TRANSCRIPTIONAL REGULATION OF HuR IN RENAL STRESS

DISSertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Sudha Suman Govindaraju

Graduate Program in Biochemistry

The Ohio State University

2014

Dissertation Committee:

Dr. Beth S. Lee, Ph.D., Advisor
Dr. Kathleen Boris-Lawrie, Ph.D.
Dr. Sissy M. Jhiang, Ph.D.
Dr. Arthur R. Strauch, Ph.D.
Abstract

HuR is a ubiquitously expressed RNA-binding protein that affects the post-transcriptional life of thousands of cellular mRNAs by regulating transcript stability and translation. HuR can post-transcriptionally regulate gene expression and modulate cellular responses to stress, differentiation, proliferation, apoptosis, senescence, inflammation, and the immune response. It is an important mediator of survival during cellular stress, but when inappropriately expressed, can promote oncogenic transformation. Not surprisingly, the expression of HuR itself is tightly regulated at multiple transcriptional and post-transcriptional levels. Previous studies demonstrated the existence of two alternate HuR transcripts that differ in their 5’ untranslated regions and have markedly different translatabilities. These forms were also found to be reciprocally expressed following cellular stress in kidney proximal tubule cell lines, and the shorter, more readily translatable variant was shown to be regulated by Smad 1/5/8 pathway and bone morphogenetic protein-7 (BMP-7) signaling. In this study, the factors that promote transcription of the longer alternate form were identified. NF-κB was shown to be important for expression of the long HuR mRNA, as was a newly identified region with potential for binding the Sp/KLF families of transcription factors. Sp1-like factors were not found to regulate HuR expression, revealing a possible KLF family member activity in regulation of the long isoform. Further
analysis revealed that at least seven potential KLF family members are expressed in LLC-PK₁ cells. One of these, Krüppel-like factor 8 (KLF8), had previously been shown to have key roles in cell proliferation, survival, and invasiveness, like HuR. Chromatin immunoprecipitation confirmed binding of KLF8 to the putative Sp/KLF binding site under both normal and stressed conditions in LLC-PK₁ cells, and overexpression of KLF8 increased HuR mRNA levels. Further, cellular stress induced by thapsigargin treatment of cultured proximal tubule cells or ischemic injury of native rat kidney resulted in a rapid decrease of KLF8 levels that paralleled those of the long HuR mRNA variant. NF-κB protein levels transiently increased during a recovery phase similar to long form HuR mRNA, suggesting it might be one of the predominant transcription factors in regulating the long variant during recovery phase following injury or stress. These results demonstrate that KLF family members, including KLF8, can participate in regulating expression of alternate forms of HuR mRNA along with NF-κB and Smad 1/5/8 under different cellular contexts. The differential expression of HuR mRNAs with alternate 5' untranslated regions and translatabilities provides a mechanism for the stress-responsive regulation of HuR expression by two distinct pathways that include Smad 1/5/8 or KLF8 and NF-κB.
Dedicated to my loving parents Govindaraju Gopala Krishna and Indira
Acknowledgments

I would like to sincerely thank my advisor Dr. Beth Lee for providing me an opportunity to pursue my graduate study under her mentorship and guidance. Her mentoring over the years taught me perseverance, inculcated passion for research and also enabled me to graciously accept success and failure in science. She has been a constant source of support, motivation and enthusiasm at difficult times in my life and I cannot be more thankful for her unconditional support.

My sincere thanks to committee members Dr. Kathleen Boris-Lawrie, Dr. Sissy Jhiang and Dr. Arthur Strauch for their valuable time, attention and excellent scientific guidance throughout my graduate career at The Ohio State University.

I am greatly indebted to Dr. Margaret Nishikawara for her generous support and commitment to benefit graduate students of the Physiology and Cell Biology department. I would also like to thank the previous and current Chair persons of the Physiology and Cell Biology department in providing financial assistance to complete final stages of my dissertation project and Ms. Mary Treon for her excellent administrative support. I would also like to thank Dr. Periasamy,
Dr. Jhiang and Dr. Davis's group members who made the deserted Hamilton hall basement corridors more human and lively and have extended their support over the years.

I would also like to sincerely acknowledge Mr. Matt Misicka, Ms. Amy Kulesza and staff from the Center for Life Sciences Education on campus who hired me as a Graduate Teaching Assistant and provided an excellent infrastructure for me to grow as an instructor in teaching college level undergraduate biology and were committed to professional development for three long years of my doctoral training.

I would like to thank current and previous lab members from the Lee lab, Dr. McMichael, Dr. Kotadiya, Dr. Jeyaraj Dr. Ayupova and Dr. Singh who all have been very helpful right from my initial days in the lab until project completion. I am also greatly indebted to my past advisor from Texas A&M university, Dr. Rafael Perez-Ballestero and co mentor Dr. Maribel Gonzalez-Garcia who have been instrumental in shaping my decision to pursue a research career.

My journey would not have been successful without an awesome support group on whom I could lean on during difficult times. My gratitude and sincere thanks to my friends here at The Ohio State University- Tapasvi Lolla, Seetha Harihara, Aishwarya Devaraj, Ramesh Venugopal, Aparna Lakshmanan, Gayathri Natarajan and Sai Kamarajugadda along with Pavan, Sowmitri and all of my Texas “family” whose continuous support and encouragement had helped me during entire course of graduate study.

vi
The list would not be complete without mentioning my gratitude to my family for their endless support and motivation- Parents Mr.Gopala Krishna and Mrs.Indira; to my brother and my sister-in-law, their angel Pratham, grandparents, my aunt Mrs.Subhadra and my dear cousin, friend Dr.Kishore Parsa and his wife Mrs.Suneeta Parsa who have been a constant source of support and inspiration.
Vita

June 1997 ........................................... Little Flower High School, Hyderabad, India.
August 2001 ...................................... B.S. Physician Assistant, BITS-Pilani, India.
August 2006 ........................................ M.S. Biology & Biochemistry, Texas A&M University, USA.
2007 to present ........................................ Graduate Research Associate, The Ohio State University.

Publications


**Fields of Study**

Major Field: Biochemistry
Table of Contents

Abstract ................................................................................................................................. ii

Dedication............................................................................................................................ iv

Acknowledgments ................................................................................................................ v

Vita ........................................................................................................................................ viii

Table of Contents ............................................................................................................... x

List of Tables ....................................................................................................................... xiv

List of Figures ....................................................................................................................... xv

List of Abbreviations .......................................................................................................... xviii

Chapter 1: Introduction ...................................................................................................... 1

1.1 Ischemia Reperfusion (IR) Injury .................................................................................. 2

1.2 Gene Expression during and after Ischemia Reperfusion Injury ............................... 5

1.3 RNA-binding Proteins in Kidney ............................................................................... 8

1.4 Human Antigen-R (HuR) / Embryonic Lethal Abnormal Vision

1 (ELAV-1) ........................................................................................................................ 10
1.4.2 Expression of HuR during Development, Aging, and Cellular Senescence ................................................................. 11
1.4.3 Regulation of HuR mRNA expression ........................................ 13
1.4.4 Regulation of HuR Translation and Protein Stability .............. 16
1.4.5 HuR Levels are Elevated in Numerous Types of Cancer .... 18
1.4.6 Regulation of HuR Expression by MicroRNAs .................. 21
1.5 Sp/Krüppel-like Family (Sp/KLF) of Transcription Factors .......... 25
  1.5.2 Krüppel-like Factor 8 (KLF8) ............................................. 28
  1.5.3 KLF8 and Oncogenic Transformation ............................... 29
1.6 Nuclear Factor Kappa B (NF-κB) ............................................. 31
1.7 Cellular Stress and Gene Regulation ..................................... 34
1.8 Endoplasmic Reticulum (ER) Stress ..................................... 36

Chapter 2: Materials and Methods ............................................. 42
  2.1 Cell culture and reagents .................................................... 42
  2.2 Reverse Transcription/ Polymerase Chain Reaction .................. 43
  2.3 Western Analysis and Antibodies ......................................... 45
  2.4 Gel Mobility Shift Assay .................................................... 46
  2.5 Chromatin Immunoprecipitation (ChIP) ............................... 47
  2.6 Northern Blotting ............................................................. 48
2.7 Ribonuclease Protection Assay ................................................................. 49

2.8 Statistical Analysis ............................................................................... 50

Chapter 3: Results ...................................................................................... 51

3.1 NF-κB is required for expression of the long HuR mRNA ..................... 52

3.2 Smad1 stimulates transcription of the HuR short form and inhibits NF-
mediated long form HuR activation .............................................................. 53

3.3 Expression of the long HuR mRNA does not parallel expression of p65
during cellular stress ................................................................................... 54

3.4 Role of Sp/KLF family members in regulation of long form HuR expression
.......................................................................................................................... 55

3.5 A KLF family member potentially regulates HuR long form expression .... 56

3.6 KLF8 binds to the HuR promoter region and increases HuR mRNA levels
.......................................................................................................................... 57

3.7 Expression of transcription factors immediately following cell stress ....... 58

3.8 Levels of p65, KLF8, and Smad 1/5/8 change in native rat kidneys
subjected to ischemia-reperfusion injury .................................................. 59

Chapter 4: Discussion ................................................................................. 84

4.1 Alternate promoter usage in mammalian cells ........................................ 84

4.2 Alternate promoters of HuR and regulation ........................................... 86

4.3 Expression of HuR and transcription factors NFkB, KLF8
in malignancies.................................................................................................. 87

4.4 Role of KLF8 in Stress / Cell Survival/ Renal health ......................... 89

4.5 Role of NF-κB in Stress / Cell Survival/ Renal health ..................... 92

4.6 Interaction between the two transcription factors Klf8 and NFκB........ 96

4.7 Role of other KLF family members in Renal homoestasis ............. 98

4.8 Smad Signaling and Interactions with KLF family members .......... 99

4.9 Post-transcriptional Regulation of HuR Expression ....................... 102

Chapter 5: Future Directions ....................................................................... 107

Bibliography .................................................................................................. 119
List of Tables

Table 2.1 Primer sequences used to detect porcine KLF members in LLC-PK₁ cells ...................................................... 44
## List of Figures

<table>
<thead>
<tr>
<th>Figures</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Basic structure of a nephron</td>
<td>39</td>
</tr>
<tr>
<td>1.2 Mechanisms in Regulation of HuR mRNA expression</td>
<td>40</td>
</tr>
<tr>
<td>1.3 Translational and post-translational regulators of HuR protein levels</td>
<td>41</td>
</tr>
<tr>
<td>3.1 Inhibition of NF-κB activity decreases expression of the long HuR mRNA</td>
<td>61</td>
</tr>
<tr>
<td>3.2 Overexpression of NF-κB p65 protein increases expression of the long HuR mRNA</td>
<td>62</td>
</tr>
<tr>
<td>3.3 Overexpression of NF-κB p65 protein does not change expression of total HuR mRNA</td>
<td>63</td>
</tr>
<tr>
<td>3.4 Smad1 overexpression increases levels of total HuR mRNA, but not levels of the long form</td>
<td>64</td>
</tr>
<tr>
<td>3.5 Smad1 overexpression inhibits NF-κB-induced increases in levels of the long HuR mRNA</td>
<td>65</td>
</tr>
<tr>
<td>3.6 Thapsigargin treatment induces p65 levels</td>
<td>66</td>
</tr>
<tr>
<td>3.7 Thapsigargin treatment inhibits expression of the HuR long mRNA</td>
<td>67</td>
</tr>
<tr>
<td>3.8 Schematic of the core promoter region of HuR</td>
<td>68</td>
</tr>
<tr>
<td>3.9 Probes used in gel mobility shift analysis</td>
<td>69</td>
</tr>
</tbody>
</table>
3.10 The putative Sp/KLF binding region in the HuR core promoter is strongly
shifted by LLC-PK₁ nuclear extracts ................................................................. 70
3.11 Binding sites for transcription factor Sp1 do not shift LLC-PK₁ nuclear
extracts or compete with their binding to Sp/KLF binding sites....................... 71
3.12 Inhibition of Sp1 does not decreased expression of the HuR long mRNA.. 72
3.13 Overexpression of Sp1 does not stimulate expression of the long HuR
mRNA .............................................................................................................. 73
3.14 LLC-PK₁ proximal tubule cells express multiple KLF family members ...... 74
3.15 KLF8, in addition to NF-κB, is capable of binding the HuR core promoter
region ............................................................................................................. 75
3.16 Further ChIP analysis to determine specificity of PCR primers for ChIP ... 76
3.17 Competitive ChIP confirms specificity of KLF8 binding to HuR promoter
region .............................................................................................................. 77
3.18 Overexpression of KLF8 stimulates expression of the HuR long mRNA .... 78
3.19 Thapsigargin treatment decreases KLF8 protein levels......................... 79
3.20 Expression of the long HuR mRNA transiently increases following
thapsigargin treatment ..................................................................................... 80
3.21 Expression of p65 transiently increases during recovery from thapsigargin
treatment ...................................................................................................... 81
3.22 KLF8 levels markedly decrease during recovery from thapsigargin treatment
..................................................................................................................... 82
3.23 Expression of HuR-regulating transcription factors in rat kidneys subjected to ischemia-reperfusion injury ................................................................. 83

5.1 Role of the HuR 3’ UTR in mRNA stability ................................................. 114

5.2 Akt over expression upregulates long form HuR ................................. 115

5.3 KLF8, p65 and Akt co-operatively upregulate long form HuR ............... 116

5.4 PI3Kinase inhibition does not significantly alter long form HuR levels .... 117

5.5 KLF15 over expression upregulates long form HuR ............................. 118
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AKI</td>
<td>Acute kidney injury</td>
</tr>
<tr>
<td>ARE</td>
<td>Adenine/Uridine rich element</td>
</tr>
<tr>
<td>AUF-1</td>
<td>ARE/poly-U binding/degradation factor</td>
</tr>
<tr>
<td>BMP-7</td>
<td>Bone morphogenic protein 7</td>
</tr>
<tr>
<td>CARM-1</td>
<td>Co-activator- associated arginine/methyltransferase 1</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immuno-Precipitation</td>
</tr>
<tr>
<td>Cdk</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclo-oxygenase</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELAV-1</td>
<td>Embryonic lethal abnormal vision 1</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extra cellular signal related kinase</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>GLP1R</td>
<td>Glucagon like peptide-1 receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
</tr>
<tr>
<td>HuR</td>
<td>Human antigen R</td>
</tr>
<tr>
<td>ICAM</td>
<td>Inter-cellular adhesion molecule</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IR</td>
<td>Ischemia reperfusion</td>
</tr>
<tr>
<td>IRP</td>
<td>Iron regulatory protein</td>
</tr>
<tr>
<td>KLF</td>
<td>Krüppel-like factor</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>miR</td>
<td>micro RNA</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metallo proteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3 kinase</td>
</tr>
<tr>
<td>RBP</td>
<td>Ribo nucleic acid binding proteins</td>
</tr>
<tr>
<td>Sp1</td>
<td>Specificity protein 1</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TIA-1</td>
<td>T-cell restricted intra-cellular antigen 1</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TTP</td>
<td>Tristetrapolin</td>
</tr>
<tr>
<td>UTR</td>
<td>Un-translated region</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>Yb-1</td>
<td>Y-box binding protein</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

The kidneys are among the most indispensable organs in the human body due to their role in maintaining homeostasis of the body’s fluids. Their critical functions include removal of waste products, maintaining electrolyte and acid-base balance, and production of hormones that regulate blood pressure, mineral balance, and erythropoiesis. Each kidney is composed of approximately one million nephrons, which represent the functional units of the kidneys. The total content of nephrons in both kidneys filters and modifies the body’s entire plasma volume about 60 times per day. Nephrons are composed of the glomerulus, where filtration of the blood occurs, and a tubular component, which consists of a single layer of epithelial cells residing on a basement membrane. The nephron tubule can be subdivided into multiple segments, each with different functions in modifying plasma composition (Figure 1.1).

Injury to the kidney may be acute or chronic in nature. Acute kidney injury (AKI) results in poor prognosis and is a risk factor for progression towards chronic kidney disease due to continuing vascular and renal injury. AKI generally
can be defined as a sudden loss of renal function, including suppression of the glomerular filtration rate and failure to eliminate nitrogenous wastes. AKI is characterized by a rapid decline in kidney function with subsequent disruption in maintenance of electrolyte, fluid, and acid-base balance and has a distinct cellular pathophysiology. AKI has a high mortality rate (30-80%), particularly in the setting of multiple organ failure. Primary causes of AKI include ischemia, hypoxia, and nephrotoxicity, all of which most severely affect the nephron segments with the highest energy requirements, namely proximal tubules (Basile et al., 2012).

