

EXPLOITING AND EXPLORING THE INTERACTIONS BETWEEN  
microRNA-122 AND HEPATITIS C VIRUS

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By

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## ABSTRACT

Hepatitis C virus (HCV) is a single-stranded plus-sense RNA virus that is transmitted by blood-to-blood contact, and infects the human liver. HCV has a unique dependence on the liver-specific microRNA miR-122, where miR-122 binds the 5' un-translated region of the viral RNA at two tandem sites and increases viral RNA abundance. The mechanisms of augmentation are not yet fully understood, but the interaction is known to stabilize the viral RNA, increase translation from the viral internal ribosomal entry site (IRES), and result in increased viral yield.

In an attempt to create a small animal model for HCV, we added miR-122 to mouse cell lines previously thought non-permissive to HCV, which rendered these cells permissive to the virus, additionally showing that miR-122 is one of the major determinants of HCV hepatotropism. We found that some wild-type and knockout mouse cell lines – NCoA6 and PKR knockout embryonic fibroblasts – could be rendered permissive to transient HCV sub-genomic, but not full-length, RNA replication upon addition of miR-122, and that other wild-type and knockout cell lines cannot be rendered permissive to HCV replication by addition of miR-122. These knockout cell lines demonstrated varying permissiveness phenotypes between passages and isolates and eventually completely lost permissiveness, and we were unable to achieve sub-genomic RNA replication in PKR knockout primary hepatocytes. Knockdown of NCoA6 and PKR in Huh7.5 cells did not substantially impact sub-genomic replication, leading us to conclude that there are additional factors within the cell lines that affect their permissiveness for HCV replication such as epigenetic regulation during passage or transformation and immortalization.

We also added miR-122 to Hep3B cells, a human hepatoma cell line lacking expression of miR-122 and previously thought to be non-permissive to HCV replication. Added miR-122 rendered the cells as highly permissive to HCV replication as the Huh7-derived cell lines commonly used to study the virus. In these cells, we were also able to observe miR-122-independent replication of sub-genomic HCV RNA. This was verified by use of a miR-122 antagonist that had no impact on the putative miR-122-independent replication, and by mutating the miR-122 binding sites to make them dependent on a single nucleotide-substituted microRNA. This replication in the absence of miR-122 was not detected in full-length HCV RNA, but was detectable using a bi-cistronic full-length genomic replicon, suggesting that the addition of a

second IRES in sub-genomic and full-genomic replicons altered replication dynamics enough to allow detectable RNA replication without miR-122 binding.

Because miR-122 has been implicated in protecting the viral RNA from destabilization and degradation by Xrn1, the main cytoplasmic 5' to 3' RNA exonuclease, we employed our miR-122-independent system to test this miR-122-mediated protection. We verified that miR-122 functions to protect the viral RNA from Xrn1, but this was insufficient to account for the overall impact of miR-122 on replication, meaning that miR-122 has further functions in the virus' life cycle. We showed that the effect of miR-122 on translation is due to stabilization of the RNA by protecting it from Xrn1, through binding at both sites. We further evaluated the role of each miR-122 binding site (S1 and S2) in the virus life cycle, and found that binding at each site contributes equally to increasing viral RNA replication, while binding at both sites exerts a co-operative effect. Finally, we determined that binding of miR-122 at site S2 is more important for protection from Xrn1, suggesting that miR-122 binding at S1 is more important for the additional functions of miR-122 in enhancing HCV RNA accumulation.

Altogether, we have shown that miR-122 is partially responsible for the hepatotropic nature of Hepatitis C virus, and that supplementation with this microRNA can render non-permissive cells permissive to viral replication. We have also identified and confirmed replication of both sub-genomic and full-length HCV RNA in the absence of miR-122. Finally, we have characterized the impact of the host RNA exonuclease Xrn1 on the HCV life cycle, and determined the roles of each miR-122 binding site in shielding the viral RNA from this host restriction factor.

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I did not become who I am on my own; I often say it took a team to raise me, and I still believe that holds true. I owe my grandmother, Lynnette McGee, a debt of gratitude for raising me to love books and ask questions, and perhaps also my grandfather, Harold, who spun tall tales to test my critical thinking – at least, that may have been how he justified them. I am forever thankful for the time I had with my dad, David McGee, who taught me by example what makes a person strong, and how to always do the right thing. I thank my mom, Karin Bayer, for teaching me that my mistakes are rarely earth-shattering, and how to grow past them, and my stepdad, Corey Hargreaves, for showing me real magic in this world. I am also grateful for the lessons my Oma and Opa, Barbara and Rolf Bayer, taught me about bettering the world we live in. And finally, although they came after me, I must acknowledge the patience I learned from my brothers and sister; Diana Bayer, Connor Hargreaves, Spencer and Paul Pilka-McGee, knowing you were watching has kept me in line more than I should admit.

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## DEDICATION

*I dedicate this thesis to my brothers and sister.*

*Diana,  
Connor,  
Spencer,  
and  
Paul*

*I hope I've merely opened the way for you each to do wonderful things.*

*This thesis is also dedicated to the memory of my father,  
David McGee,  
who never once thought to question whether I could achieve my goals,  
but just told me how proud he was when I did.*

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## LIST OF ABBREVIATIONS

Ago2 – Argonaute-2  
DAA – direct-acting antiviral  
EGFR – epidermal growth factor receptor  
eIF – eukaryotic initiation factor  
EMCV – encephalomyocarditis virus  
EphA2 – ephrin receptor A2  
FGR – full-genomic replicon  
FL – full-length  
GBV-B – GB virus B  
HBV – Hepatitis B virus  
HCV – Hepatitis C virus  
HCVcc – cell-culture derived HCV virions  
HDV – Hepatitis D virus  
HNF – hepatocyte nuclear factor  
HS-PG – heparan sulfate proteoglycan  
IFN $\alpha/\beta$  – interferon alpha/beta  
IRES – internal ribosomal entry site  
LDL – low-density lipoprotein  
LDL-R – low-density lipoprotein receptor  
MEFs – murine/mouse embryonic fibroblasts  
miR-122 – miRNA-122  
miRNA – microRNA  
miRISC – microRNA-containing RNA-induced silencing complex  
NCoA6 – nuclear receptor co-activator 6  
NPC1-L1 – Niemann-Pick C1-like 1  
NS – non-structural  
nt – nucleotide(s)  
p3, p3-4, p5, p6 – position 3 *etc.* on miR-122  
P-body – processing body



PABP1 – poly-A binding protein-1  
Peg-IFN – pegylated interferon  
PKR – protein kinase-R  
PolII – RNA polymerase II  
PPAR $\gamma$  – peroxisome proliferator activated receptor-gamma  
Rbv - ribavirin  
RISC – RNA-induced silencing complex  
RXR $\alpha$  – retinoid X receptor alpha  
S1 – miR-122 binding site 1  
S2 – miR-122 binding site 2  
SGR – sub-genomic replicon  
SR-BI – scavenger receptor B1  
TfR-1 – transferrin receptor 1  
UTR – un-translated region  
VLDL – very low-density lipoprotein

## 1.0 Literature Review

### 1.1 Hepatitis C Virus

#### 1.1.1 Viral Classification and Characteristics

Hepatitis C virus (HCV) is a member of the *Flaviviridae* family, genus *Hepacivirus*, and is an enveloped, single-stranded positive sense RNA virus (Smith et al., 2014). Within the viral species, Hepatitis C is classified into seven numbered genotypes which are further broken down by subtypes denoted by lowercase letters (*e.g.* genotype 1a, genotype 6c) (Smith et al., 2014). The *Pestivirus*, *Pegivirus*, and *Flavivirus* genera in the *Flaviviridae* family contain many other human pathogens, but HCV is the only member of the *Hepacivirus* genus currently known to infect humans (Pybus and Gray, 2013; Stapleton et al., 2011). Recently, hepacivirus sequences have been isolated from primates, dogs, horses, bats, and rodents, but no virus has been cultured yet (Burbelo et al., 2012; Drexler et al., 2013; Kapoor et al., 2011; Lauck et al., 2013; Lyons et al., 2012; Quan et al., 2013; Silva et al., 2012).

The viral genome is approximately 9.6 kilobases in length, and also directly acts as the virus' mRNA (Moradpour et al., 2007). The 5' un-translated region (UTR) of the viral RNA is comprised of several stem-loop structures essential for the virus life cycle; it bears two microRNA-122 (miR-122) binding sequences following stem-loop I, and stem-loops II and III comprise the structure of the viral internal ribosomal entry site (IRES) that drives viral translation (Hoffman and Liu, 2011; Jopling et al., 2008; Lukavsky, 2009). The viral start codon is located in stem-loop IV, and the remainder of the viral RNA is translated as a single polyprotein, which is post-translationally cleaved by host and viral proteases into ten essential proteins. Core, E1, E2, p7, and NS2 are classified as the assembly module (structural and scaffolding proteins) while NS3, NS4a, NS4b, NS5a, and NS5b are non-structural replicase proteins, although they have all been implicated in aiding assembly as well (Moradpour and Penin, 2013). Finally, the viral RNA has a 3' UTR which also contains significant RNA secondary structure critical to both viral translation and replication (Bung et al., 2010; Hoffman and Liu, 2011; Yi and Lemon, 2003).

The HCV virion is comprised of a single copy of the viral RNA genome encased in Core protein, and further protected by a lipid membrane and viral envelope glycoproteins E1 and E2. Infectious virus particles in patient serum are associated with both very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) to form a lipoviral particle (LVP) that may aid in the

early steps of attachment to target cells (Lindenbach and Rice, 2013; Vieyres and Pietschmann, 2013).

## *1.1.2 Hepatitis C Virus Life Cycle*

### *1.1.2.1 Entry*

HCV utilizes a series of host proteins to attach to and enter the host cell. Because the virion is associated with host lipoproteins and lipid droplets, attachment may be mediated by binding of the lipid droplet components to heparan sulfate proteoglycan, the low-density lipoprotein receptor (LDL-R), and scavenger receptor B1 (SR-B1), although these initial interactions may not be essential for entry (Acton et al., 1996; Albecka et al., 2012; Bartosch et al., 2003; Germe et al., 2002; Heo, 2008; Maillard et al., 2006; Mazumdar et al., 2011; Monazahian et al., 1999; Owen et al., 2009; Prentoe et al., 2014). The E2 protein of HCV specifically interacts with the cell surface protein CD81, and further signalling via receptor tyrosine kinases (epidermal growth factor receptor – EGFR and ephrin receptor A2 – EphA2) and RAS and RHO GTPases (HRas, Rac1, RhoA, and Cdc42) facilitates interaction with Claudin-1 at cellular tight junctions (Brazzoli et al., 2008; Evans et al., 2007; Farquhar et al., 2011; Farquhar et al., 2012; Harris et al., 2010; Lupberger et al., 2011; Wunschmann et al., 2000; Zona et al., 2013). E1 is not thought to interact with any of the receptors, but is implicated in modifying and maintaining the structure of E2 to permit its direct interaction with HCV receptors (Douam et al., 2014; Wahid et al., 2013). SR-B1 and Occludin, and possibly phospholipid scramblase-1 are also involved in this step, which ultimately leads to the initiation of clathrin-mediated endocytosis of the lipoviral particle (Blanchard et al., 2006; Dao Thi et al., 2012; Gong et al., 2011; Ploss et al., 2009; Sourisseau et al., 2013; Zahid et al., 2013). Additional host proteins Niemann-Pick C1-Like 1 (NPC1L1) and transferrin receptor 1 (TfR1) are likely also involved in this later stage of entry (Martin and Uprichard, 2013; Sainz et al., 2012a). Once endocytosed, lowered pH triggers the virion envelope proteins to initiate fusion with the early endosomal membrane and release the RNA genome into the cytoplasm for translation and replication (Blanchard et al., 2006; Codran et al., 2006; Collier et al., 2009; Farquhar et al., 2012; Sharma et al., 2011; Zeisel et al., 2013).

