Regulation of Cholesterol Homeostasis in Atherosclerosis and Alport Syndrome

Wen Ding
University of Miami, wding@med.miami.edu

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REGULATION OF CHOLESTEROL HOMEOSTASIS IN Atherosclerosis
AND ALPORT SYNDROME

By

Wen Ding

A DISSERTATION

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of the University of Miami
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REGULATION OF CHOLESTEROL HOMEOSTASIS IN ATHEROSCLEROSIS AND ALPORT SYNDROME

Wen Ding

Approved:

Lina Shehadeh, Ph.D.  Danuta Szczesna-Cordary, Ph.D.
Assistant Professor of Medicine  Professor of Molecular and Cellular Pharmacology

Eli Gilboa, Ph.D.  Fangliang Zhang, Ph.D.
Professor of Microbiology and Immunology  Assistant Professor of Molecular and Cellular Pharmacology

Michael Freundlich, M.D.  Guillermo Prado, Ph.D.
Professor of Clinical Pediatrics  Dean of the Graduate School
Cholesterol (C_{27}H_{46}O) is the precursor of steroid hormones, Vitamin D and bile acid and is a key component of the plasma membrane. Cholesterol levels are tightly regulated through biosynthesis, cellular uptake or efflux pathways to remain within a certain range for normal cell survival and function. Disturbed cholesterol homeostasis can be an independent risk factor for human diseases including cardiovascular disease, neurodegenerative disorder and chronic kidney disease. Understanding the molecular mechanisms that govern cholesterol homeostasis has the potential to reveal important insights into disease pathogenesis and new therapeutics.

Elevated plasma LDL cholesterol is a major risk factor for cardiovascular events and initiates the built up of atherosclerotic plaque in the arterial wall. In the arterial wall, the accumulated LDL cholesterol reacts with native oxygen species and becomes oxidized LDL that induces inflammatory responses and promote aortic Smooth Muscle Cells (SMCs) to undergo osteoblastic differentiation, proliferate, and hence contribute to the growth of atherosclerotic plaque. The first part of this study identifies microRNA-30e (miR-30e) as an OsteomiR in atherosclerosis. We present evidence that miR-30e regulates a panel of
osteogenic genes in bone marrow derived Mesenchymal Stem Cells (MSCs), reduces osteogenic differentiation in aortic SMCs and the atherogenic ApoE^{-/-} mice by directly repressing the expression of the osteogenic protein IGF2. MiR-30e also reduces calcification in SMCs. Our data reveals miR-30e-Igf2 as a novel pathway in osteogenesis-mediated atherogenic progression.

In addition to causing cardiovascular disease, altered lipoprotein metabolism has also been reported in patients with chronic kidney disease. Although the exact molecular mechanism is not clear, retention of lipid in the renal epithelial cells has been thought to promote epithelial cells to undergo epithelial-to-mesenchymal transition that causes fibrogenic responses in the kidney. The second part of this study investigates the role of LDL-c in the renal tubules in an animal model of Alport Syndrome. Alport Syndrome, characterized by hereditary progressive renal dysfunction, deafness and visual anomalies is an inherited chronic kidney disease that is caused by mutations in type IV collagen \(\alpha\) chains. Our study in the COL4A3^{-/-} “Alport mouse” reveals that Osteopontin (OPN), is highly expressed in the renal tubules of the Alport mouse and that it plays a major causative role in Alport pathology by regulation of Dynamin 3 (DNM3) and LDL receptor (LDLR) in the renal tubules of Alport mice. Our results suggest a new pathway for Alport pathology where tubular OPN causes DNM3-mediated enhanced cholesterol influx. Our data suggest that OPN may be a druggable therapeutic target for Alport Sydrome.
I dedicate this work to those who have given me constant encouragement, especially my supportive parents and grandparents.
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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>2F'</td>
<td>2 prime Fluorinated</td>
</tr>
<tr>
<td>2'O-Me</td>
<td>2 prime O-Methylated</td>
</tr>
<tr>
<td>3'UTR</td>
<td>3 prime untranslated region</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ABR</td>
<td>Auditory Brainstem Response</td>
</tr>
<tr>
<td>ACD</td>
<td>Anterior Chamber Depth</td>
</tr>
<tr>
<td>ACAA</td>
<td>Anterior Capsule Apical Angle</td>
</tr>
<tr>
<td>Bglap</td>
<td>Bone Gamma-carboxyglutamic Acid-containing Protein</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal Stem Cells</td>
</tr>
<tr>
<td>Bmp2</td>
<td>Bone Morphogenetic Protein 2</td>
</tr>
<tr>
<td>Bmp4</td>
<td>Bone Morphogenetic Protein 4</td>
</tr>
<tr>
<td>BUN</td>
<td>Blood urea nitrogen</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CCT</td>
<td>Central Corneal Thickness</td>
</tr>
<tr>
<td>Chrdl1</td>
<td>Chordin-like 1</td>
</tr>
<tr>
<td>Cnn1</td>
<td>Calponin 1</td>
</tr>
<tr>
<td>CRE</td>
<td>Creatinine</td>
</tr>
<tr>
<td>Ct-miR</td>
<td>Control microRNA</td>
</tr>
<tr>
<td>Dcn</td>
<td>Decorin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>EEG</td>
<td>Electroencephalograms</td>
</tr>
<tr>
<td>EM</td>
<td>Electron Microscopy</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ESRD</td>
<td>End Stage Renal Dysfunction</td>
</tr>
<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
</tr>
<tr>
<td>HF</td>
<td>High Fat</td>
</tr>
<tr>
<td>HMGCR</td>
<td>3-hydroxy-3-methylglutaryl CoA Reductase</td>
</tr>
<tr>
<td>Igf2</td>
<td>Insulin-like growth factor 2</td>
</tr>
<tr>
<td>Itgsf10</td>
<td>Immunoglobulin superfamily member 10</td>
</tr>
<tr>
<td>Kim-1</td>
<td>Kidney Injury Molecule 1</td>
</tr>
<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
</tr>
<tr>
<td>LDLR</td>
<td>LDL Receptor</td>
</tr>
<tr>
<td>LNA</td>
<td>Locked Nucleic Acid</td>
</tr>
<tr>
<td>Lrp6</td>
<td>Low-density lipoprotein receptor-related protein 6</td>
</tr>
<tr>
<td>MCHC</td>
<td>Mean Corpuscular Hemoglobin Concentration</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>Nfyc</td>
<td>Nuclear Transcription Factor Gamma</td>
</tr>
<tr>
<td>SMCs</td>
<td>Smooth Muscle Cells</td>
</tr>
<tr>
<td>OCT</td>
<td>Optical Coherence Tomography</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>Pparg</td>
<td>Peroxisome proliferator-activated Receptor Gamma</td>
</tr>
<tr>
<td>RCT</td>
<td>Reverse Cholesterol Transport</td>
</tr>
<tr>
<td>Runx2</td>
<td>Runt-related Transcription Factor 2</td>
</tr>
<tr>
<td>SCR</td>
<td>Scrambled Control</td>
</tr>
<tr>
<td>SMA</td>
<td>Smooth Muscle Actin</td>
</tr>
<tr>
<td>SM22α</td>
<td>Transgelin</td>
</tr>
<tr>
<td>Smad1</td>
<td>Smad family member 1</td>
</tr>
<tr>
<td>SREBPs</td>
<td>Sterol Regulatory Element Binding Proteins</td>
</tr>
<tr>
<td>SRF</td>
<td>Serum Response Factor</td>
</tr>
<tr>
<td>Synpo</td>
<td>Synaptopodin</td>
</tr>
<tr>
<td>Vcl</td>
<td>Vinculin</td>
</tr>
<tr>
<td>Wk</td>
<td>Week</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
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</table>
CHAPTER 1. Introduction

1.1 Classic Regulatory Pathways of Cholesterol Homeostasis

The initiation step in atherosclerosis is the accumulation of plasma cholesterol and abnormal cholesterol deposition on the arterial wall, which creates a lesion site. The accumulated cholesterol, namely low-density lipoprotein (LDL) cholesterol, is oxidized by interaction with native reactive oxygen species produced by endothelial cells. The oxidized LDLs recruit monocytes to the lesion site and promote their differentiation into macrophages\textsuperscript{1, 2} that take up the oxidized LDL via scavenger receptors. Oxidized LDL uptake by macrophages facilitates the formation of cholesterol-loaded foam cells\textsuperscript{2} that proliferate and die, releasing lipid content and causing a cascade of inflammatory events to the underlying smooth muscle layer. The final pathology is an atherosclerotic plaque consisting of a cholesterol-rich necrotic core surrounded by inflammatory cells. While aging, high fat diet, and genetics can cause atherogenesis, elevated plasma cholesterol is considered the major risk factor to develop atherosclerotic lesions. Therefore, finding key components in regulating cholesterol homeostasis is crucial to identifying novel therapeutic targets to prevent atherosclerosis.

1.1.1 Cholesterol Biosynthesis

Although cholesterol is synthesized in almost all cells, 50% of total body cholesterol is made by \textit{de novo} biosynthesis in the liver\textsuperscript{3}. In vertebrates, cholesterol is predominantly synthesized in the liver from acetyl-CoA through the
mevalonate pathway. The production of mevalonate is the rate-limiting step in cholesterol synthesis, which is catalyzed by 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR) and is the major pharmacologic control point. In atherosclerosis patients, reducing hepatic cholesterol synthesis and hepatic HMGCR is highly desirable. Cholesterol synthesis is transcriptionally regulated by sterol regulatory element binding proteins (SREBPs) which when stimulated by low cellular sterol levels, are cleaved and translocated to the nucleus to activate the expression of cholesterol biosynthesis genes such as HMGCR and LDL receptor (LDLR). In combination with other transcription factors such as SP1 and Nuclear Transcription Factor Y (NFYC), SREBPs are much stronger activators.

1.1.2 Cholesterol Internalization

After synthesis in the liver, cholesterol is packaged as lipoproteins and secreted into the circulation. Cells obtain cholesterol from the circulation in the form of LDL by LDLR mediate endocytosis. LDLs that are bound to LDLRs are internalized into the cell and transported to the early endosomes where low pH promotes LDLRs to dissociate from LDLs and recycled back to the plasma membrane. The remaining cholesterol molecules then proceed to late endosomes/lysosomes and upon release are transported to the plasma membrane, endoplasmic reticulum (ER), and mitochondria. Disruption of LDLR leads to a dramatic increase in plasma cholesterol levels. Such is the clinical case known as familial hypercholesterolaemia where patients have a mutation
in the LDLR gene and present a high level of plasma cholesterol. In patients with
atherosclerosis, decreasing the circulation of LDL cholesterol through LDLR is
highly desirable.

1.1.3 Reverse Cholesterol Transport
Because cells cannot degrade cholesterol, excess cholesterol is removed by
high-density lipoprotein (HDL)-mediated transportation from tissues and organs
to the liver in a process called reverse cholesterol transport (RCT)\textsuperscript{13}. In patients
with atherosclerosis, enhancing cholesterol efflux by RCT pathway is very
desirable for unloading cholesterol from foam cells.

1.2 LDL Accumulation and Osteogenic Differentiation Initiate and Drive
Atherosclerosis
Vascular calcification involves induction of an osteogenic program in smooth
muscle cells (SMCs)\textsuperscript{14, 15}. Although the origin of osteoblastic cells in
atherosclerotic lesions is debated, there is substantial evidence that SMCs enter
proliferative, migratory and synthetic states in response to atherogenic stimuli
(described in 1.1), and this includes increased expression of bone formation
markers such as alkaline phosphatase (ALP) and osteopontin (OPN)\textsuperscript{14}.
SM22α-Cre dependent lineage tracing suggests that SMCs may be precursors of
osteochondrogenic cells within calcified arterial media and atherosclerotic
lesions\textsuperscript{16}. Therefore, in this study, we analyzed both aortic SMCs and bone
marrow derived mesenchymal stem cells (MSCs) because both are possible
targets for osteogenic differentiation. While SMCs and MSCs originate from different cellular compartments, both cell types exhibit plasticity and the capability for osteogenic differentiation. To our knowledge, comparing the responses of these two cell types in parallel to osteogenic stimulation has not been studied and any distinguishing responses of either may be important to understand the mechanism, etiology and cellular origins of vascular calcification.

1.3 MicroRNAs As Novel Regulators in Cholesterol Homeostasis and Atherosclerosis

MicroRNAs (miRNAs or miRs) have recently been identified as important post-transcriptional regulators of cholesterol homeostasis. MiRs bind to 3’ untranslated region (UTR) of target genes, affecting messenger RNA (mRNA) stability and blocking protein translation, thereby repressing gene expression\(^\text{17}\). MiR-122 inhibition reduces Hmgcr mRNA expression and plasma cholesterol level\(^\text{18}\) with a marked reduction in ApoB100 protein levels\(^\text{19}\). MiR-33 regulates cholesterol efflux and high-density lipoprotein biogenesis by repressing the expression of ABCA1 and ABCG1\(^\text{20, 21}\). MiR106b decreases ABCA1-mediated cholesterol efflux from neuronal cells\(^\text{22}\). These findings support the possibility of developing miRs as therapeutic targets to treat atherosclerosis\(^\text{23}\).

Besides regulating cholesterol homeostasis, recent reports have also defined microRNAs as “osteomiRs” with impressive biomarker and/or functional roles in the osteogenic differentiation process of MSCs \(^\text{24-26}\). All members of the miR-30
family (with the possible exception of miR-30e) were shown to inhibit osteoblast differentiation of MSCs by targeting Smad1 and Runx-related transcription factor 2 (Runx2)\textsuperscript{27}. Most recently, miR-30e, located in the intron5 of Nfyc gene (Figure 1), was reported to induce adipogenic differentiation and reduce osteogenic differentiation in stromal cells by targeting Lrp6\textsuperscript{28}. Interestingly, miR-30e appears to be selectively repressed in the aortas of middle-aged ApoE\textsuperscript{−/−} mice relative to wild type (WT) mice\textsuperscript{29}.