Renal ischemia-reperfusion (IR) injury is one of the most common causes of acute kidney injury and leads to tubular damage and renal dysfunction. The molecular events leading to organ failure are very complex, although it is understood that the general cellular response to ischemia is cytoskeletal damage, altered intracellular ion concentrations, free oxygen radical formation, and changes in various gene regulatory patterns. Proximal tubule cells of the nephron are most susceptible to damage and death from ischemic events, and among proximal tubule cells, those within the renal medulla are most at risk, as the medulla is not highly vascularized and the cells do not have access to extra energy reserves.
1.1 Ischemia Reperfusion (IR) Injury

IR injury in kidney is the most common underlying pathophysiology of AKI. The damage is most likely multi-factorial and involves hypoxia and inflammatory events, leading to metabolic and immune-modulatory consequences in renal tissue (Sener et al., 2006). The structural response of renal tubular cells due to ischemic injury is dependent on the intensity and severity of ischemia and can include cell death, but may also include loss of cell polarity and de-differentiation, proliferation, and reconstitution of normal epithelium (Supavekin et al., 2003). Apoptosis is a major cause of tubular cell death following ischemic injury (Sutton et al., 1998) and the signaling events leading to tubular cell apoptosis following renal ischemia/reperfusion injury have been well characterized. One of the earliest studies describing apoptotic events was by Schumer et al (1992) and describes the appearance of apoptotic bodies as early as 12 hours after reperfusion and increasing by 24 hours. The renal cell vulnerability to apoptotic and necrotic pathways is determined by electrolyte imbalance, nucleotide deprivation, disruption of mitochondrial integrity, and reactive oxygen species (Padanilam et al., 2003). It has been documented that the genetic programming with respect to proximal tubule following an injury is different from the distal tubule of nephron and accounts for variations in response to a number of kidney pathologies (Witzgall et al., 1999).
Ischemic preconditioning is the mechanism by which initial brief ischemic events within a tissue provide resistance to subsequent ischemic episodes; although ischemic preconditioning has been well characterized in myocardium, ischemic preconditioning and reperfusion-injury with respect to kidney has been less studied. The major study with respect to renal ischemia-reperfusion (IR) injury was performed by generating knock-out mice and studying various regulator molecules involved in cell death versus cytoprotection. The results of this study revealed a complex interplay between intracellular calcium and sodium levels, the complement system, and activation of various enzyme systems (Lien et al., 2003). Although mechanisms of “remote ischemic preconditioning” are yet to be established, it has been shown that brief periods of ischemia in non-cardiac tissues such as kidney, liver, etc., could reduce the damage to myocardial tissue in subsequent ischemic events, probably through a common pathway involved in myocardial pre-conditioning (Takaoka et al., 1999). Remote ischemic preconditioning is known to reduce both renal and myocardial injury (Ali et al., 2007) by unknown mechanisms. Gho et al. have shown that in smaller mammals such as rats, brief periods of ischemia in the renal/mesenteric artery region protected myocardium against ischemia/infarction as effectively as myocardial preconditioning (Gho et al., 1996), suggesting that new studies could provide insights into developing novel molecular targets in protecting tissues against ischemic injury. Remote ischemic preconditioning is now being viewed as a general defense mechanism against ischemic injury to all the vital organs.
(Hausenloy et al., 2008). However, the molecular pathways involved in renal IR injury have not been clearly studied. Therefore, it would be critical to elucidate the molecular pathways initiated during ischemia that lead to injury of renal proximal tubule epithelial cells, and finally result in tissue death and organ failure. Hence, it is of interest to probe the molecular pathways contributing to ischemic preconditioning during the events of renal ischemia.

### 1.2 Gene Expression during and after Ischemia Reperfusion Injury

The regenerative capacity of surviving tubular epithelial cells determines the outcome of repair following ischemic tubular injury. IR injury in renal tissue induces significant changes in gene expression that eventually may enable the cells to repair and regenerate, thus requiring increased translation of genes involved in cell growth, proliferation, transcriptional processes, and cytoskeletal functions (Supavekin et al., 2003). Recently Xu et al., (2014) suggested that the cytokine IL-22 has therapeutic potential in IR injury because it increases the phosphorylation of Stat3 and Akt, cellular mediators involved in cell growth and survival. In addition, AKI upregulates anti-apoptotic genes and downregulates pro-apoptotic genes to retain functionality in proximal tubule epithelial cells. IL-11 is a well-studied hematopoietic cytokine that has been shown to be induced in renal proximal tubule cells during ischemic AKI for renal protection (Kim et al., 2013). Survivin is another caspase-inhibitory protein known to confer recovery from AKI, a mechanism mediated by Stat3 and Notch signaling in renal proximal
tubule cells (Chen et al., 2013). EGFR (Chen et al., 2012), colony stimulating factor (Zhang et al., 2012), A1 adenosine receptors (Joo et al., 2007), netrin-1 (Ranganathan et al., 2013), renal prostacyclin PGI2 (Yang et al., 2013), glucagon-1-like peptide receptor (GLP1R), and autophagy (Yang et al., 2013) have all recently been shown to confer protection to proximal tubule cells against IR injury. Activating transcription factor-3 exosomal mRNA was shown to be upregulated to as high as 60-fold compared to control, thus downregulating adhesion molecules such as P-selectin, E-selectin, inter-cellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), along with IL-6 and monocyte chemotactic protein 1 (MCP1), thus conferring protection against renal IR injury by reducing inflammation, inhibiting endothelial dysfunction as well as oxidative stress (Chen et al., 2014).

Micro-RNAs (miRNAs) also have been shown to be extensively regulated during IR injury in kidney. Godwin et al. demonstrated that miR-20a and miR-21 are rapidly upregulated in naïve kidneys immediately after IR injury; miR-146, miR-199a and miR-214 are also upregulated, but only by the third day after IR injury. MiR-187 and miR-192 are rapidly downregulated compared to controls in which these miRNAs increased and levels were stabilized. MiR-804 and miR-194 are downregulated, but to comparable levels in control kidneys. These results altogether suggest that renal IR injury alters a subset of miRNAs to confer protection of tubular epithelial cells and result in lesser tissue damage (Godwin et al., 2010). Further, Saikumar et al. demonstrated that miR-21, miR-155 and miR-
18a levels were upregulated in rat kidney following IR induced tubular injury. Upregulation of miR-21 has been shown to be a key feature in tubular epithelial cells following ischemic injury or hypoxia. MiR-21 is induced by TGF-β and may in turn upregulate Bcl-2 and confer an overall survival benefit (Saikumar et al., 2012).

TGF-β1 is a well-established master regulator of cell survival that increases the resistance of cells against pro-apoptotic and inflammatory effects induced by TNFα in renal IR injury (Guan et al., 2010). IL-1 and IL-6 have been shown to act through an autocrine loop and facilitate actions of TGF-β in renal proximal tubule cells mediated by inhibition of NF-κB signaling. This results in a switch between mechanistic roles of TGF-β from being involved in controlled wound healing to functioning as chief promoter of scarring in chronic kidney disease following AKI (Luo et al., 2009). The renal protective effects of TGF-β also have been shown to be modulated by upregulation of Smad7, resulting in NF-κB inhibition and suppression of renal inflammation. Smad 2/3 are other Smad family members that interact with TGF-β to facilitate renal fibrosis (Wang et al., 2005). MiR-146a is upregulated following renal IR injury in response to NF-κB activation and works in a negative feedback loop to downregulate TLR4 signaling that initiates NF-κB activation. This mechanism suggests that miR-146a levels are changed later in the IR injury process to facilitate repair by resolving the inflammation following renal injury (Wang et al., 2005).
Upon renal IR injury, transcription factor Sp1 is phosphorylated by ERK, resulting in its transcriptional downregulation, resulting in renal protective effects (Wu et al., 2010). Conversely, Sp1 has also been shown to act in conjunction with the HIF-1α pathway to increase resistance of proximal tubule cells to hypoxic injury and attenuate secondary damage from ER stress by upregulating glucosamine. It is also interesting to note that natural killer cells can generate significant loss of renal function following IR injury by directly inducing apoptosis of renal tubular epithelial cells (Suh et al., 2014). This mechanism was later shown to be regulated by the extracellular matrix protein osteopontin (Taganov et al., 2006). Novel potential treatment modalities such as stem cell therapy (Zhang et al., 2008) and thyroid hormone (Zhang et al., 2010) are being employed to study the comprehensive genetic profiles of proximal tubule epithelia following IR injury.

1.3 RNA-binding Proteins in Kidney

RNA-binding proteins (RBPs) largely determine the fate of mature mRNA transcripts in cells. The functions of RBPs are indispensable within the context of post-transcriptional gene regulation as they regulate the splicing, stability, and transport of nascent mRNAs. RBPs, along with micro-RNAs, are involved in complex circuits featuring reciprocal regulation (Bagul et al., 2012). RBPs are critical in determining cellular responses to stress, senescence, angiogenesis,
metastasis, cell damage, and a number of cellular processes that affect homeostasis.

HuR is a well-studied RBP involved in promoting cell survival through its regulatory effects on mRNA stability in many tissues, including kidney (Nigwekar et al., 2013). YB-1 is a multi-functional protein with RNA binding activity that is known to induce potent effects on collagen activity in renal tissue (following ischemic injury) and has a unique auto-regulatory activity similar to HuR that is modulated by Akt (Pullmann et al., 2014). YB-1 also controls the translation of TGF-β (Hanssen et al., 2011), which is critical in modulating cell survival and fibrosis following I/R injury. Tristetrapolin (TTP) is another well studied RBP with roles in biogenesis of mRNA and their degradation (Ciais et al., 2013) but further studies are needed to uncover its role in kidney function.

Iron regulatory proteins (IRP) have been shown to be upregulated during hypoxia in human embryonic kidney-293 cells (HEK-293) cells (Hanson et al., 1999). AUF-1 is an RBP that inhibits expression of inflammatory cytokines and destabilizes cell cycle check point genes (Lu et al., 2006). DJ-1 is another RBP shown to be involved in renal hypertension, and its silencing results in production of reactive oxygen species in proximal tubular cells, and also has stimulates mesangial cell hypertrophy by Akt stimulation (Das et al., 2011). T-cell restricted intracellular antigen-1 (TIA-1) was shown to be highly expressed in fetal tissue and decrease in expression in kidney with progressing age, suggesting a plausible role in maintaining renal health (Masuda et al., 2009). Baltz et al.
employed quantitative proteomics and next generation sequencing approach in HEK-293 cells and identified eight hundred RBPs, suggesting tremendous variability in post transcriptional gene regulation (Baltz et al., 2012).

1.4 Human Antigen-R (HuR) / Embryonic Lethal Abnormal Vision 1 (ELAV-1)

HuR is a ubiquitously expressed RNA-binding protein of the embryonic lethal, altered vision (ELAV) family, and is one of the best-described regulators of mRNA fate. HuR produces broad cellular effects by binding to its target mRNAs, which number in the low thousands, and by aiding in mRNA splicing, stability, and most often translation, although a small subset of targets are translationally repressed by HuR. In recent years, transcriptome analysis has identified the mammalian mRNA targets of HuR (Lebedeva et al and Mukherjee et al., 2011), and has further identified HuR as a master regulator of other RBPs. Because of its broad effects on so many aspects of post-transcriptional gene control, HuR may be considered as a “regulator of regulators” (Kullmann et al., 2002). HuR binds its mRNA targets through sequences rich in uridine or adenosine/uridine (AREs), which are most typically present in non-coding regions of the transcript, particularly introns and the 3’ untranslated region. Under normal growth conditions, HuR is present primarily in the nucleus, but can shuttle to the cytoplasm to aid in mRNA processing. Translocation to and sequestration in the cytoplasm occurs under conditions of cellular stress (e.g. UV irradiation, nutrient or energy depletion, heat shock (Meng et al., 2005, Galban et al., 2008, Durie et
al., and Srikantan et al., 2010) where it is believed to aid in coordinating mRNA turnover in a manner that protects cell survival (Uren et al., 2011). HuR is also a potent promoter of cell proliferation and survival (Abdelmohsen et al., 2012), but during lethal stresses, can aid in promoting caspase-mediated apoptosis (von Roretz et al., 2013). Thus, HuR activity is critical for regulating pathways that mediate cell survival and death. Aberrant overexpression of HuR can lead to cellular transformation, and indeed, heightened HuR levels have been observed in tumors from tissues throughout the body. Thus, tight regulation of HuR expression is key to promoting healthy cell survival while at the same time preventing pathological proliferation. Not surprisingly, regulation of HuR expression is intricately controlled at multiple levels of transcriptional, post-transcriptional, translational, and post-translational control.

1.4.2 Expression of HuR during Development, Aging, and Cellular Senescence

HuR was first described as the fourth member of the ELAV family of proteins that was originally identified in Drosophila. The three original members (currently called HuB, HuC, and HuD) were shown to have neuron-specific expression, but unexpectedly, a fourth member (originally HuA, now HuR) was predicted by PCR and low-stringency cDNA library screening of vertebrates and was expressed in all tissues tested, including brain, kidney, lung, heart, liver, muscle, skin, testis, and ovary (Good et al., 1996). Shortly thereafter, a cDNA was isolated from HeLa cells and the corresponding mRNA was similarly found
to be expressed in a wide variety of human tissues (Ma et al., 1996). A murine version was described one year later (Okano et al., 1997). Assays of mouse tissues from early embryogenesis to adulthood were performed to determine levels of HuR expression during vertebrate development. From the small number of embryonic and extra-embryonic tissues selected for assays, HuR was shown to have developmental age- and tissue-specific variability in expression. Interestingly, HuR levels strongly paralleled levels of AUF1, another RBP that binds AREs in target mRNAs, but promotes their degradation (Gouble et al., 2000 and Lafon et al., 1998). A more comprehensive examination of HuR levels in adult murine tissues showed that HuR protein was most strongly expressed in lymphoid tissues, intestine, and testes, with moderate expression in liver and uterus, and the lowest expression in brain, heart, lung, kidney, skeletal muscle, and ovary (Lu et al., 2004).

Early studies of HuR expression also examined its levels and effects on cell health during cellular aging. In multiple in vitro models of cellular senescence, HuR levels were shown to decrease, as did the half-lives of HuR’s corresponding mRNA targets. Further, HuR overexpression and knockdown of expression with antisense RNAs revealed a direct relationship between the levels of HuR and a “younger” cell phenotype (Abdelmohsen et al., 2008). However, a follow-up study examining the levels of mRNA regulatory proteins in human tissue arrays from individuals of various ages, revealed that HuR expression remained relatively unchanged with increasing age (Masuda et al., 2009).
Therefore, the significance of the cellular senescence studies as they relate to human aging is unclear. The human tissue array study also confirmed previous murine studies in demonstrating tissue-specific levels of expression and the parallel expression of HuR and ARE-binding protein AUF1. Other studies, described below, indicate the importance of maintaining appropriate balances of RBPs that both degrade and stabilize ARE-containing mRNAs (Guo et al., 2009), so the parallel expression of HuR and AUF1 is likely a mechanism to ensure an appropriate balance of these mRNA transcripts.

1.4.3 Regulation of HuR mRNA Expression

The first studies of genetic regulation of HuR were performed in 2000 when the 5' region of the mouse HuR gene was isolated and mapped. Primer extension experiments using mRNA from various tissues and cell lines revealed three products, suggesting the presence of multiple alternative transcriptional start sites. A Spel – SmaI restriction fragment containing most of exon I and a few hundred bases of upstream sequence was demonstrated to contain transcriptional activity in reporter assays (King et al., 2000). However, a definitive transcriptional activator was not identified until 2008, when it was revealed that HuR expression was mediated through the PI3K/Akt/NF-κB pathway (Kang et al., 2008). An NF-κB binding site in the HuR promoter was described as starting 133 bases upstream of the transcriptional start site, although a specific start sequence was not specified. Nonetheless, the activity of this binding site was
clearly proven in various gastric carcinoma cell lines. Later studies from our own laboratory confirmed that PI3K/Akt/NF-κB regulation of HuR is also present in renal proximal tubule cells, and that this pathway is one arm of a positive feedback loop that results not only in transcriptional activation of HuR, but also in continued increases in Akt activity (Singh et al., 2013). Therefore, without a “braking” mechanism for this signaling pathway, heightened levels of Akt and HuR can lead to tumorigenic conditions, as will be described below. A second level of regulation for HuR was identified as a consequence of the use of alternate transcriptional start sites. Our work on the role of HuR in renal proximal tubule cells during metabolic stress had identified two transcriptional start sites, at approximately 150 and 20 bases upstream of the coding region (Ayupova et al., 2009 and Jeyaraj et al., 2010). The 5’ untranslated regions of these alternate transcripts are very different; the longer mRNA contains a G+C-rich 5’ UTR with a great deal of predicted secondary structure, while the shorter mRNA contains an A+T-rich sequence with very little secondary structure. In vitro translation assays demonstrated the shorter mRNA to be much more readily translated than the longer form (Ayupova et al., 2009). During normal growth the alternate transcriptional start sites were used at roughly equal frequencies, but following metabolic stresses to kidney cells such as thapsigargin treatment or energy depletion, expression of the shorter transcript was increased (Jeyaraj et al., 2010). Expression of this transcript was found to be regulated by multiple Smad 1/5/8 binding sites that were present in the 5’ untranslated region of the longer
transcript. Expression from these sites was further shown to be responsive to bone morphogenetic protein 7 (BMP-7), which notably is a key regulator of renal development and recovery from ischemic stresses (Hruska et al., 2000, Vukicevic et al., 1998, Wang et al., 2003 and Zeisberg et al., 2003). These findings suggest that metabolic stresses may prime cells to synthesize a more readily translatable form of HuR mRNA to aid in cell survival.

Production of transcripts with alternate 3’ untranslated regions due to multiple polyadenylation sites is common in both rodents and humans, and the choice of polyadenylation sites may be used to achieve a specific biological outcome. In many cases, this choice can produce either a long 3’ UTR that contains AREs or a shorter 3’ UTR that lacks AREs (Khabar et al., 2005). In this way, it is expected that mRNAs from a single gene may be produced with lesser or greater stability. The HuR gene itself encodes two polyadenylation variants, a longer and more labile form containing functional AREs, and a shorter, more abundant form that lacks AREs (Al-Ahmadi et al., 2009). It was subsequently demonstrated that HuR autoregulates its expression by virtue of control over the production of these variants. Briefly, HuR regulates its own expression through a negative feedback loop (Dai et al., 2012). Nuclear HuR can bind its own pre-mRNA and increase production of the longer, more labile variant, thus keeping HuR levels at constant and relatively low physiological levels. These results also suggested that under conditions in which HuR is primarily cytoplasmic, the negative feedback loop may be interrupted, thus leading to increased HuR levels.
and potential oncogenic transformation of cells. Under some circumstances, HuR may also serve as a positive regulator of its own expression, as a role for HuR has been proposed in facilitating export of HuR mRNA from the nucleus in senescent cells (Wang et al., 2012). More recent studies have implicated the RNA-binding protein RNPC1 as a mediator of HuR mRNA stability. RNPC1, like HuR, can bind to AREs and regulate transcript stability or translation. While it was demonstrated that RNPC1 stabilizes HuR mRNA by binding to its 3’ UTR, it is currently unclear whether RNPC1 and HuR bind the same sequences within the 3’ UTR (Masuda et al., 2009). As will be described below, the RBP tristetraprolin (TTP) can also bind to the HuR mRNA to promote its degradation, and the cellular levels of TTP can affect promotion of a tumor phenotype (Al-Ahmadi et al., 2013). These studies indicate that regulation of HuR mRNA stability is likely to involve multiple protein binding sites and RBPs that can vary depending on the state of cellular health and growth.

