RNA Interference Pathways In Shiga toxin-treated Human Endothelial Cells

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Laboratory Medicine and Pathobiology
University of Toronto

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Abstract

Shiga toxins (Stxs) are a family of bacterial-derived protein exotoxins, implicated in the pathogenesis of Hemolytic Uremic Syndrome (HUS). Stxs have profound effects on endothelial cell (EC) gene expression. These effects include increase in the mRNA stability and translational efficiency of select mRNAs (e.g. CXCR4/SDF-1, ET-1). Here we examined whether Stx has a global effect on post-transcriptional gene regulation in ECs via RNA interference (RNAi) pathways.

Microarray analysis revealed changes in microRNA expression due to Stx, most of which were downregulated. Accordingly, we observed reduction in Dicer and Drosha, key enzymes in microRNA biogenesis. In silico analysis revealed that many of the down-regulated microRNAs target ATF3, which is highly induced by Stx. Consistent with this data, We find that Stx induction of ATF3 protein and mRNA expression is associated with an increase in ATF3 mRNA stability. These data identify an important role for Dicer and microRNA biogenesis in Stx-mediated HUS.
I would like to express my deepest appreciation to my supervisor, Dr. Philip A. Marsden, for giving me the opportunity to be a part of his talented research group and helping me with all his guidance, comments, and support. He inspired me to work and think scientifically and always aim for the best. Without his help the completion of this work would not have been possible. I would also like to thank my committee members, Dr. Andrew Advani, Dr. Kevin C. Kain, Dr. George Yousef, for their valuable input and help to improve my skills and knowledge in the current field. I would acknowledge all the staff in the department of Laboratory Medicine and Pathobiology, especially the graduate coordinator, Dr. Harry Elsholtz, for all his support and help.

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Accreditation of Work

ATF3 in situ hybridization data was provided by Tania Petruzziiello-Pellegrini.

Some ATF3 protein blots were provided by Anna Soltyk, Dr. Brunton’s laboratory.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>A/E lesion</td>
<td>Attaching and Effacing lesion</td>
</tr>
<tr>
<td>Ago2</td>
<td>Argonaute 2</td>
</tr>
<tr>
<td>ALI</td>
<td>Acute Lung Injury</td>
</tr>
<tr>
<td>ANGPTL4</td>
<td>Angiopoietin-Like 4</td>
</tr>
<tr>
<td>ARDS</td>
<td>Acute Respiratory Distress Syndrome</td>
</tr>
<tr>
<td>ARE</td>
<td>AU-Rich Elements</td>
</tr>
<tr>
<td>ASK1</td>
<td>Apoptosis Signal-regulating Kinase 1</td>
</tr>
<tr>
<td>ATF3</td>
<td>Activating Transcription Factor 3</td>
</tr>
<tr>
<td>ATF4</td>
<td>Activating Transcription Factor 4</td>
</tr>
<tr>
<td>ATF6</td>
<td>Activating Transcription Factor 6</td>
</tr>
<tr>
<td>AUF1</td>
<td>ARE-/poly (U)-binding degradation Factor 1</td>
</tr>
<tr>
<td>BBF2H7</td>
<td>BBF2 human homologue on chromosome 7</td>
</tr>
<tr>
<td>Bip</td>
<td>Binding immunoglobulin protein</td>
</tr>
<tr>
<td>bZIP</td>
<td>basic leucine Zipper</td>
</tr>
<tr>
<td>CAST/EI</td>
<td><em>Mus musculus Castaneus</em></td>
</tr>
<tr>
<td>Cat-1</td>
<td>Cationic amino acid transporter-1</td>
</tr>
<tr>
<td>CCRCC</td>
<td>Clear Cell Renal Cell Carcinoma</td>
</tr>
<tr>
<td>Ch25h</td>
<td>Cholesterol-25-hydroxylase</td>
</tr>
<tr>
<td>CHIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP-Homologous Protein</td>
</tr>
<tr>
<td>CPSF</td>
<td>Cleavage and Polyadenylation Specificity Factor</td>
</tr>
<tr>
<td>CREBH</td>
<td>Cyclic AMP-Responsive Element-Binding protein Hepatocyte</td>
</tr>
<tr>
<td>CstF</td>
<td>Cleavage stimulatory Factor</td>
</tr>
<tr>
<td>CXCL12</td>
<td>CXC Chemokine Ligand 12</td>
</tr>
</tbody>
</table>
CXCR4    CXC Chemokine Receptor 4
CXCR7    CXC Chemokine Receptor 7
DEAE    Diethylethanolamine
DGCR8    DiGeorge syndrome Critical gene 8
dsRBD    double-stranded RNA Binding Domain
dsRNA    double-stranded RiboNucleic Acid
DSE    Down Stream Element
EAEC    Enteroaggregative *E.coli*
*E.coli*    *Escherichia coli*
EDN1    Endothelin-1
EDTA    Ethylenediaminotetracetic acid
Egr1    Early growth response 1
EHEC    Enterohemorrhagic *E.coli*
eIF2    eukaryotic Initiation Factor 2
EJC    Exon Junction Complex
ELAV    Embryonic Lethal Abnormal Vision
eNOS    endothelial Nitric Oxide Synthase
ER    Endoplasmic Reticulum
ESRD    End Stage Renal Disease
ET-1    Endothelin-1
FC    Fold Change
GADD34    Growth Arrest and DNA Damage-inducible 34
GAPDH    Glyceraldehyde 3-Phosphate Dehydrogenase
Gb3    Globotrioasylceramide 3
HC    Hemorrhagic colitic
HEY1    Hairy/enhancer-of-split related with YRPW motif protein 1
HIF-2  Hypoxia inducible factor 2
HIV  Human Immunodeficiency Virus
hnRNP A1  heterogeneous nuclear Ribonucleoprotein A1
Hsp27  Heat shock protein 27
HuR  Human antigen R
HMVEC  Human Microvascular Endothelial Cell
HUS  Hemolytic Uremic Syndrome
HUVEC  Human Umbilical Vein Endothelial Cell
IC50  Inhibitory Concentration of 50%
Id-1  Inhibitor of DNA binding 1
IL6  Interleukin-6
IR  Ionizing Radiation
IRE1  Inositol Requiring Enzyme 1
ISH  In Situ Hybridization
JNK  c-Jun N terminal Kinase
KSRP  KH-type Splicing Regulatory Protein
LEE  Locus of Enterocyte Effacement
LIF  Leukemia Inhibitory Factor
LPS  Lipopolysaccharide
MMP9  Matrix Metalloproteinase 9
MMP13  Matrix Metalloproteinase 13
MYOD1  Myoblast Determination 1
MV  Mechanical Ventilation
NMD  Non-sense Mediated Decay
NPM1  Nucleophosmin 1
ORF  Open Reading Frame
PABP  Poly A Binding Protein
PAZ  Piwi Argonaute and Zwille
PERK  PKR-like Endoplasmic Reticulum Kinase
PKR  Protein Kinase R
PMR1  Polysomal Ribonuclease 1
PMSF  Phenylmethylsulfonyl Fluoride
PP1  Protein Phosphatase-1
PTC  Premature Termination Codon
qRT-PCR  quantitative Reverse Transcriptase Polymerase Chain Reaction
RIDD  IRE1 Dependent Decay
RISC  RNA Inducing Silencing Complex
RNAi  RNA intereference
SDF-1  Stromal cell-Derived Factor 1
SDS  Sodium Dodecyl Sulfate
shRNA  short hairpin RNA
siRNA  small interfering RNA
Smad  Drosophila gene 'mothers against decapentaplegic' (Mad) and the C. elegans gene Sma
snoRNA  small nucleolar RNA
SNP  Single Nucleotide Polymorphisms
snRNP  small nuclear Ribonucleic Particles
STEC  Shiga Toxin producing E.coli
Stx  Shiga toxin
TAR  Trans-Activating Region
TAZ  Transcriptional co-activator with PDZ-binding motif
TLR  Toll-Like Receptor
TNF α  Tumor Necrosis Factor alpha
TRAF2  TNFR-Associated Factor-2
TRBP  Trans-Activating Response (TAR)-Binding Protein
TTP  Tristetraproline
tRNA  transfer RNA
UPF  Up-frameshift
UPR  Unfolded Protein Response
5'/3'-UTR  5'/3'-Untranslated Region
VEGF  Vascular Endothelial Growth Factor
VEGFR2  Vascular Endothelia Growth Factor Receptor 2
VT  Verotoxin
XBP1  X-box Binding Protein1
YAP  Yes-associated protein
Chapter 1

Introduction

Part of this chapter was published in:

1 Introduction

1.1 Shiga toxin-producing *Escherichia coli (E.coli)*

*Escherichia coli (E. coli)* are Gram-negative, rod-shaped bacteria. Most strains of this species are non-pathogenic and are a part of normal intestinal flora, while some serotypes are recognized as human pathogens and cause enteric disease. One of those pathogenic groups of *E.coli* is Shiga-toxin producing *E.coli* (STEC). The ability to produce protein exotoxins, called Shiga Toxin (Stx) or Verotoxins (VT) characterizes these strains. Typically STEC are enterohemorrhagic *E.coli* (EHEC), which can cause diseases, such as diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (HUS). Different serotypes of an *E. coli* are characterized by the O (Ohne) antigen of the polysaccharide portion of cell wall lipopolysaccharide (LPS) and the H (Hauch) antigen of the flagella protein. [1] Different serotypes are associated with either sporadic disease or outbreaks, among which *E.coli* O157:H7 is considered to be most virulent. [2] The natural reservoir of EHEC is known to be the digestive tract of cattle. [3] Human infection can also result from consumption of food and water contaminated with cattle excreta, undercooked beef, or even person-to-person contact. [4]

The first host defense mechanism is gastric acidity; however, most of the strains are acid resistant. Followed by infection with enteric *E.coli*, the disease process involves intestinal colonization. Bacterial colonization is facilitated by the presence of the locus of enterocyte effacement (LEE) within the bacterial genome. [1] This locus is necessary for the formation of an attaching and effacing (A/E) lesion, which is characterized by the attachment of bacteria to the enterocytes of the colon and results in the destruction of
microvilli, and diarrhea. [5] Infected patients will develop more serious complications including hemorrhagic colitis and hemolytic uremic syndrome, which will be discussed in the following section.