![Figure 1 MiR-30e sits in intron5 of Nfyc gene. Mature miR-30e sequence is conserved across species.](figure1)

### 1.4 Alport Syndrome and Current Treatments

Alport Syndrome was first described in 1927 by Cecil A. Alport as a rare hereditary congenital hemorrhagic nephritis\textsuperscript{30}. Microscopic hematuria is the most common and earliest manifestation of the disease. Later in life, clinical implications of Alport Syndrome consist of proteinuria, hypertension, progressive renal failure, high frequency sensorineural hearing loss and ocular anomalies. A small population also presents other symptoms including dyspnea, facial puffiness, pedal edema, mental retardation and increased risk of cardiovascular events\textsuperscript{31, 32}. Mutations of type IV collagen chains, which are major components of capillary basement membranes cause Alport syndrome. Normally, embryonic α1α1α2 chain is replaced during development by α3α4α5 chain due to improved
mechanical stability. However, such a switch is absent in Alport patients\textsuperscript{31, 33, 34}, making glomerular basement membrane more vulnerable to filtration pressure and susceptible to endoproteolysis\textsuperscript{35}. Therefore, thickened glomerular (or other) capillary basement membrane is a major pathology of Alport patients. Sadly, in spite of possible early diagnosis, currently there is no therapy to prevent end stage renal failure in Alport patients and the standard care for Alport patients is limited to anti-hypertensive medications and eventually dialysis and kidney transplant.

1.5 Dynamins in Cholesterol Endocytosis

As described in 1.1.2, cells obtain cholesterol through membrane bound LDLR mediated endocytosis. After cholesterol internalization, it requires transporters to convey LDL-cholesterol to different cellular compartments where cholesterol is released for cellular functions and LDL is recycled back to the plasma membrane. Dynamin proteins including DNM3 have been shown to pinch and release clathrin-coated pit from plasma membrane, assisting LDLR-mediated cholesterol endocytosis\textsuperscript{36, 37}. After cholesterol intracellular uptake, dynamins have also been implicated in cholesterol intracellular transportation by delivery of free cholesterol from early endosome to ER\textsuperscript{37}. Inactivation of dynamins by chemical inhibitor Dynasore retards cellular cholesterol uptake and Niemann-Pick type C-like phenotype (NPC) with the accumulation of swollen late endosomes/lysosomes\textsuperscript{36}. However, the exact role of Dnm3 and its direct effects on cholesterol uptake or trafficking in renal dysfunction have not been studied.
Chapter 2. MiR-30e Targets IGF2-Regulated Osteogenesis in Bone Marrow Derived Mesenchymal Stem Cells, Aortic Smooth Muscle Cells and ApoE\(^{-}\) Mice

2.1 Background

Vascular calcification involves induction of an osteogenic program in SMCs\(^{14, 15}\). Although the origin of osteoblastic cells in atherosclerotic lesions is debated, there is substantial evidence that SMCs enter proliferative, migratory and synthetic states in response to atherogenic stimuli, and this includes increased expression of bone formation markers such as ALP and OPN\(^{14}\). SM22\(\alpha\)-Cre dependent lineage tracing suggests that SMCs may be precursors of osteochondrogenic cells within calcified arterial media and atherosclerotic lesions\(^{16}\). Therefore, in this study, we analyzed both aortic SMCs and bone marrow derived MSCs because both are possible targets for osteogenic differentiation. While SMCs and MSCs originate from different cellular compartments, both cell types exhibit plasticity and the capability for osteogenic differentiation. To our knowledge, comparing the responses of these two cell types in parallel to osteogenic stimulation has not been studied and any distinguishing responses of either may be important to understand the mechanism, etiology and cellular origins of vascular calcification.

Recent reports define “osteomiRs” as microRNAs with impressive biomarker and/or functional roles in the osteogenic differentiation process of MSCs \(^{24-26}\). All members of the miR-30 family (with the possible exception of miR-30e) were shown to inhibit osteoblast differentiation of MSCs by targeting Smad1 and Runx2\(^{27}\). Most recently, miR-30e was reported to induce adipogenic
differentiation and reduce osteogenic differentiation in stromal cells by targeting Lrp6. Interestingly, miR-30e appears to be selectively repressed in the aortas of middle-aged ApoE-/- mice relative to wild type mice. The global effects of miR-30e on the genome of SMCs or MSCs are not known. In the present study we demonstrate the following: 1) expression of miR-30e in medial SMCs, 2) global targets of miR-30e in both MSCs and SMCs, 3) down-regulation of miR-30e in aged atherosclerotic aortas, 4) binding of miR-30e to the 3' UTR of the mRNA of insulin like growth factor 2 (IGF2), and consequent regulation of gene and protein expression, 5) IGF2 rescue of miR-30e-repressed osteogenic differentiation in SMCs, 6) miR-30e mediated reduction of proliferation and induction of the smooth muscle differentiation of SMCs, and 7) induction of vascular calcification by antimiR-30e in vivo.

Together these findings support a model of miR-30e-regulation of an IGF2-dependent osteogenic transcriptional program in MSCs, SMCs, and ApoE-/- mice that develop aortic atheromas.

2.2 Materials and Methods

2.2.1 Materials and Reagents

Lentiviral vectors encoding miR-30e or a scrambled control miR sequence (ct-miR) were purchased from Open Biosystems (San Diego, California). Antibody against GAPDH (SC-25778), CD34 (SC-7324) and IGF-II (SC-5622) were bought from Santa Cruz Biotechnology (Santa Cruz, California); antibody against β-Actin (A5441) was obtained from Sigma-Aldrich (St. Louis, MO); antibody against β-gal
(ab616) was obtained from Abcam (Cambridge, MA); antibody against SM1 was obtained from Kamiya Biomedical Company (MC-352); antibody against OPN, and the IGF2, OPN and GAPDH DuoSet Elisa kits, and IGF2 (792-MG-050) and BMP2 (355-BM) recombinant protein were purchased from R&D Systems. Amersham ECL and Femto Western detection system were obtained from GE Healthcare Bio-Sciences (Piscataway, NJ). The MirVana PARIS kit used for microRNA, total RNA and protein isolations, and the reagents for real-time quantitative PCR were obtained from Life Technologies (Carlsbad, CA). Double luciferase vectors encoding \textit{Igf2}, \textit{Runx2}, or \textit{Opn} 3'UTR sequences were obtained from Genecopoeia (Rockville, MD). LNA AntimiR-30e and scrambled oligos were obtained from Exiqon (Woburn, MA). 2' O-Methyl modified (2’ Ome) antimiR-30e and scrambled oligos (used for animal injections) were obtained from IDT (Coralville, Iowa). Durascribe T7 Transcription kits were purchased from Epicentre (Madison, WI). HEPA1-6 cells were obtained from ATCC (Manassas, VA). All hepatocyte media was purchased from LifeTechnologies (Carlsbad, CA).

2.2.2 Cell Isolation and Culture

\textit{Aortic Smooth Muscle Cells (SMCs) Isolation and Culture}

SMCs were isolated from aortic explants of C57Bl6 1-month old male mice as previously described\textsuperscript{38}. Cells were passaged 5 times to enter proliferative phase as shown by reduced expressions in smooth muscle lineage proteins\textsuperscript{39} and then transduced with miR-30e or ctrl-miR lentiviral vectors. Puromycin selection was employed to create stable lines.
Bone Marrow derived Mesenchymal Stem Cells Isolation and Culture

MSCs were isolated from femurs and tibias of C57Bl6 3-month old male mice as previously described\textsuperscript{40}. Bone marrow was collected by flushing the cut femurs and tibias using a 5-ml syringe containing complete media and subsequently passing the flushed bone marrow through a 70\(\mu\)m mesh filter, and washing it with red blood cells lysis buffer to purify the MSCs. 3 hours post isolation, non-adherent cells were removed by media change. Isolated cells became more homogenous by losing hematopoietic population over sub-culture and MSCs were characterized by flow cytometry for negative expression of hematopoietic markers including CD34 and CD45 and positive expression for MSCs markers CD90.2 and CD105.

Primary Hepatocytes Isolation and Culture

C57BL6 mouse was anesthetized and Inferior Vena Cava (IVC) was exposed and cannulated. Liver was perfused through IVC with liver perfusion media at 6mL/min for 5min and then liver digestion media for 10min. Liver was excised, transferred to a 100mm culture dish, torn and shaken with forceps to free digested cells. Cells were passed through a 70\(\mu\)m strainer, centrifuged at 50g for 2min at 4\(^\circ\)C, and washed twice with William E media. Cells were then nucleofected (Amaxa, Lonza) with either control/SCR or miR-30e plasmids. Finally, transfected cells were plated on collagen I pre-coated 6-well plates. Four hours post seeding, media was changed to serum free culture media.
**Cell Culture of Human Tubular Epithelial cells, HEK293T cells and HEPA1-6 cells**

Human renal epithelial HK-2 cells, HEK293T, and HEPA1-6 cells were cultured in DMEM (Invitrogen) media supplemented with 20% FBS (Atlas Biologicals) and 1% (vol/vol) penicillin and streptomycin, and maintained under 37 °C/5% CO2 atmosphere.

### 2.2.3 Mice

All experiments involving animals were approved by the Institutional Animal Care and Use Committee at the University of Miami, conforming to NIH guidelines. ApoE−/− mice were bought from Jackson Labs and bred in-house. Young wild type mice were bought from Jackson labs, and old wild type mice were bought from the National Institute of Aging. For sacrificing all mice used in this study, isoflurane inhalation followed by cervical dislocation were used. Whole aortas were carefully dissected and pieces of the livers were excised, immediately immersed in RNALater and snap frozen in liquid nitrogen, or fixed in 10% formalin. Tissues were later homogenized in Cell Disruption Buffer (Mirvana Paris kit) for 2 consecutive 5 min in a Geno/Grinder 2000 homogenizer (OPS Diagnostics LLC, NJ), and used for RNA and protein work.

### 2.2.4 AntimiR-30e Oligo Injections

In the first experiment, 5 month old ApoE−/− male mice fed normal chow received tail vein injections of 0.6 nmoles 2′Ome antimiR-30e (n=8) or a scrambled (SCR)
control oligo (n=7) every other day for one month. A total of 16 injections per mouse were administered. Whole aortas were carefully dissected and pieces of the livers were excised, immediately immersed in RNALater and snap frozen in liquid nitrogen. Tissues were later homogenized in Cell Disruption Buffer (Mirvana Paris kit) for 2 consecutive 5 min in a Geno/Grinder 2000 homogenizer (OPS Diagnostics LLC, NJ), and used for RNA and protein work.

In the second experiment, 6 month old ApoE−/− male and female mice fed on high fat (HF) chow received tail vein injections of 3 nmoles (100nmol/kg) 2’Ome antimiR-30e (n=7) or PBS (n=6) every other day for two months. A total of 36 injections per mouse were administered. Livers were collected and processed for RNA and protein work as described above. Blood was collected and plasma CRP protein levels were measured using a CRP sandwich Elisa kit (R&D).

2.2.5 Transcribing 2’F miR-30e and miR-125b oligos

Templates for the sense and nonsense strands of premiR-30e and premiR-125b were generated as Ultramer DNA oligos (IDT), annealed at 95C for 5 mins, cooled down to room temperature, then transcribed to 2’F RNA oligos using Durascribe T7 enzyme kit following manufacturer’s protocol. Final product was DNase I digested and purified on Acrylamide/Urea gel before transfection into cells.

2.2.6 Cell Proliferation Assay

For proliferation assay, lentiviral transduced MSCs or SMCs were plated in 60 mm tissue culture dishes at a density of 5 x 10^4 per plate and counted daily for 6
days on a Coulter Counter (Beckman) or a T10 Cell Counter (Biorad). At least 3 plates were used per time point for each group.

2.2.7 RNA Isolation and Quantitative RT-PCR

For RNA isolation, cell lysates were collected in Cell Disruption Buffer (Mirvana Kit) and followed by the manufacturer’s protocol. For gene quantification, reverse transcription was performed by random hexamer primers (Applied Biosystems, Foster City, CA). cDNA was amplified using TaqMan Universal PCR master mix reagent (Applied Biosystems, Foster City, CA) and following manufacturer's conditions. For miRNA quantification, reverse transcription was performed using microRNA-specific Taqman primers, and cDNA was amplified using Taqman primers against the mature miRNA strands. Data was analyzed using the RQ Manager 1.2 from Applied Biosystems, CA. TaqMan assays were run in duplicate for each gene or microRNA in each sample (n = at least 3 biological replicates/condition) and all gene levels were normalized to 18S rRNA and all microRNA levels were normalized to sno RNA.

2.2.8 Microarrays and Bioinformatics

MSCs or SMCs were lysed using Cell Disruption Buffer (Mirvana Paris Kit) and total RNA was extracted using Mirvana Paris Kit to yield a 260–280 nm absorbance ratio of 2.0. RNA concentration and integrity was determined by using an Agilent Bioanalyzer. A 1μg aliquot of total RNA from each of the samples (3 samples per group; 2 groups per experiment) was processed using
instructions and reagents supplied by the manufacturer (Affymetrix, Santa Clara, CA). Briefly, total RNA was reverse transcribed using a T7-Oligo (dT) Promoter Primer in the first-strand cDNA synthesis reaction. Following RNase H-mediated-DNA polymerase I-second-strand cDNA synthesis, the double-stranded cDNA was purified and served as a template for in vitro transcription in the presence of T7RNA polymerase and a biotinylated nucleotide analog/ribonucleotide mix, producing biotin-labeled complementary RNA (cRNA). cRNA probes were then purified, fragmented, and hybridized on Mouse ST2 gene expression arrays (45,101 probe sets). Background noise, housekeeping gene expression and 3'/5' ratio values of all chips were within quality control limits set by Affymetrix. Expression ratios were calculated as the power-2 exponential of the log2 differences. The acceptance criteria for gene array expression changes was a minimum 2 fold change in log2 (equivalent to 4 fold) and a one-way Analysis of Variance (ANOVA) t-test p-value of <0.05. All microarray raw files were submitted to the NCBI Gene Expression Omnibus (GEO) database (GSE65435).

2.2.9 Protein Isolation and Western Blot

SMCs were lysed using Cell Disruption Buffer (Mirvana Paris Kit) and total cell lysates were subjected to SDS-PAGE and transferred to nitrocellulose membrane. Equal gel transfer was documented by Ponceau Red staining of membranes. Membranes were blocked for 1h at RT in 5 % nonfat milk in TBS-T buffer (Tris 20 mM, NaCl 137 mM, 0.5% Tween-20 pH 7.5), incubated overnight at 4C with primary antibodies, washed 3x in TBS-T for 10 mins, followed by
incubation with horseradish peroxidase-conjugated secondary antibody. After 3 final TBST washes, proteins were imaged by chemiluminescence, and bands were digitized and analyzed using Image J software (NIH, Bethesda, Maryland).