1.4.4 Regulation of HuR Translation and Protein Stability

In recent years, regulation of HuR biosynthesis by microRNAs (miRNAs) has been identified as a key process in controlling HuR levels. Multiple miRNAs, including miR-16 and miR-519, have been identified as inhibitors of HuR translation via direct binding of HuR mRNA. These miRNAs have been implicated in suppression of tumor cell growth through inhibition of HuR synthesis, and discussion of their function will be addressed below. In this
section, we will discuss mechanisms that modulate HuR expression through regulators of protein stability and cleavage.

HuR is subject to multiple levels of post-translational regulation from diverse signals. As stated above, nucleocytoplasmic shuttling is an important mechanism by which HuR can be triggered to exert differential effects in cells. As previously reviewed, phosphorylation by various kinases, including checkpoint kinase 2, Cdk1, p38, and PKC, can regulate HuR levels in the cytoplasm and/or binding to target mRNAs (Srikantan et al., 2012). HuR methylation by CARM1 (co-activator-associated arginine methyltransferase 1) can similarly affect HuR activity (Li et al., 2002). However, control of HuR protein levels and function through degradation or cleavage also has been shown to be key to HuR’s effects on cellular processes. Mild heat shock was demonstrated to rapidly decrease HuR protein without altering mRNA levels or translation rates. This loss of HuR was found to be due to ubiquitin-mediated proteolysis and is believed to enhance cell survival by altering the stability and/or translation of HuR target mRNAs (Abdelmohsen et al., 2009). Through a different pathway, ubiquitin-mediated proteolysis was also shown to cause HuR degradation when cancer cells are subjected to inhibition of glycolysis, which may represent an attempt to slow proliferation in the absence of cellular energy (Chu et al., 2012).

Post-translational regulation of HuR protein levels can be altered depending on the context of the stress. Mild stresses most often induce translocation of HuR from the nucleus to the cytoplasm, resulting in increased
cell survival. It was reported that lethal stress such as treatment with the apoptosis inducer staurosporine also results in HuR translocation to the cytoplasm; however, once there, HuR may be cleaved by caspases, leading to an enhanced apoptotic response (von Roretz et al., 2010). Similar caspase-mediated cleavage events were noted in cells subjected to chronic, but not acute, hypoxia. Further, one of the HuR cleavage products produced was demonstrated to bind the 3’ UTR of a HuR target mRNA (c-myc) and block its translation, leading to decreased cell viability (Talwar et al., 2011). A very recent study has demonstrated that under a lethal stress (staurosporine), HuR, which normally binds and stabilizes both pro- and anti-apoptotic mRNA targets, was cleaved and the resulting cleavage products bound and stabilized the pro-apoptotic mRNA caspase-9, but not the anti-apoptotic target prothymosin (von Roretz et al., 2013). These results all suggest that the cleavage of HuR under lethal stress results in products that shift HuR’s function from a pro-survival factor to a pro-apoptosis activator. Figure 1.2 summarizes the current understanding of mechanisms involved in physiological regulation of HuR expression.

### 1.4.5 HuR Levels are Elevated in Numerous Types of Cancer

The importance of HuR to cell survival and proliferation is made evident by the many tissues in which elevated HuR levels are associated with cancer. These tumor types include breast, lung, ovarian and colon cancers (Lopez de Silanes et al., 2005) and numerous other tissues. Notably, while HuR is typically...
localized primarily to nuclei, high cytoplasmic levels of HuR are known to be associated with worse prognosis in various types of cancers including human lung adenocarcinoma (Lauriola et al., 2012), gall bladder carcinoma (Sun et al., 2013), urothelial carcinoma (Liang et al., 2012), ovarian cancer (Denkert et al., 2004), breast cancer (Denkert et al., 2004 and Heinonen et al., 2005), cervical cancer (Lim et al., 2007), laryngeal squamous cell cancer (Cho et al., 2007), and colon cancer (Denkert et al., 2006). HuR has been shown to interact with and regulate a large number of mRNA transcripts with AREs that are involved in oncogenic cellular transformation. As previously reviewed, these include regulators of cell growth and division (e.g. c-Myc, cyclins), gene products involved in invasion and metastasis (e.g. MMP-9), pro-survival mediators (e.g. pro-thymosin-1), and products that can trigger local angiogenesis (e.g. VEGF, HIF-1) (Lopez de Silanes et al., 2005).

Early work to examine the mechanisms behind regulation of HuR expression in cancer was performed in gastric tumor cells that expressed high levels of HuR. No genetic or epigenetic alterations were noted in these cells, but the elevated HuR expression was found to be dependent on excessive levels of PI3K-Akt signaling. Further, NF-κB, a downstream regulator of Akt, was shown to directly activate HuR transcription through a binding site in the HuR promoter. Akt was also implicated in promoting transport of HuR from the nucleus to the cytoplasm (Kang et al., 2008). Although this study clearly implicated aberrant transcriptional control of HuR as contributing to cancer, most of our
understanding of HuR’s regulation in cancer cells comes from analysis of its interactions with miRNAs. MiRNAs are small (~22 nucleotides) noncoding RNA molecules that post-transcriptionally regulate gene expression by inducing mRNA degradation and/or suppressing translation. The interplay between HuR and microRNAs is complex, since defined miRNAs directly regulate HuR expression, and HuR is capable of inhibiting miRNA-mediated suppression or activation of target mRNAs. The latter effect occurs mostly in mRNAs that contain AREs downstream of miRNA binding sites in their 3’ untranslated regions. It is postulated that miRNA target sites extensively overlap with HuR binding sites that are observed even in the intronic regions of various growth promoting gene transcripts (Uren et al., 2011), and the relationship between HuR and various miRNAs is usually functionally antagonistic. Competitive interaction usually results in an enhanced gene expression if HuR-mRNA binding dominates. However, when both HuR and miRNAs co-operatively bind transcripts, such mRNAs are usually expressed at lower levels (Srikantan et al., 2012). MicroRNAs are differentially expressed in tumor cells and their interactions with RBPs such as HuR may eventually determine the outcome of tumor progression, chemotherapy and drug resistance. The variation of miRNA expression in primary versus metastatic tumors may partially explain the aggravated tumorigenic response in spite of interventional procedures in a number of cancers (Vang et al., 2013).
1.4.6 Regulation of HuR Expression by MicroRNAs

The first miRNA demonstrated to regulate HuR was miR-519, as predicted by sequence analysis and confirmed by experimental procedures in 2008 (Abdelmohsen et al., 2008). MiR-519 binding sites were identified in both the coding region and 3’ UTR of HuR. MiR-519 was shown to inhibit HuR expression in multiple tumor cell lines by suppressing HuR translation, but not HuR mRNA levels. Modulating the levels of miR-519 within cells affected HuR downstream targets. Not unexpectedly, decreasing the ability of miR-519 to bind HuR (through addition of antisense miR-519), increased HuR levels and the rate of cell division. In a subsequent study, HuR and miR-519 levels were examined in pairs of cancerous and adjacent healthy tissue. HuR protein, but not mRNA, levels were increased in the cancer samples, and miR-519 levels were markedly reduced. MiR-519 was also shown to inhibit tumor growth from HeLa cells injected into athymic mice, supporting the notion of miR-519 as a tumor suppressor that acts through HuR (Abdelmohsen et al., 2010). Notably, miR-519 levels were demonstrated to increase in a model of cellular senescence, suggesting that triggering of senescence through inhibition of HuR is a mechanism by which tumor suppression may occur (Marasa et al., 2010).

In the last five years, several new miRNA regulators of HuR have been identified. MiR-16 was demonstrated to translationally repress HuR in breast cancer cells by interacting with the 3’ UTR of HuR mRNA (Xu et al., 2010). This miRNA also suppresses translation of COX-2, TNF-α and Bcl-2 (Cimmino et al.
and Jing et al., 2005), which interestingly, are all tumor-promoting genes positively regulated by HuR. The complexity of the interaction between HuR and miR-16 was demonstrated to an even greater degree when it was shown that association of a HuR/miR-16 complex with AREs of several target transcripts could facilitate inhibition of miR-16 expression in colorectal cancer cells (Young et al., 2012). Thus, the tumor suppressor activity of miR-16 and the tumor-promoting activities of HuR appear to antagonize one another at multiple levels.

MiR-125a was first reported to inhibit cell growth and promote apoptosis by translationally repressing HuR in breast cancer cells (Guo et al., 2009). In another study, miR-125 was shown to inhibit phosphorylation of Akt in breast cancer cells (Scott et al., 2007). This suppression of Akt activation could interfere with the growth-promoting environment through various downstream pathways, one of which is the transcriptional activation of HuR expression through Akt/ NF-κB signaling (Kang et al., 2008 and Singh et al., 2013). Thus, miR-125 may inhibit HuR expression at multiple levels, through direct translational suppression and through indirect inhibition of transcription. Overexpression of another miRNA, miR-34a, was shown to suppress HuR protein levels in prostate cancer cells, thus modulating cell proliferation and drug resistance in those cells (Kojima et al., 2010). However, no potential binding sites for miR-34a were found by in silico analysis of the HuR 3’ UTR, suggesting that miR-34a may regulate HuR through binding in other regions of the transcript or through other mechanisms. MiR-9 similarly acts as a tumor suppressor by directly binding the 3’ UTR of HuR, thus
suppressing HuR expression and expression of its downstream targets (Leucci et al., 2012). HuR has also recently been reported to be a target of miR-146, a potent anti-inflammatory molecule (Cheng et al., 2013). HuR was shown to be a direct target of miR-146, which suppresses both HuR mRNA and protein levels. HuR is established as a regulator of mRNAs involved in inflammation (Srikantan et al., 2012 and Nabors et al., 2001), as well as a positive regulator of NF-κB activity (Rhee et al., 2010). Thus, one pathway through which miR-146 exerts its anti-inflammatory effects is through suppression of HuR.

While the miRNAs described above all directly bind to HuR mRNA and inhibit synthesis of the protein, other miRNAs can positively regulate HuR synthesis through indirect mechanisms. MiR-29a, a miRNA abundant in breast cancer cells, binds to and degrades the mRNA transcript of tristetraprolin (TTP), another RBP that works to promote decay of target mRNAs. Because HuR mRNA is a target of TTP-mediated degradation, miR-29a’s overall effects are to reduce TTP levels while increasing HuR expression. This imbalance in the HuR/TTP ratio correlated with increased expression of ARE-containing, tumor-promoting mRNAs. Importantly, inhibition of miR-29a reversed the imbalance, suggesting this microRNA as a potential target for inhibition in breast cancer (Al-Ahmadi et al., 2013). This study demonstrates that miRNA-mediated regulation of other RBPs is critical to the overall activity of HuR and provides insight into ways in which a network of RBPs can control cell fate.
The diverse molecular functions of HuR in both normal and malignant tissues have prompted researchers to probe the role of this master regulator in various types of human cancer, inflammation, and other diseases. Elucidating the mechanisms of transcriptional, post-transcriptional, translational, and post-translational regulation of HuR in both cellular stress and disease can provide critical insights into HuR’s overarching control of cellular processes. Current understanding of the transcriptional regulators of HuR expression is still limited, and it is clear that HuR itself is likely to be subjected to post-transcriptional regulation by a variety of RBPs that are still undefined. Increasing evidence demonstrates that miRNAs are involved in a complex, intricate network with HuR (Figure 1.3) to post-transcriptionally regulate genes involved in development, stress, cell cycle, and cell survival, and this interplay might very well regulate a multitude of disease pathways.

In this dissertation, I have built on previous work by the Lee laboratory to define transcription factors involved in the expression of HuR in renal proximal tubule cells, under conditions both of normal growth and cellular stress. The remaining sections of this Introduction describe these factors, which include the Sp/KLF family, particularly KLF8, and NF-κB. In addition, I introduce the current understanding of how cellular stress, including ischemic injury, can affect gene expression and cellular function.
1.5 Sp/Krüppel-like Family (Sp/KLF) of Transcription Factors

The Sp/KLF family of transcription factors consists of at least six Sp-like members and at least seventeen KLF-like members that are highly related, conserved zinc-finger proteins. These factors are critical to regulation of a large repertoire of eukaryotic GC-rich promoters. Sp/KLF family members are present in invertebrates such as C.elegans, with Drosophila expressing three family members (Kaczynsky et al., 2003). Multiple gene duplication events have resulted in the evolution of over twenty Sp/KLF family members currently identified in humans. Recently, an eighteenth KLF-like protein with a very restricted expression pattern was identified. The KLF18 gene lacks introns in its coding region and may play a significant role during embryonic development (Pei et al., 2013). The expression profiles of KLFs can range from highly tissue-restricted to ubiquitous, resulting in multiple KLFs having distinct systemic effects, thus explaining their functional diversity. All the members are characterized by a unique C-terminal DNA-binding domain containing three consecutive and conserved cysteine and histidine (C2H2) type zinc fingers. These family members are increasingly shown to critically regulate cardiovascular, respiratory, digestive, hematological and immune system physiology. They are also known to be involved in pathological states such as obesity, cardiovascular disease, cancer, and other inflammatory conditions. In addition to their involvement in tumor biology, many KLF members are known to
be involved in reprogramming somatic cells into inducible pluripotent stem cells and also in maintaining pluri-potency of embryonic stem cells (Jiang et al., 2008).

These proteins with DNA binding ability to GC-rich sequences specifically are known to affect cell proliferation, apoptosis, differentiation and various developmental processes, and have potent control over critical metabolic pathways such as adipogenesis and gluconeogenesis (Wu et al., 2013). They may regulate transcription by acting as co-activators or co-repressors, depending on the cellular context. Many KLF members have similar binding affinities to consensus motifs such as CACCC, and results from studies in fibroblasts, erythroblasts and stem cells suggest that redundancy and regulation by feedback mechanisms may occur within the KLF family. For example, depending on the tissue, KLFs 1, 2, 4 and 5 can activate KLF3 and KLF8, which then participate in a feedback mechanism and control gene activation (Eaton et al., 2008). KLFs have extensive cross-talk affecting cellular functions by modulating various signal transduction pathways. They specifically are involved in regulation of gene transcription by forming a framework of interactions with signaling molecules, nuclear receptors, multiple oncogenes and tumor suppressors, and it is this variability that results in involvement of different KLFs in altered growth regulation, cancer proliferation, metastasis, and epithelial-to-mesenchymal transition (EMT).

Numerous KLF family members have been identified in kidney, but more work is needed to definitively identify the expression patterns of all these factors.
in specific renal cell types in development and disease. However, some KLF factors are beginning to be understood in this organ system. For example, KLF15 (formerly KKLF, kidney-enriched Krüppel-like factor), is enriched in endothelial and mesangial cells of the kidney and a few nephron segments, excluding proximal tubules (Uchida et al., 2000). KLF15 has been shown to inhibit mesangial cell proliferation, promote podocyte differentiation, repress transcription of the kidney-specific chloride channel CLC-K1, and may inhibit renal interstitial fibrosis (Kojima et al., 2010, Leucci et al., 2012, Cheng et al., 2013 and Nabors et al., 2001). Another Krüppel-like factor not expressed in proximal tubules is KLF12, which is restricted to collecting ducts and promotes expression of the urea transporter UT-A1 (Suda et al., 2006). Few KLF proteins have been characterized in proximal tubules. One notable exception is KLF4, which functions as a tumor suppressor in a number of tissues, and was recently shown to be expressed in proximal tubule cells and downregulated in renal cell carcinoma tissues (Song et al., 2013). Additionally, in a screen for mRNAs that are upregulated in the earliest stages of renal ischemia/reperfusion injury, KLF6 (formerly Zf9) was detected. This factor was expressed at barely detectable levels in control kidneys, but demonstrated a rapid (within 1-3 hours) induction after ischemic injury. It also was rapidly induced in cultured proximal tubule cells subjected to ATP depletion, and its expression paralleled that of TGF-β1, a previously identified target (Tarabishi et al., 2005). Both KLF4 and KLF6 have temporal expression patterns that contrast sharply with KLF8, which is
upregulated in renal cell carcinoma, and which rapidly decreases upon ischemic injury or thapsigargin treatment.

1.5.2 Krüppel-like Factor 8 (KLF8)

KLF8 is expressed widely in human tissues at various levels, but is most abundant in kidney, heart and placenta (van Vliet et al., 2000). KLF8 was initially described as a CACCC-box binding protein that acts as a transcriptional repressor by associating with a co-repressor, the c-box terminal binding protein (van Vliet et al., 2000). However, its ability to activate transcription was later confirmed after a decade by Urvalek et al., who concluded that the amino-terminal activation domain of KLF8 recruited p300 and PCAF co-activators to promote transcription (Urvalek et al., 2010). It was later demonstrated to be a downstream mediator of focal adhesion kinase (FAK) signaling, thereby promoting cellular activities such as adhesion, migration, survival, and proliferation (Zhao et al., 2003). At this time, its role in promoting cell cycle progression was observed. Though the targets of KLF8 were not precisely defined, this study confirmed its role as a dual-function transcription factor. A role in oncogenic transformation under the control of FAK was later shown to be regulated by activation of PI3K-Akt signaling, leading to Sp1-mediated activation of KLF8 transcription in human ovarian cancer (Wang et al., 2008). KLF8 is predominantly cytoplasmic, but has two nuclear localization signals that cooperatively may determine its oncogenic role by enhancing its critical nuclear
import (Mehta et al., 2009). One signal is located in the zinc finger domain and
the second signal (unique to KLF8) is within the N-terminal activation domain.
The interaction between these two signals ultimately determines nuclear import,
which was later shown to be regulated by interaction of KLF8 with poly (ADP
ribose) polymerase-1 (PARP-1) (Lu et al., 2011).