1.1.1 Stx-mediated Hemorrhagic Colitis (HC)

In 1982, the first outbreaks of hemorrhagic colitis were reported in Oregon and Michigan. [6, 7] Laboratory investigations revealed *E.coli* O157:H7 to be the etiological agent. The clinical manifestations of HC usually begin with severe abdominal cramp along with watery diarrhea, which will become bloody within 1-3 days. This syndrome results from damage to the intestinal epithelium, which will cause edema, hemorrhage, and thrombosis of the capillaries in the cecum and colon.[6] Although HC is a self-limiting disease it can subsequently progress to a serious complication called hemolytic uremic syndrome (HUS). [7, 8]

1.1.2 Stx-mediated Hemolytic Uremic Syndrome (HUS)

Hemolytic uremic syndrome (HUS) is a potentially fatal systemic disease, defined by the triad of non-immune hemolytic anemia, thrombocytopenia, and acute renal failure. [9] In 1983 Karmali *et al.* first reported the association between STEC infection and sporadic cases of HUS. [10] The histopathologic studies revealed thrombotic microangiopathy and endothelial cell damage, especially in glomeruli, as an initial event in HUS. [11] Endothelial cells swell and detach from glomerular basement membrane, which is accompanied by capillary wall thickening and luminal occlusion.[12, 13] The thrombocytopenia is due to consumption of platelets in thrombi, which results in the
fragmentation of erythrocytes as they pass through occluded vessels. [12, 13] There are also some non-renal complications of HUS such as neurological and cardiac dysfunction, which are important determinants of mortality of HUS in children. [14-17] HUS patients may experience neurological abnormalities including seizures, coma, stroke, hemiparesis, and cortical blindness. [18] Involvement of other systems such as gastrointestinal tract, pancreas, liver, and infrequent thrombotic microangiopathy of heart and lung were also reported. [19]

HUS can be divided into two categories: diarrhea-associated typical HUS (D+ HUS) and diarrhea-negative atypical form (D-HUS). [20] Typical HUS patients, the majority of whom are children, present with diarrhea and most eventually achieve complete remission. [21] This mainly occurs by infection with enterohemorrhagic E.coli (EHEC) [22] especially the O157: H7 strain. [23] Atypical HUS (aHUS) accounts for 10% of patients presenting with similar features but is caused by deregulation of complement, mostly of the alternative pathway. [21] This form of HUS is not associated with diarrhea and carries significant risk of morbidity and mortality. [21] About 30-40% of aHUS patients have unknown mutations within the complement system, making patient management and treatment choices more challenging. [24] Recent studies also support a new role for complement activation in STEC-HUS. [25]

In September 2011, the US FDA approved Eculizumab, a humanized monoclonal antibody against complement component 5 (C5), for the treatment of aHUS. Despite its role in efficiently managing aHUS, it is currently the most expensive drug in the world. The cost may be prohibitive in terms of accessibility and as such more research is needed in order to determine optimal management strategies, which also include plasma exchange and organ transplantation. Also, the need to identify the genetic
mutations and the efficacy of Eculizumab therapy in STEC-HUS patients is important in achieving better patient outcomes. [25]

1.1.3 German outbreak: Emergence of a new pathogen

In May 2011 a huge outbreak of a dramatic disease, hemolytic uremic syndrome (HUS) and bloody diarrhea was reported in Germany due to infection with an unusual serotype (O104:H4) of Shiga toxin (Stx) producing E.coli (STEC). Contaminated sprouts were revealed to be the source of outbreak. [26] Compared to typical enterohemorrhagic E.coli (EHEC) O157:H7, the new pathogen has the characteristics of enteroaggregative E.coli (EAEC) O104:H4 which have acquired genomic elements necessary to produce Stx and extended-spectrum beta-lactamase. EAEC is a type of E.coli defined by the characteristic of a “stacked brick” pattern of adherence and colonization and is usually the cause of traveler’s diarrhea, watery diarrhea in children, and chronic diarrhea in HIV patients. [26]

The German outbreak was different compared to typical STEC outbreaks because it mainly affected adults (88%) [2] and women were represented excessively (68%).[27] This may be due to 1) the source of outbreak (salad sprouts) and the fact that women eat more vegetable or 2) the present of gender-specific biological factors that make women more susceptible to the new pathogen. The incubation period for this new strain was also longer (8 days vs. 3-5 days) with higher development of neurological complications in HUS patients. [26]
1.1.4 Shiga Toxins (Stxs)

Stxs, the key virulence factors in STEC, were first named Verotoxins (VTs) based on their cytotoxicity for Vero cells (African green monkey kidney epithelial cells). [4] Later O’Brien et al revealed that VTs are homologous to a cytotoxin produced by *Shigella dysenteriae*. This led to a change in the name of these toxins to Shiga toxins (Stx) or Shiga-like toxins (Slt). [28]

Stxs are a group of A/B5 protein toxins with two subunits: an enzymatically active A subunit (32 KDa) and a pentameric B subunit (7.7KDa) (Figure 1). [29] The B subunit is responsible for binding to the cell surface receptor, glycolipid globotriaosylceramide (Gb3), while the A subunit has N-glycosidase activity.[30] The A subunit of Stx consists of two parts: A1 fragment which has the enzymatic activity and A2 which interacts with the B subunit. [29]

There are two major groups of Stxs: Stx1 which is homologous to Shiga toxins, with identical B subunit amino acid sequences and an A subunit with only one amino acid residue different.[31] The second group is Stx2 that has 84% homology to Stx1 in the A subunit and 62% in the B subunit. [32, 33] Since STEC strains that express Stx2 are more associated with human disease [34, 35], Stx2 is considered more toxic than Stx1. [32, 33]
Figure 1. Shiga toxin structure. Stx is an A: B\textsubscript{5} bacterial exotoxin (holotoxin) consisting of a pentameric B subunit, responsible for binding to its receptor, GB3, and an enzymatically active A subunit.
1.1.5 Mechanisms of Stx-induced cellular injury

The A1 subunit of Stx inactivates ribosomes and inhibits protein synthesis by removing an adenine residue (A4324 in rats, A4565 in humans) from the 28S ribosomal RNA of the 60S subunit. [36, 37] Also, recent studies have raised the hypothesis that inhibition of protein synthesis is not the only function of Stx. It could also affect gene regulation at lower concentration with minimal effects on translation. [38] Stx also functions in a ribosomal independent manner by indirectly inducing DNA fragmentation. [39] In vitro studies have shown that Stx can also remove several adenine residues from DNA by functioning in the same manner as DNA glycosylases. [40] It has been proposed that Stx-induced cellular injury is accompanied by increased apoptosis and ultra-morphological changes. [41-43] Further, Jones et al reported that overexpression of pro-apoptotic homolog, Bcl-2-associated X protein (BAX), in Stx-treated epithelial cells led to apoptosis and caspase activation. [44]

1.1.6 Stx entry and routing

In order to enter the cell, Stx binds to its surface receptor, Gb3, through the B subunit. Once the toxin binds to Gb3; it is internalized by clathrin-coated vesicles [45] and is transported in a retrograde fashion through the Golgi apparatus to the endoplasmic reticulum (ER). It has been shown previously that Stx gets into some cells via clathrin-independent endocytosis, representing cell differences in Stx delivery. [46, 47] Inside the ER the A subunit is cleaved by an enzyme called furin. This results in the formation of the enzymatically active A1 fragment, which then is translocated across
the ER membrane (Figure 2). [48] Recently it was shown that Manganese (Mn$^{2+}$) blocks the intracellular trafficking of Stx. Mn$^{2+}$ induces degradation of cycling Golgi protein GPP130 that is interacting with the Stx B subunit and is required for its retrograde transport. GPP130 degradation leads to sorting of Stx into the lysosomes and protection against its toxicity. [49, 50] However, later in 2013 Gaston et al revealed that in a previous study Stx1 was used which is less toxic than Stx2 and that Mn$^{2+}$ failed to protect against Stx2 both in vivo and in vitro. [51] In their study they used more direct methods for assessing ribosome function and also a more sensitive model of Stx compared to the study by Mukhopadhyay et al. [44]
**Figure 2. Stx trafficking.** Stx is internalized after binding to its receptor Gb3 and reaches the golgi and ER in a retrograde manner. Within the ER the A subunit is activated by furin-dependent cleavage, thereby functioning as a translation inhibitor factor. [52]
1.1.7 Stx and endothelial gene regulation

Since Stx inhibits protein synthesis by blocking ribosome function, the mechanisms by which it induces the expression of genes that have important roles in Stx-pathobiology is of interest. It was always thought that Stx functions only by inhibiting protein synthesis; however, in 1998 Bitzan et al reported that Stx induced the mRNA transcript level of endothelin-1 (ET-1), a potent endothelial vasoconstrictor, at concentrations with minimal effects on de-novo proteins synthesis. [53] In order to find the mechanism for this induction, they performed nuclear transcription and actinomycin D experiments and found that Stx stabilized ET-1 mRNA transcript, which usually has a short half-life of less than an hour. [54]

Studies in our lab have shown that 3’- untranslated region (3’-UTR) of ET-1 mRNA is a potent regulator of its mRNA half-life and stability. [54] Using 3’-UTR deletion and mutagenesis studies, we identified a specific sequence, namely verotoxin response element, which had a role in Stx-induced mRNA stabilization. These studies confirmed that Stx can regulate ET-1 mRNA transcript at a posttranscriptional level and via Cis-regulatory elements. (Unpublished data)

Due to importance of endothelial dysfunction in HUS pathogenesis, many studies have further reported the Stx-induced changes in gene expression of cytokines, chemokines, adhesion molecules, and transcription factors in endothelial cells. [55-58] Recently, we have shown that Stx induces the mRNA levels of CXCR4/CXCR7/SDF-1, which have important function in angiogenesis and vascular remodeling. [38] Since CXCR4 mRNA has a short half-life; we decided to evaluate the effect of Stx on CXCR4 mRNA stability.
We found that Stx treatment resulted in an increased steady-state CXCR4 mRNA. We then determined the effect of Stx on RNA transcription by ChIP assay using anti-RNA polymerase II (Pol II) and interestingly, observed a higher association of RNA polymerase II with CXCR4 promoter was observed in Stx treated endothelial cells versus control. [38] Based on these observations, we concluded that the reason for the high expression of CXCR4 mRNA transcript due to Stx is because of the combination of both transcriptional and post-transcriptional mechanisms. [38] Since Stx is a potent ribosomal inhibitor, we next evaluated the effect of Stx on CXCR4 and SDF-1 mRNA translation. Polyribosome profiling indicated higher association of these transcripts with polyribosome fractions following Stx treatment. These studies suggest an increase in translation of these transcripts, despite the known role of Stx in inhibiting ribosomal function. [38]

In a different study, Page et al demonstrated the dysregulation of angiopoietin (Ang) in HUS patients and the importance of its dysregulation as a prognostic marker in children with E.coli O157:H7 infection. [59] Ang exists in two forms Ang-1 and -2 that are ligands of the endothelial Tie-2 receptor. They compete for binding to the Tie-2 receptor and regulate endothelial cell function. In this study, higher levels of Ang-2 were reported in children with HUS compared to healthy controls and correlated with endothelial dysfunction. High levels of Ang-2 compete with Ang-1 for binding to the Tie-2 receptor and sensitize endothelial cells to the effects of tumor necrosis factor (TNF) and vascular endothelial growth factor (VEGF) upregulation due to Stx. [60]

It is evident that Stx has regulatory effects on endothelial specific genes at very low concentrations with minor effects on ribosomes. The mechanisms by which Stx induces
gene expression seem to be a combination of translation, transcription, and post-transcription changes. (Figure 3)
Figure 3. Mechanisms of endothelial gene regulation by Stx. Stx affects the expression of selected mRNAs by different mechanisms, either up-regulating transcription and/or inducing mRNA stability. Stx also induces association of specific transcripts with polyribosomes. [25]
1.2 ATF3 in Endoplasmic Reticulum (ER) stress

1.2.1 ER stress

Endoplasmic Reticulum (ER) is a dynamic secretory compartment involved in protein synthesis, maturation and folding, lipid homeostasis, and calcium maintenance. [61] Proper folding of proteins within ER requires this organelle to be highly homeostatic through different cellular signals. Multiple stimuli such as oxidative stress, oxygen and nutrient shortage, and toxic agents can disrupt ER homeostasis. [62] This results in the accumulation of misfolded proteins, leading to a pathway named the unfolded protein response (UPR) or ER stress, also known to play key roles in different diseases such as, diabetes, cancer, inflammation, cardiovascular disorders, viral infection, and neurodegeneration. [63-69] The existence of the UPR pathway was first discovered by a study in which pharmacological disturbance of protein folding resulted in the activation of a subset of ER chaperones and changes in gene expression. [70] ER stress results in the activation of three stress sensors: PKR-like Endoplasmic Reticulum Kinase (PERK), Activating Transcription Factor 6 (ATF6), and Inositol Requiring Enzyme 1 (IRE1) [71] described below. Under stress conditions Binding immuglobulin Protein BiP, one of the highly abundant ER chaperones, gets released from these sensors, leading to their activation.

1.2.1.1 PERK
PERK is a transmembrane protein that has two domains: a regulatory domain within the ER lumen and a protein kinase domain in the cytoplasm, interacting with eukaryotic translation initiation factor 2 (eIF2α). Upon ER-stress and BiP dissociation from its regulatory domain PERK, it is activated via dimerization and trans-autophosphorylation. Activated PERK phosphorylates the α-subunit of eIF2α on Ser51, leading to a translational inhibition process, which results in a reduction of unfolded protein within the ER. Respectively, mRNAs with short open reading frames in their 5’-untranslated region (µORF) such as Activating Transcription Factor 4 (ATF4) are translated by phosphorylated eIF2α. ATF4 has a key role in the transcriptional regulation of a subset of pro-apoptotic and pro-survival genes including the transcription factor C/EBP-homologous protein (CHOP), growth arrest and DNA damage-inducible 34 (GADD34), and also activating transcription factor 3 (ATF3). Translational restoration is achieved by GADD34 recruiting protein phosphatase-1 (PP1) in order to dephosphorylate eIF2α.