2.2.10 Enzyme-linked Immunosorbent Assay (ELISA)
For quantification of secreted OPN and IGF2 proteins from MSC cultures, equal volumes of collected media were used. For quantification of aortic OPN protein from ApoE⁻/⁻ mice, whole aortic lysates were used and normalized to GAPDH Elisa. For quantification of aortic and hepatic IGF2 protein from ApoE⁻/⁻ mice, lysates were subjected to Elisa and readings were normalized to measured protein concentrations. In all the ELISAs, each biological sample was assayed in duplicates, and a perfect standard curve was generated for measurements and/or quality control.

2.2.11 Site-directed Mutagenesis
Two sets of primers: MUT1 - 5' CAGTGAGGGAGGTGTTCTATACAGGCTCAATTCCATCTAAGC 3' Forward; 5' GCTTAGATGGAATTGAGCCTGTATAGAACACCTCCCTCACTG 3' Reverse; MUT2 - 5'-TCTTTTCTCAGTGAGGGAGGTGTTCTATGTAGGCTCAATTCCATCTA-3' Forward; 5'- TAGATGGAATTGAGCCTACATAGAACACCTCCCTCACTGAGAAAGA-3'
Reverse were designed carrying three point mutations in the center of the primer sequences. Luciferase vector containing wild type Igf2 3'UTR sequence was
used as a template in the mutagenesis PCR using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, La Jolla, CA).

2.2.12 Firefly/Renilla Double Luciferase Assay

Luciferase vectors containing either full-length Igf2 (wt or mutant), full length Opn, or 500bp of Runx2 3' UTRs were transfected into 293T or HEPA1-6 cells stably over-expressing miR-30e or ct-miR using Lipofectamine 2000 (Invitrogen). 48 hours post-transfection, cells were assayed for luciferase activities using the Luc-Pair miR dual luciferase assay kit (Genecoepia) and a micro-plate reader (SpectraMax M5, Sunnyrale, CA). Binding activities were finally reported as Firefly to Renilla luciferase luminescence in the same cells.

2.2.13 Alizarin Red and Oil Red Staining of Cells

MSC lentiviral stables were seeded in 24 well cell culture plate and stimulated to osteogenic differentiation or adipogenic differentiation by supplementing induction media accordingly. After 10 days, 2 weeks (wks), 2.5 wks, or 4 wks induction, cells were fixed in 10% formalin for 30 minutes (min), rinsed with distilled water. For Alizarin Red staining, 2% alizarin red solution was applied to each well and left for 45 min in the dark. Finally, cells were washed with water and microscopic images were obtained. For Oil Red Staining, cells were incubated with 60% isopropanol for 5min, stained with oil red solution for 5min. Finally, cells were washed with water, and microscopic images were obtained.
2.2.14 Statistics

For all experiments, N refers to the number of individual mice or individual culture plates. All data are expressed as mean ± S.E.M. p-values were calculated using Student’s t-tests. For data in Figure 2, we employed ANOVA with Tukey Posthoc correction using SPSS 22 software, to compare gene and microRNA expression among the 4 groups. Repeated symbols represent p-values of different orders of magnitude, i.e., * p<0.05, ** p<0.01.

2.3 Results

2.3.1 MiR-30e Expression is Downregulated in ApoE-/− mouse Aorta and is Inversely Correlated to Aging and Atherosclerosis.

To study the dynamics of NFYC and hosted miR-30e expression with age and atherosclerosis, we collected the full aortas from “young” 6 month-old ApoE-/- (n=7-9) and WT (n=4) mice, and “old” 13.5 month-old ApoE-/- (n=9-15) and WT (n=3-6) mice – all on normal chow. We found by qPCR that relative to young wild type, NFYC and mature miR-30e transcripts are downregulated in old wild type and mostly old ApoE-/- aortas (Figure 2 and Table 1). We also found that the pattern of NFYC/miR-30e expression is inversely proportional to the expression of the osteogenic genes IGF2 (Figure 2-Top Panel and Table 1), Runx2 and OPN (Figure 2-Bottom Panel and Table 1). Our finding supports a previous report that miR-30e was the most downregulated microRNA in the aortas of 10 months old ApoE⁻/⁻ versus wild type mice²⁹.
Measuring aortic OPN protein and normalizing to GAPDH, both by sandwich ELISA, we found that OPN protein was significantly upregulated in ApoE−/− aortas in both young and old mice (N=3-15 per group; Figure 2-Bottom Panel). Our results confirm previous reports on over-expression of OPN in atherosclerotic arteries41-43. In addition, the increase in OPN expression at an early stage of atherosclerosis confirms previous reports on induction of osteogenesis programs during arterial calcification44.

2.3.2 MiR-30e Downregulates an Osteogenic Gene Panel in MSCs

In order to test the prediction that miR-30e controls MSC differentiation potential, we performed microarrays on MSCs stably over-expressing either a control miR (MSCs + ct-miR) or miR-30e (MSCs + miR-30e). Interestingly, the genes most downregulated by miR-30e included the bone formation/ossification genes dermatopontin (Dpt), Igf2, bone morphogenetic protein 4 (Bmp4), chordin-like 1 (Chrdl1), and immunoglobulin superfamily, member 10 (Itgsf10) – listed in Table 2. To establish the detailed dynamics of osteogenic induction in MSCs, we initially used osteogenic media that is 1000 fold less intense (in dexamethasone and β-glycerophosphate concentration) than standard osteogenic recipe. We believe that this “light” osteogenic media is more physiologically relevant and would allow to better capture the dynamics of osteogenesis. As early as 1-day after osteogenic induction and peaking at 2 weeks, MSCs over-expressed a panel of 8 osteogenic markers including Igf2 (Figure 3A). Note that Bmp4 (1.6 fold; p=0.0034) and Alp (1.6 fold; p=0.05) transcripts were significantly increased
Figure 2. NFYC and hosted miR-30e transcripts are downregulated in aortas with age and atherosclerosis. NFYC and hosted miR-30e (but not miR-30c) transcripts are downregulated with age and atherosclerosis. On the other hand, Igf2 (top panel) and Runx2, Opn (bottom panel) transcripts are upregulated in old (13.5 months) versus young (6 months) and APOE−/− versus wild type (WT) aortas as quantified by qPCR. Aortic OPN protein levels are also upregulated in APOE−/− versus age-matched wild type aortas as measured by OPN/GAPDH sandwich ELISA. Y= Young; O= Old. For Ns in all experiments: Old WT, N=4 or N=3 in OPN qPCR only; Young WT, N=4; Old APOE, N=15; Young APOE, N=9; Data are mean±SEM.*P<0.05, **P<0.01 based on ANOVA with Tukey Posthoc corrections for normalized CTs. Figure from Ding et el. Cardiovascular Research. (2015) 106 (1): 131-142.
at day 1. Starting at day 2, most of the 8 genes were up-regulated, especially Igf2 which was mostly induced at day 5 (50.1 fold; \( p=0.0003 \)) and day 14 (115.6 fold; \( p=0.0008 \); Figure 3A). This supports a very recent report on Igf2 being the most induced gene in mouse aortic SMCs after osteogenic differentiation\(^4^5\).

Most interestingly, this osteogenic panel (8 genes) was tightly regulated by miR-30e. We quantified by qPCR the gene expression of the osteogenic panel in MSCs transiently transfected with 0.1nmol/L, 1nmol/L or 10nmol/L of 2F’ miR-30e oligos. Results confirmed the reduction of the osteogenic panel (including ALP) by miR-30e in a dose-dependent manner (Figure 3B and Table 3).

Next we tested these same genes in MSCs stably over-expressing miR-30e before and after osteogenic differentiation. We found that \( Dpt \) (-11.3 fold, \( p=0.03 \)), Decorin (\( Dcn \)) (-12.9 fold, \( p=0.01 \)), \( Bmp4 \) (-5.2 fold, \( p=0.01 \)), and \( Igf2 \) (-21.7 fold, \( p=0.02 \)) transcript levels were significantly lower in MSCs over-expressing miR-30e relative to the control treated cells (Figure 4 – left panel). After 2 wk osteogenic differentiation, gene expressions of these osteogenic markers (as well as Opn, Runx2, and Bmp4) went significantly up in the control group as expected, but were only modestly increased in the miR-30e-transduced cells (Figure 4 – right panel). \( Igf2 \) transcripts were significantly down-regulated in MSCs over-expressing miR-30e relative to the controls in the differentiated cells (-37.7 fold; \( p = 0.04 \)), suggesting a leading role for IGF2 in the osteogenic panel.
2.3.3 MiR-30e Drives MSCs Towards Adipogenic Differentiation, and SMCs Towards Smooth Muscle Differentiation

We tested proliferation and differentiation potential in MSCs and SMCs stably over-expressing miR-30e versus ct-miR in normal media. First we recorded cell numbers in 6 consecutive days and found MSCs over-expressing miR-30e exhibited a significant lower proliferation rate at day 2, day 4 and day 5 (Data now shown). A slow proliferation rate suggested a tendency towards a differentiation lineage. Our microarray data confirmed adipogenic tendency, by significant over-expression of the 2 major adipogenic markers Fatty Acid Binding Protein 4 (Fabp4) and peroxisome proliferator-activated receptor gamma (Ppary) by miR-30e (Data now shown). These results are in line with a very recent report on miR-30e inducing adipogenic differentiation in bone marrow stromal cells\textsuperscript{27, 28}. Our microarray data showed that in SMCs and MSCs, miR-30e did not cause a significant change in the gene expression of Bmi1 which was reported as regulated by miR-30e*\textsuperscript{46}. Similarly, in MSCs, antimiR-30e (equivalent to miR-30e*) did not cause a significant change in the expression of Bmi gene. Therefore, our microarray data suggests that the targeting of Bmi1 by miR-30e could be cell specific and un-related to the miR-30e effects in SMCs and MSCs.

In SMCs also, miR-30e delayed proliferation rate at days 2-6 (Data now shown) and induced smooth muscle differentiation as quantified by qPCR the transcripts of smooth muscle lineage markers Smooth Muscle 22-alpha (SM22α, Calponin 1 (Cnn1)) (Figure 5A) and western blots the protein of Smooth muscle actin (SMA) were increased (Figure 5B). Our results on miR-30e inducing smooth muscle or
adipogenic differentiation while reducing osteogenic differentiation are in line with the concept of a sensitive balance/seesaw that forces the cells to choose one lineage over the other.

2.3.4 Igf2 Is Regulated by MiR-30e and AntimiR-30e in Mirror Directions

To study the effect of over-expressing and knocking down miR-30e on MSCs in an unbiased manner, we performed global gene expression microarray studies using 4 groups, MSCs stably transduced with ct-miR or miR-30e lentivirus and MSCs transiently transfected with LNA scrambled or antimiR-30e oligos (GSE65435). To identify miR-30e targets that showed reversible regulation with the antimiR-30e, we implemented Venn analysis and identified \textit{Igf2} as one of the 7 targets that are repressed by miR-30e and induced by antimiR-30e, respectively. To find common genes regulated by miR-30e in MSCs and SMCs, we also implemented microarrays (GSE65435) and Venn analysis and found that \textit{Igf2} and related genes were downregulated in both MSCs and SMCs relative to their controls (Data not shown). Using GO analysis on the genes differentially repressed by miR-30e in MSCs, we found that \textit{Kinase Related Activity}, \textit{Lectin-Like Receptor}, \textit{Insulin-Like Growth Factor Receptor}, and \textit{Protein Binding} were the four most represented molecular functions regulated by miR-30e \((p<0.05, \text{ - Data not shown})\). The microarray data further supported the role of miR-30e in regulating an osteogenic program in MSCs and specifically involving \textit{Igf2}.
Figure 3. MiR-30e reduces the gene expression of a panel of osteogenic markers in MSCs. A. An osteogenic panel comprised of 8 genes is initiated as early as one day after culturing MSCs in osteogenic media. B. Over expression of miR-30e by miR-30e 2′F oligos reduces in a dose-dependent manner the expression of the osteogenic panel in MSCs. N=3 per group; Data are mean±SEM.*P<0.05, **P<0.01 based on Student’s t-test. Figure from Ding et al. *Cardiovascular Research.* (2015) 106 (1): 131-142.
Figure 4. MiR-30e reduces gene expression of osteogenic markers before and after osteogenic differentiation in MSCs. MiR-30e lentiviral stable over-expression in MSCs causes a significant reduction in transcript levels of the osteogenic panel especially Igf2 before (left panel) and after (right panel) osteogenic differentiation as measured by qPCR. N=3 per group; Data are mean±SD. *P<0.05, **P<0.01 based on Student's t-test. Figure from Ding et al. Cardiovascular Research. (2015) 106 (1): 131-142.
qPCR validated the microarray results on Igf2; miR-30e over-expression repressed Igf2 transcripts in MSCs, SMCs, Hepa1-6 mouse hepatocarcinoma cells and primary hepatocytes (Figure 6A). AntimiR-30e, which reduced miR-30e levels (-6.0 fold, $p = 0.01$ - Figure 6B), upregulated Igf2 transcripts relative to control SCR (2.3 fold, $p=0.04$ - Figure 6C) and miR-30e oligo groups. Next we quantified IGF2 protein expression with over-expression of miR-30e. Secreted IGF2 protein levels were significantly and dramatically increased at 7day osteogenic differentiation in control MSCs (637.4 fold relative to un-differentiated group; $p=0.001$), but nearly completely repressed in miR-30e transduced MSCs.
(0.5 fold relative to ct-miR differentiated group; \( p=0.02 \) – Figure 6D). Interestingly, we found that treatment of MSCs with IGF2 recombinant protein (500ng/ml) quickly induced the expression of miR-30e (N=4/group, \( p=0.02 \) - Figure 6I). This suggests that miR-30e and IGF2 function in a feedback loop, and could explain the non-perfect correlation between miR-30e and its host gene Nfyc (Figure 6E).

To study if miR-30e regulates IGF2 in vivo, we injected 5month old ApoE\(^{-/-}\) mice with 0.6nmols/injection 2’O-Me antimiR-30e oligos (n=8) or SCR oligos (n=7). We found that antimiR-30e caused significant upregulation of Igf2 transcripts in aortas (FC=2.4; \( p=0.03 \)) and livers (FC=2.3; \( p=0.03 \)), and Igf2 protein in livers (FC=1.7; \( p=0.04 \)) relative to the Scr group (Figure 6FG). Injection of 6 month old ApoE\(^{-/-}\) mice with a higher dosage of antimiR-30e (3nmols or 100nmol/kg or 0.6mg/kg per injection, n=7) continued to induce IGF2 protein expression in the livers (Figure 6H).