Covalent addition of small ubiquitin-like modifier proteins (SUMOylation) is
one of the most common post-translational modifications within the KLF protein
family. SUMOylation has been shown to alter the protein’s ability to regulate cell
cycle progression, though there were no changes observed in KLF8’s protein
stability, or nuclear localization and DNA binding ability (Wei et al., 2006).
Interestingly, KLF8 expression is driven by two promoters containing multiple
CACCC KLF-binding sites. KLF1 (erythroid Krüppel-like factor or EKLF) was
shown to activate these two promoters, and KLF3 (basic Krüppel-like factor or
BKLF) was shown to repress this activation (Eaton et al., 2008), suggesting
feedback mechanisms.

1.5.3 KLF8 and Oncogenic Transformation

Wang et al. first described the role of KLF8 in cellular transformation of
epithelial cells leading to cancerous traits in human ovarian, breast and renal
cancers (Wang et al., 2007), by a mechanism that is critically mediated by cyclin-
D1 activation. It is interesting to note that another family member, KLF4, inhibits
cyclin-D1 to suppress tumor growth and EMT in gastrointestinal cancers (Yori et
al., 2011). KLF8 has been shown to transcriptionally inhibit E-cadherin transcriptional activity and induce epithelial-to-mesenchymal transition, thus resulting in increased migration and invasiveness of epithelial cells, leading to tumor invasion and progression (Wang et al., 2007).

Li et al. demonstrated that overexpression of KLF8 promotes cell proliferation and invasion in hepatocellular carcinoma, also causing inhibition of apoptosis (Li et al., 2010). MMP-9 was shown to be a target of KLF8, thus inducing cell invasion and metastasis in breast cancer (Wang et al., 2011) where KLF8 is expressed at high levels, though it is barely detectable in normal epithelia (Wang et al., 2007). Knockdown of KLF8 expression through various RNAi methods inhibited tumor growth in renal cell carcinoma (Fu et al., 2010), glioblastoma (Wan et al., 2012), gastric cancers (Liu et al., and Chen et al., 2012), prostate cancer (He et al., 2013), oral cancer (Bin et al., 2013), and osteosarcoma (Lin et al., 2014). KLF8 also has been shown to confer drug resistance to breast cancer cells by enhancing DNA repair following therapeutic-induced damage (Lu et al., 2012).

More recently, the role of KLF8 in other cellular pathologies such as hypoxia and cellular stress have been evaluated. Studies show that KLF8 mRNA is expressed at very high levels in human placenta (van Vliet et al., 2000 and Yamakoshi et al., 2012). During early stages of pregnancy, KLF8 is primarily expressed in the trophoblasts of chorionic villi, and its mRNA levels peak by 8 weeks at levels 18-fold higher than at 6 weeks, suggesting the critical role of this
KLF member in gestation. In pre-eclampsia, KLF8 levels are diminished and continued to stay low during re-oxygenation of hypoxic tissue, suggesting an “oxygen tension sensor” role for KLF8 in underlying pathologies that cause hypoxia-reoxygenation in placental tissue (Yang et al., 2014).

KLF8 was also shown to interact with the Wnt/β-catenin pathway and its targets in hepatocellular carcinoma (Yang et al., 2012). Yi et al. recently demonstrated that in Alzheimer’s disease (AD), both KLF8 and β-catenin are upregulated leading to NF-κB inhibition, that results in decreased accumulation of amyloid precursor protein and phosphorylation of Tau (Yi et al., 2014) suggesting attenuation of AD. KLF8 levels decreased from stage I to stage IV of the disease, confirming the ability of KLF8 in affecting disease progression by upregulating β-catenin levels and down regulating NF-κB.

1.6 Nuclear Factor Kappa B (NF-κB)

NF-κB is a stimulus-specific and stress regulated transcription factor belonging to the Rel family. It is a master regulatory protein known to play a major role in activation of genes involved in development, cell survival, cell cycle regulation, inflammation, and immune cell homeostasis. There are five known members of the NF-κB family: p50, RelA (p65), c-Rel, p52, and RelB (Baeuerle et al., 1996). Due to its importance in cell cycle and survival, dysregulation of NF-κB activity can lead to cancer and its progression, along with atherosclerosis, diabetes, and stroke (Sen et al., 1986).
In their inactive state, NF-κB proteins are sequestered in cytosol by inhibitor IκB proteins. Upon activation, IκB is phosphorylated and NF-κB dimers are released for nuclear translocation, whereupon they bind to specific target genes, activating transcription. It was shown in mouse fibroblasts that this DNA binding activity appears and disappears every 30-60 minutes owing to the repeated degradation and synthesis cycles of IκB and resulting activation and inactivation of NF-κB (Hoffman et al., 2006). Upregulation of the Rel-A/p50 canonical isoform in renal tissue, specifically within proximal tubule epithelia, podocytes, mesangial cells, and macrophages, is known to be associated with a progressive pathology in chronic kidney disease (Rangan et al., 2009). On the other hand, NF-κB was shown to play either a pro- or an anti-apoptotic role in tubular epithelia depending on the type of stimulus generated. It was shown in LLC-PK₁ proximal tubule cells that adenovirus-mediated inhibition of NF-κB or doxorubicin treatment resulted in increased cytotoxicity and apoptosis, suggesting a protective role for NF-κB (Zhou et al., 1998 and Chen et al., 2006). It is also speculated that NF-κB may elicit its pro-survival function by interacting with epidermal growth factor and cyclin-D1 in human proximal tubule cells (Haussler et al., 2005). On the other hand, NF-κB is also known to cause apoptosis by activating Fas and Fas ligands in podocytes and renal epithelia (Ross et al., 2005). The signaling pathways mediated by p65 are so complex that they have multiple nodes of crosstalk that may include a number of miRNAs, reactive oxygen species, and crosstalk by kinases such as
PI3K, p38 or GSK-3-β that may possibly affect upstream signaling events. NF-κB is also known to directly associate with other transcription factors such as p53, STAT3, ATF3, Smad3 and 4, and estrogen receptors (Hoesel et al., 2013).

Cellular ischemia normally results in modulation of expression of a number of pro-inflammatory, pro-apoptotic, and protective cytokines, all of which are critically regulated by the NF-κB family. This modulation in expression confers a survival phenotype at the cellular level and also a destructive inflammatory response following reperfusion, which is exacerbated by NF-κB expression. Antagonism of NF-κB signaling has been demonstrated to be cytoprotective by reducing infarction sizes in myocardial and cerebral ischemia (Latanich et al., 2009). Renal ischemia alone with or without reperfusion has been shown to upregulate NF-κB in rat kidneys, also accompanied by increased TNFα expression (Donnahoo et al., 2000). Peak activity of NF-κB was observed at 30 minutes into reperfusion, though results indicate that renal NF-κB is activated even before the reperfusion event is initiated, a phenomenon similar to that reported in myocardial ischemia (Li et al., 1999). Cao et al. reported that NF-κB decoy oligonucleotides, when overexpressed in kidney, reduced tubular necrosis and renal tubular damage suggesting renal protective role of NF-κB antagonism in IR injury. The role of NF-κB in ischemic preconditioning in kidney is similar to that reported in other tissues, where suppression of NF-κB in rat hearts was critical to attenuate myocardial IR injury (Zhong et al., 2004) and hepatic IR injury (Funaki et al., 2002).
1.7 Cellular Stress and Gene Regulation

Cellular stress demands rapid changes in translational machinery and alterations in post-transcriptional events to generate a rapid survival response. It is understood that continuing stress signals favor activation of apoptotic pathways accompanied by almost universal translational arrest in the cell, alterations in mRNA splicing mechanisms, and changes in mRNA stability. Morley et al. demonstrated that even though translation is strongly inhibited after commitment to apoptosis, pro-apoptotic signals may inhibit translation even in the absence of cell death (Morley et al., 2000). Stress and apoptosis critically affects translational control, wherein the global translational machinery is almost shut down, either by eIF2-α phosphorylation and/or eIF4F complex inhibition, to conserve around 50% of cellular energy (Holcik et al., 2005). eIF2-α phosphorylation occurs in apoptosis, ER stress, heat or osmotic shock, nutrient starvation, and UV irradiation, most of which are known to form stress granules except during programmed cell death, wherein granule formation is unknown. Although cap-dependent translation is severely suppressed during stress, 3-5% of mRNAs are able to be translated by a cap-independent mechanism (Johannes et al., 1999) due to the presence of internal ribosome entry sites (IRESs) in their 5’ untranslated regions.

MicroRNAs can efficiently affect protein synthesis through inhibition of translational initiation, followed by mRNA deadenylation and decay (Fabian et al.,
a process that could lead to fine-tuning the balance between repair and apoptosis during cellular stress. MiR-15 is known to induce apoptosis (Hullinger et al., 2012) in response to stress following myocardial IR injury and its effects are counteracted by miR-214 mainly through blunting of calcium overload (Aurora et al., 2012). MiR-21 was shown to be upregulated during delayed myocardial ischemic pre-conditioning and also subsequent renal IR injury (Xu et al., 2012), miR-126 was shown to promote vascularization and confer renal protection during renal IR injury and support kidney recovery (Bijkerk et al., 2014), and miR-127 was shown to protect proximal tubular cells during renal IR by regulating cellular responses (Aguado-Fraile et al., 2012). More recently, plasma miRNA levels of miR-10a, miR-192 and miR-194 were reported as biomarkers for renal IR injury in rats. MiR-10a, which was elevated within one hour of renal injury, has been suggested as a very promising molecular biomarker for kidney damage, owing to its renal specificity (Wang et al., 2014).

mRNA stability during cell stress is variable and not dependent on the global shutdown of translation during cellular stress. Research so far does not clearly define how the basal mRNA decay is initiated or how individual mRNA transcripts are identified for decay. So far the literature suggests that, following a stress event, mRNA stability and translation are uncoupled in a manner that is likely to involve the presence of stress granules. Stress granules are cytoplasmic aggregations composed of non translating mRNAs, translation initiation complexes, and additional proteins affecting mRNA function. After formation
these granules may sort the sequestered mRNAs for storage, re-initiation, or degradation and are different in response to the nature of stress stimulus.

The role of stress granules in regulating mRNA stability is not completely understood since some transcripts that are actively translated during stress are not present in the granules, whereas the transcripts of some housekeeping genes are stored in them. Stress granules are known to occur in close proximity to P-bodies, which are the sites of miRNA-dependent and -independent mRNA decay. Ibrahim et al. described a pH response element in kidney that binds to multiple RNA binding proteins such as AUF-1 and HuR in response to the onset of metabolic acidosis and activation of the ER-stress response pathway (Ibrahim et al., 2010).

1.8 Endoplasmic Reticulum (ER) Stress

ER stress is triggered by increased translation of proteins or in pathophysiological states such as hypoxia, and is characterized by cellular dysfunction resulting in translational attenuation followed by initiation of apoptosis. The ER stress response may be induced by aging, inflammation, genetic mutations, or environmental factors. Thapsigargin, a chemical inducer of ER stress, is one of the most widely used inhibitors of sarco-endoplasmic reticulum calcium ATPases (SERCA). It is a non-phorboid tumor promoter and is widely used to alter calcium storage in cells, as it induces a very rapid hormone-like elevation of cytosolic calcium and calcium depletion in the ER. This process
results in disruption of ER homeostasis, thus inhibiting protein processing and overall cellular protein synthesis (Liu et al., 1997). The interaction of thapsigargin with SERCA is rapid, irreversible and stoichiometric (Lytton et al., 1991). Hung et al. reported that treating LLC-PK1 proximal tubule cells with thapsigargin and other stress inducers caused expression of ER stress proteins. This treatment additionally preconditioned renal epithelia against future oxidative injury events by upregulation of ERK, and inhibited an increase in intracellular calcium concentration (Hung et al., 2003). In the present study, treatment of LLC-PK1 cells with thapsigargin is used to examine how HuR transcription is regulated both under conditions of normal growth and cellular stress.

In summary, renal ischemia-reperfusion injury is one of the major cellular pathologies leading to kidney disease and involves complex biochemical and physiological changes in renal tissue. The mechanisms underlying these changes involve gene regulation modulated by RNA binding proteins such as HuR, microRNAs, and signaling mechanisms involving TGFβ, bone morphogenetic proteins, and Smad transcription factors. Gaining knowledge of these mechanisms will aid us in understanding renal physiology and homeostasis in normal and diseased states. The project described here probes into transcriptional regulation of the master regulator protein HuR, and identifies mechanisms adapted by renal epithelia for survival under cellular stress. Discovering functional roles for novel transcriptional regulators in kidney such as KLF members, along with further analysis of NF-κB and other regulators of HuR,
allows us to understand how this RNA-binding protein controls cell growth and survival under both physiological and pathophysiological conditions.
Figures for Introduction

Figure 1.1 Basic structure of a nephron (Addison Wesley Longman Inc).
Figure 1.2 Mechanisms in Regulation of HuR mRNA expression. All regulators are described in the text. TTP: Tristetraprolin; Akt: Protein kinase B; NF-κB: Nuclear factor kappa B
Figure 1.3 Translational and post-translational regulators of HuR protein levels.
CHAPTER 2
MATERIALS AND METHODS

2.1. Cell Culture and Reagents

LLC-PK₁ cells from the American Type Culture Collection (ATCC), Manassas, VA, were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing penicillin/streptomycin and 10% fetal bovine serum in a humidified incubator at 5% CO₂. The cells were harvested and RNA was extracted using Trizol (Invitrogen) according to the manufacturer’s protocol. Transfections were performed using an Amaxa nucleofector with nucleofector kit L (Lonza, MD). For these experiments, 2 × 10⁶ transfected cells were plated in each well of a six-well plate and media was replaced 12 hours after nucleofection. Cells were harvested 48 hours after transfection for assays. To induce endoplasmic reticulum stress, thapsigargin (Sigma) was added to normal growth medium at 1 μM concentration for the indicated durations. Some cells were allowed to recover from thapsigargin treatment by washing with phosphate buffered saline and replacing the normal DMEM medium. The NF-κB inhibitor BAY11-7082 (Sigma) was added to cells at
2 μM, while the Sp1 inhibitor mithramycin was added to cells at 0-200 nM. Expression plasmids for p65 and Sp1 were kind gifts of Drs. Denis Guttridge and Arthur Strauch (The Ohio State University), respectively. Expression plasmids for KLF8 (IMAGE Clone-4800675) and Smad1 (IMAGE ID- BC061757) were obtained from the American Type Culture Collection.

2.2. Reverse Transcription/ Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from LLC-PK₁ cells with Trizol (Invitrogen). An internal standard for competitive RT-PCR of the long porcine HuR mRNA was synthesized using a previously described procedure (Jeyaraj et al., 2010). Ten picograms of the internal standard RNA and 2.5 μg of total LLC-PK₁ RNA were mixed and reverse transcribed using the SuperScript II First Strand System (Invitrogen). The cDNA was then subjected to PCR amplification using PrimeStar HS DNA polymerase and GC-rich buffer (Takara Bio-Clontech laboratories, CA) using the following primers and conditions: 5’-CGCGCTGAGGAGGAGCC-3’ (forward), and 5’- CCTGGGTCATGTTCTGAGGGAG-3’ (reverse); 3 minutes at 94°C followed by 40 cycles of 94°C for 10 seconds, 64°C for 15 seconds, then 72°C for 30 seconds. The resulting DNA was electrophoresed in a 2% agarose gel and visualized and quantified using a Chemi-doc image analyzer (Bio-Rad) with Quantity One software system. KLF family member transcripts were reverse transcribed using the SuperScript II First Strand System, then amplified in either
standard PCR buffer (KLFs 7, 8, 10) or in Takara GC-rich buffer and 10% dimethylsulfoxide (DMSO), using PrimeStar HS DNA polymerase (KLFs 13-16). Cycling conditions were 2 minutes at 94°C followed by 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, then 68°C for 1 minute. The primers used were as follows and were derived from previously published porcine sequences (Chen et al., 2010).

**TABLE 1**: Primer sequences used to detect porcine KLF members in LLC-PK₁ cells

<table>
<thead>
<tr>
<th></th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLF7</td>
<td>5’-ACAAACAGACCCAACTGTTGTCG-3’</td>
<td>5’-GCTTTGCACCTGTGTTTCC-3’</td>
</tr>
<tr>
<td>KLF8</td>
<td>5’-AGGCAGAGGTGATATGACATCTCC-3’</td>
<td>5’-GTCAGAGCGAGAAAAGCTA CGG-3’</td>
</tr>
<tr>
<td>KLF10</td>
<td>5’-TCAATCTGATGGCACCAGCG-3’</td>
<td>5’-TTCACAACCTTTCCAGCTAC AGC-3’</td>
</tr>
<tr>
<td>KLF13</td>
<td>5’-GAGTGCCCTCGTGTCATGTCG-3’</td>
<td>5’-TGTAGTCGCTGAGCGAGCC</td>
</tr>
<tr>
<td></td>
<td>Primer 1 (5’-3’)</td>
<td>Primer 2 (5’-3’)</td>
</tr>
<tr>
<td>---</td>
<td>----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>KLF14</td>
<td><code>5’-ACTACTTCGCCGCTGAGTG-3’</code></td>
<td><code>5’-GCTCAATCATGTCGGGATG-3’</code></td>
</tr>
<tr>
<td>KLF15</td>
<td><code>5’-GGGACCTGGCAGAACACC-3’</code></td>
<td><code>5’-TGCACCTTGACGTGCTTGG-3’</code></td>
</tr>
<tr>
<td>KLF16</td>
<td><code>5’-GCGCCAAGGCCGTACTACAAATC-3’</code></td>
<td><code>5’-AAAGCCCACACATCCCCCA-3’</code></td>
</tr>
</tbody>
</table>

### 2.3. Western Analysis and Antibodies

For LLC-PK₁ experiments, protein lysates were harvested using M-PER (Pierce). Protein extracts from rat kidneys were prepared by homogenization in 15 volumes of 50 mM Tris HCl, pH 8.0 containing 10% DMSO, 0.5 mM dithiothrietol, and 1 μM leupeptin. Lysates were then centrifuged at 10,000 rpm for 5 minutes and the supernatant was analyzed for protein concentration by BCA protein assay kit (Pierce). SDS-PAGE resolved proteins were transferred to Hybond P membrane (GE healthcare), blocked for 1 hour at room temperature with 5% milk in Tris-buffered saline-Tween incubated with primary antibodies overnight at 4°C. The membranes were then probed with polyclonal rabbit
antibodies (Sigma) at 1:1000 for anti-KLF8, anti-NF-κB p65 and H465 anti-Smad 1/5/8 (Santa Cruz Biotechnology), and at 1:10,000 for anti-GAPDH (Cell Signaling), diluted in the milk and Tris-buffered saline-Tween solution. Three washes each for ten minutes at room temperature were performed following primary antibody incubation. Primary antibodies were detected using horseradish peroxidase-conjugated secondary antibody incubation for 1 hour at room temperature and at a 1:7,500 dilution in 5% milk and Tris-buffered saline-Tween. The membranes were exposed to clarity ECL western substrate system (Bio-Rad) for antibody detection.