### 1.1.2.2 Translation

Translation of its genome is the first step that HCV must take once the virus has escaped the endosome, as no replication can occur until the requisite proteins have been synthesized. Hepatitis C virus drives its translation through a Type 3 internal ribosomal entry site (IRES) located in nucleotides 40 to 374 in the 5' UTR of its genome that functions independently of many of the initiation factors required to begin host mRNA translation (Hellen, 2009; Lukavsky, 2009; Tsukiyama-Kohara et al., 1992). The secondary structure of the HCV IRES recruits the 40S ribosomal subunit via stem-loops IIIcdef, along with a GTP-bound initiator tRNA (GTP/Met-tRNA<sup>Met</sup>) and the eukaryotic initiation factor 2 (eIF2) at the polyprotein start codon (nucleotides 342 to 344), without requirement for the vast majority of other mRNA initiation factors (Ji et al., 2004; Lukavsky, 2009). Often, eIF3(abdf) is included in this complex on the IRES, but it may also bind separately via HCV's stem-loop IIIb to stabilize the now-48S ribosome (Hellen, 2009; Ji et al., 2004; Lukavsky, 2009; Shi and Lai, 2006). By analogy to classical swine fever virus (CSFV) IRES function (a *Pestivirus* bearing another Type 3 IRES), the 48S subunit uses eIF5 to hydrolyze the GTP/Met-tRNA<sup>Met</sup> and liberate eIF2 and GDP, leading to eIF5B-mediated subunit joining by the 60S ribosomal subunit to form the functional 80S ribosome (Hellen, 2009; Locker et al., 2007; Otto and Puglisi, 2004). Upon assembly of the ribosome, translation begins at the core methionine without scanning and produces the viral polyprotein that must be cleaved by host signal peptidase and signal peptide peptidases (Core to p7), and the viral NS2 and NS3/4a proteases (NS2 and NS3 to NS5b, respectively) (Bartenschlager et al., 2010; Choo et al., 1989; Lin et al., 1994; Lohmann et al., 1996; Reynolds et al., 1996). After translation, all of these proteins are directly required for replication and packaging of the viral RNA. Viral proteins, which are produced in excess, also modulate the host cell environment to best suit the virus by modulating metabolic processes, manipulating innate immune sensing, and minimizing adaptive host-wide immune responses, among others (Alvisi et al., 2011; Macdonald and Harris, 2004; Shulla and Randall, 2012).

### 1.1.2.3 RNA Replication

Making the change from translation of the genome to replication of the RNA is managed by both host and viral proteins, although the exact mechanism is not yet known. Some likely candidate host proteins include the La autoantigen and hnRNP-D (an RNA binding protein) found to bind the 5' UTR, PCBP2 (poly-C binding protein 2), which binds the viral 5' UTR and is implicated in

circularization of the genome, PTB (polypyrimidine tract binding protein), known to bind the viral 3' UTR, and hVAP-A (human vesicle-associated membrane protein-associated protein A), which recruits NS5a to NS5b and may recruit both to NS4b (Chang and Luo, 2006; Paek et al., 2008; Ray and Das, 2011; Tu et al., 1999; Wang et al., 2011). Additionally, miR-122, which also binds the 5' UTR, has been suggested as a regulator of the switch between translation and replication, although the evidence for this is not strong (Diaz-Toledano et al., 2009). The viral NS5a protein has been heavily implicated in defining the switch to replication; in particular, the phosphorylation status of the protein is suggested to help define translation *versus* replication, but the specific kinases responsible for phosphorylation of NS5a are not all yet known (Huang et al., 2007b; Hundt et al., 2013; Macdonald and Harris, 2004). Particularly, only casein kinase-1 $\alpha$  and Polo-like kinase-1 have been shown to specifically phosphorylate NS5a, although kinase screens and inhibitor studies have implicated MAP kinases, AKT, and glycogen synthase kinase-3 (Chen et al., 2010; Coito et al., 2004; Hundt et al., 2013; Quintavalle et al., 2007; Tellinghuisen et al., 2008). Therefore, it is still unclear how the transition from translation to replication of the viral RNA is regulated.

Replication of the viral genome is dependent on the viral proteins: the non-structural proteins NS3 through NS5b are required and sufficient for replication of the viral RNA, but generally must be provided in *cis* – that is, translated from the same RNA that they will then replicate (Appel et al., 2005; Evans et al., 2004a; Herod et al., 2014). This creates an extra hurdle in exploring the process of HCV RNA replication, which is therefore still incompletely understood (Brass et al., 2009; Fournier et al., 2013; Lohmann, 2013). All of the viral non-structural proteins are membrane-bound, which presumably facilitates their efficient interactions by preventing diffusion throughout the cell. The membranous web initiated by viral proteins also serves to protect the virus from host defenses and is a scaffolding to regulate the various stages of the virus life cycle (Lohmann, 2013; Moradpour and Penin, 2013; Moradpour et al., 2007; Paul and Bartenschlager, 2013). The viral NS3/4a functions in complex as the main viral protease, cleaving the non-structural viral proteins from each other after translation of the viral polyprotein (Moradpour and Penin, 2013; Phan et al., 2011). During replication, NS3 also activates the viral polymerase and further acts as a helicase, unwinding the secondary structure and/or dsRNA replication intermediates, while NS4a is also implicated in the regulation of NS5a phosphorylation discussed previously (Binder et al., 2007; Lindenbach et al., 2007; Morikawa et al., 2011; Piccininni et al.,

2002). NS4b, in concert with NS5a, is understood to be involved in remodelling the host cell membranes to form a structures collectively known as the membranous web, which is used to house viral replication complexes (Alvisi et al., 2011; Egger et al., 2002; Gouttenoire et al., 2010). NS4b has also been implicated in catalyzing the formation of the replication complex (Gouttenoire et al., 2009; Gouttenoire et al., 2010; Jones et al., 2009). NS5a is involved in formation of the membranous web, regulation of translation and replication, and anchoring the replication complex to the membranous web (Lundin et al., 2006; Macdonald and Harris, 2004).

Finally, NS5b is the viral polymerase that is key to production of both positive and negative-sense viral genomes; NS3 and NS5a have both been shown to increase its polymerase activity (Lee et al., 2006; Moradpour et al., 2007; Piccininni et al., 2002; Shi and Lai, 2006; Shimoike et al., 2006). By analogy to other *Flaviviruses*, replication of the viral genome may also require circularization of the RNA, perhaps to catalyze assembly of the active replicase, which could be mediated by viral proteins such as NS3 and NS5a, as they are known for binding both ends of the viral genome, or by other host proteins such as those involved in translation regulation (Ray and Das, 2011; Toroney et al., 2010; Villordo and Gamarnik, 2009; Wang et al., 2011). Along with the host proteins mentioned above that potentially regulate a switch to replication such as hVAP-A and PCBP2, host protein PI4KIII is required for membranous web formation and thus replication complex assembly, while cyclophilin A is required for HCV RNA replication, and hnRNP-L and NF90 have recently also been implicated in facilitating RNA replication (Alvisi et al., 2011; Berger et al., 2009; Evans et al., 2004b; Gao et al., 2004; Li et al., 2014; Rosnoble et al., 2012; Tai et al., 2009; Tu et al., 1999; Wang et al., 2011; Weng et al., 2012).

Replication involves specific structural elements in the NS5b coding region and 3' UTR of the positive sense RNA (Cai et al., 2004; Friebe et al., 2005; Yi and Lemon, 2003; You and Rice, 2008). The resulting negative sense RNA acts as a template for more genomic RNA, and although its 3' sequence is complementary to the genomic 5' UTR, its secondary structure is significantly different and presumed to be critical for initiation of positive sense RNA production. The sole known function of the negative sense RNA is to provide a template for further positive sense RNA production, and this is supported by an observed 1:10 ratio of negative sense genomes to positive sense genomes in the host cell (Bartenschlager et al., 2010; Binder et al., 2007; Keum et al., 2012; Lohmann, 2013). The positive sense RNA genomes are then used as further templates for

translation and replication, or are packaged into virions for further infection (Bartenschlager et al., 2010; Moradpour et al., 2007).

#### *1.1.2.4 Virion Production*

Understanding of HCV infectious virion production is limited by our current cell culture systems. Egress of infectious virus particles is associated with very low-density lipoprotein (VLDL) secretion, but the major cell culture model (Huh7-derived cells) are deficient in production of Apolipoprotein B (ApoB), which is known to be associated with patient serum-derived infectious particles (Huang et al., 2007a; Icard et al., 2009). Thus, the lipovirions produced in cell culture thus have different properties from patient serum-derived virus particles, suggesting that models based on information from cell culture-derived virus are incomplete (Andre et al., 2002; Jiang and Luo, 2009; Lindenbach and Rice, 2013; Nielsen et al., 2006; Podevin et al., 2010).

The viral proteins NS2 and p7 coordinate relocation of other non-structural and structural proteins to the site of viral assembly on the endoplasmic reticulum (ER), but the order of recruitment and component assembly is not fully known (Boson et al., 2011; Corless et al., 2010; Gentzsch et al., 2013; Jirasko et al., 2010; Lindenbach, 2013; Popescu et al., 2011; Vieyres et al., 2013). Core, which functions as the nucleocapsid of the virion, is recruited to this site from cytoplasmic lipid droplets by interactions with the putative NS2-p7 scaffolding complex and the host protein DGAT1, as well as through interactions with NS3/4a and NS5a (Boson et al., 2011; Camus et al., 2013; Counihan et al., 2011; Herker et al., 2010; Ma et al., 2008; Masaki et al., 2008; Stapleford and Lindenbach, 2011). The envelope proteins E1 and E2 exist as a heterodimer localized to the ER, and are also likely brought to the site of assembly by NS2 and p7 (Brazzoli et al., 2005; Dubuisson et al., 1994; Jirasko et al., 2010; Ma et al., 2011; Popescu et al., 2011; Stapleford and Lindenbach, 2011). At some point in this process, the viral RNA is also recruited to this site, perhaps by relocation of the replicase due to interactions between structural and non-structural proteins, where it is suggested that the NS3 helicase is responsible for incorporation of viral RNA into the capsid (Bartenschlager et al., 2011; Lindenbach and Rice, 2013; Popescu et al., 2011).

The virion uses early-to-late endosomal tracking (the ESCRT pathway; the endosomal sorting complex required for transport) to mediate secretion into the extracellular milieu (Ariumi et al.,

2011b; Corless et al., 2010; Lai et al., 2010; Tamai et al., 2012). E1 and E2 become glycosylated during egress, possibly at the Golgi apparatus (Coller et al., 2012; Vieyres et al., 2010). During secretion, the p7 protein remains associated with the virion, acting as an ion channel to retain an appropriate pH balance to stabilize viral particles until egress, but it is not associated with the secreted viral particle (Clarke et al., 2006; Steinmann et al., 2007; Vieyres et al., 2013; Wozniak et al., 2010). Upon assembly of the viral structural proteins and viral RNA into a virion, the sequence of its association with lipid droplets and/or their components is likewise uncertain. Recently, both ApoB and ApoE have been found to be associated E1 and E2 at the ER membrane, as well as associated with intracellular infectious virus particles, showing that the association of the virion with lipoproteins occurs early in trafficking, and ApoE has been found to associate with NS5a on lipid droplets, suggesting that ApoE could be involved in early assembly of the virion (Benga et al., 2010; Boyer et al., 2014; Coller et al., 2012). Other results suggest that this is not the case, and that association with ApoE is unnecessary for assembly, and is only mechanistically relevant at a late stage of trafficking and egress (Hueging et al., 2014). It is therefore not certain whether virion/Apolipoprotein intracellular association is coincidental due to virion association with ApoE, ApoC, and ApoB along the same secretion pathway, or whether the virus directly and specifically associates with VLDLs during assembly and/or secretion (Gastaminza et al., 2008; Huang et al., 2007a; Jiang and Luo, 2009; Lindenbach and Rice, 2013; Merz et al., 2011; Meunier et al., 2008). There are currently two models of how this viral particle associates with the host cell lipid droplets in the serum. The secreted lipoviral particles include the virion, as well as some – but not necessarily all – components of VLDLs, but it is not yet clear whether the viral particle is encased in the lipoprotein(s) or whether it is merely associated with the outer surface of the VLDL (Catanese et al., 2013; Gastaminza et al., 2010; Lindenbach and Rice, 2013; Merz et al., 2011).