2.3.5 MiR-30e Directly Binds to Igf2 Transcripts

To determine whether Igf2 mRNA is the direct target of miR-30e, we first confirmed using RNAHybrid software that miR-30e has a predicted binding site at the murine Igf2 3’UTR (Figure 7A). Then, we launched a series of Firefly/Renilla luciferase binding assay experiments in multiple systems using the wild type and 2 mutant Igf2 3’ UTR constructs (Figure 7A). In HEPA1-6 cells, transfection with 2’F miR-30e oligos caused significant reduction in normalized luciferase activity (0.49 fold, \( p=0.0003 \)) relative to the control blank group, even more reduction than that caused by the positive control miR-125b\(^{47} \) (0.75 fold, \( p=0.04 \) - Figure 7B). HEPA1-6 cell stably over-expressing miR-30e or a control miR, luciferase -
Figure 6. MiR-30e regulates Igf2 transcripts and proteins in vitro and in vivo. A. miR-30e over-expression reduces Igf2 transcripts in MSC, SMCs, Hepa1-6 cells, mouse primary hepatocytes - all relative to their ct-miR groups – as shown by qPCR. N=3 per group. B. qPCR shows successful over-expression and knock down of miR-30e in MSCs using 2’F miR-30e or LNA antimiR-30e oligo 2-day transfections respectively. N=3 per group. C. Transfection with antimiR-30e oligos for 2 days in MSCs causes up-regulation of Igf2 transcripts as shown by qPCR. N=3 per group. D. Secreted Igf2 protein levels are significantly reduced in miR-30e-transduced MSCs after 7 days of osteogenic differentiation, as measured by sandwich ELISA. N=4 per group. E. Treatment of MSCs with IGF2 recombinant protein (500ng/ml) induces miR-30e transcripts as measured by qPCR. N=4 per group. F-H. Systematic administration of 0.6nmols antimiR-30e oligos in 6 mo ApoE-/- mice on normal chow causes significant upregulation of Igf2 aortic (F) and hepatic mRNA (G) and Igf2 hepatic protein (H) as measured by qPCR and sandwich ELISA respectively. SCR, N=4; AntimiR-30e, N=7; Data are mean±SEM.*P<0.05, **P<0.01 based on Student’s t-test. Figure from Ding et al. Cardiovascular Research. (2015) 106 (1): 131-142.
Figure 7. MiR-30e directly targets Igf2 transcripts. A. Shown is the secondary structure of the binding of miR-30e mature sequence to Igf2 3’UTR as predicted by RNAHybrid software. Arrows point to mutation sites at Igf2 3’UTR used to generate 2 mutant sequences. B. Using 2’F miR-30e or miR-125 (positive control) oligos in Hepa1-6 cells, luciferase assay shows binding of miR-30e to the Igf2 3’UTR. C-D. Using miR-30e or ct-miR lentiviral Hepa1-6 or 293T stables (D), luciferase assay shows binding of miR-30e to 3’UTR of Igf2 and Runx2 (positive control), but not OPN nor mutant Igf2 constructs. N=4 per group except N=5 for MUT1 and MUT2 groups. Data are mean±SEM. P values are as indicated on the figure and calculated based on Student’s t-test. Figure from Ding et al. Cardiovascular Research. (2015) 106 (1): 131-142.
activity was reduced in the miR group relative to the ct-miR group in a dose dependent manner with increasing concentrations of the wild type 3'UTR Igf2 construct, and also with the positive control construct Runx2 that has a predicted site for miR-30e, but not with the OPN construct (Figure 7C). Interestingly, luciferase activity showed stronger binding of miR-30e to the Igf2 3'UTR construct than Runx2 which has been reported to being regulated by the other miR-30 family members. In addition, our results show no binding of miR-30e to the 3'UTR of OPN which further suggests that the repression of OPN by miR-30e is indirect and possibly downstream of Igf2 regulation by miR-30e.

Previous investigations on microRNA binding principles revealed the importance and sufficiency of complementarity between 5' end of miRNA mature sequence and its target gene, specifically from the first to the eight base pair in the complementary sequence, recognized as seed sequence. Therefore, we generated two mutant constructs of the Ig2 3'UTR at the nucleotides (nts) corresponding to the first 3 bases of the binding miR-30e seed sequence located 1093 to 1095 nt of Igf2 3'UTR (Figure 7A). In 293T cells stably over-expressing miR-30e or a ct-miR lentivirus, luciferase activity was significantly reduced by the wt Igf2 3'UTR construct but not by the two mutant constructs (Figure 7D). These results collectively confirm that miR-30e binds directly to Igf2 3'UTR and represses Igf2 gene expression.
2.3.6 IGF2 Recombinant Protein Rescues MiR-30e-repressed Osteogenesis in SMCs

To validate that IGF2 is indeed a mechanism by which miR-30e reduces osteogenesis in SMCs, we generated SMCs stably over-expressing miR-30e or a ct-miR, and cultured them in osteogenic media for several wks. SMCs stably over-expressing miR-30e show repression of osteogenic differentiation as measured by Alizarin Red 2 and 4 wks after osteogenic induction. Addition of IGF2 recombinant protein (500μg/ml) rescued osteogenic differentiation in the SMCs over-expressing miR-30e (Figure 8A). To test the ability of another potent osteogenic agent to rescue miR-30e-repressed osteogenesis, we performed a 2.5wks osteogenic experiment in SMC stables and treated with IGF2 or BMP2 (both at 250μg/ml). Interestingly, like IGF2, BMP2 also restored osteogenesis in SMCs over-expressing miR-30e (Figure 8B). While our microarray data did not show that miR-30e regulates Bmp2 gene expression, the results from this rescue experiment suggest that IGF2 and BMP2 share common downstream osteogenic effectors that were blocked by miR-30e. This is not surprising since both IGF2 and Bmp2, for example, upregulate Runx2\textsuperscript{50,51} which is also a primary target of miR-30e (Figure 8C and Wang et al\textsuperscript{28}).
Figure 8. IGF2 Recombinant Protein Rescues miR-30e-repressed osteogenesis in SMCs. A. SMCs stably over-expressing miR-30e show repression of osteogenic differentiation as measured by Alizarin Red 2 wks (top row) and 4 wks (second row) after osteogenic induction. Addition of IGF2 recombinant protein at 500ng/ml rescues osteogenic differentiation in the SMCs over-expressing miR-30e. B. At 2.5 wks osteogenic differentiation, IGF2 as well as BMP2 recombinant protein (both at 250ng/ml) rescue osteogenic differentiation in the SMCs over-expressing miR-30e. Images are representative of 3 experiments in IGF2 and BMP2 rescue groups and 4 experiments in control without recombinant protein groups. Scale bar = 100μm. Figure from Ding et al. Cardiovascular Research. 10.1093 (2015)
Table 1. Statistical analysis of transcript changes in Old (O) and Young (Y) APOE⁻/⁻ and wild type (WT) aortas.

<table>
<thead>
<tr>
<th></th>
<th>Nfyc (Ex5-6)</th>
<th>miR-30e</th>
<th>miR-30c</th>
<th>Runx2</th>
<th>OPN</th>
<th>Igf2</th>
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<td>FC=2.84, p=0.10</td>
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<td>FC=0.94, p=1</td>
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<tr>
<td>APOE⁻/⁻ (O) vs WT (O)</td>
<td>FC=0.52, p=0.90</td>
<td>FC=2.42, p=0.17</td>
<td>FC=4.93, p=0.07</td>
<td>FC=2.82, p=0.06</td>
<td>FC=10.53, p=0.07</td>
<td>FC=1.21, p=0.98</td>
</tr>
<tr>
<td>APOE⁻/⁻ (O) vs APOE⁻/⁻(Y)</td>
<td>FC=0.03, p=0.002</td>
<td>FC=0.36, p=0.02</td>
<td>FC=2.08, p=0.40</td>
<td>FC=3.88, p=0.007</td>
<td>FC=2.47, p=0.51</td>
<td>FC=2.87, p=0.03</td>
</tr>
<tr>
<td>WT (O) vs WT (Y)</td>
<td>FC=0.05, p=0.10</td>
<td>FC=0.42, p=0.32</td>
<td>FC=0.54, p=0.86</td>
<td>FC=3.79, p=0.05</td>
<td>FC=17.75, p=0.05</td>
<td>FC=2.23, p=0.46</td>
</tr>
<tr>
<td>APOE⁻/⁻ (O) vs WT (Y)</td>
<td>FC=0.03, p=0.008</td>
<td>FC=1.02, p=1</td>
<td>FC=2.69, p=0.40</td>
<td>FC=10.69, p=0.0001</td>
<td>FC=186.86, p=0.00001</td>
<td>FC=2.69, p=0.16</td>
</tr>
<tr>
<td>WT (O) vs APOE⁻/⁻ (Y)</td>
<td>FC=0.09, p=0.11</td>
<td>FC=0.15, p=0.0004</td>
<td>FC=0.42, p=0.56</td>
<td>FC=1.37, p=0.87</td>
<td>FC=0.23, p=0.42</td>
<td>FC=2.38, p=0.25</td>
</tr>
</tbody>
</table>

Full aortas from “young” 6 month-old ApoE⁻/⁻ (n=9) and WT (n=4) mice, and “old” 13.5 month-old ApoE⁻/⁻ (n=15) and WT (n=4 or 3 in OPN group only) mice – all on normal chow were collected and transcripts were quantified by qPCR. FC = Fold Change. P-values were generated from ANOVA with Tukey Posthoc corrections using normalized CTs. Table from Ding et al. Cardiovascular Research. (2015) 106 (1): 131-142.
Table 2. Most downregulated transcripts in MSCs over-expressing miR-30e.

<table>
<thead>
<tr>
<th>FC Down</th>
<th>Gene Symbol</th>
<th>Gene Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>812.0</td>
<td>Dpt</td>
<td>Dermatopontin</td>
</tr>
<tr>
<td>45.5</td>
<td>Dcn</td>
<td>Decorin</td>
</tr>
<tr>
<td>21.7</td>
<td>C3</td>
<td>complement component 3</td>
</tr>
<tr>
<td>12.3</td>
<td>Mest</td>
<td>mesoderm specific transcript</td>
</tr>
<tr>
<td>12.1</td>
<td>Islr</td>
<td>immunoglobulin superfamily containing leucine-rich repeat</td>
</tr>
<tr>
<td>10.7</td>
<td>Igf2</td>
<td>insulin-like growth factor 2</td>
</tr>
<tr>
<td>10.7</td>
<td>Abi3bp</td>
<td>ABI gene family, member 3 (NESH) binding protein</td>
</tr>
<tr>
<td>10.6</td>
<td>Itga11</td>
<td>integrin, alpha 11</td>
</tr>
<tr>
<td>10.4</td>
<td>Cacna1g</td>
<td>calcium channel, voltage-dependent, T type, alpha 1G subunit</td>
</tr>
<tr>
<td>10.3</td>
<td>Nid2</td>
<td>nidogen 2</td>
</tr>
<tr>
<td>10.2</td>
<td>Gfra1</td>
<td>glial cell line derived neurotrophic factor family receptor alpha 1</td>
</tr>
<tr>
<td>9.4</td>
<td>Podn</td>
<td>Podocan</td>
</tr>
<tr>
<td>8.9</td>
<td>Chrdl1</td>
<td>chordin-like 1</td>
</tr>
<tr>
<td>6.5</td>
<td>Bmp4</td>
<td>bone morphogenetic protein 4</td>
</tr>
<tr>
<td>6.1</td>
<td>Igsf10</td>
<td>immunoglobulin superfamily, member 10</td>
</tr>
</tbody>
</table>

Microarrays were performed on MSCs stably over-expressing either a control miR (MSCs + ct-miR) or miR-30e (MSCs + miR-30e). Bone formation/ossification genes dermatopontin (Dpt), insulin growth factor 2 (Igf2), bone morphogenetic protein 4 (Bmp4), chordin-like 1 (Chrdl1), and immunoglobulin superfamily, member 10 (Iggsf10) were most downregulated transcripts in MSCs over-expressing miR-30e relative to control group. N=3 per group. FC = Fold Change. Supplemental able from Ding et el. *Cardiovascular Research.* (2015) 106 (1): 131-142.
Table 3. Changes in transcripts in MSCs treated with miR-30e 2’ Fluoro oligonucleotides.

<table>
<thead>
<tr>
<th></th>
<th>(0.1nmol/L miR-30e)</th>
<th>(1nmol/L miR-30e)</th>
<th>(10nmol/L miR-30e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-30e</td>
<td>FC=2.7, p=0.03</td>
<td>FC=5.3, p=0.03</td>
<td>FC=5.5, p=0.002</td>
</tr>
<tr>
<td>Dcn</td>
<td>FC=-5.3, p=0.78</td>
<td>FC=-17.3, p=0.05</td>
<td>FC=-20, p=0.03</td>
</tr>
<tr>
<td>Dpt</td>
<td>FC=-5.3, p=0.66</td>
<td>FC=-12.5, p=0.05</td>
<td>FC=-23.2, p=0.04</td>
</tr>
<tr>
<td>Opn</td>
<td>FC=-4.9, p=0.75</td>
<td>FC=-16.4, p=0.057</td>
<td>FC=-19.2, p=0.03</td>
</tr>
<tr>
<td>Alp</td>
<td>FC=-7.4, p=0.05</td>
<td>FC=-9.5, p=0.01</td>
<td>FC=-3.8, p=0.04</td>
</tr>
</tbody>
</table>

Gene expression of the osteogenic panel in MSCs transiently transfected with 0.1nmol/L, 1nmol/L or 10nmol/L of 2F’ miR-30e oligos were quantified by qPCR. Reduction of the osteogenic panel in MSCs over expressing miR-30e is in a dose-dependent manner. N=3 per group. FC=Fold Change. P values are calculated based on Student’s *t*-test. Table from Ding *et al*. *Cardiovascular Research*. (2015) 106 (1): 131-142.