2.4. Gel Mobility Shift Assay

Nuclear extracts were prepared from LLC-PK₁ cells as previously described, (Jeyaraj et al., 2010). Double-stranded oligonucleotide probes corresponding to putative Sp-KLF binding sequences in the core promoter region were end-labeled with [γ-³²P] ATP and T4 polynucleotide kinase using a Gel Shift Assay System from Promega (Madison, WI). Eight micrograms of nuclear extracts were incubated with radiolabeled probe and binding buffer from the kit at room temperature for twenty minutes prior to separation in a non-denaturing 4% polyacrylamide gel for 45 minutes at 100 volts. The gel was then fixed in 10% acetic acid + 10% methanol for 20 minutes at room temperature and exposed to autoradiography film at -80°C for 30 - 60 minutes. Oligonucleotide competition reactions were set up by pre-incubating the nuclear extracts with 100-fold excess
unlabeled oligonucleotides for 15 minutes at room temperature prior to addition of the corresponding labeled probes. For supershift assays, antibodies were incubated with the nuclear extracts and binding buffer on ice for ninety minutes, and the radiolabeled probes were then added and incubated for twenty minutes.

2.5. Chromatin Immunoprecipitation (ChIP)

Assays were performed with the Magna ChIP A kit (EMD-Millipore, CA). Untreated and treated cells (10 × 10⁶ cells) were fixed using 1% formaldehyde at room temperature, and unreacted formaldehyde was quenched using 0.125 M glycine. DNA was sheared using a Virsonic 100 sonicator, for 5 x 15 seconds at 5 watts. Fifty microliters of chromatin sample was diluted with buffer containing protease inhibitor cocktail and 1% (5 μl) of the supernatant was saved as “input” at -20 °C. The immunoprecipitating antibody and 20 μl of fully suspended protein A magnetic beads were then added to chromatin in dilution buffer and were incubated at 4 °C overnight with rotation. DNA bound to NF-κB p65 or KLF8 was detected using 10 μg of respective antibodies (Rockland Immunochemicals Inc, PA and Sigma Aldrich, MO). To amplify the NF-κB or KLF8 binding region, the precipitated DNA was subjected to PCR using PrimeSTAR Hi-fidelity DNA polymerase and GC Rich buffer (Takara Bio-Clontech laboratories, CA) with the following primers and conditions: 5’-GCCGACCCTTCTCAGGCG-3’ (forward), and 5’-TAGCGGTGGCGCAGCG-3’ (reverse); 3 minutes at 94°C followed by 40 cycles of 98°C for 10 seconds, 64°C for 15 seconds, then 72°C for 30 seconds.
Input samples were amplified using the Platinum Taq Hi-fidelity (Invitrogen) with the following primers and conditions: 5'-GGTTATGAAGACCACATGGCCG-3' (forward) and 5'-AAGCCATAGCCCAAGCTGT- 3' (reverse); 2 minutes at 94°C; then 30 cycles of 94°C for 30 seconds, 54°C for 30 seconds, 68°C for 30 seconds; followed by a final extension at 68°C for 10 minutes. Specificity of the transcription factor binding to the HuR promoter was controlled using primers designed to amplify regions of HuR exon 6 and intron 5. The primers for exon 6 were 5'-ATTGCTTTCCCAAGCAGGTCC-3' (sense) and 5'-TTGGTCCATTCCCATTGGCG-3' (antisense), while the primers for intron 5 were 5'-TCTGGCATTGCTGTGAGCTG-3' (sense) and 5'-AGGAGCTGCACCATTGAGG-3' (antisense). Competitive N-ChIP was performed similarly except that DNA-protein complexes were not cross-linked with formaldehyde prior to sonication, and 0 to 900 nM of competitor oligonucleotide was added simultaneously with the immunoprecipitating antibody.

The resulting DNA was electrophoresed in a 2% agarose gel and visualized and quantified using a Chemi-doc image analyzer (Bio-Rad) with Quantity One software system.

2.6. Northern Blotting

Forty eight hours following transfection of appropriate wild-type and truncated HuR 3' UTR constructs, RNA was harvested using Trizol (Invitrogen) and ten micrograms of total RNA from LLC-PK<sub>1</sub> cells were used to set up initial reactions.
The RNA was added to 2.5 μl formaldehyde, 5 μl formamide, and 3 μl 5X morpholine propane sulfonic aid (MOPS) buffer. The reactions were heated at 65°C for 10 minutes, 2 μl of loading dye (Ambion) were added, and samples were run in a 0.8% agarose gel at 80 volts for two hours. The gel was then treated with 50 mM sodium hydroxide for 20 minutes at room temperature, rinsed four times with diethyl pyrocarbonate-treated water and washed in 2X saline sodium citrate (SSC) buffer for 45 minutes. The same solution was used to soak the Genescreen membrane (Perkin Elmer) onto which the denatured RNA would be transferred by vacuum blotter, at room temperature for 90 minutes. The transferred RNA was cross linked to the membrane by a UV trans illuminator, and hybridization reactions were performed with γ-32P-labeled DNA probes overnight at 65°C. The blot was then washed three times with 2X SSC buffer-0.1% Sodium dodecyl sulphate (SDS) solution at 55°C, followed by one wash with 0.1X SSC buffer-0.1%SDS solution and exposed to autoradiography film at -80°C for 24 – 48 hours.

2.7. Ribonuclease Protection Assay

Ribonuclease protection assay (RPA) experiments were performed using the RPA III kit (Ambion) following the manufacturer’s specifications. For RPA of porcine HuR, a 32P-labeled probe was generated that consisted of 512 bases of 5' untranslated region and upstream sequences and 60 bases of the coding region. Total RNA from LLC-PK1 cells was isolated and reverse transcribed using
a Super Script first-strand synthesis system (Invitrogen). The resulting cDNA was subjected to PCR with the following primers: 5’-ACTTCGGAAGTGAGCGGCAG-3’ (forward) and 5’-CGTTCTCCCAATGTCACTCCCTG-3’ (reverse). The resulting PCR product was cloned into the pCR4-TOPO vector (Invitrogen), and the construct was used as a DNA template for in vitro transcription. In vitro transcription of the labeled riboprobe was performed with 40 μCi 32P-UTP (800 Ci/mmol), 15 μM UTP, and T3 RNA polymerase using a Maxiscript kit (Ambion). Transcription was terminated by adding DNase I to the reaction, and labeled RNA was purified by loading the sample onto a 8M urea - 5% acrylamide gel that was pre-run for 45-60 minutes. The labeled RNA probe was excised and treated with elution buffer overnight at 37°C. The HuR-specific probe (3-4 x 10^4 cpm) was mixed with 15-20 μg of total RNA in the presence of the hybridization buffer, heat denatured, and hybridized for 12-14 hours at 56°C. Fifteen to twenty μg of yeast tRNA was incubated with the HuR-specific probe as a control. Following digestion with RNAses A and T1, protected double-stranded RNA was precipitated, harvested by centrifugation, and run in a 5% polyacrylamide-urea gel for 4 hours at 200 V. Protected RNA fragments were visualized after exposing the gel to radiography film at -80°C for twenty four to forty eight hours.

2.8. Statistical Analysis

All statistical analyses were performed using Student’s t-test for paired samples. Statistical significance is indicated when $P < 0.05$. 

50
Chapter 3

RESULTS

Proximal tubule cells of the kidney are particularly sensitive to cell stress due to an extreme need for ATP that supports their high levels of ongoing solute reabsorption and secretion. In previous studies, the Lee lab has used two proximal tubule cell lines, the porcine LLC-PK₁ and the human HK-2, in well-established models of cell stress to study HuR expression (Jeyaraj et al., 2005, 2006, 2010, Ayupova et al., 2009 and Singh et al., 2013). However, HuR is not expressed at high levels in kidney cells. Although detection of the two mRNA forms that differ in their 5’ untranslated regions is possible by ribonuclease protection assays and other means, the extremely high G+C content of the 5’ UTR of the long form makes these assays difficult and prone to artifacts. Therefore, for these studies we have identified RT-PCR conditions that can distinguish the long transcript of HuR. Using RT-PCR, we are unable to directly detect the short form, since it is just a truncated version of the longer transcript and contains no unique sequences.
3.1 NF-κB is required for expression of the long HuR mRNA

As described in the Introduction, earlier studies (in gastric tumor cells) identified a functional NF-κB binding site in an appropriate position to promote transcription of the HuR long form mRNA. However, no similar studies were performed in renal epithelial cells; therefore, to determine whether NF-κB regulates expression of this variant, LLC-PK₁ cells were incubated with the NF-κB inhibitor BAY11-7082 and the mRNA was detected by competitive RT-PCR. BAY11-7082 is a selective and irreversible inhibitor that blocks TNFα-induced phosphorylation of IκB-alpha. As shown in Figure 3.1, incubation in the inhibitor for 60 minutes was sufficient to almost completely abolish the long form HuR transcript. A similar trend was also observed for total HuR levels (not shown). These results indicate that NF-κB is required for transcriptional activation of long form HuR in proximal tubule epithelial cells and its inhibition is detrimental to HuR mRNA expression.

It has been clearly established that NF-κB is a critical transcriptional regulator of a number of genes involved in homeostasis, while other studies suggested the role of NF-κB in activating the HuR promoter in gastric cancer cell lines. Our inhibitor studies suggested that long form HuR is downregulated by inhibition of NF-κB, so the converse experiment was performed to determine if overexpression of this transcription factor alters long form HuR levels. Overexpression of the NF-κB subunit p65 by nucleofection increased HuR long form expression in a dose-dependent manner (Fig. 3.2). In contrast,
overexpression of p65 did not change levels of total HuR mRNA (the sum of both forms, Fig. 3.3). Because p65 increased expression of the long form and the long and short HuR mRNAs are expressed in LLC-PK₁ cells at similar levels (Jeyaraj et al., 2010), this experiment suggests that NF-κB increases levels of the long transcript while decreasing levels of the short transcript. This finding is consistent with our previous results demonstrating a reciprocal relationship in expression between the two forms (Jeyaraj et al., 2010). Data from these experiments suggest that overexpression of the p65 subunit of NF-κB stimulates expression of the long form HuR mRNA in LLC-PK₁ cells exclusively.

3.2 Smad1 stimulates transcription of the HuR short form and inhibits NF-κB mediated long form HuR activation

Previous studies in the Lee lab identified a region of Smad 1/5/8-binding sites downstream of the transcriptional start site for the HuR long form, but positioned properly to promote expression of the HuR short form (Jeyaraj et al., 2010). This region was shown by gel mobility shift analysis and chromatin immunoprecipitation to bind Smad 1/5/8. In addition, overexpression of Smad1 in LLC-PK₁ cells was demonstrated to increase levels of total HuR mRNA (Figure 3.4A). However, Smad1 overexpression did not increase levels of the HuR long form mRNA (Figure 3.4B), indicating that this family of transcription factors stimulates expression of the short form exclusively.
The previously described published studies demonstrated a reciprocal relationship between levels of the isoforms of HuR mRNA in stress and recovery conditions. That is, increased expression of the short form during recovery from stress also resulted in decreased expression of the long form (Ayupova et al., 2009). The reason for this reciprocal relationship is not understood. To more directly determine whether interference between transcriptional regulators might account for this relationship, the effect of Smad1 on p65 activation was tested. As shown in Figure 3.5, Smad1 co-transfection inhibited induction of the long form by p65. This is likely explained by the proximity of the NF-κB and Smad 1/5/8 binding sites, which are separated by less than 100 bases (see Figure 3.8).

3.3 Expression of the long HuR mRNA does not parallel expression of p65 during cellular stress.

We next examined how NF-κB and HuR levels may change during cellular stress. LLC-PK₁ cells were incubated with 1 μM thapsigargin, a treatment that was previously shown to decrease levels of the long HuR transcript (Jeyaraj et al., 2010). Western analysis demonstrated that total p65 levels increased during this stress (Figure 3.6). In contrast, competitive RT-PCR confirmed that thapsigargin treatment over a 6 hour period markedly decreased levels of the long HuR transcript (Fig. 3.7). These results suggest that other transcription factors may predominate in regulating expression of this mRNA during stress.
3.4 Role of Sp/KLF family members in regulation of long form HuR expression

Analysis of the HuR 5' flanking region indicated potential additional transcription factor binding sites near the NF-κB binding sequence. One region, positioned just upstream of the long form transcriptional start, contained two putative core Sp/KLF binding sites (GC boxes, Fig. 3.8). Gel mobility shift analysis was performed on nuclear extracts from LLC-PK₁ cells, using probes and competitive oligonucleotides shown in Figure 3.9. As shown in Figure 3.10, a probe containing the full Sp/KLF region, but not the NF-κB or Smad 1/5/8 sites, was capable of creating a band shift (lane 4). In contrast, probes containing either the individual (lanes 2 and 5) or mutated (lanes 3 and 6) GC boxes did not. These results indicate that transcription factor binding requires both Sp/KLF core sequences. To better determine the identity of the protein binding this region, gel mobility shift analysis using additional probes and cold competitor oligonucleotides (in 100-fold excess) was performed. Figure 3.11 shows that addition of an unlabeled Sp1 binding oligonucleotide was unable to inhibit protein binding to the Sp/KLF region (lane 5). In addition, a commercial, labeled Sp1 binding sequence did not produce shifted bands when combined with the LLC-PK₁ extracts (lane 7). Attempts to either supershift the protein complex by using anti-Sp1 antibodies or detect binding of probe containing the full Sp/KLF core sequence to recombinant Sp1 protein (Promega) were unsuccessful (not shown). These results suggest that a putative Sp/KLF binding region just upstream of the
transcriptional start site binds a factor that is not Sp1. These results are consistent with findings demonstrating that Sp1 is expressed at very low levels in mature proximal tubule cells (Cohen et al., 1997).

To confirm that HuR is not regulated by Sp1-like factors, LLC-PK₁ cells were alternatively treated for 24 hours with varying concentrations of mithramycin, an inhibitor of Sp1 (Fig. 3.12), or were transfected with a plasmid expressing Sp1 (Fig. 3.13). Neither treatment resulted in changed levels of either the long HuR transcript, as shown or total HuR mRNA levels (not shown). These results together demonstrate that Sp1 does not significantly contribute to regulation of the HuR transcript within the conditions tested.

3.5 A KLF family member potentially regulates HuR long form expression

We next turned our attention to determining what KLF family member(s) might be involved in HuR regulation. The KLF family is large, with 17 members, and previous studies had identified at least eight of these in the kidney (McConnell et al., 2010). To determine which KLF factors were present in LLC-PK₁ cells, primers were designed against KLFs 7-16. RT-PCR resulted in detection of mRNA for KLF 7, 8, 10, and 13-16, though signals for KLF15 and KLF16 were very weak (Figure 3.15). Others had previously demonstrated the presence of KLF 4 and KLF6 in proximal tubule cells (Tarabishi et al., 2005 and Song et al., 2013). A review of the literature indicated that KLF8 in particular shared many functional attributes with HuR, including promotion of tumor
development, growth, and metastasis (Lahiri et al., 2012) and this particular KLF family member is expressed in highest levels in placenta, kidney and heart. KLF8 also is regulated by PI3K-Akt pathway, which was recently shown by the Lee laboratory to be involved in a feedback loop mechanism with HuR involving NF-κB and an adaptor protein Grb10. We therefore focused our attention on this transcription factor.

3.6 KLF8 binds to the HuR promoter region and increases HuR mRNA levels

The data so far suggested involvement of both KLF8 and NF-κB in regulation of long form HuR mRNA, but direct interaction of these proteins with their consensus sequences in the 5’UTR promoter flanking region was yet to be confirmed. Therefore, oligonucleotide primers were designed that spanned both the NF-κB and KLF8 binding sites in the HuR promoter. Chromatin immunoprecipitation (ChIP) of either p65 or KLF8 was able to pull down this region of the genome, as assessed by PCR (Figure 3.15). Binding of both factors could be observed in normal, stressed and recovered conditions, though these experiments were not performed in a quantitative manner. The specificity of this reaction is demonstrated by the failure of either magnetic beads alone (Figure 3.16-lane 1) or irrelevant antibody (lane 2) to precipitate a product. Further, only primers against the HuR promoter were able to amplify DNA of the correct size, while primers designed against irrelevant regions of chromatin (HuR exon 6 and
intron 5) did not detect co-precipitated DNA. In addition, the S/K double-stranded oligonucleotide was capable of competitively inhibiting a native ChIP assay (N-ChIP), while the mutant S/K oligonucleotide was not (Figure 3.17). These reactions definitively demonstrate the ability of KLF8 to specifically bind the HuR promoter. Further, overexpression of KLF8 in LLC-PK1 cells caused an increase in the HuR long form transcript (Figure 3.18). Finally, KLF8 levels were found to decrease during thapsigargin treatment, with kinetics similar to that of the HuR transcript (Figure 3.19, compare to Figure 3.7). These results are consistent with KLF8 playing a dominant role in expression of the HuR long form mRNA during cellular stress. Data from these experiments suggest that both KLF8 and NF-κB bind to the HuR core promoter and stimulate expression of the long HuR mRNA, but that different factors may predominate under conditions of growth and cell stress.