### *1.1.3 Treatment and Prevention of Hepatitis C Virus Infection*

#### *1.1.3.1 Epidemiology and Transmission*

Hepatitis C virus infects an estimated 150 million people world-wide, and over 250,000 people in Canada (Myers et al., 2014; WHO, 2012). Many who are infected are unaware of their status; the virus is spread by blood-to-blood contact (parenteral exposure), which can include exposure to improperly sterilized medical equipment, contaminated blood or blood products, sexual contact, or sharing needles and other drug paraphernalia (Denniston et al., 2012; Ghany et al., 2009). In



Canada, the major populations at-risk for acquiring or harbouring the virus are people who inject drugs, adults born between 1945 and 1975, and immigrants from countries with high HCV prevalence (Cornberg et al., 2011; Grebely et al., 2013; Myers et al., 2014; Ward, 2013). World-wide, there are an estimated 3-4 million new infections with HCV every year (Mohd Hanafiah et al., 2013).

### *1.1.3.2 Clinical Infection*

Infection with HCV is largely asymptomatic; although acute infection may initially present with non-specific flu-like symptoms (fever, malaise, aches, *etc.*), cases of fulminant, symptomatic hepatitis from infection with HCV are very rare (Thomas, 2013; Thomas and Seeff, 2005). The acute phase of infection can last up to 6 months, and a minority of patients (approximately 20%) can spontaneously clear the infection during this time (Cox et al., 2005). Thereafter, the infection becomes chronic, which can last up to three decades; spontaneous clearance during the chronic phase is less common (Cox et al., 2005; Thomas and Seeff, 2005). Chronic infection can lead to a variety of clinical sequelae: liver-related outcomes include steatosis, fibrosis and cirrhosis, decompensated liver disease and failure, and hepatocellular carcinoma (Thomas, 2013; Zampino et al., 2013). Other effects of a chronic HCV infection include metabolic disease; mixed cryoglobulinemia and other lymphoproliferative disorders; glomerulonephritis; autoimmune sequelae such as hypothyroidism and rheumatic disorders; cardiovascular diseases; and a host of neuropsychiatric symptoms (Zampino et al., 2013; Zignego et al., 2012).

### *1.1.3.3 Treatment*

All genotypes of Hepatitis C virus can be treated with a combination therapy of the general antiviral drug ribavirin (Rbv) and pegylated interferon alpha or beta (peg-IFN $\alpha$  or  $\beta$ ); the peg-IFN is delivered by weekly injection, and Rbv is taken orally twice per day, with therapy lasting from 24 to 48 weeks, depending on viral genotype and host factors – including change in viral load at defined points during therapy (Sherman et al., 2007). This treatment, however, has varying effectiveness depending on viral genotype and host genetics; it can also have severe side-effects that reduce patient adherence to treatment, particularly since the therapy (and side-effects) can last for nearly a year (Lieveld et al., 2013). Because genotype 1 infections are more prevalent in developed nations than other genotypes, and because genotype 1 infections respond relatively

poorly to peg-IFN/Rbv therapy (approximately 50-60% effective), initial direct-acting anti-viral (DAA) drug development has targeted genotype 1 infections. In 2011, two protease inhibitors (boceprevir and telaprevir) were approved for use in combination with peg-IFN/Rbv therapy to treat genotype 1 infections, increasing response to treatment in naïve patients by 30% and in previous non-responders by up to 60% (Conteduca et al., 2014; Myers et al., 2012). Additionally, a third protease inhibitor (simeprevir) was approved in 2013 for use in genotype 1, 2, and 4 infections, in combination with peg-IFN/Rbv (Conteduca et al., 2014). These drugs all select for pre-existing resistance mutations, however, which is why they must still all be used as part of combination therapy (deLemos and Chung, 2014). Unfortunately, these protease inhibitors also come with their own host of side-effects that can lead to cessation of treatment with these DAAs.

Because many of the viral proteins differ widely between genotypes, early-generation DAAs target a limited range of viral genotypes. However, a fourth DAA – sofosbuvir – was approved in late 2013 that is both pan-genotypic (approved for use in genotypes 1-4), and can be used with only ribavirin in patients with genotype 2 (94% effective) and genotype 3 (62%) infections when interferon is contraindicated (Conteduca et al., 2014; deLemos and Chung, 2014; Lawitz et al., 2013). Sofosbuvir targets the active site of the NS5b polymerase, which is much more conserved across viral genotypes, and is less able to generate resistance mutations (Asselah, 2014). It is also very effective when used in combination with peg-IFN/Rbv (Lawitz et al., 2013).

Future DAAs are anticipated to reduce side-effects while increasing effectiveness – both in treatment-naïve patients and in previous non-responders – and decreasing length and frequency of treatment (Conteduca et al., 2014; Grebely et al., 2013). When used in combination with other DAAs targeting viral proteins (NS3/4a, NS5a, and NS5b) and/or with drugs targeting essential host factors (miRNA-122, cyclophilin A, and TLR-7), new treatment regimens should no longer require use of either interferon or ribavirin, reducing many of the issues of treatment toxicity and patient adherence (Conteduca et al., 2014; deLemos and Chung, 2014; Feld, 2014).

#### *1.1.3.4 Prevention*

Hepatitis C virus infection is currently prevented by minimizing risk of exposure: avoiding needle-sharing, screening blood and blood products, properly sterilizing medical equipment, and not re-using needles and syringes (Thomas, 2013). Additionally, when exposure (such as a needle-stick injury in a healthcare provider) is expected but infection has not yet been detected, post-

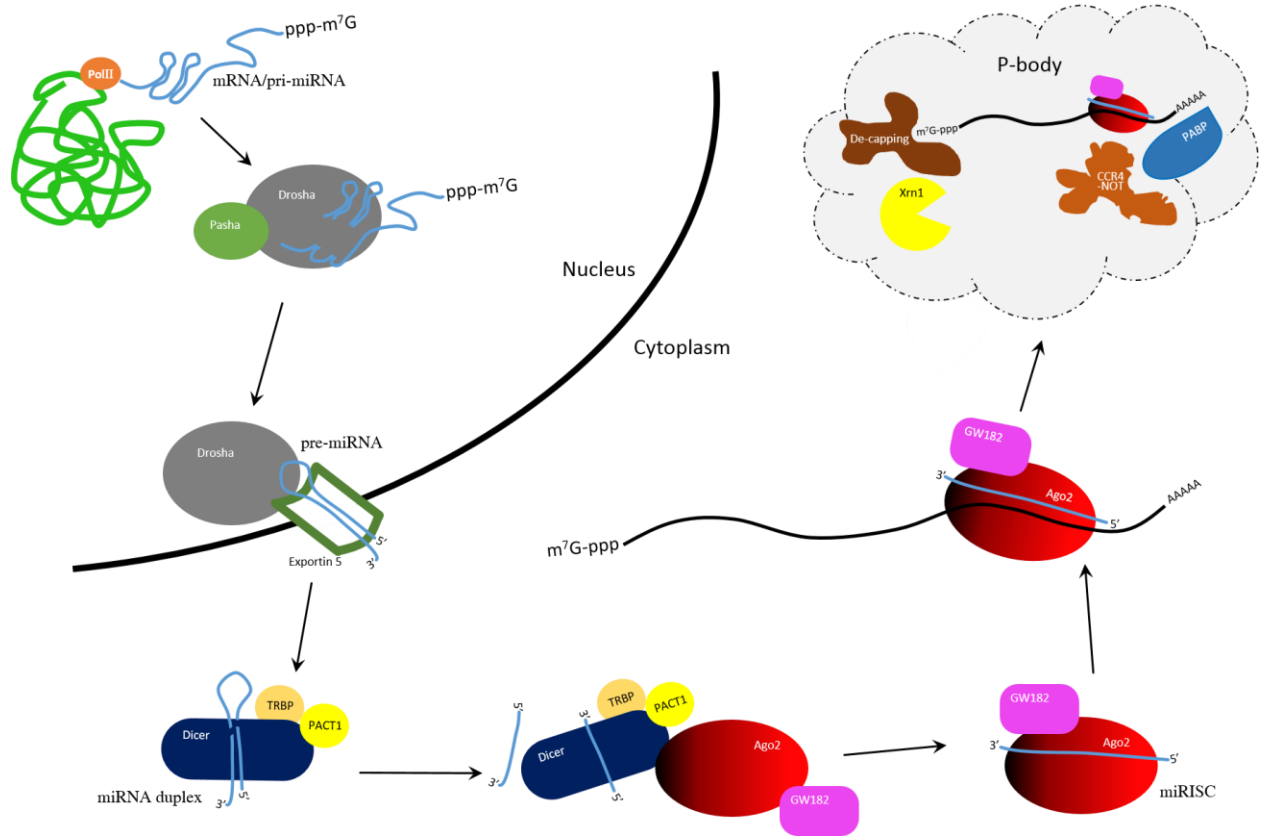
exposure prophylaxis with peg-IFN/Rbv can also be employed to prevent establishment of infection (Myers et al., 2012; Thomas, 2013).

Unlike Hepatitis A and B, no vaccine is available for Hepatitis C virus. Although patients and chimpanzees who spontaneously clear infection may retain some immunity to re-infection, this is not always the case, and what, precisely, defines their ability to resist re-infection is controversial (Fauvelle et al., 2013). Because of this, and because of the broad range of genotypes and antigenicity the vaccine needs to cover, developing a preventative vaccine is difficult (Shi and Ploss, 2013). Both neutralizing antibodies and broad-epitope CD4 and CD8 T-cell responses are expected to be important for a putative vaccine, but defining specific antigens and formulations to drive the desired responses remains challenging (Fauvelle et al., 2013). Some vaccines are currently in Phase I clinical trials to assess safety and immunogenicity; combined with *in vitro* testing, there is hope that these trials and others soon to follow will provide insight into an effective preventative HCV vaccine (Fauvelle et al., 2013; Shi and Ploss, 2013).

## **1.2 *microRNA-122***

### **1.2.1 *microRNA Pathway and Functions***

microRNAs (miRNAs) are short, double-stranded non-coding RNAs that post-transcriptionally regulate translation of cellular mRNAs (Sand, 2014). They play a regulatory role in essentially every cellular process, targeting an estimated 30-60% of the mRNAs encoded by the human genome (Friedman et al., 2009; Lewis et al., 2005). Mature miRNAs are between 17-23 nucleotides (nt) in length, with a 2-nt 3' overhang on each strand; the passenger strand of the miRNA is discarded, while the guide strand is loaded into an RNA-induced silencing complex (RISC) to target specific mRNAs for translational silencing (Sand, 2014). As depicted in Figure 1.1, pri-miRNAs are encoded in transcripts from RNA polymerase II (PolII) – meaning they are capped and poly-adenylated – of both coding and non-coding genes (Kim and Kim, 2007). The pri-miRNA hairpins are excised from the transcript in the nucleus by a microprocessor complex containing Drosha (an RNaseIII enzyme) and Pasha (DGCR8, an RNA binding protein implicated in regulating Drosha activity) (Gregory et al., 2004); the miRNAs can be encoded within both introns and exons, and in coding transcripts, pri-miRNA excision may occur prior to or during intron processing and still permit the full transcript to function as an mRNA afterwards (Kim and Kim, 2007). PolII can also produce poly-cistronic pri-miRNA transcripts, which are a series of



**Figure 1.1** miRNA processing pathway.

miRNA hairpins on the same dedicated transcript that are processed by Drosha into several different pre-miRNAs (Hammond, 2006; Sand, 2014).