2.4 Discussion

We found that *NFYC* gene and hosted miR-30e transcripts are downregulated in aged mouse atherosclerotic aortas. Interestingly, the *NFYC* gene was also reported to be downregulated in the blood of patients with coronary artery disease (CAD) and predicted the extent of CAD\(^52\). Our results are consistent with previous reports that miR-30e is downregulated in aortas of middle-aged ApoE\(^{-/-}\) mice\(^{29}\), human thoracic dissections\(^{53}\), and brains of calorie-restricted aging mice\(^{54}\). We report for the first time that age and atherosclerosis simultaneously regulate miR-30e. Global gene expression in SMCs and MSCs that over- or under-express miR-30e indicate that miR-30e regulates a unique osteogenic panel of at least 8 genes in MSCs, with Igf2 being the shared target across
several cell types (MSCs, SMCs, hepatocellular carcinoma cells, and primary hepatocytes). Our results on the anti-osteogenic effects of miR-30e are consistent with the functions of miR-30e in osteogenesis and adipogenesis reported by others\textsuperscript{27,28}.

A novel finding is that miR-30e is pro smooth muscle differentiated phenotype, lack of which leads to pathogenesis of vasculo-proliferative disease such as atherosclerosis and restenosis. In SMCs, miR-30e reduces proliferation and organelle count (Data now shown), and increases smooth muscle lineage markers (Figure 5), thereby inducing smooth muscle differentiation.

One of the most interesting findings in our study is the identification of IGF2 as a primary target of miR-30e, and that IGF2 rescued the osteogenic phenotype repressed by miR-30e in SMCs. IGF2 is a protein hormone that has been implicated in atherogenesis. IGF2 knock-out ApoE\textsuperscript{−/−} mice showed a significant attenuation of atherogenic plaque formation and development, while IGF2 transgenic ApoE\textsuperscript{−/−} mice showed increased atherosclerotic lesions\textsuperscript{55}. We show that antimiR-30e induces expression of IGF2 in aortas and livers of ApoE\textsuperscript{−/−} mice (Figure 6F-H), accompanied by calcification of the aortic valves (Data not shown). IGF2 is a major aging and osteogenic factor\textsuperscript{50,56-59}, making it a clinically desirable target for treating age-related calcification. Moreover, it has been proposed that IGF2 contributes to the development of a variety of seemingly unrelated cancers that appear with advanced age\textsuperscript{60}. Our results on miR-30e directly binding and/or regulating IGF2 in various cell lines and primary cells, as
well as in vivo in atherogenic ApoE^{-/-} mice suggest that miR-30e can be a therapeutic target for IGF2-regulated diseases.

2.5 Summary

Activation of an osteogenic transcriptional program contributes to the initiation of aortic plaque calcification in atherosclerosis. In aortas of wild-type mice, we found that miR-30e is highly expressed in medial SMCs. In aortas of old ApoE^{-/-} mice, we found that miR-30e transcripts are down-regulated in an inverse relation to the osteogenic markers Runx2, Opn, and Igf2. In vitro, miR-30e over-expression reduced the proliferation of MSCs and SMCs while increasing adipogenic differentiation of MSCs and smooth muscle differentiation of SMCs. In MSCs and SMCs over-expressing miR-30e, microarrays and qPCR showed repression of an osteogenic gene panel including Igf2. Inhibiting miR-30e in MSCs increased Igf2 transcripts. In SMCs, IGF2 recombinant protein rescued miR-30e-repressed osteogenic differentiation. Luciferase and mutagenesis assays showed binding of miR-30e to a novel and essential site at the 3{'UTR of Igf2. In ApoE^{-/-} mice, injections of antimiR-30e oligos increased Igf2 expression in the aortas and livers and significantly enhanced OPN protein expression and calcium deposition in aortic valves. In conclusion, miR-30e represses the osteogenic program in MSCs and SMCs by targeting IGF2 and drives their differentiation into adipogenic or smooth muscle lineage, respectively. MiR-30e may play an important role in calcification in atherogenic plaque.
Chapter 3. Osteopontin Deficiency Ameliorates Alport Pathology by Preparing DNM3-mediated Cholesterol Influx

3.1 Background

Alport Syndrome is a rare hereditary renal dysfunction characterized by high frequency sensorineural hearing loss and ocular anomalies\(^{30,61}\). The disease is caused by mutations in Type IV collagen α chains. Mutation of col4a5 causes X-linked Alport Syndrome while mutations of col4a3 and col4a4 cause autosomal recessive inheritance. The type IV collagen is an essential component of the basement membranes\(^{62}\). Mutation of col4a3 causes capillary basement membrane thickening in kidney glomeruli\(^{63}\), as well as in the inner ear of Alport mice\(^{64}\). Besides impaired kidney functions and hearing inabilities, Alport patients also present symptoms including hypertension and vision deficits. Current treatment of Alport Syndrome is limited to reducing hypertension by ACE inhibitors\(^{65,66}\) alone or combined with multiple drugs for proteinuria\(^{67}\) in Alport patients or to reducing fibrosis in Alport mice\(^{68}\). There is still no cure to Alport Syndrome nor a potent treatment that can ameliorate the multiple Alport symptoms.

The \textit{Col4a3}^-/- “Alport” mouse is an acceptable model for Alport Syndrome\(^{61,64,69}\) as the mouse demonstrates many symptoms of an Alport patient. The Alport mouse gradually develops severe proteinuria, glomerulosclerosis and tubulointerstitial fibrosis, and dies at approximately 10 wks of age. Otologic assessments have shown that the Alport mouse tends to have elevated hearing thresholds with age, as seen in the human syndrome.\(^7\) Ocular abnormalities have
not been investigated previously in the Alport mouse. Here, we further characterize the Alport mouse on mixed genetic background and show previously un-reported clinically relevant phenotype including hypertension, lenticonus, thickened corneal capillary basement membrane, and an immensely increased renal cholesterol influx.

OPN is a secreted phosphoprotein that plays important roles in regulating bone morphogenesis (described in Chapter 2)\textsuperscript{70}, inflammation, heart failure, and tumor metastasis.\textsuperscript{71, 72} Renal OPN mRNA and protein are increased in animal models of albuminuria and urinary OPN levels may be useful predictor of steroid treatment response in pediatric nephrotic patients.\textsuperscript{73} In cultured murine podocytes, OPN recombinant protein increased expression of matrix-degrading enzymes including MMP-2 and MMP-9, and podocyte motility, which has been strongly linked to the development of proteinuria\textsuperscript{73-75}. In this study, we report a novel OPN-driven signaling pathway that plays a potentially central role in Alport pathology. We show that OPN is highly expressed in Alport renal tubules where it regulates Dynamin-3 (DNM3)-mediated increased LDL cholesterol influx.

3.2 Materials and Methods

3.2.1 Animals

All experiments involving animals were approved by the Institutional Animal Care and Use Committee at the University of Miami, conforming to NIH guidelines. Col4a3\textsuperscript{-/-} Alport mice on 129X1/SvJ background, OPN\textsuperscript{+/+} and LDLR\textsuperscript{-/-} mice on
C57Bl/6 background were purchased from Jackson Lab and inter-bred at least 10 times.

3.2.2 EdU Injections
5-ethynyl-2’-deoxyuridine (EdU) (A10044, LifeTechnologies) was prepared at 1mg/ml stock and filtered through 0.2μm Nalgene syringe filter. 100μl (100μg) EdU stock was administered daily to each mouse through intraperitoneal injections for up to 30 days to identify mitotic cells. EdU incorporation was visualized by EdU fluorescent staining on paraffin kidney sections.

3.2.3 Dil-LDL Injections
150μl (150 μg) of Dil-LDL cholesterol (L3482, ThermoFisher Scientific) were injected into 10 wks old of wild type or Col4a3-/- mice via tail vein injections. After 2 hours, mice were perfused through cardiac puncture with 10ml PBS followed by 10ml 4% PFA. Kidneys and livers were collected for histological studies on paraffin sections.

3.2.4 Blood Pressure Recording
Blood pressure measurements were carried out using CODA mouse tail-cuff system (Kent Scientific). Anesthesia was induced with 5% vaporized isoflurane at 0.8 L/min flow rate and maintained with 1% isoflurane during blood pressure recordings. Heart rate was monitored and body temperature was controlled within 37-39°C. Two tail cuffs: occlusion and VPR were equipped onto the tails.
Occlusion tail cuff was inflated and deflated to control blood flow. VPR tail cuff was incorporated with specially designed differential pressure transducer that measured the systolic and diastolic blood pressure by determining the blood volume in the tail. Animals were trained 3 times to minimize stress-related blood pressure changes caused by the system prior to recording the final blood pressure values. 20 systolic and diastolic blood pressure readings were recorded from each mouse and the 15 most consistent readings were used for analysis.

3.2.5 Hearing Testing

Auditory Brainstem Response (ABR) was performed on anesthetized mice at 8-9 wks of age. ABR subdermal electrodes were placed on the vertex, both mastoids, and the left hind leg (ground). Using a commercial system (Intelligent Hearing Systems, Miami, FL), electroencephalograms (EEG) were amplified 100,000 times, band-pass filtered between 30 Hz and 1500 Hz and acquired using a sampling frequency of 10 Ksps in 102.4 ms long epochs. Acoustic stimuli using tone bursts at 4, 8, and 16 kHz were delivered with insert ear phones. For each frequency and epoch, eight amplitude-modulated tone bursts were presented to each ear at an average stimulation rate of 78.13. The raw EEGs were averaged in blocks of 64 epochs to obtain the auditory evoked potential responses. Each epoch lasted 102.4 ms and contained eight tone pips modulated by a 3 ms trapezoidal envelope (1 ms rise time, 1 ms plateau, and 1 ms falling time). ABR hearing threshold was defined as the minimum intensity
required for an ABR response that was identifiable and consistent. Intensity levels of stimuli were tested in 10 dB SPL intervals.

3.2.6 Anterior Segment Imaging
Anterior-segment images were captured by optical coherence tomography (OCT) using a SD-OCT system (Bioptigen, NC). Central corneal thickness (CCT), anterior chamber depth (ACD) and anterior capsule apical angle (ACAA) were determined using Image J software (ver. 2.0.0). Central corneal thickness was defined as the distance from the corneal epithelial surface to the corneal endothelium. Anterior chamber depth was defined as the distance from the corneal endothelium to the anterior surface of the lens capsule. Anterior capsule apical angle was defined as the angle formed by the anterior lens protrusion. A schematic of these measures is shown in Figure 12A.

3.2.7 Triglycerides Quantification Assay
100mg of snap frozen kidney tissues collected from non-fasted Col4a3−/−, Col4A3−/−OPN+/- or Col4A3−/−OPN−/- mice were homogenized in a hypotonic (15 mM KCl, 1.5 mM MgCl₂, 1 mM DTT and 10 mM HEPES, ph 7.0) in a Dounce homogenizer. 200ul homogenized solution was extracted with heptane/isopropanol (60% v/v) and incubated on ice for 5min. After centrifugation (2000g x 10 min), the top organic layer was collected, dried under N2 gas flow, and reconstituted in 100ul isopropanol. Triglycerides were quantified by using 5ul
extract lipids in a triglyceride quantification kit (Ab65336) following the manufacturer instructions.

3.2.8 ELISA

Albumin concentration was measured by ELISA (E90-134, Bethyl Laboratories, Montgomery, TX) using 1:500 – 1:20,000 diluted urine samples collected from 8-9 wks old mice. Creatinine concentration was measured using 1:20 diluted urine samples by ELISA (1012, Exocell, Philadelphia, PA). Albumin concentrations (mg/mL) were then normalized to Creatinine (mg/mL) for analysis of albuminuria. Plasma Galectin-3 levels were measured by ELISA (DY1197, R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer’s instruction.

3.2.9 Blood Pathology

Blood samples were collected into Capiject tubes (T-MLHG, Terumo, Elkton, MD) by cardiac puncture and centrifuged to separate plasma from blood cells. Mean corpuscular hemoglobin concentration (MCHC), blood urea nitrogen (BUN) and Creatinine (CRE) levels were analyzed by the Pathology Core Lab at University of Miami.

3.2.10 Histochemistry

De-paraffinized 4 μm kidney sections were processed for antigen retrieval using citrate buffer in a steamer for 45 min, permeabilized using 0.2% triton for 15 min, and blocked in 10% donkey serum in 1% TBST for 1 hour. EdU-555 was stained
for 30 min as recommended by the manufacturer. Sections were then counterstained for the glomeruli marker Synaptopodin (Synpo) (sc-21537, Santa Cruz Biotechnology, Santa Cruz, California) at 4C overnight, and staining was detected using anti-Goat Alexa Fluor-488 at RT for 30 min. Dnm3 and LDLR were stained overnight and detected using biotinylated anti-Rabbit (BA-1000, Vector Laboratories, Burlingame, CA) at RT for 30 min followed by DAB peroxidase (HRP) amplification (SK-4100, Vector Laboratories, Burlingame, CA).

For OPN or kidney injury molecule (KIM-1) staining, 10 μm frozen kidney sections were air dried, rinsed with PBS, permeabilized with 0.2% triton for 3 min and blocked with 10% donkey serum for 30min at RT. OPN (AF808, R&D, Minneapolis, MN) or Kim-1 (AF3689, R&D, Minneapolis, MN) antibodies were used at 4C overnight and detected using anti-Goat or anti-Rat Alexa Fluor-488 at RT for 30 min. For immunostaining, primary and secondary antibody incubations were done in 10% donkey Serum in 1% TBST. For Oil Red staining, frozen sections were processed as described above and incubated with 60% isopropanol for 5min followed by oil red solution for 45min. Tunel staining was performed on frozen kidney sections following the manufacturer's instructions (AB66110, Abcam, Cambridge, MA). Dnm3, LDLR and Oil Red staining images were captured on a Zeiss microscope using a 32x objective and Tunel images were captured using at 40x objective. OPN, Kim-1 and EdU fluorescent images were captured on a Zeiss LSM710 confocal microscope using z-scanning at 40x magnification. 3-6 kidneys/mice per group were used and 5-10 images per kidney were acquired and quantified. For immunostaining, images were
quantified using Fiji ImageJ. For Oil Red staining, a plugin segmentation-Weka trainable segmentation, was used to classify Oil Red color and such classifier was applied to all Oil Red images for quantification of Oil Red intensity. For Tunel staining, apoptotic cells were counted per field at 40x objective and 5 different fields were used for quantification.

3.2.11 Electron Microscopy

Kidneys, cochlea and eyes were fixed for 24 hours in 10% formalin, followed by 24 hours in 2% glutaraldehyde, rinsed in wash buffer three times, and then post-fixed in 2% osmium tetroxide in 0.1 M phosphate buffer overnight. After buffer rinses, tissues were dehydrated through a series of graded ethanols and embedded using Embed/Araldite (Electron Microscopy Sciences, Hatfield, PA) overnight in a 64°C oven. Silver/gold sections were cut on a Leica Ultracut R (Leica) and stained in uranyl acetate and lead citrate. Images were captured by a Gatan Orius SC 200D CCD camera (Gatan, Pleasanton, CA, USA) in a JEM 1400 electron microscope (JEOL, Peabody, MA, USA).

