iron levels may cause increased retinal iron-induced oxidative stress levels. Elevated systemic iron levels, whether caused by genetics or diet, may increase the risk of age-related retinal disease, including age-related macular degeneration. In support of this possibility, people eating diets high in red meat, which causes high levels of iron absorption by the gut, have an increased risk of AMD (Chong et al., 2009).
CHAPTER 4

Brief summary of major findings

The accumulation of iron in the human retina is associated with the development of AMD, a disease characterized by central vision loss in millions of elderly individuals. In AMD, one site of major pathology is the retinal pigment epithelium (RPE) cell layer. Increased iron in the RPE cell and other cell types has been shown previously by our group to lead to some pathological features of AMD. In mouse models of systemic iron overload and of RPE-specific iron overload, both developed by our group, there are more deposits of complement component 3 (C3) protein fragments in the RPE. C3 is a central molecule in the innate immune complement system, which in recent years has become a focus of genetic, biochemical, and functional studies in the context of AMD with the aim of revealing novel targets for therapy.

As part of our effort to understand how iron may act as a driver for AMD development, we discovered that increased iron within RPE cells can trigger the transcription, and subsequently secretion and activation of C3 through modulation of the TGF-β pathway. We detail several signaling intermediates, notably ERK1/2, SMAD3, and C/EBP-δ that mediate this effect. However, the iron-dependent signal that results in ERK1/2 phosphorylation has not been fully characterized. In fact, an excess dose of free radical scavenger is unable to mitigate iron-induced C3 up-regulation, though a supra-physiologic dose of peroxide, to the detriment of the cultured ARPE-19 cells, is able to induce C3 up-regulation. In all, we have not examined in detail as to how the iron-sensing and/or oxidative stress pathways may contribute to iron-induced C3 up-regulation. Nevertheless, the above-mentioned signaling intermediates and endogenous TGF-β ligands may have the potential to become therapeutic targets for the prevention and/or treatment of some forms of AMD.

In terms of the contribution of systemic iron versus dysregulation of local iron homeostasis to retinal iron accumulation, we found that high systemic iron levels, rather than a reduction in local
retinal Hamp levels, was chiefly responsible iron overload in the RPE cell in Bmp6 KO mice. As such, there was no difference in RPE Hamp mRNA levels between Bmp6 KO mice and WT, indicating that in vivo, local retinal Hamp expression may be subject to other layers or types of regulation, not the Bmp6 axis as initially elucidated in hepatic cells/tissues.

**Future directions**

**The iron-induced C3 pathway in an alternative cell line and in vivo**

In Chapter 2, we detailed a putative mechanism for iron-induced C3 up-regulation in the immortalized human RPE cell line, ARPE-19. In order to determine if this pathway is more broadly applicable in other cell lines of epithelial origin, it is advisable to conduct a similar set of experiments, minimally encompassing the C3 gene expression and p-SMAD3 Western blot studies. We will use other adherent, non-cancer cell-derived cell lines, such as the hepatic epithelial cell line THLE-2/3. We have tried the hepatic adenocarcinoma cell line HepG2 cells and did not see a significant up-regulation of C3 despite much lowered TFRC mRNA levels with iron treatment (data not shown). The reasons for this finding are likely many, including but not limited to insufficient expression of iron-C3 pathway components and the lack of iron-induced pathway induction due to other effects of cellular transformation.

We acknowledge that one of the major shortcomings of our studies in Chapter 2 is the absence of substantial evidence for pathway activation in vivo. Although we show in Fig. 2.10 the elucidation of the iron-C3 pathway, the work is done entirely in ARPE-19 cells. Since our ARPE-19 cells experienced a sub-acute influx (2d to 4d) of iron, we attempted to generate a sub-acute in vivo model of RPE iron overload by performing FAC and sham injections intravitreally in WT C57BL/6 mice. At several time points following injection (i.e. 2d, 4d, 8d) using an approximate effective dose of 250 μM or lower (25 and 50 μM), C3 mRNA levels in isolated mouse RPE cells were highly inconsistent and variable, despite standardization of the technique. Focal iron-induced injury to the RPE may not have been widespread enough to detect global changes when we isolated all RPE cells from the mouse eyes for analysis.
Our studies may be better suited in genetic models of iron overload, such as the Cp-/HephSla mice. However, this would not be the sub-acute iron influx model that we used in ARPE-19 cells and signaling changes may be different or difficult to capture using established antibody-based techniques. Furthermore, using the genetic mouse model would certainly require substantial more effort on animal husbandry and waiting time. At the current juncture, we don’t have a satisfactory model for studying sub-acute iron-induced complement dysregulation in vivo.

**CRISPR/Cas9**

One of the major questions that remain to be answered in Chapter 2 of this thesis is the molecular composition of the 150 kDa, p-SMAD3 (S213)-containing species. Currently, our knowledge extends to the finding that C3 up-regulation is highly correlated with increased formation or production of this 150 kDa species. Since this 150 kDa species is highly resistant to dissociation of SDS and reducing agents such as DTT and TCEP, the logical approach to determining the binding partners of SMAD3 in this protein species would be to perform immune-precipitation, preferably with a tagged protein for enhanced antigen recognition and specificity. Overexpression of FLAG (DYKDDDDK)-SMAD3 prevented the time-dependent formation of the 150 kDa protein species following FAC treatment. We hypothesize that the FLAG tag, although only 8 aa in length, may have prevented the interaction of SMAD3 and molecule X in the 150 kDa protein species. Furthermore, the intracellular stoichiometry of SMAD3 is perturbed substantially in the context of overexpression, which may have untoward effects on the iron-induced formation of the 150 kDa complex.