1.2.1.2 IRE1

IRE1 is a ubiquitously expressed transmembrane glycoprotein with a cytoplasmic domain containing both endoribonuclease and kinase functions. In response to ER stress, dimerization, oligomerization and trans-autophosphorylation of IRE1 lead to its activation. Respectively, IRE1 excises a 26-nucleotide intron of the X-box binding protein (XBP1) RNA precursor through its endoribonucleolytic activity. This splicing leads to the formation of a mature XBP1 mRNA with a new open reading frame, which
is more stable. [73] XBP1 protein then translocates to the nucleus to induce multiple ER stress response genes. [74] In order to regulate gene expression, IRE1 also interacts with some of the adaptor proteins and regulators such as protein TNFR-Associated Factor-2 (TRAF2), which results in the activation of apoptosis signal-regulating kinase 1 (ASK1) and c-Jun N terminal kinase (JNK). [75] The last mechanism of function of IRE1 is through a process called Regulated IRE1 Dependent Decay (RIDD) in which subsets of mRNAs are degraded by IRE1 endoribonuclease activity.

1.2.1.3 ATF6

ATF6 belongs to a family of basic leucine zipper (bZIP) transcription factors including ATF6α, ATF6β, LUMNA (known as CREB3), cyclic AMP-responsive element-binding protein hepatocyte (CREBH, also known as CREB3L3), old astrocyte specifically induced substance (OASIS), BBF2 human homologue on chromosome 7 (BBF2H7), and CREB4. [76] Upon ER stress BiP dissociates from ATF6 and this results in the translocation of ATF6 to the Golgi. Within the Golgi site-1 (S1P) and site-2 (S2P) proteases cleave ATF6 at its transmembrane domain, which has a specific cleavage site for S1P and S2P. [77] The N-terminal cytosolic fragment of ATF6 (ATF6c, 50KDa) [73] is a transcription factor that translocates to the nucleus and regulates the expression of a subset of genes responsible for ER stress, such as genes involved in ERAD pathway, where it also regulates XBP1 mRNA levels. [78, 79]
Each of these three pathways results in adaptation to stress stimuli and cellular reprogramming. Under conditions in which ER stress cannot be alleviated, pro-apoptotic signaling pathways become activated to control cell fate and inducing cell death. [71]

1.2.2 Stx and ER stress

Since Stx functions by depurinating 28S rRNA and inhibiting protein synthesis, this results in changes within the structure of ribosomes and rRNAs and also in the production and accumulation of misfolded proteins, which all lead to the activation of ER stress. During Stx translocation, Stx A fragment binds to ER chaperone proteins such as BiP, supporting the idea that there would be a conformational change in the Stx A1 fragment that also promotes the activation of ER stress pathways. [80, 81]

There is little known about the role of Stx in ER stress in a variety of cell types. Lee et al have shown that a human monocytic cell line (undifferentiated, THP-1) was sensitive to Stx and treatment of THP-1 with Stx led to the activation of all three ER stress sensors. [82] In that study, CHOP was highly induced following Stx1 treatment, which regulated the expression of death receptor 5 (DR5, also known as TRAIL-R2) and Bcl-2 an anti-apoptotic protein. The authors also showed that the Stx B subunit alone as well as the enzymatically inactive A subunit had no effect on the activity of ER stress sensors. [82] Additionally, gene expression profiling in human brain microvascular endothelial cells (HBMVEC) treated with Stx2 revealed induction of stress response genes such as, PERK, CHOP and ATF4 [83] It is been shown that Stx1 and Stx2 differentially regulate stress response pathways in human renal tubule epithelial cell line HK-2. [84] Here, the
authors reported that Stx1 mostly activated ATF6, while Stx2 was more effective at phosphorylating PERK and IRE1.

Prolonged activation of ER stress by Stx may result in apoptosis. Thus, characterizing Stx-induced stress pathways will help us to better identify therapeutic targets and to prevent disease progression.

1.2.3 Activating transcription factor 3 (ATF3)

1.2.3.1 ATF3 cloning

The family of activating transcription factor (ATF)/cAMP responsive element binding (CREB) proteins were first discovered in 1987 by Lee et al based on their ability to bind to specific sequences within DNA. [85] The ATF consensus sequence (TGACGT(C/A)(G/A)), was identified by the ability of ATF(s) to bind to identical sequences on viral promoters such as, adenovirus early promoters E2, E3 and E4 as well as CRE on the somatostatin promoter. [86] Screening of λgt11 expression library, using a DNA probe containing three tandem ATF/CRE sites, resulted in the identification of eight different cDNA clones, ATF-1 through ATF-8. [87] ATF-1 through ATF-6 proteins was further analyzed and it was revealed that they all have a homologous basic region-leucine zipper (bZip) motif. [87] This sequence is required for the ability of these groups of proteins to bind DNA as well as the ability to form heterodimers and homodimers. There are more than 20 different mammalian ATF/CREB cDNA clones that can be sub-grouped based on the amino acid similarity in
the bZip region. Members of each group share similarity both inside and outside the bZip motif, while proteins of different groups share similarity only within this domain. [88] In 1989, activating transcription factor 3 (ATF3) was first isolated from Hela cells induced by tetradecanoylphorbol acetate (TPA). [87] This partial cDNA clone was later resulted in the isolation of the full-length human ATF3 cDNA clone encoding a protein of 22 kDa. [89] The human ATF3 gene is mapped to chromosome 1q32.3, consisting of five exons (namely A, B, C, D, and E) spanning approximately 16 kb. [85] Exon A encodes the 5'-untranslated region (5'-UTR). Exon B contains the start codon, AUG, and encodes the N-terminal 80 amino acids. Exon C mostly encodes the basic region. Exon D encodes an in-frame stop codon leading to the formation of a truncated ATF3 protein that has no leucine zipper domain and no C-terminal tail. Exon E encodes the leucine zipper domain, the 3'-untranslated region, and the C-terminal 30 amino acids. [90] (Figure 4A)

Analysis of the ATF3 promoter revealed multiple transcription factor binding sites such as ATF/CRE, AP-1, NF-κB, and also some that are implicated in cell cycle regulation including Myc/Max, E2F, and p53 binding sites. [90] An alternate promoter of ATF3 was identified as P1, which is located 43.5 kb upstream of the canonical P2 promoter in human. P1 is highly conserved among human and mouse and has several transcription factor binding motifs for ATF/CRE, AP-1, NF-κB, E2F, and P53. The novel P1 promoter of the ATF3 gene is activated at transcriptional level but in a different manner compared to P2. The P1 promoter is constitutively active in human cancer cells such as LNcaP prostate and Hodgkin cancer cells, and its chromatin structure is modified to an active configuration. [91]
1.2.3.2 ATF3 isoforms

Alternative splicing results in the formation of several isoforms of ATF3, which include ATF3ΔZip [92] ATF3ΔZip2a, ATF3ΔZip2b [93], ATF3ΔZip2c, ATF3ΔZip3 [94], and ATF3b. [95] ATF3ΔZip lacks the leucine zipper region and cannot bind DNA. The role of this isoform is to antagonize ATF3 transcriptional activity probably by sequestering co-factors away from the promoters. [92] ATF3ΔZip2a and ATF3ΔZip2b isoforms occur due to an alternative 3’ splice acceptor site upstream of exon D and they both have a truncated leucine zipper region and are incapable of binding the ATF/CREB site in DNA. [93, 96] ATF3ΔZip2c is the result of alternative splicing within exon B and lacks 29 amino acids near the N-terminal sequence. [94] In ATF3ΔZip3 isoform, exon C and D are not spliced and this results in addition of extra sequence, termed C’, giving rise to a truncated leucine zipper region. [94] ATF3b occurs due to partial deletion within exon B leading to an early in-frame stop codon. ATF3b protein lacks the N-terminal 57 amino acids due to translational initiation from an alternate in-frame start codon. In contrast to other isoforms, ATF3b contains a bZip domain, can bind to DNA and is known to function in a similar way as full-length ATF3. [95] (Figure 4B)
Figure 4. ATF3 gene and protein isoforms. A) The structure of the ATF3 gene and its splice variants. Boxes represent exons A-E. Star codon and all the stop codons are
shown on top of each boxes. B) The protein structure for each of the ATF3 mRNA variants is shown with each boxes represent a specific functional domain. (For more details about exon nomenclature refer to ref. [94, 97])
1.2.3.3 ATF3 function

Within normal conditions ATF3 steady state mRNA and protein levels are low and are rapidly induced in response to a variety of stimuli both in vivo and in vitro, usually within hours. In vivo, it has been reported that ATF3 is induced in the liver by acetaminophen, cycloheximide, and carbon tetrachloride, in heart by ischemia and ischemia coupled with reperfusion, [89] in brain by seizure, and in vitro by stress signals such as UV light, serum, ionizing radiation (IR), and proteasome inhibitors. [98] ATF3 induction is not stimulus specific and the response is the same in all tissues or cell types. [99] ATF3 was first known to be a stress-inducible gene [100]; however, since most of the stimuli such as MCF-7 breast cancer cells exposed to adipokines [101] and G1 to S transition are not among stress signals, the term adaptive-response gene was suggested to better describe ATF3. [99]

ATF3 functions by binding to an ATF/CREB consensus on the promoter of its target genes. In contrast to its name, Activating Transcription Factor 3 mainly functions by repressing the expression of its downstream targets as a homodimer. However, it has been shown that ATF3 forms heterodimers with Jun proteins, leading to transcriptional activation. (For a list of genes that are regulated by ATF3 refer to Table 1). [102] Since ATF3 has an ATF/CREB site within its promoter and is considered a transcriptional repressor; the proposed mechanism for its transient expression is by binding to its promoter and regulating its expression by autorepression. [103] ATF3 has been demonstrated to have dual functions in a variety of cellular processes including growth arrest and apoptosis versus inducing proliferation and cell survival. [91] This diversity in ATF3 functions is probably cellular context dependent and might be due to the presence of other factors and proteins within a given cell type interacting with ATF3.
<table>
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<tr>
<th>Target gene</th>
<th>Transcriptional regulation</th>
<th>Ref</th>
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<tr>
<td>IL6, IL12b</td>
<td>Repression</td>
<td>[104]</td>
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<tr>
<td>Id-1</td>
<td>Repression</td>
<td>[105-107]</td>
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<tr>
<td>Gadd153/CHOP10</td>
<td>Repression</td>
<td>[108]</td>
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<td>P53</td>
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<td>ATF3</td>
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<td>[103]</td>
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<td>Egr1</td>
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<td>MMP9</td>
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<td>HIF-2α</td>
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<td>Hsp27</td>
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<td>MMP13</td>
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<tr>
<td>SDF-1α</td>
<td>Induction</td>
<td>[116]</td>
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_Table1. A subset of genes that are regulated by ATF3._
1.2.3.4 ATF3 in diseases

Since ATF3 functions in response to changes in cellular homeostasis and regulates gene expression, one would expect that it might have an important role in the development of disease. ATF3 over-expression was reported to down regulate p53 expression in human umbilical vein endothelia cells (HUVECs) and cardiac myocytes, protecting them from TNFα and doxorubicin-induced apoptosis, respectively. [109, 117] In diabetes, ATF3 overexpression was reported to suppress cell growth and induce pathological angiogenesis in NP31 endothelial cells. Among those genes that are thought to be involved in diabetic angiopathy integrinα1 subunit, p8, PAI-1, and metalloprotease 13 (MMP13) demonstrated ATF3-dependent up regulation. [118]

In another study, Akram et al demonstrated that ATF3 protects against ventilator-induced lung injury. [119] Acute respiratory distress syndrome (ARDS) is a serious form of acute lung injury (ALI). ALI results in the release of inflammatory mediators and chemokines, oxidative stress, leaky vessels, and apoptosis. [120] The need for mechanical ventilation (MV) exposes patients to higher risk of lung injury due to repetitive cyclic stretch (CS) and over-inflation. [120, 121] Some pathogens like lipopolysaccharide (LPS) or even infectious diseases such as, sepsis and pneumonia induce pulmonary inflammation and increase the risk of ARDS/ALI. [122] [123] ATF3 also has a critical role in innate immune disorders. During the immune response, foreign agents are recognized by Toll-like receptors (TLRs), leading to the activation of macrophages and secretion of cytokines such as IL-6 and IL-12b. [104] Endotoxin (LPS) is an agonist of TLR4 that has been shown to induce an immune response and ATF3 expression. ATF3 was reported to negatively regulate levels of IL-6 and IL-12b by binding to ATF/CREB consensus sequences within their promoters. Additionally, in
ATF3 knockout mice, circulating LP-activated levels of IL-6 and IL-12b were increased more than 10-fold compared to wild type animals. [104] This highlights the important role of ATF3 in regulating TLR4- stimulated inflammation and protection against LPS-induced inflammatory cytokines in vivo. ATF3 was reported to be involved in a variety of cancers such as, colorectal cancer [124], prostate cancer [125, 126], and breast cancer [127, 128], functioning as an oncogene and tumor suppressor. The conflicting functions of ATF3 in cancer cells are probably dependent on cancer type and stage.