3.2.12 Lentiviral Infections

293T and HK-2 cells were seeded at 2.5 x 10^5 cells per dish and infected with either a CMV-scrambled-GFP (LVP015-G, ABM) or a CMV-DNM3-GFP (LVP140689, ABM) lentivirus at MOI=1 overnight. The next day, infection media was changed to fresh culture media and 5μg/ml puromycin was supplemented for stable selections. After expansion, the cells were sorted for the top 5% GFP-
positive cells. Selected cells were maintained in 5μg/ml puromycin supplemented culture media at all stages during experiments.

3.2.13 Microarrays and Bioinformatics

Kidneys were lysed using Cell Disruption Buffer (Mirvana Paris Kit) and total RNA was extracted using Mirvana Paris Kit to yield a 260–280 nm absorbance ratio of 2.0. RNA concentration and integrity was determined by using an Agilent Bioanalyzer. A 1µg aliquot of total RNA from each of the samples (3 samples per group; 2 groups per experiment) was processed using instructions and reagents supplied by the manufacturer (Affymetrix, Santa Clara, CA). Briefly, total RNA was reverse transcribed using a T7-Oligo (dT) Promoter Primer in the first-strand cDNA synthesis reaction. Following RNase H-mediated-DNA polymerase I-second-strand cDNA synthesis, the double-stranded cDNA was purified and served as a template for in vitro transcription in the presence of T7RNA polymerase and a biotinylated nucleotide analog/ribonucleotide mix, producing biotin-labeled complementary RNA (cRNA). cRNA probes were then purified, fragmented, and hybridized on Mouse ST2 gene expression arrays (45,101 probe sets). Background noise, housekeeping gene expression and 3’/5’ ratio values of all chips were within quality control limits set by Affymetrix. Expression ratios were calculated as the power-2 exponential of the log2 differences. The acceptance criteria for gene array expression changes was a minimum 2 fold change in log2 (equivalent to 4 fold) and a one-way Analysis of Variance
(ANOVA) t-test p-value of <0.05. All microarray raw files were submitted to the NCBI Gene Expression Omnibus (GEO) database (GSE).

3.2.14 Western Blots
Protein concentration was measured using a Bradford assay. Samples were prepared and separated by a 4-12% Novex mini 15-well gradient gel (Bolt System, Life Technologies), and probed with OPN (AF808, R&D, Minneapolis, MN), Dnm3 (ab3458, Abcam, Cambridge, MA), Kim-1 (AF3689, R&D, Minneapolis, MN) and GAPDH (sc-25778, Santa Cruz Biotechnology, Santa Cruz, California) antibodies. Signals were detected by chemiluminescence (Femto, Thomas Scientific) on photographic films. Digitized images were analyzed using Image J (NIH). Protein band densitometry was normalized to that of GAPDH, and the averaged results were plotted as normalized densitometry units (n.d.u.).

3.2.15 Statistics
For all experiments, N refers to the number of individual mice or individual culture plates. All data are expressed as mean ± S.E.M. P-values were calculated using Student’s t-tests. Where indicated, p-values were calculated using ANOVA and corrected for multiple comparisons using Tukey Posthoc correction in GraphPad Prism. Repeated symbols represent p-values of different orders of magnitude, i.e. *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001.
3.3 Results

3.3.1 OPN is substantially expressed in the tubules of Col4a3\textsuperscript{+/} mice

To characterize the role of OPN in Alport Syndrome, we studied OPN expression by immunostaining and western blots in kidney samples from wild type and Col4a3\textsuperscript{-/-} mice. Immunostaining shows that OPN is substantially expressed in the renal tubules of Col4a3\textsuperscript{-/-} mice (Figure 9A). Western blots revealed a dramatic increase of OPN expression in kidneys (Figure 9B) of Col4a3\textsuperscript{-/-} mice.

3.3.2 OPN deficiency increases life span and attenuates Alport pathology in Col4a3\textsuperscript{-/-} mice

Alport mice develop severe renal dysfunction and die approximately at 10 wks of age. In order to test whether OPN deficiency increases lifespan in Alport mice, we recorded survivals of Col4a3\textsuperscript{+/-}, Col4a3\textsuperscript{+/-}OPN\textsuperscript{+/} and Col4a3\textsuperscript{+/-}OPN\textsuperscript{-/-} animals and body weights at 9-12 wks of age. Kaplan Meier survival curves show that the Col4a3\textsuperscript{+/-}OPN\textsuperscript{+/} and Col4a3\textsuperscript{+/-}OPN\textsuperscript{-/-} mice significantly outlived the Alport mice (Figure 10A), and that the body weights were significantly increased in OPN deficient animals (Figure 10B). Impressively, two Col4a3\textsuperscript{+/-}OPN\textsuperscript{+/} mice lived up to 20 wks (Figure 10A), doubling the lifespan of Alport animals. To study the functional effects of OPN deficiency on Alport pathology, urine and blood samples from animals at 8-9 wks were analyzed. At 8-9 wks of age, Alport mice develop severe proteinuria, renal dysfunction, hearing deficits and eye abnormalities. Using sandwich Elisa, we quantified urinary albumin/creatinine
levels, and found that they were markedly increased in Alport animals by 161 times compared to wild type (as expected), and significantly decreased by 50% -

**Figure 9. OPN expression is increased in Alport mice.** A. OPN expression is substantially elevated in the renal tubules of Alport versus Wild Type (WT) mice as shown by immunostaining and confocal microscopy. Images are representative of 6 mice per group. Nuclei are shown in blue. Scale bar = 20 μm. B. Western blots and corresponding densitometry shows elevated OPN expressions in Col4a3^−/−~ (n=5) versus WT (n=3) or Col4a3^+/−~ (n=3) mouse kidneys. Quantification is based on three independent experiments. Data are mean±SEM. *P < 0.05; **P < 0.01 based on Student’s *t*-test. n.d.u = normalized densitometry units.
Figure 10. OPN deficiency increases lifespan and attenuates Alport pathology in Col4a3-/- mice. OPN deficiency in Alport mice causes an increase in lifespan by Kaplan Meier survival curve (A) (Col4a3-/-, N=14; Col4a3-/-OPN+/-, N=9; Col4a3+/OPN-/-, N=3), a reduction in body weight loss (B) (WT, N=32; OPN-/-, N=42; Col4a3+/-, N=22; Col4a3+/OPN+, N=13; Col4a3-/OPN-/-, N=33), a reduction of albuminuria as measured by Albumin and Creatinine ELISA (C) (WT, N=21; OPN-/-, N=19; Col4a3+/-, N=12; Col4a3-/-OPN+/-, N=21; Col4a3+/OPN-/-, N=15), a decrease in plasma BUN (D) (WT, N=21; OPN-/-, N=18; Col4a3+/-, N=24; Col4a3+/OPN+, N=24; Col4a3-/OPN-/-, N=14) and creatinine (E) levels (WT, N=20; OPN-/-, N=16; Col4a3+/-, N=20; Col4a3+/OPN+, N=20; Col4a3-/OPN-/-, N=11), an increase in blood MCHC (F) (WT, N=23; OPN-/-, N=5; Col4a3+/-, N=15; Col4a3+/OPN+, N=18; Col4a3-/OPN-/-, N=11), an increase in plasma Gal-3 as measured by ELISA (G) (WT, N=9; OPN-/-, N=10; Col4a3+/-, N=17; Col4a3+/OPN+, N=15; Col4a3+/OPN-/-, N=7), reductions in systolic (I) and diastolic (H) blood pressures as recorded by tail-cuff blood pressure system (WT, N=11; OPN-/-, N=11; Col4a3+/-, N=11; Col4a3-/OPN-/-, N=13; Col4a3+/OPN-/-, N=7) – all relative to Alport mice. Data were collected from animals at 8-9 wks of age. ALB/CRE = Albumin/Creatinine, BUN = Blood Urea Nitrogen, CRE = Creatinine, Gal-3 = Galectin-3, MCHC = Mean Corpuscular Hemoglobin Concentration, SBP = Systolic Blood Pressure, DBP = Diastolic Blood Pressure. Data are mean±SEM. *P < 0.05; **P < 0.01 based on ANOVA with Tukey multiple correction.
in Col4a3<sup>-/-</sup>OPN<sup>+/+</sup> and Col4a3<sup>-/-</sup>OPN<sup>-/-</sup> mice as compared to Col4a3<sup>-/-</sup> mice (Figure 10C). We also assessed other renal dysfunction parameters, plasma BUN and Creatinine showed a modest decrease in Col4a3<sup>-/-</sup>OPN<sup>+/+</sup> and Col4a3<sup>-/-</sup> OPN<sup>-/-</sup> mice as compared to Col4a3<sup>-/-</sup> mice (Figure 10DE). We show that blood MCHC levels were decreased in Col4a3<sup>-/-</sup> mice, suggesting anemia, which is often seen in patients with chronic kidney disease as well as in patients with Alport disease. Our data show that OPN deficiency significantly improved MCHC levels in Alport mice (Figure 10F). Interestingly, we found Galectin-3, whose expression correlates with end-stage renal dysfunction (ESRD), was expressed two times higher in Col4a3<sup>-/-</sup> mice than wild type controls and significantly decreased to control levels in Col4a3<sup>-/-</sup>OPN<sup>+/+</sup> and Col4a3<sup>-/-</sup>OPN<sup>-/-</sup> mice (Figure 10G). In addition, using the CODA mouse tail-cuff system (Kent Scientific), here we report that the Col4a3<sup>-/-</sup> mice develop moderate hypertension relative to wild type mice and that the hypertension is markedly reduced in Col4a3<sup>-/-</sup>OPN<sup>+/+</sup> and Col4a3<sup>-/-</sup>OPN<sup>-/-</sup> mice (Figure 10HI). Our data suggest that OPN deficiency reduced high blood pressure, which is a major and early pathology in Alport patients.

3.3.3 OPN deficiency improves high frequency hearing threshold and reduces cochlear pathology in Col4a3<sup>-/-</sup> mice

Otopathology of Alport syndrome is characterized by high frequency sensorineural hearing loss. In order to examine the effects of OPN deficiency on hearing ability of Alport mice, we recorded the responses to a series of clicks and pure tone stimuli in an ABR test. Col4a3<sup>-/-</sup> animals showed retarded respons-
Figure 11. OPN deficiency does not significantly improve hearing threshold in Col4a3\(^{-/-}\) mice but reduces capillary basement membrane pathology of inner ear. A. Auditory brainstem response (ABR) thresholds across stimulus frequencies and clicks are shown. OPN deficiency caused an improvement of hearing ability of Alport mice at the 8 and 16 KHZ stimuli. Mice per group: WT and OPN\(^{+/+}\), N=11; Col4a3\(^{-/-}\), N=8; Col4a3\(^{+/-}\)OPN\(^{+/+}\), N=12; Col4a3\(^{-/-}\)OPN\(^{-/-}\), N=6. Data are mean±SEM. *P < 0.05 based on ANOVA with Tukey multiple correction. B. Representative electron microscopy (EM) images show thickened basement membrane in Alport cochleas - a pathology that is reduced with OPN deficiency. Insets are shown at a higher magnification on the bottom.
-es to clicks and pure tone stimuli. Interestingly, we found that Col4a3\(^{+/-}\)OPN\(^{+/-}\) but not Col4a3\(^{-/-}\)OPN\(^{-/-}\) mice showed improved hearing ability in response to pure tones and 8, 16 KHZ click stimuli (Figure 11A) as compared to Alport mice despite that the hearing threshold were not significantly reduced in Col4a3\(^{+/-}\)OPN\(^{+/-}\) group. However, the striking morphological changes of thickened basement membrane in the stria vascularis of Col4a3\(^{+/-}\) mice\(^{64}\) were ameliorated in the cochlea of Col4a3\(^{-/-}\)OPN\(^{-/-}\) mice (Figure 11B).

3.3.4 OPN deficiency reduces ocular pathology in Col4a3\(^{-/-}\) mice

Cornea hydration is tightly regulated by corneal endothelium, and is usually evaluated in patients by measuring central corneal thickness (CCT)\(^{79}\). In order to study the vision pathology of Alport mice, we performed Optical Coherence Tomography (OCT) studies to visualize the structure of the cornea as shown in the schematic in Figure 12A. We analyzed the CCT and anterior chamber depth (ACD), but we did not observe a difference among the different genotypes (Figure 12BC). Next we examined anterior capsule apical angle (ACAA), which correlates with anterior lenticonus in Alport patients\(^{80}\). Anterior lenticonus is a rare congenital anomaly where the anterior surface of the crystalline lens of the eye is conical. In the case of anterior lenticonus, ACAA is decreased. From OCT analysis, we observed that ACAA was significantly reduced in Col4a3\(^{-/-}\) mice and was rescued to control level in Col4a3\(^{+/-}\)OPN\(^{-/-}\) mice (Figure 12BC). EM revealed a dramatic thickening of the retinal capillary basement membrane in Col4a3\(^{-/-}\) mice, that was improved in Col4a3\(^{-/-}\)OPN\(^{-/-}\) mice (Figure 12C). Our data suggested a tendency to develop anterior lenticonus in Col4a3\(^{-/-}\) mice that
phenocopied symptoms recorded from human Alport patients. The results also showed that OPN deficiency improved anterior lenticonus and retinal capillary basement membrane pathology that was developed in Col4a3-/ mice.