In light of the experimental difficulties observed with using traditional cell and molecular biology approaches, we would like to, as an immediate next step, express SMAD3 at endogenous levels, though still with a small protein tag. We propose using CRISPR-Cas9 technology to selectively knockin the FLAG tag at the *SMAD3* locus, but with a small buffer, or linker region between the tag and the SMAD3 protein proper. To enhance FLAG flexibility and minimize steric hindrance, we propose using a 4X Gly sequence as the linker. To enhance the probability of success, that
is, formation of the 150 kDa protein species with iron treatment, the FLAG tag would be engineered at the N- and C-termini, separately, to determine which would least affect the formation of the 150 kDa species.

We anticipate, but hope to minimize, the off-target effects of the Cas9 enzyme, which would result in engineering the FLAG tag at other loci. We would determine site specificity by correlating FLAG and SMAD3 immunoblotting. The main techniques and procedures used will be derived from the a classic introduction on genome engineering using the CRISPR-Cas9 system (Ran et al., 2013).

**Tail vein injection of venofer in WT and Cfh mutant mice**

Individuals who harbor the disease risk-conferring polymorphisms in complement-related genes may be more vulnerable to pathological progression in the context of iron insult. We alluded to this hypothesis in Chapter 2. As a continuation of efforts to further understand the effect of labile iron on complement gene regulation and protein activity, we are IV injecting iron supplement Venofer, a drug approved for human therapy, into both WT and Cfh knockin mice. The Cfh mutant mice harbor a D1210C mutation and have an impaired ability to bind and inactivate C3b. These mice, therefore, have systemic over-activation of the alternative complement pathway, which presents as a potentially useful model for studying retinal pathology in the context of iron insult with Venofer treatment. We look forward to determining if there is a pathological phenotype. If so, this would support disease relevance of the mechanistic work we described in Chapter 2.

**Therapeutic implications of our findings**

The work described is basic science in nature but has potential to translate into a novel therapeutic approach to prevent or slow the progression of geographic atrophy (RPE cell degeneration) in the dry form of AMD. To date, most of the high-profile anti-complement therapies for AMD target the activation of complement. Understandably, activation of complement leads to cell damage and inflammation, which are hallmark features of AMD. In this
effort, pharmaceutical companies such as Alexion has developed a neutralizing antibody against complement component 5 (anti-C5 Nab) and Roche/Genentech has, in clinical trials, a promising drug called lampalizumab, which is also a neutralizing antibody but directed against Factor D (anti-Factor D), an activator of the alternative complement pathway known to be highly associated with AMD. On the other hand, our work delineates a mechanism of C3 expression, a process that must occur before activation. The pathway involved, TGF-β signaling, is an important and well-studied one implicated in many disease processes such as chronic inflammation, cell fate, and malignancy. Our rationale is to target complement dysregulation in the context of AMD further upstream, before protein activation in the complement cascade. To our knowledge, there is currently no major drug in the pipeline for the treatment of dry AMD that targets complement gene expression. The development of therapies targeting C3 expression may result in more effective therapies or in the least, provide an effective additional, or adjunctive therapy to existing regimens. We envision that the eventual therapy to modulate TGF-β signaling in the diseased RPE would be in the form of small molecule delivery by sustained release in the ocular micro-environment, protein-based therapy, antibody-based therapy, or gene therapy. Small molecules may be pharmacologic inhibitors or miRNA’s that modulate the activity and/or expression of pathway signaling intermediates. Ophthalmologists who specialize in the posterior segment of the eye could implant or inject drug-containing sustained delivery devices in proximity to the outer retina, near the RPE cell layer for patients who are either at risk for developing dry AMD due to their genetic risk profile (a service already available) or at risk for further progression of RPE atrophy and continued loss of central vision.


Seddon, J.M., Yu, Y., Miller, E.C., Reynolds, R., Tan, P.L., Gowrisankar, S., Goldstein, J.I.,
Triebwasser, M., Anderson, H.E., Zerbib, J., et al. (2013). Rare variants in CFI, C3 and C9 are
associated with high risk of advanced age-related macular degeneration. Nat Genet 45, 1366–
1370.


Soe-Lin, S., Apte, S.S., Andriopoulos, B., Andrews, M.C., Schranzhofer, M., Kahawita, T., Garcia-


Neurosci. 5, 24.

administration of the iron chelator deferiprone protects against light-induced photoreceptor

administration of the iron chelator deferiprone protects against light-induced photoreceptor

Tan, J.S.L. (2007). Smoking and the Long-term Incidence of Age-Related Macular Degeneration:


Thurman, J.M., Renner, B., Kunchithapautham, K., Ferreira, V.P., Pangburn, M.K., Ablonczy, Z.,

Pathologic features of vascular endothelial growth factor-induced retinopathy in the nonhuman


cardiovascular disease as risk factors for age-related macular degeneration. Ophthalmology 112,
2076–2080.