The newly-processed pre-miRNA (typically a ~70nt hairpin with a characteristic 2-nt 3' overhang) is exported from the nucleus to the cytoplasm by Exportin-5, where it is collected by Dicer for further processing (Lee et al., 2002). Along with its co-factors TRBP and PACT-1, Dicer utilizes its RNaseIII activity to cleave the pre-miRNA into a mature miRNA of ~22 nt by removing the hairpin loop and leaving the dsRNA stem with both ends displaying a 2-nt 3' overhang (Kok et al., 2007). This complex of proteins then selects the guide strand of the miRNA (based on activity of the co-factors and thermodynamic stability of the strands' 5' end) to load into the RISC and produce the miRISC, discarding the passenger strand (often denoted with an asterisk, *e.g.* miR-199a\*) (Ambros et al., 2003; Noland and Doudna, 2013). The minimal functional components of the miRISC are considered to be the guide strand of the miRNA and an Argonaute (Ago1, 2, 3, or 4) protein, but TNRC6A (GW182) has recently been implicated as essential to RISC function as well, by mediating interactions between the RISC and the downstream silencing machinery (Carthew and Sontheimer, 2009; Pfaff and Meister, 2013; Takahashi et al., 2014). Dicer and its binding partners, as well as several other proteins have been associated with the RISC as well, although they may not be critical for the complex's ability to silence RNA translation (Carthew and Sontheimer, 2009; Takahashi et al., 2014). One of the Argonaute proteins cradles the guide strand of the miRNA and uses its seed sequence (the first 7-9 nt of the guide strand) to target mRNAs for translational suppression by imperfect sequence complementarity to the target sequence on the mRNA (Carthew and Sontheimer, 2009; Sashital and Doudna, 2010). GW182 mediates the interaction between the mRNA-RISC complex and components such as poly-A binding protein (PABP1) and the CCR4-NOT complex in a cytoplasmic processing body (P-body), where the mRNA can be either stored or degraded (Fabian et al., 2010; Liu et al., 2005; Pfaff and Meister, 2013; Zekri et al., 2013). P-bodies have a variable composition that includes proteins that sequester and store mRNAs for future translation, as well as components of several different mRNA degradation pathways such as Xrn1 that permanently remove the mRNA from translation (Decker and Parker, 2012).

miRNAs typically bind the 3' un-translated region of their target mRNAs and lead to translational suppression and/or degradation as described, but they can also function by binding elsewhere on the mRNA, and there is evidence that in some cases, miRNAs can cause increased

translation instead of translational suppression when the cell undergoes certain stresses (Fabian et al., 2010; Ørom et al., 2008; Vasudevan et al., 2007). There are also alternative pathways for biogenesis of certain miRNAs that bypass different steps in canonical biogenesis, and some of these may come into play when the cell is under particular stresses, but the vast majority of miRNAs identified so far are produced in this canonical manner (Yang and Lai, 2011; Yeo and Chong, 2011).

### *1.2.2 Functions of microRNA-122 in the Host*

Human hsa-miR-122 is produced in a canonical manner by PolII from the non-coding *hcr* gene on chromosome 18 (Chang et al., 2004; Jopling, 2012; Li et al., 2011b). microRNA-122 (miR-122) levels are highest in the liver, representing approximately 70% of its total small RNA transcripts, but expression has also been detected in nervous, respiratory, and hematopoietic tissues (Chang et al., 2004; Landgraf et al., 2007). Transcription of miR-122 appears to be driven by several liver-enriched transcription factors, including hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ), HNF1A, HNF3A and HNF3B, and to a lesser extent HNF4G and HNF6 (Coulouarn et al., 2009; Li et al., 2011b; Xu et al., 2010). Peroxisome proliferator activated receptor-gamma (PPAR $\gamma$ ) and retinoid X receptor alpha (RXR $\alpha$ ) can activate transcription of miR-122, while the circadian rhythm regulator REV-ERB $\alpha$  suppresses transcription at specific times in the cell's "day" (Gatfield et al., 2009; Song et al., 2013). Like many miRNAs, miR-122 is believed to play a role in embryonic development by driving differentiation of hepatocytes (Chang et al., 2004). CUTL1, a repressor of genes required for hepatocyte differentiation, is targeted for post-transcriptional suppression by miR-122 (Xu et al., 2010). Additionally, in a positive-feedback loop, HNF6 promotes transcription of miR-122, which in turn enhances expression of HNF6 and the other liver-enriched transcription factors to stimulate terminal differentiation of hepatocytes (Kymrzi et al., 2006; Laudadio et al., 2012).

In the mature liver, miR-122 is a regulator of lipid metabolism, and in mice, chimpanzees, and humans, antagonism of miR-122 leads to a reduction in serum cholesterol (Elmen et al., 2008; Janssen et al., 2013; Lanford et al., 2010). Use of microarrays has shown that antagonism of miR-122 causes down-regulation of mRNAs for major players in cholesterol synthesis, fatty acid synthesis, and lipid metabolism, suggesting that miR-122 targets and down-regulates one or more transcriptional suppressors of lipid metabolism (Esau et al., 2006; Norman and Sarnow, 2010).

Two of the known direct targets for miR-122 are involved in lipid metabolism: AGPAT1 stimulates the conversion of fatty acids into triglycerides (Hsu et al., 2012; Ruan and Pownall, 2001), while CIDEC is differentially expressed during fasting and is associated with lipid droplets and regulates triglyceride storage and fatty acid oxidation (Hsu et al., 2012; Keller et al., 2008; Vila-Brau et al., 2013).

miR-122 is also known to be involved in maintaining liver homeostasis. Cationic amino acid transporter-1 (*CAT-1*) was the earliest discovered mRNA to directly interact with miR-122: this interaction functions to regulate liver homeostasis by preventing degradation of plasma arginine under most conditions, and *CAT-1* is only relieved from miR-122-mediated suppression when the hepatocyte requires an influx of lysine and arginine for increased protein synthesis (Bhattacharyya et al., 2006; Chang et al., 2004). miR-122 depletion has been shown to cause iron deficiency: miR-122 directly binds and suppresses both the hemochromatosis (*Hfe*) and hemojuvelin (*Hjuv*) mRNAs; these proteins activate hepcidin and result in degradation of an iron efflux channel (ferroportin), and when this is suppressed by miR-122 more iron can be released from storage into the body (Castoldi and Muckenthaler, 2012; Castoldi et al., 2011). Precursor miR-122 (pre-miR-122 and pri-miR-122) levels fluctuate with the circadian rhythms of the cell, and although mature miR-122 levels do not vary significantly, this still affects downstream targets of the miRNA (Gatfield et al., 2009). Interestingly, some of the direct miR-122 binding targets identified when analyzing the circadian pattern of miR-122 levels included Nocturnin, Smarcd1/Baf60a, and PPAR $\beta/\delta$ , which are nuclear proteins that are also implicated in regulating lipid metabolism (Gatfield et al., 2009; Kojima et al., 2010).

Finally, miR-122 has been identified as a tumour suppressor. Most recently, miR-122 knockout mice were generated independently by two different research groups who both observed a drastic increase in liver cancer in these mice (Hsu et al., 2012; Tsai et al., 2012). Genes pinpointed in these mice, combined with previous research suggesting a role for miR-122 in reducing cellular proliferation and limiting fibrosis, and the observation that miR-122 expression is reduced or absent in many cases of liver cancer, has led to a view of miR-122 as a key player in suppressing development of liver cancer (Bai et al., 2009; Coulouarn et al., 2009; Fan et al., 2011; Fornari et al., 2009; Gao et al., 2009; Gramantieri et al., 2008; Hsu et al., 2012; Jung et al., 2011; Kutay et al., 2006; Li et al., 2013b; Tsai et al., 2009; Tsai et al., 2012; Xu et al., 2012). Both cell cycle and growth dysregulation, and regulation (or de-regulation) of the immune response are associated

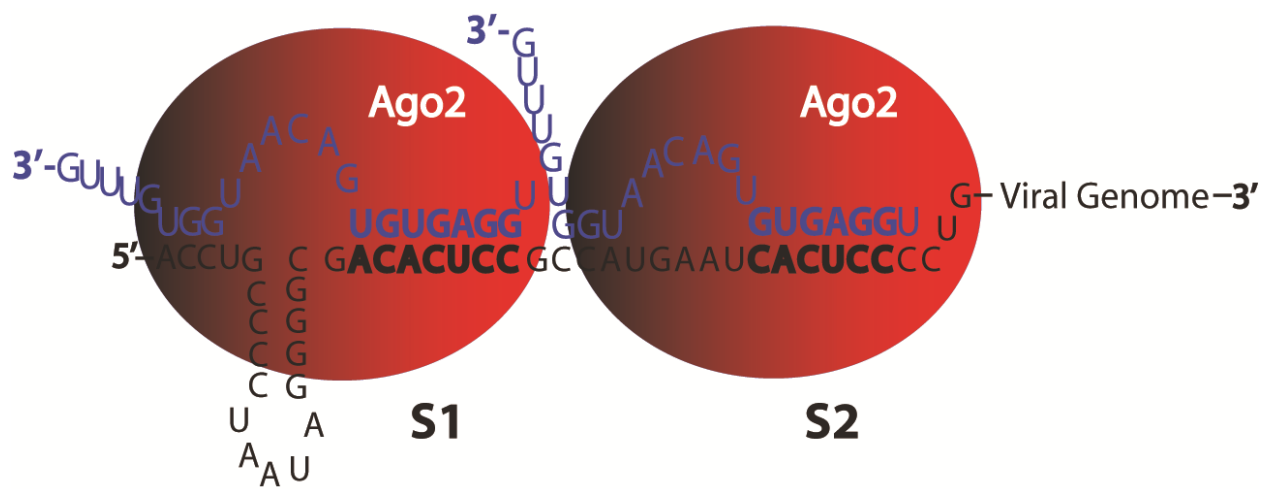
with carcinogenesis, and miR-122 is implicated in both processes. (Li et al., 2013a; Pedersen et al., 2007; Qian et al., 2010). As examples, levels of mature miR-122 fluctuate in the cell throughout cell cycle progression (from high levels during G<sub>0</sub> to low levels during S-phase), and miR-122 can promote Type I interferon production by suppressing SOCS1, “suppressor of cytokine signaling-1” (Fehr et al., 2012; Li et al., 2013a).

Interestingly, restoration of miR-122 expression to hepatocellular carcinoma cells also increases their sensitivity to chemotherapeutics such as doxorubicin, adriamycin, and vincristine; this was also marked by miR-122-mediated reduction of cell cycle progression proteins, aiding in induction of cell cycle arrest (Fornari et al., 2009; Xu et al., 2011). miR-122 is believed to aid many chemotherapeutic agents in hepatocellular carcinoma by down-regulating the unfolded protein response, permitting therapeutic apoptosis that would otherwise be suppressed in cancer cells (Yang et al., 2011). The level of miR-122 in human serum is also being explored as a potential biomarker to detect hepatocellular carcinoma development and progression, as well as an indicator of other sources of liver damage (Bala et al., 2012; Bihrer et al., 2011; Cermelli et al., 2011; Chen et al., 2012; Ding et al., 2012; Farid et al., 2012; Hu et al., 2013; Laterza et al., 2009; Laterza et al., 2013; Starkey Lewis et al., 2011; van der Meer et al., 2013; Waidmann et al., 2012; Zhang et al., 2012).