Figure 12. OPN deficiency attenuates tendency of lenticonus in Col4a3-/- mice. A. Schematic of Optical coherence tomography (OCT) measurements studied. B-C. OCT measurements show significant improvement in Anterior Capsule Apical Angle (ACAA) in Alport OPN deficient relative to Alport mice. ACD = anterior chamber depth; CCT= central corneal thickness. Mice per group: WT, N=10; Col4a3-/-, N=10; Col4a3-/-OPN+/-, N=3; Col4a3-/-OPN-/-, N=2. Data are mean±SEM. *P < 0.05; **P < 0.01 based on ANOVA with Tukey multiple correction. D. Electron Microscopy shows disrupted basement membrane of cornea in Col4a3-/- mice – a pathology that is reduced with OPN deficiency. Insets are shown at a higher magnification on the bottom.
3.3.5 OPN deficiency reduces tubular cell proliferation, lipid accumulation and kidney pathology in Col4a3-/- mice

KIM-1, a type 1 transmembrane protein whose expression is markedly up-regulated in the proximal tubules in acute tubular necrosis in rats and human patients, was found dramatically increased in Col4a3-/- Alport mice and decreased in Col4a3-/-OPN-/- mice (Figure 13AB). A significant decrease of KIM-1 was also observed in Col4a3-/-OPN+/ mice (FC=0.41, p=0.05) as compared to the Alport, similar to that observed in Col4a3-/-OPN-/- mice (FC=0.36, p=0.02). Because KIM-1 can promote renal fibrosis, decreased Kim-1 in Col4a3-/-OPN-/- mice suggests an anti-fibrotic effect of OPN deficiency in Alport mice. To study pathologic cell proliferation, we intraperitoneally injected EdU, a thymidine analogue that incorporates into newly synthesized DNA, into wild type, Col4a3-/- and Col4a3-/-OPN-/- mice at a 100 μg/100ul concentration daily. Animals were sacrificed after a total of 30 injections. EdU incorporation was visualized by EdU fluorescent staining on paraffin kidney sections. To distinguish, we co-stained the kidney tissues with Synaptopodin, a glomeruli protein. The majority of EdU was present in tubules and interstitial space. In Col4a3-/- mice, extensive EdU incorporation was observed outside the glomeruli while wild type and Col4a3-/-OPN-/- mice had almost undetectable level of EdU (Figure 13CD). These data suggest that OPN expression in the injured Alport tubules is correlated with high tubular fibrotic proliferation.

Accumulated lipids and foam cells have been implicated in many forms of fibrosis, nephrotic glomeruli, and Alport nephropathy. In order to
determine if there is increased lipids accumulation in Alport mice, using Oil Red, we stained for lipids in frozen kidney sections collected from three genotypes. Impressively, we found extensive lipids deposited on tubules and interstitial tubules in Col4a3\(^{-/-}\) mice while in Col4a3\(^{-/-}\)/OPN\(^{-/-}\) mice the staining of oil red was significantly less (Figure 13EF). In addition, we quantified triglycerides in whole kidney lysates. However, there was no increase in total kidney triglycerides in Alport mice compared to wild type (Figure 13H) suggesting that the increased tubular lipids accumulation observed by Oil Red staining are other forms of lipids contents rather than triglycerides.

Electron microscopy showed thickened basement membrane and podocytes effacement in the Col4a3\(^{-/-}\) mice as other groups have reported\(^{61,69}\). In Col4a3\(^{-/-}\)/OPN\(^{-/-}\) mice, structure of basement membrane and morphology of podocytes are much improved relative to the Alport mice (Figure 13G).

Because OPN regulates macrophage function\(^{90}\), and interstitial inflammation is a key feature of progressive renal dysfunction including Alport Syndrome\(^{91,92}\). Therefore, we investigated macrophage infiltration by staining for CD68 which has been reported in foam cells in Alport patients\(^{93}\). Quantification of the CD68 macrophage marker showed that there is no reduction of macrophage infiltration in the Alport kidneys on OPN knockout background (Figure 14A), suggesting OPN deficiency does not reduce Alport pathology by suppressing inflammatory responses. Similarly, we studied apoptosis in Aport mice with or without OPN deficiency\(^{94}\). TUNEL staining showed that apoptosis was less prominent in the OPN deficient Alport kidneys (Figure 14B).
Figure 13. OPN deficiency reduces tubular cell proliferation and prevents severe renal pathology in Alport mouse kidneys. (AB) Kim-1 expression is elevated in renal tubules of Alport mice and reduced in Alport OPN deficient mice as shown by immunofluorescent staining and corresponding quantification. (CD) Extensive EdU incorporation is found in Alport mouse kidneys but not in Col4a3-/OPN-- mouse kidneys. DAPI/nuclear staining is shown in blue. Synpo outlines glomeruli structure. EdU staining is quantified outside glomeruli and represents tubular proliferation. To be Continued on the Next Page-
(EF) Immunostaining and western blots show an increased Dnm3 protein expression in Alport kidneys that is significantly reduced in Col4a3+/−OPN+/− mouse kidneys. (GH) Oil red staining and corresponding quantification shows extensive lipids accumulation in tubules of Col4a3−/− mice, that is dramatically reduced in Col4a3+/−OPN+/− mouse kidneys. For all staining, N=6 mice per group except for oil red staining N=3 mice per group, and 5 (immunostaining) or 10 (oil red staining) images were quantified per mouse. Scale bar = 20 μm. (I) Renal triglyceride quantification in kidneys of non-fasted mice does not show differences between Alport and Col4a3+/−OPN−/−. Mice per group: WT and Alport, N=8; Col4a3+/−OPN+/−, N=4. TG = Triglycerides. (J) Representative electron microscopy (EM) images show podocytes effacements and disruption of basement membrane in Col4a3+/− mice as compared to normal or restored morphology in WT or Col4a3+/−OPN−/− mice. Insets are shown at a higher magnification on the bottom. In the zoomed in images, scale bar = 20 μm. Data are mean±SEM. *P < 0.05; **P < 0.01 based on Student’s t-test.

Figure 14. OPN deficiency does not prevent renal inflammation nor apoptosis in Col4a3+/− mice. A. CD68 expression is dramatically increased in kidneys of Col4a3+/− mice and the elevated level of CD68 expression is retained in both Col4a3+/−OPN+/− and Col4a3+/−OPN−/− mouse kidneys. B. TUNEL staining (red) and corresponding quantification shows apoptotic cells are significantly increased in Col4a3+/−, Col4a3+/−OPN+/− and Col4a3+/−OPN−/− mouse kidneys. DAPI/nuclear staining is shown in blue. Overlap of TUNEL and nuclei is shown in pink. CD68 staining, N=6 mice per group; TUNEL staining, N=4 mice per group, and 5 images were quantified per mouse. Scale bar = 20 μm. Data are mean±SEM. **P < 0.01 relative to each of the 3 other groups based on ANOVA with Tukey multiple correction.
In order to study the mechanism by which OPN reduces tubular proliferation and lipids accumulation in Alport animals in an unbiased manner, we performed global gene microarray studies in kidney samples from wild type, \textit{Col4a3}^{+/−} and \textit{Col4a3}^{−/−} \textit{OPN}^{+/−} mice. We found that 11 genes were upregulated and 4 downregulated in Alport relative to wild type kidneys, and then normalized by OPN deficiency. Interestingly, we found that Dynamin3 (Dnm3) was significantly downregulated in \textit{Col4a3}^{−/−} \textit{OPN}^{+/−} mice as compared to \textit{Col4a3}^{−/−} mice (Table 4). To validate the expression pattern of Dnm3, we performed immunohistochemistry on kidney sections and found that Dnm3 was highly expressed in renal tubules of \textit{Col4a3}^{−/−} mice and then normalized in the \textit{Col4a3}^{−/−} \textit{OPN}^{+/−} mice (Figure 13I). This was confirmed by western blot quantification (Figure 13J).

3.3.6 Dnm3 is Positively Regulated by OPN and Contributes to Cholesterol Influx in Alport Renal Tubules

Our \textit{in vivo} data revealed that expression of DNM3 was increased in Alport mouse kidneys and normalized in OPN deficient mouse kidneys. It’s been known that Dynamin oligomerization ameliorates chronic kidney disease via restructuring podocytes\textsuperscript{95}. However, only DNM1 and DNM2 were implicated in that report. Whether DNM3 is involved in renal pathology through the same pathway remains unclear. In order to answer these questions, we first investigated if the expression of OPN is correlated to that of DNM3 \textit{in vitro}. We chose human renal epithelial HK-2 cells as the \textit{in vitro} model as our most
important findings of OPN deficiency ameliorates Alport pathologies were present in tubules (Figure 13). HK-2 cells were treated daily with 5ng/ml TGFβ recombinant protein to induce stress as in an Alport environment\textsuperscript{96, 97}. The stressed cells were treated with OPN (1\textmu g/ml) recombinant protein every 24h and were collected 48h after the first treatment. In comparison to the stressed cells without OPN treatment, we found that DNM3 protein levels were significantly increased in OPN recombinant protein-treated HK-2 cells (Figure 15AB). Therefore, OPN protein causes induction of DNM3 protein levels.

Since Dnm3 is a member of enzymatic GTPase family that regulates clathrin vesicle mediated cholesterol endocytosis\textsuperscript{98}, we hypothesized that the increased DNM3 protein in Alport kidneys contributed to renal epithelial cholesterol influx that was reversed by OPN deficiency. To directly test this hypothesis, we infected 293T and HK-2 cells with a CMV-DNM3 or scrambled control lentivirus and validated by qPCR the over-expression of DNM3 transcripts in both stable cell lines (Figure 15C). Most interestingly, we found that in the DNM3-overexpressing HK-2 cells, LDLR protein was significantly increased (Figure 15DE). We then validated by immunostaining in Alport mice on OPN positive or negative backgrounds, that LDLR was dramatically increased in Alport renal tubules and significantly decreased in \textit{Col4a3}⁻/⁻\textit{OPN}⁺/⁻ and \textit{Col4a3}⁻/⁻\textit{OPN}⁺/⁺ renal tubules (Figure 15FG). Changes in LDLR protein levels may suggest dynamic changes in cholesterol influx rates. To validate if cholesterol influx is increased in Alport kidneys, Alport or wild type mice were injected with Dil-LDL for two hours, and kidneys and livers were studied histologically. We found a remarkable 45 fold
increase in Dil-LDL influx in the Alport renal tubules relative to the wild type group (Figure 15H) - suggesting that this increased/defective rate of cholesterol influx in the renal tubules of Alport mice may be the reason for the observed tubular lipid accumulation described above (Figure 15EF). To validate this observation, we crossed the Alport mice with the LDLR−/− mice that have defective cholesterol influx and generated \( \text{Col4a3}^{-/-}\text{LDLR}^{+/+} \) mice\(^99\). We found that disruption of LDL influx by genetic deletion of LDLR improved (though modestly) the lifespan of Alport mice (Figure 15I). These data suggest a novel pathological pathway in Alport renal tubules by which OPN induces DNM3 expression, which causes increased expression of LDLR and hence cholesterol influx.

### 3.4 Discussion

We present for the first time Osteopontin as a novel therapeutic target of Alport Syndrome by comprehensive evaluation of Alport pathologies in the \( \text{Col4a3}^{+/+} \) Alport mouse on OPN positive and negative backgrounds. Importantly, to mimic the diversity of human population, all mice used in our study were littermates of multi-strain background. Although increased OPN has been found in an albuminuria animal model\(^73\), OPN has never been implicated in Alport Syndrome. Here we are the first to report that OPN is substantially over-expressed in the renal tubules of Alport mice and that OPN has a major causative role in Alport pathology by driving metabolic pathways.
Figure 15. OPN deficiency reduces DNM3-mediated cholesterol influx. (AB) OPN recombinant protein treatment causes increase of DNM3 protein expression in HK2 cells. N=4 per group. (CD) DNM3 was over-expressed by lentivirus in 293T and HK2 cells. (E) DNM3 over-expression in HK2 cells causes LDLR over-expression. N=3 per group. n.d.u = normalized densitometry units. Data are mean±SEM. P < 0.05; **P < 0.01 based on Student’s t-test. (FG) LDLR protein expression is increased in Alport kidneys and normalized in Col4a3+/−OPN−/− kidneys. Data are mean±SEM. P < 0.05; **P < 0.01 based on ANOVA with Tukey multiple correction. For mice per group: WT and Col4a3−/−, N=6, Col4a3+/−OPN+/− and Col4a3+/−OPN−/−, N=4, and 4 images were quantified per mouse. (H) Infusion of Dil-LDL in Alport mice shows a significant 45.9 fold increase in LDL uptake by Alport renal tubules. N=3 mice per group, and 3 images were quantified per mouse. (I) Heterozygous deletion of LDLR in Alport mice improves survival as shown by Kaplan-Meier curve. Alport group includes littermates and non-littermates. Col4a3−/−LDLR−/− N=13, Col4a3−/− N=8.
Table 4. Transcriptional alterations in WT and Col4a3^-/-OPN^-/- as relative to Alport mice.

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Microarray was performed on kidney samples from WT, Col4a3^-/- and Col4a3^-/-OPN^-/- mice. We found that 11 genes were upregulated and 4 downregulated in Alport relative to WT kidneys, and then normalized by OPN deficiency. N=3 per group.
The Alport mouse suffers from severe proteinuria and end stage renal disease with an average lifespan of 10 wks\textsuperscript{69}. Our study shows an extension of the lifespan of OPN deficient Alport mice for up to 22 wks. The improved survival in OPN deficient mice is due to an improved metabolic condition and renal function as reflected by a significant increase in the body weight and decrease in albuminuria in OPN deficient Alport mice. While plasma BUN and CRE levels can also be used to assess improvement in kidney function in Alport mice\textsuperscript{61, 100}, we observed only a mild decrease of plasma BUN and CRE in OPN deficient mice. This may be due to persistent renal inflammation in Col4a3\textsuperscript{-/-}OPN\textsuperscript{-/-} mice (figure 14A). Interestingly, OPN deficiency also increases mean corpuscular hemoglobin concentration. This is clinically relevant as patients with chronic kidney disease often suffer from anemia\textsuperscript{101}. Although the Col4a3\textsuperscript{-/-} mouse is known as a non-hypertensive model of renal fibrosis with reported mean arterial blood pressures (MAP) close to that of the control groups\textsuperscript{102-104}, our blood pressure measurements show a mild yet significant increase in systolic and diastolic pressures in the Col4a3\textsuperscript{-/-} compared to wild type animals. Notably, a moderately high though non-significant MAP of 122±22 mmHg (vs 107±12 mmHg in controls, N=5 per group) is reported by Gross et al\textsuperscript{104} for Col4a3\textsuperscript{-/-} mouse which is consistent with our measurements of MAP in Col4a3\textsuperscript{-/-} mouse; 126.4±4.5 mmHg (vs 93.46 ±3.53 mmHg in controls, N=11 per group, p=0.0000016). This discrepancy could be due to different mouse strains used (129/SvJ in the published papers versus mixed background in this study), or different sample sizes; however, more sensitive techniques to measure blood
pressure in physiologic conditions without exogenous stress such as telemetry may be utilized to precisely assess hypertension in this model.