1.3 Regulation of gene expression by microRNAs

1.3.1 Mechanisms of gene regulation

Regulation of gene products at both the transcriptional and post-transcriptional levels is of importance since mis-regulation could result in disease progression. Transcription is the first step in gene expression that is highly regulated and determines the rate of RNA that is being produced. However, there are also an increasing number of studies focusing on the importance of post-transcriptional gene regulation. These include mechanisms functioning on both mRNA and protein such as, mRNA processing, mRNA localization, translation, and decay. All of these mechanisms are required for maintenance of mRNA stability and translation. [129]

1.3.1.1 mRNA processing

Within the nucleus, precursor mRNAs (pre-mRNAs) are transcribed by RNA polymerase II before transportation to the cytoplasm where they undergo some
processing steps that happen co-transcriptionally. These steps include 5’-capping, 3’-end cleavage and polyadenylation, and splicing. [129]

The first nucleotide of pre-mRNA at the 5’-end is covalently modified by the addition of 7-methylated guanosine triphosphate (m7G, also known as 5’ cap) by 5’-5’ linkages. The 5’ cap has an important role in mRNA stability and in preventing exonucleolytic degradation. In the nucleus, a complex of proteins called cap binding complex bind m7G, which consists of two subunits and protects mRNA from decapping. [130]

Polyadenylation at the 3’ end of the pre-mRNA occurs via two steps: 3’ end cleavage and the addition of a poly (A) tail. [130] The 3’-end processing requires a multi-protein polyadenylation complex that cleaves pre-mRNA at a specific site between an AAUAAA hexamer and a GU-rich downstream element (DSE). A variety of protein factors are involved in pre-mRNA cleavage such as the cleavage and polyadenylation specificity factor (CPSF) that binds to upstream signaling region, AAUAAA, and the cleavage stimulatory factor (CstF), which binds to the DSE. In addition, there are some other factors that have important roles in pre-mRNA cleavage and poly (A) addition such as cleavage factor I and II (CF I, CF II) and poly (A) polymerase (PAP). [131-133] Poly (A) binding proteins (PABPs) bind to the poly (A) tail and are required for mRNA nuclear export and mRNA stability by affecting the length of the poly (A) tail. [127-[134] PABPs also protect mRNA from nuclease cleavage since it has been shown that mRNAs for β-globin, chloramphenicol acetyltransferase, and simian virus 40 virion proteins were degraded faster in PABP depleted extracts compared to those with high levels of PABP. [135] The initial length of the poly (A) tail is approximately 250 nucleotides (nts) in human and 90 nt in yeast; however, it becomes progressively shortened over time, and has likely to be 50-100 nt by the time the mature mRNA reaches the cytoplasm. [136]
The last step in nuclear processing is splicing, in which introns that interrupt exons in pre-mRNA are spliced out in order to generate functional mRNA. This process requires a catalytic multi-protein complex known as a spliceosome, consisting of five small ribonucleoprotein particles (snRNPs): U1, U2, U4, U5 and U6. [137] Moreover, different patterns of splicing by alternative use of exons (alternative splicing) can produce multiple isoforms and proteins with different functions and sizes. [138]

1.3.1.2 mRNA decay

A variety of pathways are involved in mRNA degradation. Initially, mRNA degradation involves 3’-5’ exonucleolytic deadenylation to partially or completely remove the poly (A) tail. [129] Following deadenylation, mRNA is degraded by two mechanisms: either 5’-3’ decapping via exoribonuclease or 3’-5’ decay by a complex of exonucleases known as an exosome. [139] Some mRNAs are also degraded via deadenylation- independent mechanisms such as, endoribonucleolytic decay. In this process mRNA is cleaved internally which leads to the production of two fragments with unprotected ends exposed to exonucleases. Studies have identified some of these endonucleases including polysomal ribonuclease 1 (PMR1), IRE1, and short interfering RNA (siRNA). [139] PMR1 and IRE1 both bind to actively translating mRNAs and endonuclease activity of IRE1 has been shown in response to ER stress via splicing of XBP1 mRNA. [79]

The other pathway for mRNA decay is known as Nonsense-mediated decay (NMD). It functions as a quality control mechanism to identify mRNAs with a premature termination codon (PTC). These truncated mRNAs can result from frame-shift mutations, inefficient processing and extended 3’UTR that leads to the production of
aberrant proteins. [139-141] There are three main trans-acting proteins functioning in NMD including up-frameshift 1 (UPF1), UPF2, UPF3. [142] During splicing in eukaryotic cells, spliceosomes deposit a complex of proteins, known as exon junction complex (EJC) along with UPF2 and UPF3, 20-24 bases upstream of an exon-exon junction. [143] During translation, ribosomes are stalled when encountering PTC, following which the SURF complex (including SMG1, UPF1, and peptide-release factors) bind the stalled ribosome. EJC also recruits UPF1 to bind to UPF2 and that leads to the formation of a bridge between EJC and PTC. Consequently, formation of this complex leads to mRNA degradation via an uncertain mechanism. [144, 145] Positioning of EJC downstream of PTC is not the only mechanism for triggering NMD since it was discovered that D. melanogaster and S. cerevisiae mRNAs with long 3'-UTRs are also subjected to NMD. [146] An extended 3’ UTR increases the distance between PTC and the poly (A) tail. Since translation termination occurs far from the 3’-end, this inhibits normal interaction between ribosomes and PABP leading to NMD. [144, 146-148]

1.3.1.3 mRNA stability

Changes in gene expression mostly occur at the level of mRNA stability. [144] Commonly, assays that are used to measure mRNA stability and half –life involve transcription inhibition using drugs such as, Actinomycin D, or by mutating RNA pol II. [149] mRNA decay rates vary between different transcripts and depend on the function of their products. mRNAs encoding proteins that function as transcription factors, or that are involved in ribosome biogenesis, translation, cell cycle, development and differentiation usually have a short half-life while transcripts involved in metabolic pathways have long-lived mRNA. [150-152]
mRNA stability is determined by cis-regulatory sequences that are bound by different trans-factors. These factors include RNA binding proteins (RBPs) such as, AU-rich binding proteins and small RNAs including microRNAs and small interfering RNAs (siRNAs). Most of the cognate cis-regulatory response elements are located within the 3'-untranslated region (UTR) and few of them within the 5'-UTR. [129, 153, 154]

1.3.1.4 AU-rich binding proteins

AU-rich elements (AREs) are within the 3’-UTR and highly affect the steady-state level and translation of mRNA via binding of ARE-binding proteins. First, it was discovered that the AUUUA motif had a destabilizing role even though AREs with only this motif are not completely functional. [151, 155] Further, it was revealed that at least the presence of UUAUUUUUUU or UUAUUUA (U/A)(U/A) is of essence for binding of destabilizing factors and mRNA degradation [156, 157] Three different types of ARE-binding proteins have been well identified so far including, the ARE-/poly (U)-binding degradation factor (AUF1), tristetraproline (TTP), and human antigen R (HuR). [129] AUF1 was first isolated by an in vitro mRNA decay system that bound to c-myc mRNA and led to its degradation. [158] Also, AUF1 binds to a variety of AREs and mostly functions as an mRNA destabilizing factor, probably by interacting with components of the proteasome pathway. [159]

Tristetraproline (TTP) is a zinc finger protein with two copies of a Cys-Cys-Cys-His (CCCH) zinc finger domain. [160] In vivo studies have shown that TTP destabilizes TNF-α mRNA by binding to AREs, since TTP-/− mice exhibited an elevated level of TNF-α leading to an inflammatory syndrome with arthritis, cachexia, and autoimmunity. [161, 162] It also binds IL-2, IL-3 and c-fos mRNAs and promotes their decay. [161, 163-165]
HuR (Human antigen R) is a member of the embryonic lethal abnormal vision (ELAV) family of RNA binding proteins in Drosophila. [166] [167] HuR binds to the ARE of a variety of mRNAs and stabilizes those mRNA by competing with other destabilizing factors such as, AUF1 and TTP. [168, 169] It is mostly localized in the nucleus and is translocated to the cytoplasm by different stimuli including heat shock, UV irradiation, and actinomycin D. [170-172] Studies have shown increased interaction of HuR with AU-rich elements within the 3’-UTR of ATF3 mRNA and cationic amino acid transporter-1 (Cat-1) mRNA during amino acid starvation and ER stress. This interaction lead to increased mRNA stability of those genes. [173, 174] HuR may also function in translational control and translation re-initiation of repressed mRNAs since it associates with polysomes and also results in relocation of mRNAs from P bodies to polysomes. [129, 175] Thus, HuR stabilizes various transcripts either by competing with destabilizing factors, including deadenylases and endonuclease, or by displacing them from site of decay. [139, 176]

1.3.1.5 RNA interference

The discovery of RNA interference (RNAi) by Andrew Fire and Craig Mello (awarded the Nobel Prize in 2006) in C.elegans revealed a new mechanism for gene regulation and sequence specific gene silencing. In their research they found that injection of either sense or anti-sense mRNA strands against a specific endogenous gene in C. elegans elicited no response while injection of double stranded RNA (dsRNA) was more effective in interfering with gene expression. [177] Recent studies have focused on the significance of small (~ 20-30 nucleotide) non-coding RNAs as important regulators of gene expression. Since these molecules generally function as inhibitory regulators, they
are identified as a part of an RNA interference (RNAi) pathway. [178] There are two main categories of small RNAs: short interfering RNAs (siRNAs) and microRNAs (miRNAs).