### *1.2.3 Role of microRNA-122 in the Hepatitis C Virus Life Cycle*

miR-122 has a unique relationship with Hepatitis C virus. By interacting with two binding sites in the 5' UTR (Figure 1.2) of the viral RNA, it positively impacts viral RNA stability, translation, and RNA accumulation, which is entirely different from canonical miRNA activity. Furthermore, the interaction between HCV and miR-122 is unique in the known world of virus-host interactions, as well. Some viruses encode their own miRNAs (primarily DNA viruses) to directly manipulate host mRNAs, or produce decoy transcripts to “soak up” cellular miRNAs, while other viruses take a less-direct approach of inducing or suppressing select miRNAs to modify the host cell environment, and still other viruses take a broad-spectrum approach of hindering or altering global miRNA biogenesis by targeting pathway proteins like Dicer or Argonaute (Carnero et al., 2011; Green et al., 2014; Guo and Steitz, 2014; Haasnoot and Berkhout, 2011; Roby et al., 2014). In these instances, the purpose of the interaction between viral proteins or transcripts and miRNA(s) appears to have been to achieve the ideal host cell environment. In contrast, the direct interaction





**Figure 1.2 miR-122 binding at the 5' UTR of the Hepatitis C virus genome.** miRNA-122 (blue) requires Ago2 (red) to facilitate its binding at two sites (S1 and S2) on the HCV 5' UTR (black).

of the HCV genome with miR-122 has been deemed essential for efficient HCV RNA accumulation, and is considered a major determinant of the virus' liver tropism. miR-122 was first identified as binding via 7 nt of its seed sequence to one site on the HCV 5' UTR (now known as Site 1 or S1), 21 nt from the 5' end of the viral RNA and just upstream of the virus' IRES (Jopling et al., 2005). miR-122 was not originally detected to have any impact on translation, but did positively modulate accumulation of the viral RNA, as detected by northern blot. Abolition of its binding reduced RNA accumulation by 80% using an antagonist of cellular miR-122, or rendered it undetectable by mutating a single nucleotide in the predicted miR-122 binding sequence, and RNA accumulation was restored to wild-type levels in the binding site mutants by providing a synthetic miR-122 with the matching mutation that reinstated binding (Jopling et al., 2005). This mutational analysis also verified that it was a direct effect of miR-122 binding, rather than an indirect effect that miR-122 exerted on the cell. The same group later identified a second miR-122 binding site just a few nt 3' to the first, designated Site 2 or S2, and again showed that binding at this second site was also critical for detectable HCV RNA accumulation by generating point mutations in the binding site(s) that abolished RNA accumulation, and rescuing the mutants by supplying synthetic miR-122 with the concomitant mutation (Jopling et al., 2008). These seminal papers determined that binding of miR-122 at both S1 and S2 was required for detectable HCV RNA accumulation. Since this discovery, additional research has shown that binding of miR-122 occurs outside the seed sequences as well, with the nucleotides 13-20 of miR-122 also interacting with the viral genome both between the two miR-122 binding sites, and also covering the very 5' nucleotides of the viral genome, depicted in Figure 1.2 (Machlin et al., 2011; Mortimer and Doudna, 2013; Shimakami et al., 2012b).

miR-122 has at least two other putative binding sites on the HCV genome, but these have not been as well-studied. Jopling *et al.* found a potential binding site in the 3' UTR of the HCV genome that was conserved among viral genotypes, but abolition of this sequence by mutagenesis had no observable effect on viral replication or translation, indicating that this site does not interact functionally with miR-122 (Henke et al., 2008; Jopling et al., 2005; Nasheri et al., 2011). However, miR-122 has also been shown to bind to a seed sequence in the NS5b coding region of the viral RNA, with potential interactions between 3' nucleotides on the miRNA and the genome similar to that found for binding at sites S1 and S2 (Nasheri et al., 2011). This interaction results in a reduction of HCV RNA accumulation – perhaps through translational suppression; such an

interaction has been proposed to reduce pathogenicity or immunogenicity of other chronic infections by reducing viral titres, but since this sequence is not conserved among all HCV genotypes, it is uncertain how large of a role it has in Hepatitis C virus infection (Mahajan et al., 2009; Nasheri et al., 2011).

Because the net effect of miR-122 on HCV RNA accumulation is contrary to typical miRNA functions, the precise mechanism(s) of this effect are uncertain. Further research comparing canonical miRNA functions to the effect of miR-122 on HCV has shown that the same biogenesis and RISC proteins involved in miRNA-mediated silencing are also involved in miR-122-mediated enhancement of HCV RNA accumulation. Particularly, Ago2 (and to a lesser extent, Ago1, 3, and 4), GW182, and TRBP have been shown to be involved in the effect of miR-122 on HCV RNA accumulation, while Dicer has been implicated in aiding HCV RNA accumulation through mediating miR-122 biogenesis (Bukong et al., 2013; Conrad et al., 2013; Roberts et al., 2011; Roberts et al., 2014; Shimakami et al., 2012a; Thibault et al., 2013; Wilson et al., 2011). P-body components have also been implicated in aiding HCV RNA accumulation, both negatively (Xrn1) and positively (DDX6, LSm1, PatL1, ATX2, PABP1), although not all have been shown to be associated with the effect of miR-122, and some – like DDX6 – have been shown to impact HCV replication independently of miR-122 (Ariumi et al., 2011a; Huys et al., 2013; Li et al., 2013d; Pager et al., 2013; Roberts et al., 2014; Scheller et al., 2009).

Since miRNAs are known to affect translation, other researchers revisited the possible effect of miR-122 on HCV IRES-driven translation, and found that miR-122 binding to the HCV genome did, in fact, increase the translation of the viral RNA by two to three-fold (Henke et al., 2008; Huys et al., 2013; Niepmann, 2009; Roberts et al., 2011; Wilson et al., 2011; Zhang et al., 2012). It was suggested that the sole function of miR-122 for the virus was to increase translation, resulting in a higher concentration of viral proteins that over time could increase replication efficiency and RNA accumulation, but evaluation of a viral translation mutant showed that the magnitude of the effect of increased translation could not account for the overall replication phenotype (Henke et al., 2008). This was later verified by showing that a protein (Lsm1) involved in mediating the effect of miR-122 on translation had no impact on the ability of miR-122 to increase replication, emphasizing separate functions (Roberts et al., 2014). More recently, it has been suggested that the increase in translation is merely an indirect effect of stabilization of the viral RNA, with the implication being that when miR-122 binds, less viral RNA is degraded, which results in more

viral RNA available for translation (Conrad et al., 2013; Li et al., 2013d; Mortimer and Doudna, 2013; Shimakami et al., 2012a; Shimakami et al., 2012b). The host 5' to 3' RNA exonuclease Xrn1 has been implicated in this destabilization and degradation of the viral RNA; although early reports using siRNA to knock down Xrn1 showed no effect on HCV RNA accumulation, more in-depth analysis has shown that abolishing Xrn1 does permit increased HCV RNA accumulation (Jones et al., 2010b; Li et al., 2013d; Mortimer and Doudna, 2013). Most recently, miR-122 has been shown to be the mediator of protection from degradation by Xrn1 (see also Chapter 7), and has been shown to have no effect on translation in the absence of Xrn1, confirming that the increase in translation observed by adding miR-122 was due to stabilization and protection of the viral RNA from Xrn1 (Li et al., 2013d). However, just as the effect of miR-122 on translation was insufficient to explain the overall impact of miR-122 on HCV RNA accumulation, knockdown of Xrn1 was not able to rescue HCV RNA accumulation in the absence of miR-122, showing that miR-122 must have an additional function in enhancing HCV RNA accumulation (see also Chapter 7) (Li et al., 2013d). There is an additional possibility, however; the substrate for Xrn1-mediated degradation is a 5' monophosphate, but the HCV genome requires a 5' tri-phosphate to replicate, which suggests that in order for the viral RNA to be susceptible to Xrn1 in the first place, it must first be modified by a host pyrophosphatase (Li et al., 2013c; Li et al., 2013d; Nagarajan et al., 2013). The possibility that miR-122 also protects the HCV genome from a pyrophosphatase remains to be explored.

miR-122 has also been suggested to act as a molecular switch for the viral RNA, where its binding defines whether the RNA is available for translation, or is instead being used as a template for RNA replication (Diaz-Toledano et al., 2009). Although structural studies suggest that miR-122 binding does change the secondary RNA structure of the 5' UTR – including the availability of the viral IRES – these proposed changes require additional sequences and structures in the core protein coding region of the genome (Diaz-Toledano et al., 2009; Mortimer and Doudna, 2013; Pang et al., 2012). However, sub-genomic HCV constructs respond to miR-122 binding similarly to full-length constructs, but they lack the core protein coding region suggested to be important in this switch, indicating that if miR-122 binding does act as a switch, this is not the mechanism involved (Thibault et al., 2013).

It is possible that miR-122 directly affects replication of the viral RNA. This is the most challenging role to examine, because unlike other RNA viruses like polio, HCV requires all the

replicase components to be expressed in *cis* from the very RNA that is to be replicated, rendering it very difficult to separate translation from replication *in vivo*. Thus far, there is little evidence for or against miR-122 participating in replication. Mortimer and Doudna noted that miR-122 could still bind and stabilize HCV RNA in *in vitro* assays when point mutations were introduced to the miRNA that abolished its ability to affect replication, suggesting that the binding requirements for its effect on replication may be different than those for its effect on stability (Mortimer and Doudna, 2013). More directly, Norman *et al.* evaluated the synthesis of new viral RNA when the cells were treated with a miR-122 antagonist, but did not see a reduction in RNA synthesis (in fact, they observed a slight *increase* in RNA synthesis), while Villanueva *et al.* isolated replication complexes from cell culture before adding or antagonizing miR-122 and observed no change (Norman and Sarnow, 2010; Villanueva *et al.*, 2010). With the replication complexes isolated from cells, the researchers could only evaluate the elongation phase of replication, and not initiation of the polymerase; however, they were also unable to detect miR-122 in these complexes, suggesting it may not be there at all (Villanueva *et al.*, 2010). Thus, although the current consensus is that the role for miR-122 is not solely due to its function in stabilizing the viral RNA, it is still unclear what other mechanism miR-122 has for enhancing HCV RNA accumulation.

#### 1.2.4 Other miRNAs Binding to the HCV Genome

At least four other miRNAs have been confirmed to interact with the Hepatitis C virus genome: miR-199a\*, miR-196, let-7b, and miR-448 (Conrad and Niepmann, 2014; Singaravelu *et al.*, 2014). Unlike the overall impact of miR-122 binding to the HCV genome, each of these miRNAs impacts HCV in a more canonical manner, repressing translation of the viral genome and ultimately resulting in reduced replication (Cheng *et al.*, 2012; Hou *et al.*, 2010; Murakami *et al.*, 2009; Pedersen *et al.*, 2007). These miRNAs may not only suppress translation but could also lead to cleavage and degradation of the viral genome; this was only investigated – and found to occur – with let-7b, and it is not known whether the others do so (Cheng *et al.*, 2012). Since HCV has an error-prone polymerase, each of these sites would have been lost very quickly if they were detrimental. This suggests either that these miRNAs do not impact the virus *in vivo*, or that their suppressive effect benefits the virus – again, perhaps by reducing viral titres and limiting pathogenicity and immunogenicity (Mahajan *et al.*, 2009). It is also important to note that knockdown of the proteins involved in miRNA-mediated translational suppression (such as Ago2)

has a net negative impact on HCV replication, indicating that the effect of these miRNAs on HCV RNA accumulation does not supersede the positive impact of miR-122 (Bukong et al., 2013; Conrad et al., 2013; Roberts et al., 2011; Shimakami et al., 2012a; Wilson et al., 2011).