Because of the importance of type IV collagen in maintaining basement membrane structure and function, Col4a3 mutation causes dramatic morphological changes in the stria vascularis in the cochlea of Col4a3/- mice. We show by EM that OPN deletion ameliorates the thickened basement membrane pathology in the Alport's stria vascularis. Hearing loss is among the early symptoms in Alport patients where it becomes evident at later childhood or early adolescence. Our investigations on the hearing threshold of Col4a3/- mice in responses to both pure tone (low frequency) and click stimuli validate that Alport mice present diminished hearing ability and show that OPN heterozygous deletion has the potential to improve hearing deficit at 16 kHZ. Since Alport patients present high frequency sensorineural hearing loss, future studies including higher frequency assessment (i.e. 24 and 32KHZ) are likely needed to further investigate the effects of OPN deficiency on hearing in Alport mice. Identification of potential therapeutic targets for treatment of sensorineural hearing loss is a priority in otological research, and our present work implicates OPN in cochlear pathogenesis.

Anterior and posterior lenticonus have been observed in Alport patients but have not yet been characterized in Alport mice. Here we present the first evidence of anterior lenticonus examinations in Alport mice by using OCT measurements. Our analysis of the OCT images shows a significant decrease in anterior capsule apical angle (ACAA) in Alport mice that is rescued in Alport OPN
deficient mice. Interestingly, central cornea thickness (CCT) and anterior chamber depth (ACD) remain the same in Alport versus wild type mice, pointing to the alternation of lens pathology that is causing the anterior lenticonus in Alport mice. Our EM images reveal dramatically thickened membranes in the corneal capillaries that are rescued by OPN deficiency. However, corneal hydration, and hence, transparency, were not affected. Additional studies to evaluate posterior segment structures would be also interesting.

We show that KIM-1 expression is elevated in the renal tubules of Alport mice and significantly normalized in Col4a3−/−OPN−/− mice. EdU incorporation suggested a dramatic increase in interstitial cell proliferation in Alport kidneys. This was markedly reduced in OPN deficient Alport mice. In addition, apoptotic cells were reduced in the OPN deficient Alport kidneys. Interestingly, unlike expected, OPN deficiency did not reduce inflammation. Glomerular basement membrane thickness was ameliorated in the OPN deficient Alport kidneys. In addition, we report extensive lipid accumulation in the renal tubules of Alport mice. The lipid accumulation was significantly reduced in OPN deficient Alport mice. This is in line with a recent report on how OPN deficiency reduced lipid accumulation in kidneys of atherogenic APOE mice110, presenting OPN as a regulator of lipid homeostasis. These data provide a new insight into mechanism of OPN function in renal diseases which is different from its more well-known roles in regulating cell motility and inflammation111-113 and activation of NFκB in albuminuria animals73. Our findings emphasize the importance of tubules in renal injury. In fact, tubular epithelial cells have already been implicated in renal
fibrosis through epithelial-mesenchymal transition (EMT)\textsuperscript{114, 115}. Our data on tubular lipid accumulation and cell proliferation provide the first evidence that tubular lipid homeostasis is strongly associated with tubular epithelial fibroblastic proliferation that leads to the progression of Alport Syndrome and end stage renal dysfunction. Our quantification on renal triglycerides level did not reveal any differences suggesting that the lipid content is mainly cholesterol.

Our microarray on the kidney tissues revealed a list of genes that were significantly upregulated in Alport mice and downregulated in OPN deficient Alport mice. Among these potential participants in Alport pathology development, DNM3 was the most relevant because of its regulatory role in lipid metabolism. Although DNM3 has been shown as expressed in brain and heart tissues, our microarray, western blot and immunostaining results showed significant increase of DNM3 RNA and protein in the kidneys of Alport mice compared to wild type mice. This overexpression was normalized in the OPN deficient Alport mice. Since DNM3 is a member of GTPase family that regulates clathrin vesicle mediated cholesterol endocytosis, we hypothesized that it may contribute to Alport pathology by activating cholesterol influx pathway in the Alport renal tubules. Our in vivo data showed an increased level of LDLR protein in Alport kidneys that was normalized in OPN deficient Alport mice, further supporting the hypothesis that increased cholesterol influx contribute to Alport pathology. Our in vitro data showed that lentivirus-mediated induction of DNM3 protein significantly upregulated LDLR expression. Furthermore, injection of Dil-LDL showed increased level of cholesterol influx into Alport renal tubules. Our data showing
OPN regulation of DNM3 and/or LDLR in vivo in Alport kidneys and in vitro in HK2 cells, and DNM3 regulation of LDLR in vitro in HK2 cells suggest that OPN upregulates DNM3 which in turn regulates LDLR and subsequent LDL influx. This was validated by a remarkable 45 fold increase in Dil-LDL influx in Alport kidneys, and improved survival of the Alport mouse when crossed with LDLR knockout mice.

3.5 Summary

Alport syndrome is a rare hereditary renal disorder with no etiologic therapy. We found that Osteopontin (OPN) is highly expressed in the renal tubules of the Alport mouse and plays a causative pathological role. OPN genetic deletion ameliorated albuminuria, hypertension, tubulointerstitial proliferation, renal apoptosis, and hearing and visual deficits in the Alport mouse. We found extensive cholesterol accumulation and increased protein expression of DNM3 and LDLR in Alport renal tubules. Increased pathological cholesterol influx was confirmed by a remarkably increased uptake of injected Dil-LDL cholesterol by Alport renal tubules, and by the extended lifespan of the Alport mice when crossed with the \(LDLR^-\) mice with defective cholesterol influx. In human renal epithelial cells, over-expressing DNM3 resulted in elevated LDLR protein expression. Our results suggest a new pathway in Alport pathology where tubular OPN causes DNM3-mediated enhanced cholesterol influx.
Chapter 4. Conclusions and Future Directions

4.1 Overview

The aim of this dissertation was to investigate cellular mechanism of dysregulated cholesterol metabolism in atherosclerosis and Alport Syndrome. The first part of my work highlighted miR-30 as a major regulator of osteogenic differentiation in MSCs and SMCs by targeting IGF2 through a novel miR-30 binding site in the 3'UTR of IGF2 mRNA. I used qPCR and Western Blots to study the regulatory role of miR-30 for a panel of osteogenic proteins, Alizarin and Oil Red stainings to study the function of miR-30 over-expression in MSCs and SMCs, and Mutagenesis and Luciferase assays to reveal a novel binding site of miR-30 in Igf2 transcript. The second part of my work shows that OPN deficiency in the Alport mouse rescues Alport pathologies including capillary basement membrane morphology in kidney, inner ear and eye, life-span, lens structure, hearing ability, and renal functions through reduced DNM3-mediated cholesterol influx in renal tubules. In this part of study, I performed immunostainings to study expressions of OPN, DNM3 and LDLR in the renal tubules, and Oil Red staining and triglycerides assays on kidney tissues to study the component of renal lipids. In addition, I used lentiviral infections to study the influence of DNM3 over-expression on LDLR protein in vitro and fluorescent microscopy to track LDL influx after systematic injections. This work provides insights into the mechanisms of cellular cholesterol metabolism and how that influences Alport pathology progression. The work reveals a novel pathway of DNM3-mediated cholesterol influx in Alport renal tubules.
4.2 MiR-30e Functions as an OsteomiR in MSCs, SMCs and ApoE−/− Mice

In atherosclerosis, aortic SMCs become de-differentiated under stimuli including growth factors and cytokines released by foam cells. The de-differentiated SMCs expressed less lineage proteins such as SM22α and Cnn1, and could undergo osteoblastic differentiation become osteoblasts that contribute to the plaque growth and vascular calcification. The process of SMCs losing its lineage markers and tendency of it becoming osteoblasts makes it present characteristics of MSCs, thus making MSCs an accessible tool for our in vitro studies. In MSCs, miR-30e regulated a panel of osteogenic genes in MSCs in a dose dependent manner and reduced Igf2 transcripts after 2wks of osteogenic differentiation, suggesting miR-30e participating osteogenesis by regulating expressions of osteogenic genes including Igf2. In SMCs, miR-30e over-expression increased transcripts of SM22α and Cnn1, protein of SMA and decreased osteogenic protein RUNX2 expressions, indicating miR-30e drove SMCs towards a healthy phenotype and away from osteogenic differentiation. In SMCs over-expressing miR-30e, we also found reduced calcification that was rescued by IGF2 recombinant protein treatments, suggesting miR-30e reduced osteogenic differentiation in SMCs was through repressing IGF2. In vitro in aortic SMCs, bone marrow derived MSCs, liver carcinoma transformed HEPA1-6 cells and primary hepatocytes, miR-30e reduced Igf2 transcripts and in vivo in ApoE−/− mice, antimiR-30e injections increased aortic and hepatic Igf2 transcripts and hepatic IGF2 proteins. All these findings revealed that miR-30e functions as an
OsteomiR to reduce osteogenic differentiation by down-regulations of essential osteogenic genes especially *Igf2* during osteogenesis induction.

### 4.3 MiR-30e Reduces Osteogenic Differentiation By Directly Binding to IGF2

Before this work, IGF2 was not a predicted target for miR-30e in any database. Our findings of miR-30e regulated IGF2 *in vitro* and *in vivo* in mirror directions, and IGF2 rescued miR-30e-reduced osteogenic differentiation in SMCs made us hypothesize that miR-30e directly binds to Igf2 transcripts. I launched a series of luciferase assays by transfecting either miR-30e oligos while using same dosage of miR-125b oligos as positive control, or *Igf2* 3'UTR constructs into miR-30e over-expressing stable lines using *Runx2* 3'UTR as positive control and *Opn* 3'UTR as negative control. Significant reductions of firefly/renilla luciferase activities revealed miR-30e bound to *Igf2* 3'UTR. In order to further validate the binding and also with the purpose to reveal possible binding base pairs, I generated two mutated sequences of *Igf2* 3'UTR using site-directed mutagenesis and by transfecting the mutants versus a wild type *Igf2* 3'UTR for luciferase assays, we found reduced luciferase activity only in the wild type but not the two mutant sequences. Our study provides evidence that IGF2, a calcification-, atherogenic-, cancer-, proliferation-, and aging-related molecule, is a primary, novel and clinically desirable target of miR-30e. Therefore, regulation of the miR-30e pathway could be critical for multiple diseases.
4.4. OPN is Substantially Expressed in the Tubules of Alport Mice

Although an increased level of renal OPN mRNA was implicated in an albuminuria animal model\textsuperscript{73}, no one has studied OPN expressions in Alport mice. Here we were the first to report increased renal tubular OPN in Alport mice relative to wild type mice by western blotting and immunostaining.

4.5 OPN Deficiency Reduces Alport Pathologies

We evaluated therapeutic values of OPN deficiency in ameliorating Alport pathologies in the \textit{Col4a3\textsuperscript{-/-}} “Alport” mice\textsuperscript{61, 64, 69}. The most important parameter was to assess survival as Alport mice die early at an average lifespan of about 10wks. OPN deficiency significantly extended the lifespan of Alport mice as shown by the survival curves. \textit{Col4a3\textsuperscript{-/-} OPN\textsuperscript{-/-}} and \textit{Col4a3\textsuperscript{-/-} OPN\textsuperscript{+/+}} significantly outlived the Alport mice and two of the \textit{Col4a3\textsuperscript{-/-} OPN\textsuperscript{+/+}} mice survived for up to 20wks, doubling the average lifespan of an Alport mouse. Like Alport patients, \textit{Col4a3\textsuperscript{-/-}} mice showed hearing and vision impairment. OPN deficient \textit{Col4a3\textsuperscript{-/-}} mice showed improved hearing ability and significant amelioration in anterior lenticonus tendency. In assessment of renal functions, OPN deficiency significantly decreased urinary Albumin/Creatinine levels in Alport animals by 50% in \textit{Col4a3\textsuperscript{-/-} OPN\textsuperscript{+/+}} and \textit{Col4a3\textsuperscript{-/-} OPN\textsuperscript{-/-}} mice as compared to \textit{Col4a3\textsuperscript{-/-}} mice, and modestly decreased the plasma Blood Urea Nitrogen (BUN) and Creatinine levels. We characterized \textit{Col4a3\textsuperscript{-/-}} mice as hypertensive mice by showing increased systolic and diastolic blood pressures relative to wild type mice. OPN deficient Alport mice showed reduced systolic and diastolic blood pressures.
Interestingly, OPN deficiency also significantly improved MCHC levels which were reduced in the Alport mice suggesting that the Alport mice are anemic as in patients with Alport disease. Some of our findings such as improved life-span, kidney function, albuminuria, and glomerular capillary membrane thickness were also seen in the antimir-21 treatment study by Gomez et al. It would be interesting to study in the future the therapeutic effect of a combined treatment of Alport mice with antimiR-21 and a blocking OPN agent such as an antibody or aptamer to have the more powerful reduction of Alport pathologies than single therapy.

**4.6 OPN Deficiency Reduces DNM3-mediated LDL Influx in Alport Renal Tubules**

We found extensive lipid accumulation in the renal tubules of Alport mice by Oil Red staining and quantification of renal triglycerides showed no differences in Alport mice versus wild type mice, suggesting that the increased lipid content may be accumulation of cholesterol. Microarray revealed that DNM3 expression is increased in the Alport kidneys and decreased in Col4a3^+/OPN^-/- kidneys. *In vitro* in human renal epithelial cells, OPN recombinant protein increased DNM3 protein, indicating that OPN protein expression is positively correlated to that of DNM3 expression. Because DNM3 participates in clathrin-coated vesicle mediated receptor endocytosis, increased DNM3 in Alport mice lead us to hypothesize that the accumulated renal cholesterol was through abnormally increased tubular cholesterol influx. Fluorescently labeled LDL molecules (DIL-
LDL) were injected through tail vein into Alport mice and tracked by fluorescence microscopy. We observed a significant increase in the internalized Dil-LDL in renal tubules of Alport mice as relative to wild type mice, thus validating our hypothesis that DNM3-mediated LDL influx into Alport renal tubules causes lipid accumulation that was reversed in the OPN deficient Alport mice.

Our findings revealed a novel regulatory role of DNM3 in LDL-cholesterol influx in Alport renal tubules, providing grounds for future investigations of developing DNM3 as therapeutic target in dysregulated cholesterol-associated metabolic syndromes including kidney diseases like Alport Syndrome.
References


3. Dietschy JM, Turley SD and Spady DK. Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. J Lipid Res. 1993;34:1637-59.