In 1993 Ambros et al discovered the first small RNA, lin-4 in C.elegans, which controls the timing of larval development. [179] Studies thereafter discovered a great number of microRNAs in other organisms including animals, plants, and viruses being involved in a variety of cellular processes. [180, 181] Despite some similarities between siRNA and microRNA biogenesis and mode of action, there are also significant differences between their cellular origins and double-stranded RNA (dsRNA) precursors. First, microRNAs are derived from the endogenous genome while siRNAs are considered as either endogenous or exogenous origins such as, viruses or transposons. [182] [178] Second, microRNAs are processed from RNA hairpin structures with mismatches, while siRNAs are processed either from long bimolecular duplexes or from synthetic short hairpin RNA (shRNA). [178, 182] RNAi functions by being incorporated into a multi protein complex, known as RNA inducing silencing complex (RISC), a main component of which is Argonaute 2 (Ago2) protein, which binds to its target and leads to target mRNA degradation. [182]

As RNAi technology advances as a gene-silencing tool and in therapeutic approaches, there are still some barriers to the efficient use of RNAi especially in vivo. [183] In a study by Grimm et al, viral vectors were used as a means of shRNA delivery in the liver of mice. The authors found that 36 out of 49 constructs resulted in dose-dependent tissue injury and fatality and this was reported to be due to competition between shRNA and endogenous microRNAs in using the same nuclear-export pathway, and via exportin-5. [184]
1.3.2 microRNAs and their biogenesis

Since discovery of lin-4 in 1993, microRNAs and their role in regulating gene expression have become the main focus of most studies. microRNAs are short, single-stranded non-coding RNAs, \( \sim 22 \) nt in length, that are derived from stem-loop hairpin precursors. microRNAs function by Watson-Crick base pairing to the 3'-untranslated region (3'-UTR) of their target and repress expression by either translational inhibition or mRNA degradation. Studies have shown that microRNAs can also bind to other regions of mRNA such as protein coding regions and 5'-UTR. [185, 186] Studies have predicted that over 60% of human coding genes are targeted by microRNAs. [187]

Within the nucleus microRNAs are transcribed, usually by RNA polymerase II (Pol II), as primary microRNAs (pri-microRNAs), consisting of one or multiple hairpins. pri-microRNA is further trimmed and cropped by a microprocessor complex. This multiprotein complex consists of Drosha, a key RNase III enzyme, and DiGeorge syndrome critical gene 8 (DGCR8) that has two double-stranded RNA binding domains (dsRBD). [180] DGCR8 binds pri-microRNA and is required for proper positioning of Drosha and its endonucleolytic cleavage activity. Drosha cleavage results in the production of a \( \sim 65- \) to 70 nt precursor microRNA (pre-microRNA) with 5' phosphate and 2 nt 3' overhang. [182] The nuclear export of pre-microRNA is then mediated via Exportin-5, a member of the nuclear transport receptor family. Exportin -5 binds to its cofactor RanGTP and leads to pre-microRNA transport by hydrolyzing GTP in the cytoplasm. After its transport, pre-microRNA is further processed by an RNase III enzyme called Dicer. This enzyme plays a key role in processing both siRNAs and mature microRNAs. It recognizes the double-stranded region of pre-microRNA and cleaves the stem loop off to form microRNA:microRNA* duplex. Dicer also helps in
loading the microRNA duplex into RISC. The microRNA duplex contains mature microRNA (guide strand) with a less stable base-paired 5’ end and microRNA* (passenger strand) with more stable 5’ end. The passenger strand is usually targeted for degradation. [180, 182] The guide strand microRNA, with the help of RISC, exerts its functions, which are mRNA degradation and/or translational inhibition. (Figure 5)

Compared to the canonical biogenesis pathway, there are other classes of microRNAs that are processed in a Drosha-independent manner, such as miRtrons, tRNAZ, and small nucleolar RNA (snoRNA). [178, 188-191] Further, Cifuentes et al have identified a Dicer-independent pathway for the processing of a blood specific microRNA, miR-451 that was dependent on Ago2 catalytic activity. [192]

Another class of microRNAs that are of recent interest includes circulating microRNAs in extracellular body fluids, namely serum, plasma, and urine. The levels of these microRNAs can change in a variety of pathological conditions and are considered as promising biomarkers. [193, 194] They are proposed to be released via three different routes: 1) passive leakage from broken cells such as fractionated platelets, 2) active secretion that requires energy and is either via shedding vesicles or 3) via RNA binding proteins including Ago2, Nucleophosmin 1 (NPM1), or even in association with high density lipoprotein (HDL). [195, 196]

Recently, Lorenzen et al analyzed the circulating microRNA levels in the plasma of patients infected with STEC O104:H4. A global microRNA expression analysis revealed deregulation in the level of circulatory microRNAs in HUS patients compared to healthy controls. The level of two endothelial-enriched microRNAs, namely miR-126 and miR-24, was validated. Further the correlation between the level of these microRNAs, neurological symptoms, and platelet count was evaluated. These data provided
evidence for the possible role of altered microRNAs in the pathogenesis of Stx-mediated HUS and endothelial injury, the level of which could be used as prognostic and therapeutic biomarkers. [197]

Since microRNAs control key aspects of cell biology, identification of the mechanisms that regulate their levels is of importance. Currently, it is known that microRNAs can be regulated at both transcriptional as well as post-transcriptional levels. [198, 199] Similar to protein coding genes, RNA polymerase II (RNA pol II) also has an important role in the transcription of most of the microRNAs. Many RNA pol II associated transcription factors can regulate the level of microRNAs in a tissue and developmental specific manner. [200] For instance, it is known that myogenin and myoblast determination 1(MYOD1) activate the transcription of miR-1 and miR-133 during myogenesis. [198, 199, 201] In addition, the tumor suppressor p53 and proto-oncogene c-Myc induce miR-34 and the cluster of miR-17-92, respectively. [202-204] Epigenetic control of microRNAs by histone modification and DNA methylation is another key concept in the transcriptional control of these small RNA molecules. [205, 206]

Post-transcriptional regulation of microRNAs can occur at different stages of their biogenesis. The proper positioning of Drosha cleavage affects microRNA processing and stability. There are different co-factors associated with Drosha that regulate the accumulation of microRNAs either negatively or positively. Some of these accessory proteins include, DGCR8, Dead-box RNA helicase p69 and p72 [207, 208], and Smads. [209] There are also a subset of RNA binding proteins such as, KH-type splicing regulatory protein (KSRP) [210], Lin-28 [211-214], and heterogeneous nuclear ribonucleoprotein A1(hnRNP A1) [212], which affect Drosha and Dicer processing of microRNAs. There are also other factors such as, single nucleotide polymorphisms
(SNPs), point mutations [206], and even the concentration of mRNA targets [211] that affect microRNA processing and their stability.
**Figure 5. microRNA biogenesis pathway.** microRNAs are transcribed as pri-microRNAs which are then processed by Drosha to form pre-microRNAs. Pre-microRNAs are then transported to the cytoplasm via Exportin-5. Dicer will further process pre-microRNAs to form mature microRNAs and also help load them into RISC. RISC-associated microRNAs will then function by either inhibiting translation or inducing mRNA degradation.
1.3.3 Role of Dicer in endothelial cells

After being translocated into the cytoplasm, Dicer, a key RNase type III enzyme, involved in the generation of mature microRNAs, further processes microRNAs. This evolutionarily conserved enzyme is composed of the following domains: the DEXH-box helicase, DUF283, double-stranded RNA binding domain (dsRBD), a PAZ domain, and two RNase III domains. [214] Dicer also helps in the incorporation of microRNAs into RISC, consisting of Ago2, Dicer, and TAR RNA binding protein (TRBP). Recently, Sinkkonen et al proposed a novel role for Dicer in the maintenance of the integrity of ribosomal DNA repeats. [215] Analysis of Dicer mRNA species identified diversity only within the 5’-UTR and not the open reading frame (ORF). [216] Other alternatively spliced leader exons were also identified that indicated a tissue-specific distribution. In vitro studies have shown that these diversities can affect the translational efficiency of Dicer mRNA. [216] Given the important role of Dicer in microRNA biogenesis and even development, the maintenance of its activity at normal levels is crucial. Dicer deficiency was reported to cause lethality in mice at embryonic day 7.5 (E7.5) as a result of pluripotent stem cell depletion. [217] In a different study it has been shown that mice with hypomorphic expression of Dicer died between embryonic days 12.5 and 14.5 due to defects in blood formation. [218] In addition, Dicer knockout resulted in embryonic lethality in zebrafish with phenotypic defects observed in gastrulation, brain morphogenesis, and cardiac development. [219] Inactivation of Dicer and its importance in a tissue-specific manner was further studied in a variety of tissues such as germ cells, neurons, skeletal muscles, T-cell development, and cardiac function and development. [220] Selective ablation of Dicer in mouse podocytes resulted in defects in glomeruli including podocyte foot
process effacement, glomerulosclerosis, disruption of glomerular basement membrane, apoptosis, and proteinuria, which further progressed to end-stage kidney disease and lethality after 6-8 weeks. [221-223]

Dicer knockdown has significant consequences on endothelial angiogenesis as well. In vitro studies have shown that conditional endothelial depletion of Dicer led to a reduction in tube formation, capillary sprouting, and migration of endothelial cells. [220, 224-226] Altered expression of key regulators of endothelial biology such as Tie-2/TEK, vascular endothelial growth factor receptor 2 (VEGFR2), endothelial nitric oxide synthase (eNOS), interleukin-8, and angiopoietin-like 4 (ANGPTL4) was also observed in Dicer knockout endothelial cells. [220, 225]

Low Dicer expression was also reported in a variety of human cancers including ovary, lung, and breast, which correlates with cancer progression and poor survival. [227-231] Dicer also functions as a haploinsufficient tumor suppressor gene. Studies in mouse models of cancer have shown that monoallelic loss of Dicer increases tumor progression while biallelic loss of Dicer inhibits tumorigenesis. [232, 233] Due to these results, it is important to identify factors that affect Dicer levels in both health and disease. Dicer expression has been reported to be regulated with its associated proteins such TRBP and Ago2. [234] In addition, studies have shown that Dicer expression can be regulated differentially in response to different stimuli including, reactive oxygen species, interferons, hypoxia [235], serum withdrawal, and by Von-Hippel-Lindau (VHL) protein in clear cell renal cell carcinoma (CCRCC). [206, 236, 237] Dicer may also be regulated by a negative feedback loop via microRNA let-7. [238] It has been shown previously that cell density affects Hippo pathway effector molecules localization, known as transcriptional co-activator with PDZ-binding motif (TAZ) and
Yes-associated protein (YAP), which could regulate dicer at post-transcriptional level through let-7. [239] Furthermore, a recent study has identified the interaction of Dicer with cytoskeleton-linking endoplasmic reticulum (ER) membrane protein of 63 kDa (CLIMP-63), which affects the stability of Dicer. [240] Bennasser et al also identified another novel post-transcriptional regulatory mechanism for Dicer mRNA expression. Their results identified an interaction between Dicer mRNA and Exportin-5, which was further out competed by over expressing pre-microRNAs and viral RNAs and led to down-regulation of Dicer expression. [241] A comprehensive understanding of the mechanism that regulate Dicer expression and function will further elucidate the role of this enzyme in both normal and pathological conditions.

1.3.4 microRNAs in ER stress

The importance of microRNAs in ER stress has become of interest to researchers since the discovery of localization of Ago2-associated microRNAs in stress granules and P-bodies upon ER stress. [242, 243] Specifically localization of Ago2 protein within the stress granules was reported to be microRNA dependent. Stress granules are cytoplasmic aggregates harboring stalled translational mRNA/protein complexes and in this case miRNA targeted mRNAs under stress conditions. [244] Differential expression of microRNAs due to ER stress was reported in many studies and in a variety of cell types including HEK293T cells [245], mouse embryonic fibroblasts [246], and human airway epithelial cells (Calu-3) [247]. microRNAs can target different effector molecules of the ER stress pathway. For instance, down regulation of miR-30d, miR-181a, and miR-199–5p, which target Bip expression, was shown in prostate, colon, and bladder cancers. [248] In addition, it was shown that miR-214 targets ATF4 and XBP1, a
mechanism of which is not fully understood. [249, 250] These data demonstrate that microRNAs might have dual roles as either effectors or regulators of ER stress with differential effects on cellular function in both health and disease. [71]

1.4 Rationale

Because of the shared effect of Stx on mRNA stability and translational efficiency of target mRNAs in endothelial cells we are motivated to define whether Stx has a global effect on post-transcriptional gene regulatory pathways. Also, because of the role of Dicer in microRNA processing it is considered as a critical regulator of post-transcriptional gene silencing. Increases and decreases in Dicer expression have broad functional effects on cellular phenotype. For example, knockdown of Dicer in mature endothelial cells is associated with altered levels of several key regulators of endothelial biology and angiogenesis, such as VEGF [217, 218]. These findings suggest that functional deficiency of Dicer can regulate gene expression and function in endothelial cells through changes in the processing of microRNAs.