#### 1.2.5 *miR-122 in Other Viral Infections*

miR-122 has been implicated to play a role in at least two other viral infections in humans; surprisingly, only one of these is a liver disease. Hepatitis B virus (HBV) is a small, partially double-stranded DNA virus that is transmitted by blood and sexual contact, and causes a chronic liver infection in about 5% of adult acute infections, although the risk is much higher in children and infants (Kuo and Gish, 2012). One of the potential complications of HBV infection is the risk of accidental insertion of the viral DNA into the host's genome, which could cause dysregulation of proliferation (for example) and lead to tumourigenesis (Kuo and Gish, 2012). Like an infection with Hepatitis C, HBV also causes long-term inflammation and damage that is associated with the development of liver cancer (Fallot et al., 2012; Kuo and Gish, 2012). Loss of miR-122 in Hepatitis B virus infection leads to increased viral replication; miR-122 directly suppresses CyclinG1 expression, which results in the release of the antiviral and anti-tumour protein p53 (Wang et al., 2012). Conversely, addition of miR-122 inhibited HBV replication, and was proposed to limit HBV-associated hepatocellular carcinoma by directly suppressing translation of NDRG3 (Fan et al., 2011). miR-122 has also been shown to directly bind to conserved HBV sequences, suppressing viral replication; in apparent response to this, the HBx viral protein inhibits miR-122 expression by binding PPAR $\gamma$ , a transcriptional activator of miR-122 (Chen et al., 2011; Song et al., 2013). In addition, HBV appears to overproduce the targeted transcript to act as a miR-122 sponge (Li et al., 2013b).

The interaction between miR-122 and Borna disease virus (BDV) is less well-studied. BDV is a neurotropic virus, but it has been shown to have miR-122-complementary sequences, and functional assays have determined that miR-122 exerts a direct suppressive effect on both viral replication and translation (Qian et al., 2010). miR-122 also appears to indirectly impact viral replication by enhancing interferon signaling (Qian et al., 2010).

Recently, several other *Hepaciviruses* have been discovered that bear putative miR-122 binding sites, and it is possible that the miRNA may play a key role in the replication of these viruses too, although no functional assays have been published to determine this (Burbelo et al.,

2012; Drexler et al., 2013; Kapoor et al., 2011; Kapoor et al., 2013; Lauck et al., 2013; Lyons et al., 2012). GB virus B is the closest relative to HCV that has an active culture system; it, too, bears two miR-122 binding sites in its 5' UTR, and binding at these sites also positively modulates RNA accumulation of sub-genomic constructs of this virus (Sagan et al., 2013). Unfortunately, not enough is known about the origins of GB virus B to provide an explanation of its dependence on miR-122 and relation to Hepatitis C virus. The miR-122 sequence is highly conserved among mammals; since miR-122 is expressed at highest levels in the liver, and to a much lesser degree in other tissues such as nervous tissue, determining the infected tissues and developing culture models for these newly-discovered *Hepaciviruses* – and any others identified in the future – may provide some answers about the origins of Hepatitis C virus, and some explanations for its dependence on miR-122 and hepatotropism in humans (Chang et al., 2004; Jopling et al., 2005).

### **1.3 Model Systems for Hepatitis C Virus**

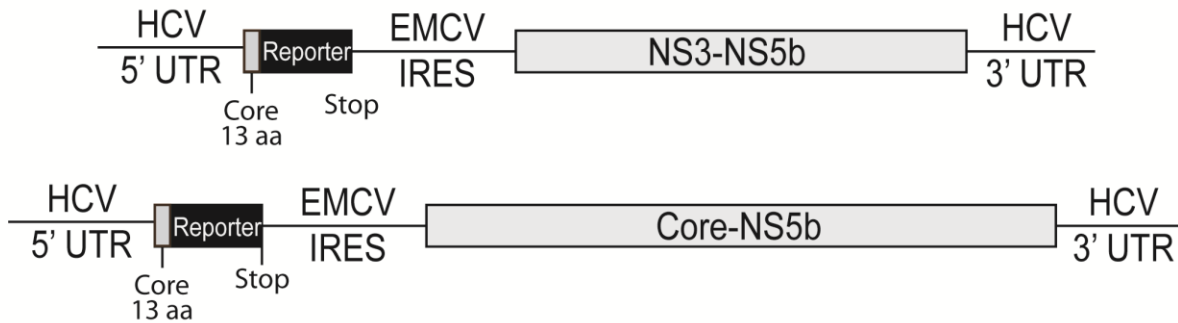
#### **1.3.1 Virus Constructs**

Early research in Hepatitis C virus was hindered by an inability to achieve the complete virus life cycle in a single system; workarounds included using bi-cistronic sub-genomic RNA constructs to assay replication and retroviral particles coated with HCV envelope proteins to study entry (Wang, 2013). In 2005, the first HCV isolate was identified that could complete the full viral life cycle, from entry and uncoating, translation and replication, and production of infectious virus particles, in a single system, and could further be used to infect animal models (Wakita et al., 2005). This genotype 2a isolate was known as JFH-1 (for Japanese fulminant hepatitis), and was later modified through adaptive mutations to increase titers, and then by replacing the structural proteins with those from the J6 isolate, another genotype 2a virus (Date et al., 2004; Lindenbach et al., 2005). Additional chimeras have since been developed using the structural proteins from other genotypes to evaluate particle production and entry, but the JFH-1 isolate has been most-heavily utilized as a base for evaluating the complete virus life cycle because it replicates to high levels in cell culture. Other isolates commonly used include the Con1 (Construct 1) and NC1 genotype 1b constructs, the H77 (Hutchinson strain 77) genotype 1a construct, and a new genotype 2a construct JFH-2 (Vieyres and Pietschmann, 2013). Although these isolates do not reach the same titers as the JFH-1-based constructs, they are still often used to understand genotype-specific aspects of the virus' life cycle and verify pan-genotypic findings.

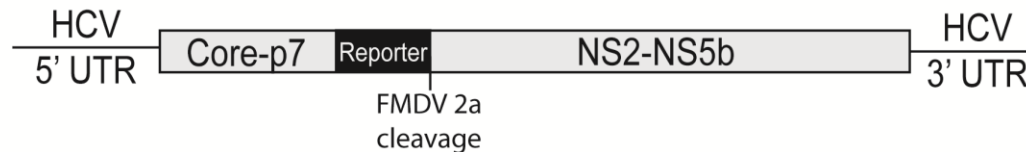
Many of the viral RNA constructs used in cell culture also bear reporter genes. There are three strategies for inserting a reporter gene in an HCV construct (depicted in Figure 1.3): the first is the generation of a bi-cistronic construct (Figure 1.3A), where the reporter is translated from the HCV IRES and has a functional stop codon, while the viral genome (either full-length or only the essential non-structural proteins) is translated from a second IRES – typically the encephalomyocarditis virus (EMCV) IRES (Lohmann et al., 1999; Sheehy et al., 2007; Vieyres and Pietschmann, 2013). This requires that the reporter gene have the first 39 nucleotides (13 amino acids) of the Core protein fused to its N terminus, since the HCV IRES cannot initiate translation without them; this also then separates the translation of the viral non-structural proteins from the control of the HCV5' UTR and IRES, which can be beneficial when attempting to separate translation and replication, but may introduce artefacts (Lohmann et al., 1999; Lu and Wimmer, 1996; Reynolds et al., 1995). The second strategy is to insert the reporter gene in frame with the virus polyprotein to produce a mono-cistronic construct (Figure 1.3B), utilizing the virus' existing cleavage sequences to separate the C terminus of the preceding viral protein from the N terminus of the reporter, and then append a FMDV 2a slippage site in place of a stop codon (Jones et al., 2007a). This ensures that translation of the viral polyprotein continues uninterrupted after the reporter, but the slippage site separates the C terminus of the reporter protein from the N terminus of the following viral protein. In the most-commonly-used J6/JFH-1 chimeric genome, reporter genes such as Renilla or firefly luciferase, Gaussian (secretory) luciferase, neomycin phosphotransferase, and fluorescent proteins have been inserted between p7 and NS2 (Jones et al., 2007a; Sheehy et al., 2007; Vieyres and Pietschmann, 2013). This is considered a less-artificial construct as it does not have the second IRES, but both the bi- and mono-cistronic constructs containing reporter genes are necessarily longer than the un-manipulated genome, which could affect replication efficiency. Finally, it is possible to insert a reporter or tag within the non-structural coding region of either mono- or bi-cistronic constructs in the C-terminus of the NS5a coding region while maintaining the endogenous protease cleavage site between NS5a and NS5b; this has been particularly used to tag NS5a with green fluorescent protein or an immunological tag for intracellular tracking and pulldowns (Figure 1.3C) (Jones et al., 2007b; McCormick et al., 2006; Moradpour et al., 2004; Wu et al., 2011).



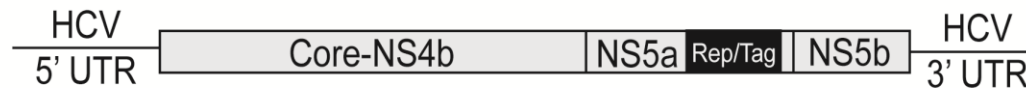
**A** Reporter - separate ORF (bi-cistronic)



**B** Reporter in-frame (mono-cistronic)



**C** Reporter or tag fusion (mono-cistronic)



**Figure 1.3 HCV constructs containing reporter genes.**

**A.** General structure of HCV replicons containing a reporter gene or selection marker in the bi-cistronic arrangement. Boxes represent open reading frames, while linear sections represent non-coding RNA sequences. Top: Sub-genomic construct lacking structural genes. Bottom: Full-genomic construct with all viral genes. Note that any gene product translated from the HCV IRES requires the first 39 nucleotides (13 amino acids) of the Core coding sequence for the IRES to function. **B.** General structure of HCV constructs containing excisable in-frame reporter gene or selection marker. The stop codon of the reporter is removed, and replaced with the short FMDV2a cleavage sequence that allows the reporter peptide to be cleaved from the following viral protein (e.g. NS2) without interrupting translation of the viral protein. **C.** General structure of HCV constructs with a reporter or tag within the non-structural proteins. The C-terminus of NS5a accommodates insertion without significant impairment of viral replication.

### 1.3.2 *Cell Culture Models*

The first cell culture models for propagation of Hepatitis C virus made use of freshly-isolated human cells such as primary human hepatocytes, as standard lab cell lines were not amenable to HCV infection or replication – due in part, we now know, to their lack of miR-122 expression (Sheehy et al., 2007; Vieyres and Pietschmann, 2013). Huh7 was the first cell line identified to permit HCV replication and quickly became the key system for studying the HCV life cycle. Although culture methods for primary human hepatocytes have improved, rendering them useful for the study of serum-derived HCV, Huh7-derived cells hold an advantage over primary cell systems because they can be readily propagated and are relatively consistent from one experiment to the next (Lohmann and Bartenschlager, 2013; Lohmann et al., 2003; Lohmann et al., 1999; Molina et al., 2008; Nakabayashi et al., 1982). These cells are a polyclonal human hepatoma cell line, and are one of the few hepatoma cell lines that retained some miR-122 expression upon transformation and immortalization, which is likely why they were readily permissive to HCV replication when so many other cell lines tested were not (Jopling et al., 2005; Thibault et al., 2013; Varnholt et al., 2008). Since then, Huh7 cells have been selected and manipulated to a variety of ends by different research groups to analyze different aspects of the viral life cycle, whether selected for better permissiveness to HCV by maintenance of viral genomes expressing a neomycin resistance gene, or transduced by lentiviral vector to express a gene or shRNA to abolish expression of a particular gene (Jopling et al., 2005; Vieyres and Pietschmann, 2013). For example, Huh7.5 cells are a derivative of Huh7 cells generated by using a viral construct containing a neomycin resistance gene; cells were selected with G418 (an antibiotic antagonized by the neomycin resistance gene), such that those with greater permissiveness for replication would have greater resistance to G418 treatment (Blight et al., 2002). After several rounds of selection, clones were isolated and cured of the viral RNA by prolonged interferon treatment – much like a patient. The selected Huh7.5 clone was more permissive to HCV replication than the parent Huh7 cell line (Blight et al., 2002). In addition, a Huh7-derived reporter cell line was generated by selecting for lentiviral transduction with a construct bearing a fluorescent reporter tagged with a nuclear localization sequence and an NS3/4a cleavage site also found on the cellular IPS-1 protein (Jones et al., 2010a). When these cells are infected with HCV, NS3/4a cleaves the reporter construct, revealing the nuclear localization sequence and allowing the fluorescent marker to translocate from

the mitochondrial membrane to the nucleus; this translocation is the indicator of infection and can be read independent of active replication (Jones et al., 2010a).