3.7 Expression of transcription factors immediately following cell stress

To determine what factors may be involved during immediate recovery from cellular stress, LLC-PK1 cells were incubated for 2 hours with thapsigargin, but were allowed to recover in growth medium for several hours more. Levels of the long HuR mRNA transcript transiently increased 2 to 3 hours after treatment but returned to their baseline levels (Figure 3.20). Similarly, total p65 levels transiently increased within 2-3 hours after thapsigargin treatment, although with somewhat slower kinetics (Figure 3.21). Although the finding that p65 levels
increase after HuR may initially appear counter-intuitive, this result may be explained by our previous discovery that HuR promotes NF-κB activity in a positive feedback loop (Singh et al., 2013). In contrast, KLF8 levels markedly decreased in the hours after thapsigargin treatment, demonstrating the sensitivity of this transcription factor to stress (Figure 3.22).

3.8 Levels of p65, KLF8, and Smad 1/5/8 change in native rat kidneys subjected to ischemia-reperfusion injury.

We previously showed that in native rat kidneys transiently subjected to ischemic stress for 30 minutes, expression of the long HuR mRNA (and HuR protein) in the renal cortex approximately doubled by 14 days of reperfusion, when damaged proximal tubules have regenerated and are proliferating (Ayupova et al., 2009). To understand the expression of the HuR-regulatory transcription factors in native kidneys, protein lysates from the cortex of rat kidneys that had been subjected to temporary ischemic injury were analyzed by Western blot. As shown in Figure 3.22A, p65 expression remained relatively constant immediately following ischemia but increased during reperfusion. However, KLF8 expression was more sensitive to ischemic injury and diminished markedly but began to recover within 1 hour. After 14 days, expression of KLF8 also appeared to have increased over baseline, although this difference did not reach statistical significance (Figure 3.22B). In contrast, levels of Smad1/5/8, which promotes expression of the shorter HuR mRNA, was quite variable among
individual rats, but tended to sharply decrease during ischemia and rapidly increase following reperfusion. The reason for the variability of Smad proteins among rats is not clear, but it is possible that an analysis of activated Smads may produce more consistent results. Nonetheless, these findings are consistent with the *in vitro* studies that revealed KLF8’s sensitivity to cellular stress. Further, both p65 and KLF8 levels also appear to correlate with that of the HuR long mRNA in rat kidneys before and after renal injury, suggesting that these factors are likely to play important roles in stress response and possible pre-conditioning mechanisms in native kidney.
Figures demonstrating Results

Figure 3.1 Inhibition of NF-κB activity decreases expression of the long HuR mRNA. LLC-PK₁ cells were treated with the NF-κB inhibitor BAY11-7082 (2 μM) for the indicated times, and relative expression of the long HuR mRNA was measured by competitive RT-PCR. The gel from a single experiment is shown at the top, while composite data from at least three independent experiments are shown at the bottom. Asterisks indicate values significantly different from the “0” timepoint ($P < 0.05$).
Figure 3.2 Overexpression of NF-κB p65 protein increases expression of the long HuR mRNA. Competitive RT-PCR was used to measure relative expression of the long HuR mRNA following transfection with a plasmid expressing p65. The gel from a single experiment is shown at the top, while composite data from at least three independent experiments are shown at the bottom. Asterisks indicate values significantly different from the untransfected control ($P < 0.05$).
Figure 3.3 Overexpression of NF-κB p65 protein does not change expression of total HuR mRNA. Competitive RT-PCR was used to measure relative expression of total HuR mRNA following transfection with a plasmid expressing p65. The gel from a single experiment is shown at the top, while composite data from at least three independent experiments are shown at the bottom. No statistically significant differences were noted.
Figure 3.4. Smad1 overexpression increases levels of total HuR mRNA, but not levels of the long form. (A) Nanogram levels of a Smad1 expression vector were sufficient to induce expression of total HuR mRNA, while an empty vector control did not (*, P < 0.05). (B) Transfection of the same Smad1 expression vector did not induce expression of the long HuR mRNA, as shown by competitive RT-PCR.
Figure 3.5 Smad1 overexpression inhibits NF-κB-induced increases in levels of the long HuR mRNA. Varying levels of a Smad1 expression plasmid were co-transfected into LLC-PK₁ cells with two micrograms of a p65 expression plasmid. Smad1 inhibited the expected p65-mediated stimulation of long form HuR mRNA expression, as measured by competitive RT-PCR.
Figure 3.6 Thapsigargin treatment induces p65 levels. LLC-PK₁ cells were treated with 1 μM thapsigargin (TG) for the indicated times, and Western blot analysis was used to quantify cellular p65 levels. At least three independent experiments like that shown at the top were quantified to produce the graph at the bottom (*, P < 0.05). GAPDH was used as a loading control.
Figure 3.7 Thapsigargin treatment inhibits expression of the HuR long mRNA. Following thapsigargin treatment (1 μM) for the indicated times, competitive RT-PCR was used to measure levels of the long HuR mRNA. At least three independent experiments were performed (*, P < 0.05).
**Figure 3.8 Schematic of the core promoter region of HuR.** This schematic illustrates the relative positions of the known NF-κB and Smad 1/5/8 binding regions, along with the putative Sp/KLF binding site. Positions of the transcriptional start sites for the long and short HuR mRNAs are shown.
Figure 3.9 Probes used in gel mobility shift analysis. The sense strand of each double-stranded probe is shown. Underlined sequences indicate core GC boxes, while bolded letters indicate introduced mutations.

S/K 5’-GCTGAGCAGGGGCGTGTCTGGCCGCGGGCGGGGAGCGGTCGT-3’
mut 5’-GCTGAAATATATATATATGCTGGCCGATATACATATAGCCGGTCGT-3’
Δ1 5’-GCTGAGCAGGGGCGTGTCTGGCCCG-3’
Δ2 5’-GTCTGGCGCCGGGCGGGGAGCG-3’
Δ2mut 5’-GTCTGGCGCCGCATATCCGGGGAGCG-3’
Sp1 5’-ATTCGATCGGGCGGGCGAGC-3’
Figure 3.10 The putative Sp/KLF binding region in the HuR core promoter is strongly shifted by LLC-PK₁ nuclear extracts. A probe spanning the full Sp/KLF binding region strongly shifts nuclear protein, but individual or mutated GC boxes do not. The sequences of probes used in this experiment are shown in Figure 3.9.
Figure 3.11 Binding sites for transcription factor Sp1 do not shift LLC-PK\(_1\) nuclear extracts or compete with their binding to Sp/KLF binding sites. Competition and direct probes demonstrate that the shifted band produced by the Sp/KLF probe is not Sp1. Sequences used in this experiment are indicated in Figure 3.9.
Figure 3.12 Inhibition of Sp1 does not decrease expression of the HuR long mRNA. Addition of the Sp1 inhibitor mithramycin at varying concentrations for 24 hrs did not inhibit expression of the long HuR mRNA, as assayed by competitive RT-PCR.
Figure 3.13 Overexpression of Sp1 does not stimulate expression of the long HuR mRNA. Transfection of an Sp1 expression plasmid at varying concentrations did not increase levels of the long HuR mRNA in LLC-PK1 cells, as assessed by competitive RT-PCR. Western blot demonstrated Sp1 protein expression and also confirmed endogenous levels of this protein are almost undetectable in proximal tubule cells.
Figure 3.14 LLC-PK1 proximal tubule cells express multiple KLF family members. RT-PCR primers as described in the Methods were used to detect expression of KLF family members in LLC-PK₁ cells.
Figure 3.15 KLF8, in addition to NF-κB, is capable of binding the HuR core promoter region. Chromatin immunoprecipitation and PCR was used to detect binding of p65 and KLF8 in the HuR proximal promoter in untreated, thapsigargin (TG)–treated, and recovered samples. The antibody control lane shows lack of immunoprecipitation of the HuR promoter when an irrelevant antibody is used.
Figure 3.16 Further ChIP analysis to determine the specificity of PCR primers for ChIP. A primer set encompassing the HuR promoter, and two negative control primers designed against HuR exon 6 and intron 5 were used. For each primer set, lane 1 is an immunoprecipitation control with magnetic beads only; lane 2 is an immunoprecipitation control with magnetic beads plus an irrelevant antibody (rabbit anti-IgG); and lane 3 is magnetic beads complexed to antibodies against KLF8 or p65. The input DNA lanes show PCR amplification of HuR as a loading control. The bottom panel demonstrates that the primer sets for exon 6 and intron 5 are capable of amplifying genomic DNA in spite of their negative reactivity in the ChIP assay. The expected sizes of the primer products are as follows: promoter, 224 bp; exon 6, 195 bp; intron 5, 191 bp.
Figure 3.17 Competitive ChIP demonstrating the specificity of KLF8 binding to HuR promoter. Specificity of KLF8 binding to the HuR promoter was further demonstrated by competitive N-ChiP, which shows loss of KLF8-promoter interaction when a competitor oligonucleotide containing the S/K binding site is present, but not when a mutated form of the oligonucleotide is present.
Figure 3.18 Overexpression of KLF8 stimulates expression of the HuR long mRNA. Transfection of LLC-PK₁ cells with KLF8 results in increasing levels of the long HuR mRNA, as measured by competitive RT-PCR. At least three independent experiments were performed (*, $P < 0.05$).
Figure 3.19 Thapsigargin treatment decreases KLF8 protein levels. Addition of thapsigargin (1 μM) to LLC-PK₁ cells results in a loss of KLF8 expression, as measured by Western blot. At least three independent experiments were performed (*, P < 0.05).
Figure 3.20 Expression of the long HuR mRNA transiently increases following thapsigargin treatment. LLC-PK₁ cells were treated with 1 μM thapsigargin for 2 hours, then cultured in normal growth medium for varying times. Competitive RT-PCR demonstrates that the HuR long form transiently increases when cells are placed in normal medium. At least three independent experiments were performed (*, P < 0.05).
Figure 3.21 Expression of p65 transiently increases during recovery from thapsigargin treatment. Cells were treated as in Figure 3.19, and p65 protein levels were assessed by Western blot. GAPDH is shown as a loading control. At least three independent experiments were performed (*, P < 0.05).
Figure 3.22 KLF8 levels markedly decrease during recovery from thapsigargin treatment. Cells were treated as in Figure 3.19, and KLF8 protein levels were assessed by Western blot. At least independent experiments were performed (*, \( P < 0.05 \)).
Figure 3.23 Expression of HuR-regulating transcription factors in rat kidneys subjected to ischemia-reperfusion injury. Protein lysates from rat kidney cortex (where proximal tubule cells predominate) were Western blotted and probed for p65, KLF8 and Smad 1/5/8. Three kidneys were obtained for each time point. Beta-actin was probed for loading control. Levels of p65 and KLF8 are shown graphically below (*, \( P < 0.05 \)).
4.1 Alternate Promoter Usage in Mammalian Cells

HuR is a ubiquitously expressed RNA binding protein that increasingly is being considered a master regulator of cellular homeostasis, owing to the number of targets it is known to bind and regulate. HuR controls thousands of target genes by affecting post-transcriptional processes such as mRNA trafficking, mRNA stability, translation, alternative splicing (Zhu et al., 2007) and polyadenylation (Lebedeva et al., 2011). The post-transcriptional regulatory role of HuR is highlighted by a recent study of Sindbis virus, which effectively sequestrates HuR into the cytoplasm, thus acting to stabilize its own RNAs that contain high-affinity HuR binding sites. Because of this cytoplasmic sequestration, post-transcriptional processing of cellular mRNAs, including polyadenylation and splicing, is extensively altered (Barnhart et al., 2013).

Large-scale characterization of full-length cDNAs has estimated that about half of human genes are regulated by alternate promoters (Kimura et al., 2006). Transcription from alternate promoters has been demonstrated in multiple cellular contexts, including differentiation, growth, and stress, that enable diverse
forms of gene regulation with their differences in transcriptional initiation and translational efficiencies. Alternative promoters also have been demonstrated to possess different functional strengths. For example, the alpha-amylase gene possesses a weak downstream promoter that is active in liver and a strong, upstream promoter that is active in the parotid gland (Schibler et al., 1983). Such promoters are at times characterized by varied responsiveness to different stimuli, as illustrated by the glucokinase gene that uses two widely separated promoters that are activated in different tissues by diverse hormonal stimuli (Iynedijian et al., 1993). Another example of alternative promoter usage is the Hox-5.1 gene, which is characterized by a promoter switch leading to mRNA isoforms that differ in their 5’-untranslated region, subcellular distribution, and stability in response to induction by retinoic acid (Cianetti et al., 1990). C-myC is a well-studied proto-oncogene that expresses alternate mRNA isoforms with different half-lives and translatabilities (Atwater et al., 1990). The sodium dependent phosphate co-transporter Npt2a in renal proximal tubule cells also is expressed from two promoters that regulate variant transcripts (Yamamoto et al., 2005). These examples show similarities to the regulation of HuR, demonstrating that alternate 5’ sequences in mRNAs can affect transcript turnover and translatability.
4.2 Alternate Promoters of HuR and regulation

The existence of alternate HuR transcripts with very different 5’ untranslated regions and consequently distinct translatabilities offers cells a means to keep HuR protein levels stable under a variety of conditions. A moderate level of HuR expression is required for cell survival, since its absence results in cell death (Ghosh et al., 2009). Yet, its overexpression is well-established to play a role in oncogenic transformation and tumor invasiveness (Lopez de Silanes et al., 2005, Govindaraju et al., 2013, Xu et al., 2010, Kang et al., 2008, Danilin et al., 2010, Denkert et al., 2006, Lim et al., 2007 and Sun et al., 2013). Therefore, even under conditions of cell stress, when normal translational regulation may be impaired, it is critical that HuR expression is maintained at appropriate levels. Our studies indicate that the longer form of HuR mRNA is translated under normal growth conditions, but during a stress event, translation of the shorter form predominates. This shorter form has a ~20 base, AT-rich, unstructured 5’ untranslated region, which permits rapid translation in in vitro assays. In contrast, the longer HuR mRNA is much more poorly translated under the same conditions (Ayupova et al., 2009). In this respect, the longer form of HuR mRNA is similar to class I mRNAs, which have been defined as those encoding transcription factors, growth factors and their receptors, proto-oncogenes, and similar transcripts that are not well translated under normal conditions. This class of mRNAs is notable in that >90% of their 5’ UTRs are
longer than 100 bases and have extensive secondary structure (Davuluri et al., 2000). In contrast, the shorter HuR transcript is similar to class III mRNAs, which consists of highly expressed genes whose levels of expression are controlled primarily at the transcriptional level and are thought to be efficiently translated. These mRNAs were found to have short 5’ UTRs that were free from secondary structure (Davuluri et al., 2000). A stress-induced increase in the shorter transcript would permit ready translation of HuR under poor cellular conditions. Indeed, we previously showed that HuR translation actually increased in renal proximal tubule (LLC-PK1) cells that were undergoing an almost total depletion of ATP (Jeyaraj et al., 2006). However, upon return of the cells to normal growth medium, HuR levels returned to baseline. The differential expression of HuR mRNAs with alternate 5’ UTRs and translatabilities provides a mechanism for this stress-responsive regulation of HuR expression.

4.3 Expression of HuR and Transcription Factors NF-κB and KLF8 in Renal Malignancies

Although regulation and expression of HuR are critical for kidney homeostasis, overexpression of this protein in renal tissue has deleterious effects, such as promoting renal cell carcinoma (RCC). Initially, HuR was thought to elicit a tumor suppressor effect, by increasing von-Hippel-Lindau (VHL) tumor suppressor protein levels that mediated p53 activation and decreased tumor growth (Galban et al., 2003). However, one of the first studies reporting the role
of HuR in the progression of RCC was by Stickle et al., where the authors demonstrated that HuR binds and stabilizes mutant p53 transcripts, leading to resistance to traditional therapy (Stickle et al., 2005). HuR was later shown to affect VHL mRNA stability in RCC by destabilizing its interaction with hypoxia induced vascular permeability factor (Datta et al., 2005), and also to interact with parathyroid hormone related protein, resulting in suppression of the anti-tumorigenic functions of VHL (Danilin et al., 2009). Cytoplasmic HuR expression, along with increased COX-2 levels, has been confirmed to be a poor prognosis for RCC patients, indicating advanced stage of the disease (Ronkainen et al., 2011). Danilin et al. later demonstrated that depleting HuR in human RCC cell lines negatively regulated the expression of VEGF, TGFβ, HIF2-α, and constitutive activation of PI3K-Akt, NF-κB and MAPK pathways (Danilin et al., 2010). It is of note that our group later demonstrated the role of HuR in constitutive activation of PI3K-Akt/ NF-κB pathways by at least one mechanism, which seemingly has potential to be applicable in multiple human cancers (Singh et al., 2013).

Multiple studies also suggest that NF-κB plays a critical role in progression of renal cell carcinoma. The anti-apoptotic response mediated by NF-κB seems to be inhibited by the VHL tumor suppressor protein in disease progression (Heng et al., 2003); loss of VHL was shown to result in HIF1-α induced NF-κB activation (Pantuck et al., 2010), leading to conferral of drug resistance, and promoting epithelial-mesenchymal transition in renal cell carcinoma (Morais et
NF-κB was also shown to promote tumor angiogenesis in clear cell RCC by activation of VEGF and EGF (Meteoglu et al., 2008), and Bera et al. demonstrated that miR-21 and NF-κB mediate activation of cyclin-D1, resulting in RCC proliferation (Bera et al., 2013). Several of the genes discussed above, namely VHL, HIF1-α, cyclin-D1, and miR-21 are known to be post-transcriptionally activated by HuR protein, confirming a potential role for the HuR-NF-κB axis in regulation of transcripts in RCC.