1.5 Hypothesis

Shiga toxin-mediated effects on post-transcriptional gene regulatory pathways in vascular endothelium are mediated via decreased Dicer function.

1.6 Specific Aims
Aim 1: To define the effect of Stx on Dicer protein and RNA levels in human microvascular endothelial cells. (HMVECs)

Aim 2: To evaluate the effect of Stx on microRNA expression in human microvascular endothelial cells (HMVECs).
Chapter 2

Role of microRNA and Dicer in Stx pathobiology
2.1 Introduction

Hemolytic Uremic Syndrome (HUS) is a serious disease and major concern for public health, and is characterized by non-immune hemolytic anemia, thrombocytopenia, and acute renal failure. [9] HUS is most common in children and mainly follows as a result of digestive infection by shiga toxin producing \textit{E.coli} (STEC), especially the O157:H7 strain. [23] However, recently other strains were reported to be responsible for HUS as well.

In May 2011 a huge outbreak of HUS and bloody diarrhea was reported in Germany due to infection with an unusual serotype of STEC (O104:H4). It mainly affected adults [2] and women [27] compared to the typical STEC serotype (O157:H7), which affects children. Analysis of this strain has shown that O104:H4 is an enteroaggregative \textit{E.coli} that had acquired genomic elements necessary to produce Stx. [3]

STEC produce a group of bacterial exotoxins, known as shiga toxins (Stxs). Stxs were first named verotoxins based on their cytotoxicity for Vero cells (African green monkey kidney epithelial cells) [251]. O’Brien et al have shown that VTs are similar to a cytotoxin produced by the Shigella dysenteriae serotype, therefore VTs became known as Shiga toxins or Shiga-like toxins (SLTs). [28] Stx has two subunits with specific functions: The A subunit with N-glycosidase activity that cleaves an adenine residue from 28S rRNA and a pentameric B subunit, binding to the Stx receptor, globotriaosylceramide (Gb3), following which the A subunit becomes internalized. [36] Vascular endothelium and endothelial cell activation plays an important role in Stx
pathobiology. Previously it has been shown that Endothelin-1 (ET-1) mRNA is stabilized by low concentrations of Stx, resulting in an increase in mRNA levels. [53] This suggests that post-transcriptional mechanisms regulate ET-1. There are different mechanisms to regulate mRNA half-life, one of which is the presence of cis-elements within the 3′-untranslated region. The 3′-UTR has a destabilizing effect on ET-1 expression. [54]

We recently comprehensively characterized the gene expression profile of human microvascular endothelial cells (HMVEC) after treatment with 10 fM Stx2, which produces about 10% inhibition of protein synthesis. [38] It is interesting that this extremely low concentration of Stx has a potent and reproducible effect on the phenotype of these microvascular endothelial cells. A variety of mRNA species were upregulated at these low concentrations of Stx. For example, Stx activation of HMVEC implicated a role for activation of CXCR4/CXCR7 and their shared cognate chemokine ligand, stromal cell-derived factor-1 (SDF-1). [38] Changes in gene expression were not observed with the B subunit of Stx alone and required a catalytically active A subunit. Like ET-1, CXCR4 has a short-lived mRNA. We found that Stx stabilizes CXCR4 mRNA levels through post-transcriptional mechanisms. [38]

MicroRNAs (miRNAs) are short noncoding RNAs that have been shown to regulate gene expression. Inside the nucleus these RNAs are transcribed as larger molecules as primary microRNAs (pri-microRNAs), which are then processed into hairpin RNAs by Drosha. Exportin-5 transports these pre-microRNAs to the cytoplasm where Dicer, an endoribonuclease, cleaves them. The mature microRNAs are then incorporated into the RNA induced silencing complex (RISC), which can regulate gene expression through translational inhibition and targeted mRNA degradation. MicroRNAs play critical roles in
the control of physiological and pathophysiological pathways. [252]

Dicer is a key endoribonuclease that processes pre-microRNAs into mature microRNAs and cleaves double-stranded RNAs into small interfering RNAs. [200] Dicer also aids in the incorporation of microRNAs into the RNA-induced silencing complex (RISC) [253], which silences gene expression via changes in target mRNA stability and/or translation. Recent studies in our lab have shown that chronic hypoxia impairs Dicer expression and activity, which results in changes in the global microRNA pathway. We have shown, for the first time, that there is an interaction between the Dicer-dependent microRNA pathway and the cellular hypoxia response pathway. [236]

Because of the effect of Stx on mRNA stability in endothelial cells we elected to assess the effect of Stx on post-transcriptional gene regulation via changes in microRNA biogenesis pathway. We identify in our in vitro model of human endothelial cells that Stx impairs Dicer expression with significant effects on microRNA biogenesis.

ER is the main organelle involved in both protein synthesis and secretion. The maintenance of homeostasis within this compartment is necessary for the proper folding of proteins. Stress stimuli can affect the folding capacity of ER, leading to the activation of ER stress sensors including IRE1, PERK, and ATF6. Phosphorylation of each of these sensors results in changes in gene expression and activation of a subset of transcription factors such as ATF3. [61, 71] ATF3 is an adaptive response transcription factor that is highly induced in response to stress signals and regulates the expression of a variety of genes. Here, we show that Stx induces ATF3 expression as well, and most of the significantly down-regulated microRNAs target that gene. We find that Stx results in the overexpression of ATF3 via increasing its protein and mRNA level as well as affecting its mRNA stability at a post-transcriptional level. We conclude that induction
of ATF3 is in part via microRNAs and most importantly in a Dicer-dependent manner, understanding the mechanism of which will help us to better identify therapeutic approaches to overcome HUS.
2.2 Methods

2.2.1 Toxin purification

Wild type Stx2 was purified as explained before. [38] Recombinant *E.coli* strains were cultured and used as a host for Stx2 expression. Holotoxin was extracted from cultures using lysozyme 10 µg/ml and colimycin 0.1 mg/ml in PBS in the presences of 5mM EDTA and PMSF 0.1 g/l (Sigma-Aldrich). Centrifugation was used for clearing extracts, which were further dialyzed against 50 mM acetate buffer pH 5. DEAE Sepharose Fast Flow (Amersham Biosciences) was applied for protein elution using a NaCl gradient 0-0.5 M in 50 mM acetate buffer. Fractions containing Stx2 were precipitated with 80% ammonium sulfate, followed by backwashes with 0.8 M ammonium sulfate in 50 mM acetate buffer pH 5, and diluted to 0.6 M ammonium sulfate in 50 mM acetate buffer pH 5. Fractions were loaded on Phenyl Sepharose HP (Amersham Bioscience) and Stx2 was eluted with a decreasing salt gradient, dialyzed against 50 mM ammonium bicarbonate, lyophilized for storage, and resuspended in PBS to remove remaining LPS.

2.2.2 Cell Culture

Human Dermal Neonatal microvascular endothelial cells (HdMVEC), isolated from different donors, were purchased from Lonza, Walkersville, MD, cultured at a density of 40,000 cells/ml on 0.2 % gelatin-coated plates, and were maintained in EGM-2MV medium (Lonza). Cells at passages 5 and 6 were used in these studies. Two days after reaching confluence, medium was replaced and monolayer cells were treated with 1000
fM Stx2 for 24 hour. Total RNA and protein were extracted using mirVana PARIS Kit (Ambion) according to the manufacturer’s protocol.

### 2.2.3 Protein synthesis assay

Metabolite incorporation assay was performed to determine the dose-dependent effect of Stx2 on global protein synthesis using L-[3, 4, 5-^3^H (N)] leucine (>140 Ci/mmol) (purchased from NEN-Dupont). Cells were cultured on gelatin-coated 24 well plates and treated with different concentrations of Stx2 for 24 hr. One hr before harvest, 1 µCi/ml of [^3^H] leucine was added to each well. Following this cells were washed with ice-cold PBS and incorporated radioactivity was precipitated using 15% ice-cold TCA for 20 minutes. Cells were then washed with water and solubilized with 0.1 M NaOH-0.1 % SDS. Radioactivity incorporation was determined using a scintillation counter. Each experiment was performed in triplicate and data were normalized to vehicle-treated control wells.

### 2.2.4 Quantitative Real Time PCR (qRT-PCR)

First strand cDNA was synthesized using random-primed hexamers and SuperScript III reverse transcriptase (Invitrogen), according to the manufacturer’s recommendation. qRT-PCR was performed in triplicate using ABI 7900HT Sequence Detection System (Applied Biosystem) and SYBR Green master mix. For microRNA measurements,
TaqMan human microRNA Assays and TaqMan microRNA Reverse Transcription kit (Applied Biosystems) were used based on the manufacturer’s protocol. TaqMan microRNA Assays are pre-formulated primer and probe sets designed to detect and quantify mature microRNAs. Pre-microRNAs have a stable hairpin in their structure, which was used for primer design. Results were normalized to a housekeeping gene, GAPDH, and relative fold changes were calculated using either serial dilutions of GAPDH plasmid and running a standard curve (absolute quantification) or the comparative Ct method. A pre-determined amount of in vitro synthesized luciferase mRNA was added to each sample before RNA extraction and quantified by qRT-PCR as a control for RNA extraction efficiency and first strand cDNA synthesis. A complete list of primers used in these studies is shown in Table 2.

2.2.5 Western Blot

Total cellular protein was resolved on NuPAGE Novex 4-12% Bi-Tris or 3-8 % Tris acetate gels (Invitrogen) and transferred onto nitrocellulose membranes using XCell II Blot Module (Invitrogen) according to the manufacturer’s protocol. Membranes were blocked using 5% non-fat milk and probed with the following antibodies: anti-Dicer (13D6) (ab14601, Abcam), anti-Drosha (ab12286, Abcam), anti-Exportin-5 (ab31351, Abcam), anti-Argonaute2 (Ago2) (ab57113, Abcam), Anti-ATF3 (C-19) (sc-188, Santa Cruz Biotechnology), and anti-α tubulin (T9026, Sigma-Aldrich). Secondary antibodies that were used are as follow: Horse Radish Peroxide (HRP)-conjugated rabbit anti-mouse IgG (heavy and light chain) (ab6728, Abcam) and HRP-conjugated goat anti-
rabbit IgG (sc-2004, Santa Cruz Biotechnology). Blots were developed with
Amersham™ enhanced chemiluminescence (ECL™) Prime Western Blot Detection
Reagent (GE HealthCare). Image quantification was performed using NIH Image J and
results were normalized to loading control.

2.2.6 Global microRNA expression profiling
Confluent HMVEC cells were treated with Stx (1000fM-24 hr) and total RNA was
extracted using mirVana PARIS Kit (Ambion). Microarray analysis of global microRNA
expression was performed by Exiqon using miRCURY LNA Array (7th Gen). Samples
were labeled using the miRCURY LNA™ microRNA Hi-Power Labeling Kit,
Hy3™/Hy5™ (Exiqon, Denmark) and hybridized on the miRCURY LNA™ array. The
Hy3™-labeled samples and a Hy5™-labeled reference RNA sample were mixed pair-
wise and hybridized to the miRCURY LNA™ microRNA Array 7th Gen (Exiqon,
Vedbaek, Denmark), which contains capture probes targeting all microRNAs for human,
mouse or rat registered in the miRBase 18.0. After hybridization, the microarray slides
were scanned and stored in an ozone free environment (ozone level below 2.0 ppb) in
order to prevent potential bleaching of the fluorescent dyes. The miRCURY LNA™
microRNA Array slides were scanned using the Agilent G2565BA Microarray Scanner
System (Agilent Technologies, Inc., USA) and the image analysis was carried out using
the ImaGene® 9 (miRCURY LNA™ microRNA Array Analysis Software, Exiqon,
Denmark). The quantified signals were background corrected (Normexp with offset
value 10, see Ritchie et al. 2007) and normalized using the global Lowess (Locally
Weighted Scatterplot Smoothing) regression algorithm.
2.2.7 mRNA half-life measurements

To determine the effect of Stx2 on mRNA half-life, HMVEC were treated with either vehicle or 1000 fM Stx2 for 20 hr and then treated with actinomycin D (Sigma – Aldrich) at a final concentration of 10 µg/ml. After which total cellular RNA was extracted at 0, 5, 15, 30, 60, 120, 180, 270, and 360 minutes. Levels of target transcript were assessed by RT-PCR. mRNA decay rates were determined using an exponential regression model.