Huh7 cells and their derivatives originate from a 57-year-old Japanese man with hepatocellular carcinoma; recent publications have shown that other human hepatoma cell lines with diverse genetic backgrounds can be made to permit the complete virus life cycle as well, such as HepG2, Hep3B, and PLC/PRF-5 cells (Aden et al., 1979; Alexander et al., 1976; Date et al., 2004; Kambara et al., 2012; MacNab et al., 1976; Narbus et al., 2011; Sainz et al., 2012b; Thibault et al., 2013; Watashi et al., 2007; Windisch et al., 2005; Zhu et al., 2007). In particular, addition of miR-122 to HepG2 or Hep3B cells was necessary or aided in permitting efficient replication of Hepatitis C virus RNA (Kambara et al., 2012; Narbus et al., 2011; Thibault et al., 2013). Hep3B and HepG2 cell lines transiently or stably expressing miR-122 have been touted as especially useful new culture systems because they can be polarized; since several of the HCV entry factors are tight junction proteins, fully understanding the entry process requires polarized cells (Mee et al., 2009; Narbus et al., 2011; Vieyres and Pietschmann, 2013; Zigmond et al., 2008).

In addition to this, since the identification of miR-122 as a key requirement for HCV RNA replication, many others have explored addition of miR-122 to non-liver and non-human cell lines in an attempt to develop more diverse models for the study of HCV. Non-liver human cell lines have been shown to permit low levels of HCV RNA replication with heavy selection and/or when miR-122 is supplemented: HeLa cells, HEK-293 cells, FU97 cells, and neuroepithelioma cells (Ali et al., 2004; Da Costa et al., 2012; Fletcher et al., 2010; Kato et al., 2005b; Shiokawa et al., 2014; Zhu et al., 2003). A wide array of murine liver and fibroblast cell lines have also been employed – with or without addition of miR-122 – in an attempt to study host requirements and restriction factors, and to develop a small animal model for the study of the HCV life cycle (see also Chapter 3) (Chang et al., 2006; Frentzen et al., 2011; Frentzen et al., 2013; Lin et al., 2010; Long et al., 2011; Thibault and Wilson, 2014; Uprichard et al., 2006; Vogt et al., 2013; Yeh et al., 2008; Zhu et al., 2003). Particularly, innate immune knockout mouse embryonic fibroblasts (MEFs) such as IRF-3 knockouts, or NCoA6 knockout MEFs – a gene in liver-X receptor signaling, unrelated to innate immune signaling – have been rendered permissive for transient sub-genomic HCV RNA replication upon supplementation with miR-122 (Lin et al., 2010; Thibault and Wilson, 2014). Since mammalian liver cells – such as the IPS-1, IRF-3, and IFNAR (interferon- $\alpha/\beta$  receptor) knockout mouse liver cells used by Frentzen *et al.* – have mature miR-122 that is identical to that

found in humans, mouse liver cell lines do not require supplementation with miR-122 unless transformation or immortalization results in loss of its expression (Chang et al., 2004; Frentzen et al., 2013).

Primary cell lines, human embryonic stem cells and induced pluripotent stem cells, liver slices and explants, and PBMCs still have important roles to play in our understanding of HCV infection (Lagaye et al., 2012; Molina et al., 2008; Roelandt et al., 2012; Schwartz et al., 2012; Vieyres and Pietschmann, 2013; Wu et al., 2012). They act as the bridge between the *in vitro* cell culture models and the *in vivo* animal models in understanding the pathology of the virus, as well as its molecular virology. For example, virus particles produced from cell lines discussed above do not have the same density profile nor lipoprotein content as serum-derived HCV particles, indicating that while secretion from these cells produces infectious virus particles, they are imperfect models for *in vivo* particle production (Lindenbach et al., 2006; Molina et al., 2008; Podevin et al., 2010; Vieyres and Pietschmann, 2013). In addition to this, more sophisticated culture methods have been employed to mimic the complete liver environment, including 3D culture, micropatterned co-cultures, and organoid cultures of both Huh7-derived cell lines and primary hepatocytes (Aly et al., 2009; Lohmann and Bartenschlager, 2013; Molina-Jimenez et al., 2012; Ploss et al., 2010; Sainz et al., 2009). As might be expected, with some of these systems there are trade-offs in terms of viral RNA production and titer, but these *ex vivo* models provide another system to develop new avenues of research, as well as to verify or refute findings in cell culture that could otherwise prove to be artifactual (Steinmann and Pietschmann, 2013; Vieyres and Pietschmann, 2013).

### 1.3.3 Animal Model Systems

Chimpanzees were the first animal model to permit HCV infection, and were in fact instrumental to the discovery of the causative agent of non-A non-B Hepatitis (Choo et al., 1989; Houghton, 2009). To date, they are still the only demonstrated immunocompetent animal model for HCV infection for that analysis of the adaptive immune response and vaccine development (Bukh, 2012; Park and Rehermann, 2014). However, chimpanzees imperfectly mimic the disease course of HCV in humans, as a much smaller percentage of them progress from acute infection to chronic infection, and they do not show the fibrosis or cirrhosis observed in chronically-infected humans (Lanford et al., 2001). Additionally, chimpanzee research has always been expensive and fraught with ethical concerns, and most recently, it has all but been halted in most of the world due

to the ethical ramifications of using an endangered species for biomedical research (Harrington, 2012; Maily et al., 2013; Wadman, 2013).

Much effort has gone into the development of a small animal model for HCV, particularly a mouse model because of the availability of genetic knockouts. One of the most successful models has been the human liver xenograft SCID (severe combined immune-deficient)-uPA mouse: this mouse is deficient in both B- and T-cells and therefore lacks an adaptive immune system, and is also transgenic for the urokinase-type plasminogen activator that caused degeneration of the mouse's hepatocytes. This mouse is then implanted with human liver cells – because of its lack of an adaptive immune system, it cannot reject the xenograft – that take over the function of the mouse's liver and can be infected with Hepatitis C virus (Mercer et al., 2001b). This mouse model, and another similar to it where mouse hepatocyte death is held off by a drug treatment until the time of xenografting, supports efficient infection with cell-culture and patient-derived viruses and demonstrates viremia similar to that in humans (Bissig et al., 2010; Mercer et al., 2001b). These mice have been valuable for the study of direct-acting antivirals and innate immune responses to the virus, along with the various stages of the virus' life cycle *in vitro*; their major disadvantages are the heterogeneity of the human liver samples, and their lack of adaptive immune system that precludes the study of immunopathogenesis (Bukh, 2012; Meuleman and Leroux-Roels, 2008; Sandmann and Ploss, 2013).

Other small animal models have been developed in an attempt to overcome these disadvantages: for example, the AFC7/huHSC-hep mice lack their own immune system, but are partially reconstituted with a human immune system (human hematopoietic stem cells), and human hepatocyte progenitors (Washburn et al., 2011). These mice do not produce detectable viremia, as human hepatocyte engraftment efficiency is quite low, but HCV infection has been confirmed, and these mice do reproduce cell-mediated responses, along with hepatitis and fibrosis associated with immunopathology of infection (Bukh, 2012; Washburn et al., 2011). Another interesting small animal model, the tree shrew (*Tupaia belangeri*), also mimics much of the pathogenesis of infection, including progression to chronicity, fibrosis and cirrhosis, and hepatocellular carcinoma (Amako et al., 2010; Bukh, 2012; Sandmann and Ploss, 2013). Viremia is low but detectable, and the animal is fully immunocompetent. However, immune responses and effects of antiviral therapy have not yet been examined in this model to validate it for all aspects of infection (Amako et al., 2010; Xu et al., 2007).

Since the finding that mouse liver cells could permit replication of HCV RNA, a major remaining hurdle for an all-mouse model of HCV infection was the inability of the virus to use murine receptors – namely, CD81 and Occludin (Dorner et al., 2011; Frentzen et al., 2011; Frentzen et al., 2013; Ploss et al., 2009). Dorner *et al.* verified this by infecting mice with adenovirus expression vectors for CD81 and Occludin, and then infecting the mice with HCV (Dorner et al., 2011). While replication could not be detected in these mice, infection (that is, entry, uncoating, and translation) was detected using a reporter as described for Huh7 cells above, where infection causes NS3/4a-mediated cleavage of the IPS-1 sequence and translocation of fluorescence to the nucleus (Dorner et al., 2011). Transduction efficiency of liver cells with all receptors in the adenovirus-infected mice was very inefficient, and the adenovirus vectors themselves induced interferon responses that could have inhibited Hepatitis C virus, so these researchers then generated mice transgenic for the required entry receptors and verified their increased permissiveness for entry (Dorner et al., 2013b). By crossing these transgenic mice with Stat1<sup>-/-</sup> mice, they could be rendered susceptible to the complete virus life cycle, and infection was responsive to treatment with direct-acting antivirals (Dorner et al., 2013a). Although viral titers were very low in these mice, the infection persisted for 90 days in most mice before becoming undetectable; it is not yet clear whether the mice mounted a successful immune response to the virus and cleared it, or whether they are still deficient in some human factor the virus requires for chronic infection. These mice still have an adaptive immune system, but because the complete virus life cycle was best achieved by crossing with innate immune knockout mice, it is not yet known if these mice will truly recapitulate the immune responses and immunopathology of Hepatitis C virus infection in humans (Dorner et al., 2013a).

#### **1.4 Conclusions**

Systems to study the infection and life cycle of Hepatitis C virus have improved drastically in the last 10 years, and with them have come valuable advances in understanding virus-host interactions and the development of direct-acting antiviral drugs and early attempts at vaccines, as described above. These same drugs and vaccines also offer hope for eventual eradication of Hepatitis C virus infection. The identification of miR-122 as a required host factor for HCV replication has aided in development of both new cell culture systems and antiviral therapies, and has opened up a new avenue of investigation into the way a virus can hijack host pathways for its

own ends. Understanding the various functions of miR-122 in the HCV life cycle will provide more information on the relatively-unclear regulation and process of HCV RNA replication, and may also provide additional insight into mechanisms of miRNA functions in eukaryotic cells. The link between miR-122 and cancer may also be exploitable to understand HCV-associated hepatocellular carcinoma and prevention of carcinogenesis in chronically infected patients. As new relatives to Hepatitis C virus are discovered, the maintenance of one or both miR-122 binding sites may also provide clues as to the origin of HCV and other Hepaciviruses and their spread in their respective hosts.

## **2.0 Hypotheses and Objectives**

### **2.1 Rationale and Hypothesis**

microRNA-122 has been deemed crucial to the Hepatitis C virus life cycle through its binding to two sites on the 5' UTR of the viral RNA, which has a drastic positive impact on HCV RNA accumulation. miR-122 is highly abundant in the liver, and found only at much lower levels in other body tissues, and has been implicated as a major determinant in Hepatitis C virus' liver tropism. In addition, there are limited cell culture and animal model systems that permit the full Hepatitis C virus life cycle or infection. Thus, we set out to explore the possibility that we could exploit miR-122 to expand the tropism of HCV and broaden the repertoire of cell culture systems to study Hepatitis C virus. We further aimed to use our new system(s) to better understand the mechanism of the effect of miR-122 on the Hepatitis C virus life cycle. We hypothesized that addition of miR-122 to non-permissive non-human and human cell lines would render them permissive for part or all of the HCV life cycle, and we further hypothesized that these cell lines would be useful for providing new insights into how miR-122 promotes the Hepatitis C virus life cycle.

### **2.2 Objectives**

- i. Supplement wild-type and HCV restriction factor (PKR and NCoA6) gene knockout murine embryonic fibroblasts (MEFs) with miR-122 and test for permissiveness to sub-genomic and full-length HCV replication.
- ii. Test hepatocytes from most-permissive knockout mice (PKR) for permissiveness to HCV replication.
- iii. Supplement Hep3B cells (a human hepatocellular carcinoma cell line) with miR-122 and test for permissiveness to sub-genomic and full-length HCV replication.