KLF8 is known to promote cell proliferation in RCC, and siRNA-mediated knockdown of KLF8 decreases tumor growth in the 786-0 human RCC cell line (Fu et al., 2010). It is noteworthy that HuR, KLF8, and NF-κB proteins by themselves and with cross-talk through common targets are involved in tumor growth and progression to epithelial-mesenchymal transition. Identification of the roles of HuR mRNA variants and their transcriptional control is a promising area that needs to be validated in human cancers.

4.4 Role of KLF8 in Cell Stress and Survival

KLF8 initially was described as a transcriptional repressor, but was later established as a transcriptional activator and discovered to be critically involved in oncogenic transformation and metastasis. Knowledge of signal transduction pathways that regulate KLF8 and its target genes has begun to emerge only in the last decade. KLF8 is one of a family of zinc-finger transcription factors that are highly conserved across species. Seventeen Krüppel-like factors have been
identified that share homologous C-terminal zinc-finger domains, and each possesses a distinct amino-terminal region that determines its specificity for binding partners (McConnell et al., 2010). Sp1 also shares homology with the KLFs in its zinc-finger domains, resulting in their binding to similar GC-rich sequences. However, some distinctions do exist in binding preferences, with the KLFs having a preference for 5′-CACCC-3′ (or 5′-GGTG-3′) sequences (Miller et al., 1993). This core sequence is not present in the region of the HuR promoter tested, though chromatin immunoprecipitation studies readily demonstrated the binding of KLF8 in these studies. Although kidney cells express mRNAs for many members of the KLF family, we focused on KLF8 for a number of reasons. First, though KLF8 is expressed at low levels in many tissues, it is at its highest levels in kidney (van Vliet et al., 2000). Further, the influence of KLF8 on cellular activity is similar to the influences of HuR. Overexpression of KLF8 has been associated with oncogenic transformation in a number of cell types, including renal cells (Fu et al., 2010), as has HuR (Danilin et al., 2010). Additionally, many of KLF8’s transcriptional target genes are also regulated by HuR’s post-transcriptional effects. These include cyclin D1 (Li et al., 2010, Lal et al., 2004 and Zhao et al., 2003), Bcl-2 family members (Abdelmohsen et al., 2007, Li et al., 2010 and Liu et al., 2012), and MMP9 (Li et al., 2010 and Akool et al., 2003), which regulate cell proliferation, survival, and motility/invasion. Further, expression of KLF8 is upregulated by activation of PI3K/Akt signaling (Wang et al., 2008), as is expression of HuR (Kang et al., 2008 and Singh et al., 2013). KLF8 expression
was recently shown to be sensitive to oxygen tension in placenta, indicating its role as a stress-responsive regulator of cellular function (Yang et al., 2014). Interestingly, knockout of HuR in mice showed HuR to be critical to appropriate placental morphogenesis (Katsanou et al., 2009). Schaecher et al. demonstrated that decreased HuR levels in placental trophoblasts alter placental growth factor expression in pre-eclampsia, causing diminished placental perfusion and renal systemic effects, altogether contributing to the pathophysiology (Schaecher et al., 2006). Our findings, together with the placental studies involving hypoxia and pre-eclampsia, suggest that HuR might serve as an oxygen sensor and alter target gene expression. These findings suggest that KLF8 works in concert with HuR to regulate cellular activities and might indeed be involved in HuR expression. KLF8 probably elicits many target effects through upregulation of pro-survival and growth proteins such as HuR. The results shown in the current study demonstrate that KLF8, along with NF-κB, promotes expression of the long HuR transcript within the context of cellular stress, but the significance of this regulatory mechanism needs further investigation in inflammation, oncogenesis and other metabolic dysfunction pathways.

KLF family members have been grouped into classes based on their structural homology and N-terminal interaction domains. Group 1 (KLFs 3, 8, 12) is characterized by interaction with the carboxy-terminal binding protein (CtBP) and may serve as transcriptional repressors. Members of group 2 (KLFs 1, 2, 4, 5, 6, 7) bind and are regulated by histone acetylases and act primarily as
transcriptional activators. Members of group 3 (KLFs 9, 10, 11, 13, 14, 16) are characterized by interaction with the transcriptional co-repressor Sin3A and usually are involved in multiple protein-protein interactions (Nagai et al., 2009). All three groups are represented in LLC-PK1 cells, allowing for independent regulation and coordinated cross talk between the family members. Multiple KLF members are known to interact and regulate common intracellular pathways by modulating transcriptional networks in response to stimuli such as stress and inflammation. It is likely that HuR levels are regulated by KLF members other than KLF8, and studying the interactions among these members would appear to be a promising avenue for understanding renal cell health.

4.5 Role of NF-κB in Stress, Cell Survival, and Renal Health

NF-κB is a master regulatory transcription factor that is known to have potent effects on distinct subsets of genes in a specific stimulus-dependent manner. In kidney, NF-κB was originally considered to be a pro-fibrotic and pro-inflammatory cytokine aiding in progression of kidney disease, but a recent study suggested that NF-κB is a pro-survival factor that is inhibited by collagen-I and other factors to induce apoptosis in renal mesangial cells, a mechanism that fine tunes the balance between survival and apoptosis (del Nogal et al., 2012). New roles are being attributed, owing to involvement of this transcription factor in reperfusion, ischemic preconditioning, and cellular repair mechanisms as discussed below. Models of experimental hypertension demonstrated that NF-κB
activation and apoptosis are simultaneously induced in renal tubular interstitium (Quiroz et al., 2003). Further, ischemic treatment for as short as fifteen minutes results in NF-κB activation, which peaks at thirty minutes and remains through one hour of reperfusion (Donnahoo et al., 2000), indicating that distinct mechanisms have evolved to rapidly upregulate NF-κB in response to renal I/R injury. Bitzer et al. demonstrated that NF-κB inhibits the TGFβ pathway by modulating Smad7 levels critically regulating cell proliferation and apoptosis (Bitzer et al., 2007). Thus, NF-κB may be required for attenuation of TGFβ-mediated kidney fibrosis following IR injury. Along with AP-1, NF-κB has been shown to be cyto-protective to LLC-PK1 cells in response to oxidative stress, through the MAPK pathway that reverses the ROS-induced suppression of NF-κB (Ramachandiran et al., 2002). Further, in IR injury in mice, NF-κB is upregulated within 3 hrs of reperfusion, implying rapid onset of pro-survival mechanisms (Supavekin et al., 2003).

A recent study by He et al. demonstrated that NF-κB mediated regulation of hypoxia inducible factor (HIF-2α) induces nitric oxide synthase (NOS) expression, thus improving micro-circulation in experimental renal ischemia-reperfusion (He et al., 2014). NO and NOS are recognized as mediators of pathophysiological responses following renal I/R injury and they have been shown to be involved in inhibiting or accelerating apoptotic processes that involves complex interactions with p53, Bcl-2, cyt-c, TNF-α, and NF-κB (Chung et al., 2001). Interestingly, mRNAs of both HIF-2α and NOS are known targets of
HuR and are stabilized upon HuR binding. Although KLF8 has not been reported to regulate NOS or NO levels, other family members such as KLF2 (SenBenerjee et al., 2004), KLF4 (Feinberg et al., 2004), and KLF10 (Mitsumoto et al., 2003) have been known to activate NOS promoter region in angiogenesis, vascular repair, and stress. These findings suggest that the master regulatory protein HuR and KLF members may be involved in intricate cross-talk or common signaling pathways in regulating target genes that are critical for cell survival. These studies along with our experimental data validate pro-survival effects of this cytokine-mediated transcription factor in renal epithelia.

On the other hand, NF-κB activation has been shown to cause pathological changes that mediate renal tubular injury by mechanisms such as cytokine activation and macrophage infiltration (Rangan et al., 1999). NF-κB activation in glomerular epithelial cells was observed in HIV-associated nephropathy that contributes to disease severity by adversely affecting the kidney microenvironment (Martinka et al., 2006). Meldrum et al. reported that NF-κB is activated in simulated ischemia in renal tubular cells and that NF-κB induces cellular apoptosis (Meldrum et al., 2002). In another study, one hour of ischemic injury resulted in activation of NF-κB in renal tubular cells, and ATP was demonstrated to inhibit NF-κB in a manner that protected tubular cells (Lee et al., 2005). However, the most detrimental effects of NF-κB activation in injured kidneys may come not from renal tissue but from immune and inflammatory cells. Specific NF-κB inhibition only in murine T-cells was demonstrated to be markedly
protective against renal IR injury, when compared to mice with systemic NF-κB inhibition (Xue et al., 2014). While most studies have focused on the canonical pathways of NF-κB activation in renal IR injury, activation of a non-canonical pathway was also demonstrated in tubular epithelial cells for up to twenty four hours following treatment with the cytokine TNF-like weak inducer of apoptosis (TWEAK, Poveda et al., 2013).

HuR fits into NF-κB regulatory pathways in promoting cell survival and activation of pro-inflammatory responses that have been studied in detail. The role of NF-κB-mediated HuR regulation was not discovered until recently (2008), in which NF-κB was demonstrated to bind to the HuR promoter and promote metastasis in gastric tumor cells. In a later study by our group, it was demonstrated that HuR is central to cell proliferation and growth due in part to its amplification of Akt signaling (Singh et al. 2013). In proximal tubule cells, Akt activation stimulated NF-κB activity, thereby increasing HuR expression. HuR then bound to and stabilized Grb10 mRNA, resulting in its increased expression. Grb10, an adaptor protein, stimulated Akt function by translocating Akt to the plasma membrane where it was phosphorylated and activated by PI3K, resulting in a positive feedback loop. This positive feedback mechanism was shown to be critical for cell survival. These findings, together with the current study, could help elucidate the molecular basis of pro-survival and apoptotic roles of the NF-κB – HuR axis, in the context of proximal tubule cell survival following ischemic injury or other stresses.
4.6 Interaction Between KLF8 and NF-κB

Although the current project has not yet explored potential interactions between KLF8 and NF-κB, it is clear that both factors are individually involved in promoting transcription of the long HuR mRNA. It is of interest that expression of KLF8 was previously demonstrated to be upregulated by PI3K/Akt signaling in human ovarian cells (Wang et al., 2008). HuR expression was also demonstrated to be stimulated by PI3K/Akt signaling in both renal proximal tubule cells (Singh et al., 2013) and gastric tumor cells (Kang et al., 2008). As described above, we found that HuR participates in a positive feedback loop for Akt signaling, although only total HuR mRNA was studied, and the alternate transcripts were not distinguished. We do not know how KLF8 might integrate into this signaling pathway in our model system. However, it seems unlikely to be part of this feed-forward mechanism, given that its expression decreases in the context of cellular stress, when Akt activation occurs. More studies are required to delineate its responses to PI3K activity in the kidney. Recent studies by Zhang et al. demonstrated that KLF6 acts as co-activator of NF-κB and is recruited to promoters of target genes in a highly specific p65-dependent manner. Although this is an obligatory mechanism for NF-κB mediated transcriptional activation, it has been validated only for selected downstream targets after TNF-α stimulation (Zhang et al., 2014). KLF13 has been shown to be required for p65-mediated regulation of the CCL5 chemokine response following transplants and in
generation of an immune inflammatory response during renal allograft rejection (Krensky et al., 2007). KLF13 is also required for expression of several oncogenes, which possibly are regulated by NF-κB. Similar studies would further elucidate the interdependency of transcription factors KLF8 and p65 in transcriptional activation of HuR, as individual overexpression studies suggest stimulation of long form HuR.

We did not observe a role for Sp1-like transcription factors in regulation of HuR, and this was consistent with findings that Sp1 is expressed at very low levels in mature proximal tubule cells (Cohen et al., 1997). Even overexpression of Sp1 in LLC-PK₁ cells did not promote expression of the long HuR transcript, indicating a role for KLF family members in this core promoter region of the HuR gene. We tested the possible role of another Sp-family member, Sp3, in the regulation of long form HuR. Our preliminary studies involving over expression of Sp3 in LLC-PK₁ cells did not result in significant changes in long form expression. Further, supershift assays with Sp3 antibody did not generate any shifting of the DNA-protein complexes (data not shown), suggesting that Sp3 is likely not involved in regulating this HuR mRNA. However, possible interactions with other Sp or KLF members cannot be overlooked, considering Sp family members act in synergy or antagonistically with KLF family members in transcriptional regulation.
4.7 Role of KLF Family Members in Renal Homeostasis

Numerous KLF family members have been identified in kidney, but more work is needed to definitively identify the expression patterns of all these factors in specific cell types in development and disease. However, some KLF factors are beginning to be understood in this organ system. For example, KLF15 (formerly KKLF, kidney-enriched Krüppel-like factor), is enriched in endothelial and mesangial cells of the kidney and a few nephron segments, excluding proximal tubules (Uchida et al., 2000). KLF15 has been shown to inhibit mesangial cell proliferation, promote podocyte differentiation, repress transcription of the kidney-specific chloride channel CLC-K1, and may inhibit renal interstitial fibrosis (Uchida et al., 2000, Hong et al and Mallipattu et al., 2012, Gao et al., 2013). Another Krüppel-like factor not expressed in proximal tubule cells is KLF12, which is restricted to collecting ducts and promotes expression of the urea transporter UT-A1 (Suda et al., 2006). Only a few KLF proteins have been characterized in proximal tubules. One example is KLF4, which functions as a tumor suppressor in a number of tissues, and was recently shown to be expressed in proximal tubule cells and down regulated in renal cell carcinoma tissues (Song et al., 2013). Additionally, in a screen for mRNAs that are upregulated in the earliest stages of renal ischemia/reperfusion injury, KLF6 (formerly Zf9) was detected. This factor was expressed at barely detectable levels in control kidneys, but demonstrated a rapid (within 1-3 hours) induction
after ischemic injury. It also was rapidly induced in cultured proximal tubule cells subjected to ATP depletion, and its expression paralleled that of TGF-β1, a previously identified target (Tarabishi et al., 2005). Both KLF4 and KLF6 have temporal expression patterns that contrast sharply with KLF8, which is upregulated in renal cell carcinoma, and which rapidly decreases upon ischemic injury or thapsigargin treatment. It is clear that individual KLF family members adopt distinct spatio-temporal patterns in kidney cells, and comprehending how these factors are expressed will give us a clearer understanding of how the regulation of their target genes.

4.8 Smad Signaling and Interactions with KLF Family Members

Regulation of the short HuR mRNA by BMP-dependent Smad 1/5/8 was previously demonstrated by our group (Jeyaraj et al., 2010), and our current studies suggest that Smad1 overexpression interferes with p65-mediated upregulation of long form HuR mRNA. This result is consistent with our previous studies showing that the long and short mRNAs appear to be regulated in a reciprocal manner, at least in the LLC-PK1 cell culture model used for these experiments. Therefore, it was of interest to determine how p65, KLF8, and Smad1 are regulated during IR injury in native rat kidneys. Figure 3.22 demonstrates that there is variability of Smad 1/5/8 expression among rats, but nonetheless suggests that one hour ischemic injury sharply diminishes their levels. Further, protein levels gradually increase from 1 hour through 14 days of

99
reperfusion, suggesting Smad1 involvement in eliciting the pro-survival and preconditioning role of HuR, likely through increasing levels of the short HuR transcript. However, transcriptional and translational control mechanisms in native rat kidneys need to be explored further in order to precisely define the regulatory mechanisms of both HuR isoforms.

The interaction between Smad and KLF family members seems to be critical in multiple disease processes such as abnormal growth and inflammation, as reported by Subramaniam et al., where it was demonstrated that KLF10 is a critical mediator of TGF-β cellular responses through regulation of Smad signaling (Subramaniam et al., 2010). KLF10 was shown to be regulated by the TGF-β – Smad 2/3 pathway and also modulate inflammatory response and proliferation signals, traits similar to both KLF8 and HuR, except for its tumor suppressor role. KLF10 and potentially KLF11 have been demonstrated to inhibit cell proliferation and tumor invasiveness in various cancers by inducing apoptosis (Tachibana et al., 1997 and Song et al., 2012). KLF10 immunostaining was undetected in human kidney, though it was reported that KLF10 is detected in human epithelial cells of placenta, breast and numerous other tissues. However, our study suggests KLF10 is expressed in LLC-PK₁ cells, so its role in kidney requires further investigation. Stress in neural tissue also has been reported to alter KLF10 activity (Alme et al., 2007), suggesting that in addition to KLF8, other KLF family members might be involved in stress responses.
It is possible that Smads may play a role in regulating KLF-mediated transcriptional activation of the HuR long form mRNA. Several published studies have suggested interaction among Smads and KLF family members. KLF1 has been reported to be transcriptionally activated by Smads 5/4 in hematopoietic development, a mechanism which is inhibited by Smad6 (Kang et al., 2012). In addition, Smads 1/5/8 have been shown to induce EKLF/KLF1 in association with BMP4 during embryoid body differentiation, suggesting that Smad1 interaction with KLF members is critical from the initial stages of development (Adelman et al., 2002).

TGF-β1 is the most abundant isoform of TGF-β family members and is secreted by all cell types in renal tissue. It also has been implicated as the central regulator of renal fibrosis, independent of the cause of kidney disease (Bottinger et al., 2007). Even though it is still debated, existence of EMT in renal tissue has been shown to be primarily regulated by TGF-β1 because of the cytokine’s ability to transform fully differentiated tubular epithelial cells to a mesenchymal phenotype by gaining migration capabilities and de novo expression of α-smooth muscle actin (Liu et al., 2010).