2.2.8 RNA in situ hybridization

A series of PBS and Stx-treated CAST/Ei mice were examined. These mice were treated with PBS, Stx treatment (2400 pg/g) for 1 day, or Stx treatment for 3 or 4 days. All study tissues were cryosectioned at 8-10 µm and mounted on either gelatin-coated slides, or slides coated with UV-activatable resin (Cryojane, Instrumedics, USA) and stored at -80° C. Before ISH, sections were fixed in 4% formaldehyde (freshly made from paraformaldehyde; Sigma Aldrich, P6148) in phosphate buffered saline (PBS), treated with triethanolamine/acetic anhydride, washed and dehydrated in a series of ethanols. Sections were hybridized and washed as described previously. [38] Slides were then dehydrated, exposed to Kodak BioMaxMR x-ray film for 4 days, dipped in Kodak NTB nuclear track emulsion and exposed in light-tight boxes with desiccant at 4° C for 10 days. ATF3 riboprobe templates were provided as linearized templates. Cold riboprobes were first synthesized to validate that the templates would produce riboprobes of the expected length. Both antisense (769 nucleotides-representing exons 2-4 of ATF3) and sense radiolabeled riboprobes (781 nucleotides) were synthesized in
vitro according to the manufacturer’s specifications (Ambion) and labeled with $^{35}$S-UTP ($> 1,000$ Ci/mmol; Cat. #NEG039H, PerkinElmer LAS Canada, Inc.).

2.2.9 Statistical analysis

Data points represent the mean ± SEM of at least three independent experiments, unless otherwise stated. Statistical significance was assessed using paired t-test. In addition, the Benjamini and Hochberg multiple testing adjustment method has been applied to the p-value from the microarray data to control for false positive rates.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer sequence (5'-3')</th>
<th>Reverse primer sequence (3'-5')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dicer</td>
<td>ACG TTT TCC CAC CAT ATG T</td>
<td>CTG CAT TTA GGA GCT AGA TGA</td>
</tr>
<tr>
<td>Drosha</td>
<td>GGC CCA CCC TGA CCG ACT TCA TGA T</td>
<td>TGG CCT TTG CGC TGC ATT TGC AGA GT</td>
</tr>
<tr>
<td>ATF3</td>
<td>CCT CGG GGT GTC CAT CAC AA</td>
<td>CTG CAG GCA CTC CGT CTT CT</td>
</tr>
<tr>
<td>Pre-miR-874</td>
<td>GGC CCC ACG CAC CAG GGT AA</td>
<td>GGC CAG GGC AGG AAG CGA GA</td>
</tr>
<tr>
<td>Pre-miR-513a-5p</td>
<td>GTG CCT TTC ACA GGG AGG</td>
<td>GCA GTG CAT GCT GTA CAT TAC</td>
</tr>
<tr>
<td>Pre-miR-126</td>
<td>GGC GAC GGG ACA TTA TTA C</td>
<td>TCA CGG TAC GAG TTT GAA GTG</td>
</tr>
<tr>
<td>Pre-miR-16</td>
<td>GCC TTA GCA GCA CGT AAA TAT</td>
<td>CTT CAG CAC AGT TAA TAC TG</td>
</tr>
<tr>
<td>Pre-miR-4290</td>
<td>GGG TCA GTC CCA ATC TGA AT</td>
<td>AGA GGT GAG GGA AGA AAG GA</td>
</tr>
<tr>
<td>Pre-miR-483-3p</td>
<td>GGG AAG ACG GGA GGA AAG A</td>
<td>AGG AGT GAG GAG GCG TGA TG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAA GGT GAA GGT CGG AGT C</td>
<td>GAA GAT GGT GAT GGG ATT TC</td>
</tr>
<tr>
<td>Luciferase</td>
<td>ACT CCT CTG GAT CTA CTG GTC</td>
<td>GTA ATC CTG AAG GCT CCT CA</td>
</tr>
</tbody>
</table>

**Table 2. List of primer sequences used in this study.** (All the sequences are for human genes).
2.3 Results

2.3.1 HMVEC sensitivity to Stx

To determine the cytotoxic activity of Stx2 on our previously established in vitro model of HMVECs (TP’s), leucine incorporation assay was performed to measure inhibition of protein synthesis. HMVEC were seeded at a concentration of 40,000 cells/ml in 24 well plates. Confluent monolayers of cells were treated with different concentrations of Stx2 and their sensitivity was evaluated after 24 hr. Experiments were done in triplicate and data were normalized to untreated samples. Results revealed a concentration-dependent inhibition of leucine incorporation into de novo synthesized proteins following Stx2 exposure (Figure 6). Consistent with our previous data [34], the 50% inhibitory concentration (IC50) for leucine incorporation was calculated and reported to be 139 fM of Stx2. Our result was in accordance with previous studies demonstrating higher sensitivity of HMVEC to Stx2 compared to endothelial cells from different vascular beds. [254, 255] It has been reported that HMVEC express a 50-fold or higher amount of Gb3 compared to HUVECs [254] and this heterogeneity in Gb3 expression of endothelial cells highlights the importance of appropriate use of an in vitro model in studying Stx-mediated HUS. In addition, we have previously reported low levels of cell death in HMVECs treated with 1000 fM of Stx for 24 hr. [34]
Figure 6. In vitro model of HUS using HMVEC. Cell were plated at 100% confluence and treated with indicated concentrations of Stx for 24 hr. 1µCi $[^3]$H leucine was added to the samples one hour before harvest for further determination of newly synthesized proteins. (N=4)
2.3.2 Effect of Stx on microRNA expression

We have shown previously that Stx can affect endothelial gene expression at concentrations that have minor effects on protein synthesis. Global gene expression analysis on HMVECs treated with Stx indicated that 86.2 % of differentially regulated genes were up-regulated while only 13.8% were down-regulated. (Reported previously in [38]) We have shown evidence for the role of activation of CXCR4/CXCR7/SDF-1 and its contribution to HUS pathophysiology. Our results revealed that Stx along with its enzymatically active A subunit induced gene expression via increasing both transcription and mRNA stability. [38] microRNAs are critical regulators of gene expression at post-transcriptional levels, mediated through sequence-specific recognition of their mRNA targets.

Since altered expression or function of microRNAs is associated with critical clinical outcomes, we decided to determine the effect of Stx on microRNA expression and their role in post-transcriptional gene regulation. HMVECS were treated with 1000 fM of Stx for 24 hr as we have shown previously that this concentration of Stx has minimal effects on cell death.[38] We performed a microRNA microarray analysis on HMVEC treated with Stx. Out of 2084, 554 microRNAs indicated detectable expression above background and were analyzed by miRCURY LNA Array. Volcano plot analysis revealed 35 microRNAs with differential expression pattern (P<0.01), out of which 24 microRNAs (75%) were down-regulated compared to only 8 microRNAs (25%) that were up-regulated (Figure 7).
Figure 7. Effect of Stx on microRNA expression. Volcano plot of 554 detectable microRNAs that are isolated from HMVEC (1000FM Stx for 24 hr Vs. Vehicle) and analyzed by microarray. (N=4) The volcano plot is constructed by plotting the negative log of the p-value on the y-axis (base 10). This results in data points with low p-values appearing towards the top of the plot. The log fold-change (logFC) between the two experimental groups is plotted on the x axis. (The horizontal line represents P=0.01)
2.3.3 Identification and validation of microRNAs of interest

The major microRNAs whose processing was impaired by Stx were cross-referenced with those that target the mRNAs we have shown previously to change in response to Stx. [38] microRNAs that target selected genes were identified using two different algorithms, TargetScan and Miranda (Table 3). These algorithms use different criteria such as cross-species conservation, the degree of complementarities between the seed sequence (nucleotide 2-8) of microRNA and its target and also binding energy. Since none of these algorithms are complete it is recommended to use multiple algorithms to increase the specificity of prediction. [256, 257]

The expressions levels of four Stx-regulated microRNAs were validated using qRT-PCR along with two internal controls (miR-126 and miR-16). (Figure 8) As discussed earlier, microRNA maturation occurs through three steps: pri-microRNA, pre-microRNA, and mature microRNA. Thus, the levels of the corresponding precursors were also measured using microRNA-specific stem loop primers. To our interest, results revealed up-regulation in the level of precursors for chosen microRNAs due to Stx treatment except for one microRNA (miR-874) (Figure 9 A). Our results suggested that microRNA biogenesis is indeed impaired during Stx exposure, which was further evaluated by measuring the ratio of precursor versus mature microRNAs (Figure 9 B).
Table 3. Stx-regulated microRNAs and their mRNA targets. Two different algorithms were used to identify Stx-regulated microRNAs that target 8 highly up-regulated mRNA transcripts in HMVECs treated with Stx (1000 fM-24 hr). The numbers of target sites are represented by ✓ (✓: one target site, ✓✓: two target sites).
Figure 8. Validation of microarray results. Data represent mean ± SEM of 3-4 independent experiments. (* denotes p<0.05 vs. Vehicle)
Figure 9. Relative expression of pre-microRNAs and the ratio of pre/mature. A)
Relative expression of selected pre-microRNAs and B) the ratio of pre/mature in HMVECs treated with 1000 fM Stx-24 hr. Data represent mean ± SEM of 3-4 independent experiments. (* Denotes p<0.05 vs. Vehicle)
2.3.4 Stx affects microRNA biogenesis pathway

As mentioned before key enzymes such as, Drosha and Dicer, have critical roles in processing microRNAs. Dicer and Drosha control key aspects of endothelial biology [252, 258] and germline Dicer (-/-) ablation was reported to be embryonic lethal. [217] Studies have shown that the levels of these enzymes vary between tumour tissues, which correlate with cancer progression and poor survival. [227-231] Recently, Ho et al revealed evidence of altered expression of Dicer in human umbilical vein endothelial cells (HUVECs) under chronic hypoxia with consequences on global microRNA expression. [236]

In our study microarray analysis and qRT-PCR results revealed changes in microRNAs and their intermediate (pre-microRNAs) expression levels, based on which we hypothesized that Stx might affect enzymes with a significant role in microRNA biogenesis. The level of Dicer protein in Stx-treated HMVECs was measured using Western blot. Interestingly, we observed a significant decrease in Dicer protein expression with 1000 fM of Stx for 24 hr (Figure 10). To assess the effect of Stx on other proteins that are involved in microRNA biogenesis, we further quantified the expression of Drosha, Ago2, and Exportin-5 proteins in Stx- treated HMVECs. While treatment of HMVECs with 1000 fM of Stx for 24 hr had no effect on Ago2 and Exportin-5 protein expression, a reduction of 50% in Drosha protein level was seen. (Figure 11) The mRNA level of Dicer and Drosha was also assessed by qRT-PCR and no significant change was observed. (Figure 12) These data confirmed that Stx significantly affects the expression of Dicer and Drosha at protein levels only.
Figure 10. Stx impairs Dicer protein expression. Representative immunoblots of human microvascular endothelial cells (HMVEC) treated with Stx. Quantification of A) Dicer immunoblots in B). Data represent mean ± SEM of 8 independent experiments. * denotes statistical significance (p<0.05) compared to Vehicle.
Figure 11. Effect of Stx on key regulators of microRNA biogenesis pathway. A) Immunoblots of Exportin-5, Ago2, and Drosha. B) Quantification of the protein blots. Data represent mean ± SEM of 3-4 independent experiments. (* denotes p<0.05 vs. Vehicle)
Figure 12. Effect of Stx on Dicer and Drosha mRNA expression. mRNA level of Dicer and Drosha in HMVECs treated with 1000 fM of Stx-24 hr were measured using qRT-PCR. Data represent mean ± SEM of 3-4 independent experiments.
2.3.5 Stx induces ATF3 via enhanced mRNA stability

Among genes highly induced by Stx we decided to further study activating transcription factor (ATF3), which increased with a fold change of 25.88. ATF3 has a long 3'-UTR of 1219 nt that has multiple target sites for various microRNAs, among which are significantly down-regulated ones, miR-874 and miR-513a-5p. (Figure 8) Within normal conditions the baseline ATF3 mRNA level is low; however, it increases significantly due to stress signals such as ER stress, oxidative stress, UV light, cytokines, and renal ischemia/reperfusion (I/R) injury. [98] Recent studies have also shown that ATF3 induces apoptosis in human brain microvascular endothelial cells via activation of stress inducible C/EBP homologous protein (CHOP) due to infection with Stx2. [83]

First, we confirmed our microarray analysis from our previous study [38], using qRT-PCR, and results revealed a significant increase of 70-fold in ATF3 mRNA expression levels. We also measured ATF3 protein levels, and observed a time and concentration dependent increase in its protein expression compared to vehicle-treated cells with almost no expression of ATF3 (Figure 13).