BMP-7, on the other hand, is required for normal renal development and is expressed at high levels in the medullary tubules, glomerular epithelial cells, podocytes, and renal artery adventitial cells in healthy adult kidney. Ischemic acute kidney injury has been shown to significantly downregulate renal BMP-7 expression (Simon et al., 1999). Consistent with its renal protective role, BMP-7
has been shown to inhibit TGF-β1 triggered EMT \textit{in vitro} in mouse tubular epithelial cells and also to reverse chronic injury by repairing damaged renal proximal tubular cells (Zeisberg et al., 2003). Another interesting study reported that BMP-7 might potentially block macrophage infiltration into kidney during renal fibrosis (Zhang et al., 2005), a mechanism known to be activated by NF-κB (Lee et al., 2004). It is worthwhile to mention that previous studies from our group demonstrated the role for BMP-7 as a transcriptional activator of HuR (Jeyaraj et al., 2010), and current data suggest a potential role for NF-κB in transcriptional activation of the longer HuR isoform. BMP-7 inhibition studies by chordin or noggin, might provide valuable insights into the regulation of both HuR isoforms and their defined roles in renal protection. It will be worthwhile to study these potential interactions between BMP-7, NF-κB and HuR isoforms that may contribute to regeneration after renal IR injury, in cell survival, or during progress to a chronic injury phase.

4.9. Post-transcriptional Regulation of HuR Expression

Al-Ahmadi et al. reported alternatively polyadenylated variants of HuR mRNA with different lengths, AREs, abundance and stabilities, and also demonstrated that HuR binds and stabilizes its own transcripts and also competes with another RBP, tristetrapolin (TTP), to bind to HuR mRNA to confer stability. A 6-kb HuR mRNA variant was identified that contained AREs in its 3′ untranslated region. This longer alternate polyadenylation variant was shown to
be less abundant and stable (in the absence of ectopic HuR expression), suggesting an auto-regulatory role of HuR (Al-Ahmadi et al., 2009). Briefly, HuR was shown to regulate its own expression through a negative feedback loop. Nuclear HuR can bind its own pre-mRNA and increase production of the longer, more labile variant, thus keeping HuR levels at constant and relatively low physiological levels (Dai et al., 2012). Thus, existence of alternate polyadenylation variants determine relative HuR abundance in tissues and may play a critical role in maintaining the normal state of the cells.

The first miRNA demonstrated to regulate HuR expression was miR-519, as predicted by sequence analysis and confirmed by experimental procedures in 2008 (Abdelmohsen et al., 2008). MiR-519 binding sites were identified in both the coding region and 3' UTR of HuR. MiR-519 was shown to inhibit HuR expression in multiple tumor cell lines by suppressing HuR translation, but not HuR mRNA levels. Modulating the levels of miR-519 within cells affected HuR downstream targets. Not unexpectedly, decreasing the ability of miR-519 to bind HuR (through addition of antisense miR-519), increased HuR levels and the rate of cell division. In a subsequent study, HuR and miR-519 levels were examined in pairs of cancerous and adjacent healthy tissue. HuR protein, but not mRNA, levels were increased in the cancer samples, and miR-519 levels were markedly reduced. MiR-519 was also shown to inhibit tumor growth from HeLa cells injected into athymic mice, supporting the notion of miR-519 as a tumor suppressor that acts through HuR (Abdelmohsen et al., 2010). Notably, miR-519
levels were demonstrated to increase in a model of cellular senescence, suggesting that triggering of senescence through inhibition of HuR is a mechanism by which tumor suppression may occur (Marasa et al., 2010).

In the last five years, several new miRNA regulators of HuR have been identified. MiR-16 was demonstrated to translationally repress HuR in breast cancer cells by interacting with the 3' UTR of HuR mRNA (Xu et al., 2010). This miRNA also suppresses translation of COX-2, TNF-α and Bcl-2 (Cimmino et al and Jing et al., 2005), which, interestingly, are all tumor-promoting genes positively regulated by HuR. The complexity of the interaction between HuR and miR-16 was demonstrated to an even greater degree when it was shown that association of a HuR/miR-16 complex with AREs of several target transcripts could facilitate inhibition of miR-16 expression in colorectal cancer cells (Young et al., 2012). Thus, the tumor suppressor activity of miR-16 and the tumor-promoting activities of HuR appear to antagonize one another at multiple levels.

miR-125a was first reported to inhibit cell growth and promote apoptosis by translationally repressing HuR in breast cancer cells (Guo et al., 2009). In another study, miR-125 was shown to inhibit phosphorylation of Akt in breast cancer cells (Scott et al., 2007). This suppression of Akt activation could interfere with the growth-promoting environment through various downstream pathways, one of which is the transcriptional activation of HuR expression through Akt/ NF-κB signaling (Kang et al., 2008 and Singh et al., 2013). Thus, miR-125 may inhibit HuR expression at multiple levels, through direct translational suppression
and through indirect inhibition of transcription. Overexpression of another miRNA, miR-34a, was shown to suppress HuR protein levels in prostate cancer cells, thus modulating cell proliferation and drug resistance in those cells (Kojima et al., 2010). However, no potential binding sites for miR-34a were found by \textit{in silico} analysis of the HuR 3’ UTR, suggesting that miR-34a may regulate HuR through binding in other regions of the transcript or through other mechanisms. MiR-9 similarly acts as a tumor suppressor by directly binding the 3’ UTR of HuR, thus suppressing HuR expression and expression of its downstream targets (Leucci et al., 2010). HuR has also recently been reported to be a target of miR-146, a potent anti-inflammatory molecule (Cheng et al., 2013). HuR was shown to be a direct target of miR-146, which suppresses both HuR mRNA and protein levels. HuR is established as a regulator of mRNAs involved in inflammation (Srikantan et al., 2012 and Nabors et al., 2001) as well as a positive regulator of NF-κB activity (Singh et al., 2013 and Rhee et al., 2010). Thus, one pathway through which miR-146 exerts its anti-inflammatory effects is through suppression of HuR.

While the miRNAs described above all directly bind to HuR mRNA and inhibit synthesis of the protein, other miRNAs can positively regulate HuR synthesis through indirect mechanisms. MiR-29a, a miRNA abundant in breast cancer cells, binds to and degrades the mRNA transcript of tristetraprolin (TTP), another RBP that works to promote decay of target mRNAs. Because HuR mRNA is a target of TTP-mediated degradation, miR-29a’s overall effects are to
reduce TTP levels while increasing HuR expression. This imbalance in the HuR/TTP ratio correlated with increased expression of ARE-containing, tumor-promoting mRNAs. Importantly, inhibition of miR-29a reversed the imbalance, suggesting this microRNA as a potential target for inhibition in breast cancer (Al-Ahmadi et al., 2013). This study demonstrates that miRNA-mediated regulation of other RBPs is critical to the overall activity of HuR and provides insight into ways in which a network of RBPs can control cell fate.

Mechanisms contributing to renal homeostasis are evidently complex and intricate, and are dictated by normal physiological response along with continuous restoration of structural and functional integrity of kidney. This is achieved by integration and regulation of the survival pathways and inflammatory responses following ischemia and IR injury, including cytokines, RNA binding proteins, cross talk between TGF-β -BMP and other super-families of proteins, Smad signaling pathways that regulate each other, transcription factors such as NF-κB, Sp/KLF family members, microRNA dynamics, and fine-tuning of all these elements contributing to normal renal function. Enhanced understanding of these physiological and biochemical mechanisms may help in delaying the onset, slowing the progression, or improving the epidemiological outcome of chronic kidney disease.
5.1. 3′-Untranslated Region of HuR and mRNA Stability

mRNA stability is an important means of regulating gene expression. Hence, its study is critical for understanding intracellular pathways and subsequently, the molecular mechanisms of various underlying pathologies. Post-transcriptional control of mRNA is also known to be a significant mechanism in gene regulation, and AU-rich elements are the best characterized ‘cis’ elements in regulating stability of eukaryotic mRNAs. mRNA stability is controlled by a number of regulatory pathways that involve interactions between various proteins and conserved, highly specific AU-rich elements, usually in the 3′ untranslated regions of these transcripts. These cis-trans interactions are regulated by a number of cytokines, hormones, and hypoxic conditions (Hollams et al., 2002). Genetic polymorphisms in 3′ UTRs are often associated with disease conditions and this is mainly attributed to unstable mRNA transcripts. As
a stabilizer of thousands of cellular mRNAs, particularly mRNAs involved in cell
growth and survival, HuR has the potential to be involved in the pathophysiology
of many disease states, and is well-established to be dysregulated in numerous
cancers.

We began studies to determine whether the stability of the HuR mRNA
itself is a factor in its expression. Deletion mutants of the 3’ UTR of HuR mRNA
were generated in order to identify the potential regions conferring stability to
HuR mRNA. These mutants were expressed pair-wise in LLC-PK₁ cells to
compare their stability. Plasmids expressing the full-length HuR cDNA and
another lacking the 3’ UTR were transfected in equal amounts in LLC-PK₁ cells.
Because transcription of the cDNAs was driven by the same promoter (CMV),
any differences in mRNA expression levels must come from differences in
stability. Lanes 1 and 2 of Figure 5.1 show the sizes of the individual full-length
and truncated HuR mRNAs, while lane 3 shows the relative expression of the two
cop-transfected forms. As shown, deletion of the 3’ UTR increased HuR stability
suggesting the presence of potential stability elements in the 3’ UTR of porcine
HuR transcripts, which may also play a role in regulating its expression under
cellular stress in kidney. Subsequently, Al-Ahmadi et al. identified two
polyadenylation variants of HuR and suggested a role for auto-regulation of HuR
through its own 3’ UTR in HEK-293 and HeLa cells (Al-Ahmadi et al., 2009). It is
possible that renal ischemia and other cellular stresses might affect these auto-
regulatory pathways and modulate HuR expression. Molecules that interfere with
HuR binding to the 3'UTR of HuR seem to be possible therapeutic targets in a number of conditions associated with dysregulated HuR dynamics in various cellular pathologies.

5.2. Role of PI3K-Akt Pathway and mTOR Signaling in HuR mRNA Expression

Previous findings from the Lee laboratory confirmed the role of the PI3K-Akt pathway in regulation of total HuR levels in both human and porcine proximal tubule cell lines (Singh et al., 2013). However, this study did not distinguish between the long and short HuR mRNAs. Currently, our preliminary studies suggest that overexpression of constitutively active Akt (CA-Akt) results in upregulation of long form of HuR, possibly a downstream effect of KLF8 promoter activation (Figure 5.2). Along similar lines, co-transfection of plasmids expressing constitutively active CA-Akt, KLF8 and NF-κB p65 in LLC-PK1 cells yielded high levels of long form HuR mRNA, suggesting a synergistic upregulation event (Figure 5.3). Previous studies suggested that inhibition of PI3K by LY294002 down-regulates KLF8 promoter activity, and conversely, overexpression of CA-Akt stimulates KLF8 expression in human ovarian epithelial and cancer cells (Wang X et al., 2008). In preliminary experiments, we found that treatment of LLC-PK1 cells with 25 – 100 μM LY294002 for 24 hours sharply decreased KLF8 mRNA levels. In the same conditions, we did not see loss of HuR long form mRNA, but without Western analysis of KLF8 protein, it is unclear whether KLF8
protein diminished during the treatment period. However, from our published studies (Singh et al., 2013), it is clear that HuR is part of a positive feedback loop with Akt signaling, and this pathway deserves further investigation in studies of renal health.

One downstream effector of Akt signaling, the mammalian target of rapamycin (mTOR), is a well-studied kinase that is involved in a number of pathways that maintain cellular homeostasis, such as metabolism, response to stress, and growth. It is implicated in a vast number of diseases such as metabolic syndrome and type II diabetes mellitus, and mTOR kinase has been implicated in inhibiting autophagy of proximal tubule epithelial cells and podocytes, a mechanism shown to maintain normal physiology of these cells (Inoki et al., 2013). Multiple stimuli activate the Akt - mTOR pathway through PI3K, including EGF, IGF-1, nutrients such as glucose, amino acids, and oxygen, and elicit their functions in cell proliferation and growth (Lieberthal et al., 2009).

Normal mTOR levels in kidney are very low or absent and have been shown to increase rapidly following renal IR injury (Lieberthal et al., 2001). mTOR has been shown to be involved in chronic kidney diseases such as diabetic nephropathy and polycystic kidney disease, and delays progression of renal fibrosis (Eddy et al., 2006). Though mTOR and HuR have been known to regulate common transcripts in malignancies such as breast cancer and others, associations between mTOR signaling and HuR are largely unknown. However, it has been reported that mTOR inhibition by rapamycin confers protective effects
against IR injury by attenuating oxidative stress (Das et al., 2014). Preliminary data from our experiments are only suggestive at this point, but they indicate that further probing of mTOR signaling in ATP depletion and renal I/R injury could provide insights into cell survival mechanisms in kidney that may possibly involve HuR interaction or regulation.

5.3. Roles of Other KLF Members in HuR mRNA Expression

There are a number of KLF members expressed in porcine epithelial cells whose roles in regulation of HuR are yet to be identified. KLF15 (formerly KKLF, kidney-enriched Krüppel-like factor), is a KLF member known to be involved extensively in lipid metabolism and regulation of vascular response to smooth muscle injury (Lu et al., 2010), and act as a negative regulator of stress-responsive cardiac remodeling and fibrosis (Wang et al., 2008). It is enriched in endothelial and mesangial cells of the kidney and a few nephron segments, excluding proximal tubules (Uchida et al., 2000). Interestingly, oxidative stress, TGF-β and TNF-α are all known to downregulate KLF15 expression through the TNF-α receptor-1 and NF-κB (Gao et al., 2011). KLF15 has been shown to inhibit mesangial cell proliferation, promote podocyte differentiation, repress transcription of the kidney-specific chloride channel CLC-K1, and may inhibit renal interstitial fibrosis by regulating ERK/MAPK and JNK/MAPK pathways (Uchida et al., 2000, Hong et al., 2012, Mallipattu et al., 2012 and Gao et al., 2013).
Even though KLF15 is expressed at very low levels in LLC-PK₁ cells (Figure 3.15), and some other groups reported no detection of this KLF member in proximal tubule cells, preliminary data from over-expression studies of KLF15 so far suggest a stimulatory role in transcription of the long form of HuR mRNA and an inhibitory role in regulation of KLF8 levels (Figure 5.5). ATP depletion studies in LLC-PK₁ cells suggest no significant change in KLF15 levels in TG stress, suggesting no significant role for this KLF member in the stress response, though involvement of KLF15 in regulation of long form HuR independent of KLF8 is still plausible. The KLF members are known to have extensive cross talk among each other and to promote and repress each other's expression levels. Potential regulation of KLF8 by KLF15 and other factors (or vice-versa) in renal homeostasis would appear to be a promising area of research.

5.4. Potential Interaction Between NF-κB and KLF Members in Regulation of HuR

Our work has been the first to demonstrate KLF regulation of HuR in kidney or other tissues, and KLF8 interaction with NF-κB is yet to be identified within any cellular context. There have been several studies suggesting interaction between Sp-KLF transcription factors and NF-κB in regulating transcriptional activity of targets. Sp1 and NF-κB have been demonstrated to interact and regulate Neph3 gene activity in podocytes of kidney (Ristola et al., 2009), and Sp1 and KLF family members interact extensively in regulating a
number of other targets. KLF4 in macrophages has been shown to interact with NF-κB and cooperatively activate pro-inflammatory genes, and also compete with Smad3 and inhibit anti-inflammatory genes (Autieri et al., 2008). Das et al., reported that KLF2 inhibits transcriptional activity of NF-κB and may thus regulate monocytic activation (Das et al., 2006). KLF15 has been shown to interact with NF-κB and alter its acetylation status and control vascular inflammation (Lu et al., 2013). KLF7 is also known to positively regulate NF-κB signaling pathways when overexpressed during progenitor cell formation (Schuettppelz et al., 2012).

It has been shown that HuR knockdown results in increased half-life of KLF2 mRNA and inhibited phosphorylation of NF-κB and IκBα in mediating an inflammatory response in human endothelium (Rhee et al., 2009). This suggests a potential crosstalk between NF-κB, HuR, and KLF members during mechanical and biochemical stress. It will be interesting to pursue this cross talk mechanism in other cellular pathologies such as stress and cancer.
Figures for future direction studies

Figure 5.1 Role of the HuR 3’ UTR in mRNA stability. Full-length or truncated HuR cDNAs were transfected either individually (lanes 1 and 2) or together (lane 3) in LLC-PK1 cells, and the resulting mRNAs were identified by Northern blot. In four individual experiments, the form lacking the 3’ UTR showed greater stability than the full-length form.
Figure 5.2 Akt over expression upregulates long form HuR. Varying levels of a constitutively active (CA) - Akt expression plasmid were transfected into LLC-PK₁ cells and the long HuR mRNA was detected by competitive RT-PCR. These data suggest that Akt overexpression at higher concentrations stimulates long form HuR mRNA expression.
Figure 5.3 KLF8, p65, and Akt upregulate long form HuR. KLF8, CA-Akt and p65 (2.5 μg each) were co-transfected into LLC-PK₁ cells. The preliminary data suggest that overexpression of all three factors might result in a higher activation of long form HuR mRNA expression, as measured by competitive RT-PCR.
Figure 5.4 Inhibition of PI3K decreases KLF8 mRNA expression. Addition of the PI3 kinase inhibitor LY294002 at varying concentrations for 24 hrs inhibited KLF8 mRNA expression, but did not alter expression of the long HuR mRNA.
Figure 5.5 KLF15 overexpression alters expression of KLF8 and long form HuR mRNA. Varying levels of a KLF15 expression plasmid were transfected into LLC-PK₁ cells. KLF15 overexpression at higher concentrations stimulated long form HuR mRNA expression as measured by competitive RT-PCR, but downregulated KLF8 mRNA levels.


Donnahoo, K. K., Meldrum, D. R., Shenkar, R., Chung, C. S., Abraham, E., & Harken, A. H. (2000). Early renal ischemia, with or without reperfusion,


Xue, ChengBiao; Liu, Yong; Li, Chao; Li, Yao; Yang, Tao; Xie, Lin; Zhou, P. (2014). Powerful Protection Against Renal Ischemia Reperfusion Injury by T Cell–Specific NF-κB Inhibition. *Transplantation, 97*(4).