Since ATF3 exhibited high levels of expression compared to normal conditions we hypothesized that this could be due to an increase in both its transcription and its mRNA stability. Actinomycin D (ActD), which blocks transcription, was added to HMVECs and the rate of mRNA decay was assessed at indicated time points. ATF3 has an mRNA with a short half-life of 1.40 mins; however, the half-life of its mRNA was extended to 99.02 hr upon Stx exposure (Figure 14). These observations confirm that Stx affects ATF3 expression at a post-transcriptional level by increasing its mRNA stability.
Figure 13. Stx induction of ATF3 expression. A) Representative immunoblots of ATF3 in HMVECs treated with Stx. (TNFα was used as a positive control) B) ATF3 mRNA levels in Stx-treated HMVECs. Data represent mean ± SEM of 3 independent experiments.* denotes statistical significance (p<0.05 compared to Vehicle)
Figure 14. ATF3 mRNA half-life measurements in Stx-treated HMVECs compared to vehicle. Stx increased mRNA stability of ATF3 from being 1.40 min in vehicle to 99.02 hr. Data represent mean ± SEM of 3-4 independent experiments. (* denotes p<0.05 compared to vehicle)
2.3.6 Effect of Stx exposure on ATF3 expression in a murine model

Most lab mouse strains injected with Stx do not recapitulate the hallmarks of HUS. It has been shown that in inbred strains the main source of Gb3 expression is tubular epithelium. [259, 260] This leads to renal tubular damage rather than the glomerular damage. [261] It is thus important to choose proper in vivo models to better study the pathogenesis of Stx-mediated HUS.

Thrombotic thrombocytopenic purpura (TTP) is a serious disease with the same clinical characteristics as HUS. TTP is due to deficiency in an enzyme called ADAMTS13, a von Willebrand Factor (vWF) protease. In a study by Motto et al, the authors showed that mice deficient in ADAMTS13 are viable when introduced in the common lab inbred strain, C57BL/6. The investigators proposed that these mice are resistant to developing TTP due to the presence of other genetic modifying agents. However, they were successful in generating mice susceptible to TTP by introducing ADAMTS13 in a wild-derived Mus musculus castaneus background (CAST/Ei). [262] Accordingly, we used CAST/Ei mice for our in vivo studies. [38] The expression of ATF3 in a series of PBS-treated and Stx2-treated adult mice at 1 and 3 days was assessed using in situ hybridization.

ATF3 was expressed at low levels in the hepatocytes of PBS-treated animals, however; it increased significantly in the livers of animals after 4 days of Stx exposure. High levels of ATF3 labeling were also observed in the tubules of the kidney cortex at day 3 of Stx treatment (Figure 15). There were tissues showing relatively stable levels of ATF3 mRNA concentration (thymus, lung, small intestine), suggesting independence of Stx
treatment. Other tissues such as the heart, liver, stomach and kidney respond to Stx treatment first by down-regulation on day 1 and thereby up-regulation on day 3 (Figure 15). These data demonstrate a heterogeneous expression of ATF3 in mouse models (Figure 16).
Figure 15. In vivo expression of ATF3. ATF3 mRNA expression was measured in liver (A) heart (B) and kidney (C) using in situ hybridization (magnification 72X). Abbreviations: CM-cardiac muscle; H-heart; S-sense probe.
Figure 16. Comparative ATF3 expression in vivo. (A) PBS-treated, (B) Stx-treated for 1-day (VTD-1), (C) Stx-treated for 3 days (VTD-3). Abbreviations: Li: liver; Lu: lung; Sin: small intestine; St: stomach. (Magnification: 2.4 X)
Chapter 3

Discussion and Future Directions
3.1 Discussion

Stx mediated HUS belongs to the category of thrombotic microangiopathies, and is a common cause of renal failure mostly in children, the incidence of which is increasing around the world. We have shown previously that treatment of HMVEC with low concentration of Stx had significant effects on gene expression with a special focus on the expression of the ET-1 and CXCR4/CXCR7/SDF-1 pathways. [38, 53] The mechanisms of regulating gene expression were reported to be via an increase in transcription and most importantly in mRNA stability. In the current work we used our previously established in vitro model of HMVEC based on the cells’ higher sensitivity to Stx and also higher expression of Gb3 compared to endothelial cells residing in other vascular beds such as, HUVEC. [254, 255] Our study is the first to report the role of microRNAs in Stx pathology in vascular endothelium. Dicer, a key enzyme in microRNA biogenesis, has been shown to be under the regulation of different stress stimuli. [235] Here, we have found that Stx affects the expression of microRNAs by decreasing Dicer protein levels. Significantly, reduction in Dicer levels results in the down-regulation of most of mature microRNAs as well as changes in the level of their precursors. Our results revealed that changes in microRNA expression, due to altered levels of Dicer, might be functionally involved in Stx-induced endothelial injury. (Figure 17) Although Dicer is down-regulated, we did not observe a reduction in the level of all microRNAs. There are possible explanations for this: 1) Regulation of microRNAs by Dicer-
independent pathways [263-265] 2) Regulation of the steady-state level of microRNAs based on the abundance of their mRNA targets. [266]

Stx-induced expression of ET-1 and CXCR4/CXCR7/SDF-1 was reported to be due to increase in their mRNA half-lives. It was reported that Stx1 treatment of a myelogenous leukemia cell line THP-1 resulted in the induction of ER stress sensors IRE1, PERK and ATF6. [82] Our results show that Stx2 also induces ATF3, a down-stream effector molecule of PERK sensor in ER stress, by up-regulating its mRNA as well as protein levels. Like ET-1 and CXCR4, ATF3 also has a short-lived mRNA; however, following Stx an induction in the steady state of ATF3 mRNA level is reported. [38] These data explain the marked fold change of ATF3 seen in our previously published microarray data. [38] Previously, Fujii et al identified that activation of CHOP led to Stx2- induced apoptosis in human brain microvascular endothelial cells (HBMVEC). [83] A possible mechanism for increased expression of the CHOP could be induction in the ATF3 level followed by its binding to the CHOP promoter. [267] Most of the significantly down-regulated microRNAs target ATF3 3'-UTR, using multiple prediction algorithms and further highlights the importance of Dicer- dependent microRNAs in maintaining the expression of ATF3 in cellular response to Stx via post-transcriptional mechanism.

One of the highly down-regulated microRNAs is miR-874 with two binding sites within the 3'-UTR of ATF3. miR-874 resides in intron 10 of the kelchlin-like-3 (KLHL3) gene, having a significant role in hypertension and cardiovascular disease. [268] Our microarray data revealed a 50% reduction in the expression of KLHL3 mRNA [38], which would be a possible explanation for the significant decrease in the level of pre-miR-874.
To evaluate the effect of Stx on ATF3 expression we used our in vivo model of CAST/Ei mice. The heterogeneous expression of ATF3 and most importantly the highest expression in kidney further confirmed this tissue as a major site of injury by Stx2 treatment.

Taken together, our results provide insight into the role of Dicer-regulated microRNAs in post-transcriptional gene regulation in STEC-HUS patients that could be used as a potential diagnostic and therapeutic marker.

3.2 Future Directions

Our study opened up a new area in the molecular events responsible in the pathogenesis of Stx-mediated HUS. The microRNA expression profile explained in Chapter 2 provides evidence of how microRNA can be differentially regulated in Stx-treated endothelial cells versus controls. Our data provide evidence for the role of Dicer ablation in changes in microRNA expression. However, defining the possible mechanisms responsible for Dicer down-regulation need further studies. These will include measuring Dicer protein half-life and also evaluating whether proteasomes are important in Dicer down regulation following Stx. We hypothesized that Stx results in the activation and phosphorylation of PERK and this leads to post-translational modification of Dicer. This could be further validated by measuring Dicer expression in Stx-treated HMVECs exposed to PERK inhibitors.
We have identified an increase in the steady state level of ATF3 mRNA and also validated a subset of microRNAs that target ATF3. However, confirming that these microRNAs indeed target ATF3 will further support our hypothesis of the possible role of microRNAs in post-transcriptional regulation of ATF3 expression. To test this, HMVECS will be transfected with chimeric luciferase reporter constructs representing the 3’-UTR of ATF3 located downstream of the open reading frame of luciferase and treated with either Stx or vehicle and the activity of the luciferase construct will be measured. An alternative method would be to overexpress microRNAs targeting ATF3 by using microRNA mimics, known as agomirs, and validating the expression of the luciferase construct. In addition, it’s important to determine the effect of Dicer down regulation on the ability of HMVECs to respond to Stx-induced ER stress. For this purpose, HMVECs will be transfected with Dicer plasmid along with control plasmids and the effect of forced overexpression of Dicer on ATF3 as well as its down stream targets such as, SDF-1 will be measured.

Furthermore, measurements of Dicer protein levels and microRNA expression in different mouse tissues will establish the in vivo relevance of our data. Since ATF3 has a dual function as both a pro-survival and anti-survival transcription factor, which is cell and tissue dependent, it is of importance to identify the physiological relevance of ATF3 induction in Stx pathogenesis. Under normal conditions, ATF3 knockout mice develop normally with no major phenotypical changes [269] and could be used as an in vivo model to identify the specific role of ATF3 in Stx pathology.

Taken together our data indicate an important role for Dicer and microRNA biogenesis in HUS associated with STEC. Here we found that Stx leads to down regulation of
Dicer, which affects global expression of microRNAs. This leads to down regulation of most of Dicer-dependent microRNAs. Stx damages host cells by interfering with ribosomal function and thereby inhibiting translation. This results in the activation of a cascade of cellular events and ER stress. We have shown in our previous work that ATF3 is among genes that were highly up regulated by Stx. ATF3 is an important down stream effector molecule of PERK sensor in ER stress. Our in silico analysis revealed that ATF3 is a potential target of most of the microRNAs. We have also shown that Stx affects ATF3 by increasing its mRNA level, protein expression, and its mRNA stability. These highlight the importance of microRNAs in regulating Stx-induced genes at a post-transcriptional level. (Figure 17) HUS is a serious life-threatening illness and currently there is no effective therapeutic approach for preventing this disease. Thus, It is important to understand the mechanism of action, intracellular trafficking, and activation of signaling pathways and molecular events by Stx in order to develop preventive strategies.
Figure 17. Functional deficiency of Dicer in response to Stx. Stx affects Dicer expression and results in its down-regulation. This leads to a reduction in the level of a subset of Dicer-dependent mature microRNAs. Some of these microRNAs are regulators of ATF3 at post-transcriptional level. This model represents the importance of dicer-dependent microRNAs in maintaining the expression of ATF3 and its targets (e.g. SDF-1) in response to Stx.
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