Based on results from experiments in Hep3B cells, additional objectives were defined:

- iv. Verify miR-122-independent replication of sub-genomic HCV RNA replication in Hep3B cells and Huh7.5 cells (Huh 7.5 cells are the widely-accepted model system to study the HCV life cycle).
- v. Examine the role of each miR-122 binding site in the known functions for miR-122 in the Hepatitis C virus life cycle.



### **3.0 Transient Replication of Hepatitis C Virus Sub-genomic RNA in Murine Cell Lines is Enabled by miR-122 and Varies with Cell Passage**

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Key words: microRNA-122, miR-122, Hepatitis C virus, HCV, mouse model, murine embryonic fibroblasts, PKR, NCoA6, Sub-genomic Replicons

Running Title: HCV Replication in Mouse Cells

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### **3.1 Abstract**

Hepatitis C virus (HCV) is a serious global health problem, infecting almost 3% of the world's population. The lack of model systems for studying this virus limit research options in vaccine and therapeutic development, as well as for studying the pathogenesis of chronic HCV infection. Herein we make use of the liver-specific microRNA miR-122 to render mouse cell lines permissive to HCV replication in an attempt to develop additional model systems for the identification of new features of the virus and its life cycle. We have determined that some wild-type and knockout mouse cell lines – NCoA6 and PKR knockout embryonic fibroblasts – can be rendered permissive to transient HCV sub-genomic RNA replication upon addition of miR-122, but we did not observe replication of full-length HCV RNA in these cells. However, other wild-type and knockout cell lines cannot be rendered permissive to HCV replication by addition of miR-122, and in fact, different NCoA6 and PKR knockout cell line passages and isolates from the same mice demonstrated varying permissiveness phenotypes and eventually complete loss of permissiveness. When we tested knockdown of NCoA6 and PKR in Huh7.5 cells, we saw no substantial impact in sub-genomic HCV replication, which we would expect if these genes were inhibitory to the virus' life cycle. This leads us to conclude that along with the influence of specific gene knockouts there are additional factors within the cell lines that affect their permissiveness for HCV replication; we suggest that these may be epigenetically regulated, or modulated by cell line immortalization and transformation.

### **3.2 Introduction**

Hepatitis C is a blood-borne viral disease prevalent worldwide; current estimates suggest approximately 150 million people are infected (WHO, 2012). Hepatitis C virus (HCV) becomes chronic in approximately 70% of acute infections, and can lead to the development of various cancers, liver steatosis, and numerous other complications throughout infection (Strader et al., 2004). A member of the Flaviviridae family, HCV is an enveloped virus with a single-stranded RNA genome of positive orientation, and is a member of the genus *Hepacivirus*. The HCV genome is approximately 9.6kb in length, and consists of an uncapped 5' un-translated region (UTR) containing significant secondary RNA structure deemed essential for viral RNA replication and an internal ribosomal entry site (IRES) essential for translation, followed by a single open reading frame (ORF) containing all the viral genes, and completed by a 3' UTR with further secondary

RNA structures necessary for genome replication (Bartenschlager et al., 2004). The single ORF of the virus is translated as a polyprotein that is then cleaved into the individual viral proteins by both cellular and viral proteases (Bartenschlager et al., 2004).

At least two aspects of the viral life cycle are major determinants of host range and cell specificity for Hepatitis C virus. Entry factors appear to define host range of the virus, as has been demonstrated both *in vitro* and *in vivo*: addition of human CD81 and human Occludin to murine cells permits entry of the virus, while generation of mice transgenic for these factors has permitted infection (Ploss et al., 2009; Vogt et al., 2013). However, these and other identified receptors – SR-B1, Claudin-1, Tfr1, NPC1-L1, Syndecan-1, EGFR, and EphA2 – do not appear to define the liver specificity of HCV, as they can be found on and in many other human cell types as well (Dorner et al., 2013a; Dorner et al., 2013b; Evans et al., 2007; Lupberger et al., 2011; Martin and Uprichard, 2013; Pileri et al., 1998; Ploss et al., 2009; Sainz et al., 2012a; Scarselli et al., 2002; Shi et al., 2013; Vogt et al., 2013). ApoE expression (murine or human), which is liver-specific, does appear to be a requirement for production of infectious virions following infection, but is not required in the host cell for earlier stages of entry or RNA replication (Chang et al., 2007; Frentzen et al., 2013; Vogt et al., 2013).

A host factor that likely influences liver-specificity of HCV replication is miR-122, a liver-specific microRNA that binds to two sites on the 5' UTR of the HCV genome (Jopling et al., 2006; Jopling et al., 2008; Jopling et al., 2005). This miRNA is highly abundant in the liver, accounting for approximately 70% of the small regulatory RNAs in hepatocytes; while its role in the liver is not yet completely defined, it does appear to regulate typical aspects of liver cell function such as cholesterol production and secretion, and it is also implicated as a tumour-suppressor microRNA (Bai et al., 2009; Esau et al., 2006; Norman and Sarnow, 2010; Xu et al., 2012). Interestingly, we and others have shown that expression of miR-122 in human liver cell lines previously considered refractory to HCV replication renders the cells permissive to HCV replication (Kambara et al., 2012; Narbus et al., 2011; Thibault et al., 2013). This has also been demonstrated in other non-human and non-liver cell lines, mostly through use of stable replicons rather than transient replication (Chang et al., 2008; Fukuhara et al., 2012; Lin et al., 2010).

There is a need for more and better model systems in HCV research. Although we have recently begun adding other human and hepatocyte-derived cell culture systems to the predominant Huh7-derived cell lines, non-human – particularly murine – cell lines remain a critical stepping-stone to

animal model development. A series of efforts using knockout mouse cells, selectable virus replicons, and human factor transduction has recently culminated in multiple research groups achieving the complete virus life cycle in mouse cells expressing the required entry and liver factors (Frentzen et al., 2013; Nandakumar et al., 2013; Vogt et al., 2013), but animal models of HCV infection are still limited (MacArthur et al., 2012). HCV mouse models relying on SCID-uPA mice supporting xenografted human liver tissue can be infected, and virus-induced pathogenesis or drug response studied, but the mice have limited life spans, lack immune systems, and are expensive to produce (Mercer et al., 2001a). Similar mice have only recently been developed to contain a humanized immune system, but the expense of these animals, as well as the fact that their livers and immune systems come from humans and vary between each xenografted mouse, remains an obstacle for their widespread use in HCV studies (Bility et al., 2012; Robinet and Baumert, 2011). Most recently, the human CD81/Occludin transgenic mouse model developed by Dorner *et al.* has been demonstrated to support the entire viral life cycle, but since it relies on innate immune gene knockouts such as Stat1 or IFN $\alpha/\beta$ , it is as yet unknown how much of the immune-mediated pathology will be recapitulated (Dorner et al., 2013a). The chimpanzee best represents the disease in humans, but they are currently not available for most research, and present both ethical and financial obstacles (MacArthur et al., 2012; Ploss and Rice, 2009; Wadman, 2013). Tree shrews (*Tupaia belangeri*) have also been identified as a potential model for HCV chronicity and pathogenesis, but they have also not yet been evaluated for immunological aspects of the infection (Amako et al., 2010; Xie et al., 1998; Xu et al., 2007).

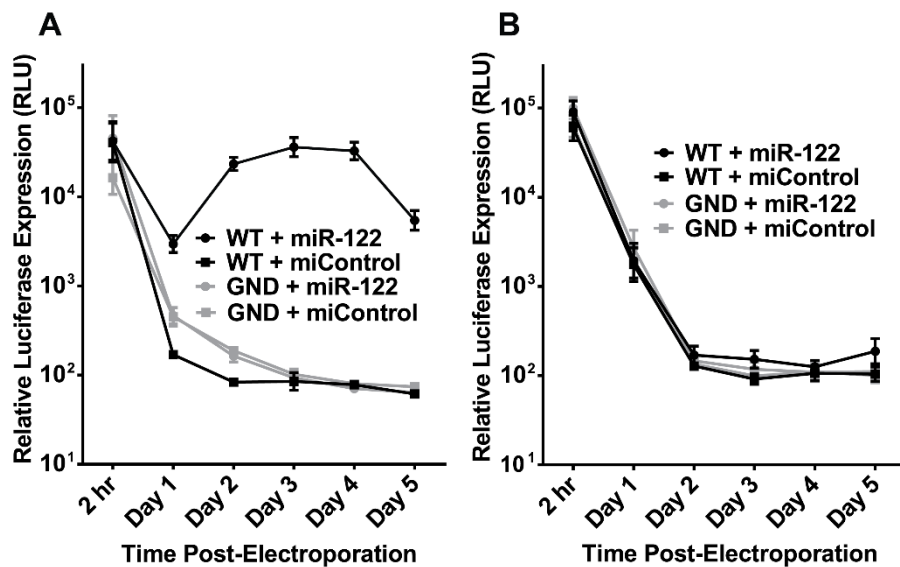
Thus, we chose to examine mouse cell lines to identify other genetic knockouts that could render mouse cells, and potentially the mice of origin, permissive to HCV replication. We identified one wild-type and two knockout mouse embryonic fibroblast (MEF) cell lines, “GH” wild-type, and NCoA6 and PKR knockout cells, that were permissive to transient, unselected sub-genomic HCV RNA replication when supplemented with miR-122. GH wild-type MEFs permitted only low levels of sub-genomic HCV RNA replication, while PKR MEFs initially supported high levels of sub-genomic RNA replication, but later passages, alternate isolates, and alternate sources of PKR knockout cells did not display a permissive phenotype. NCoA6 knockout cells also supported sub-genomic HCV RNA replication, although not to the levels seen in PKR MEFs, and HCV RNA replication in these cells also varied with cell passage. Since PKR and NCoA6 knockdown in Huh7.5 cells had only a minor impact on sub-genomic RNA replication, we

conclude that the permissiveness of our mouse cell lines, while possibly modulated by the gene knockout, was also defined by other aspects of the cell. We speculate that cell line modifications induced by culturing, immortalization, transformation, or other epigenetic changes also impact permissiveness of cells to HCV replication.

### **3.3 Results**

#### *3.3.1 One wild-type MEF cell line is permissive to HCV replication when supplemented with miR-122, while others are not.*

Wild-type MEFs from multiple sources were tested for permissiveness to HCV replication by electroporating cells with either wild-type (WT) or replication-incompetent (GND) sub-genomic HCV RNA, and supplementing the cells with either a control miRNA (miControl) or miR-122. Since the HCV RNA expresses a firefly luciferase reporter gene, replication was monitored by assaying firefly luciferase expression; firefly reporter luciferase expression from the wild-type viral RNA was compared to the non-replicating polymerase GND mutant to confirm replication, and electroporation efficiency was similar in all cases as determined by co-electroporation of a Renilla luciferase mRNA (data not shown). The MEFs depicted in Figure 3.1A were permissive to low levels of sub-genomic HCV RNA accumulation when electroporated with synthetic miR-122; however, the raw luciferase expression was approximately a thousand-fold lower than is typical in Huh7.5 cells, which was reflected in our inability to detect RNA accumulation via northern blot (data not shown). In a different wild-type MEF cell line, sub-genomic HCV RNA was unable to replicate in any detectable manner regardless of miR-122 supplementation (Figure 3.1B). In fact, of the five wild-type MEF cell lines we tested, as well as commonly-available NIH 3T3 cells (data not shown), only those shown in Figure 3.1A demonstrated detectable HCV replication. We therefore conclude that some wild-type MEFs are permissive to low levels of transient sub-genomic HCV replication when supplemented with miR-122. As we later observed with other knockout mouse cell lines, permissiveness of these MEFs varied through passage, although not in a predictable manner.



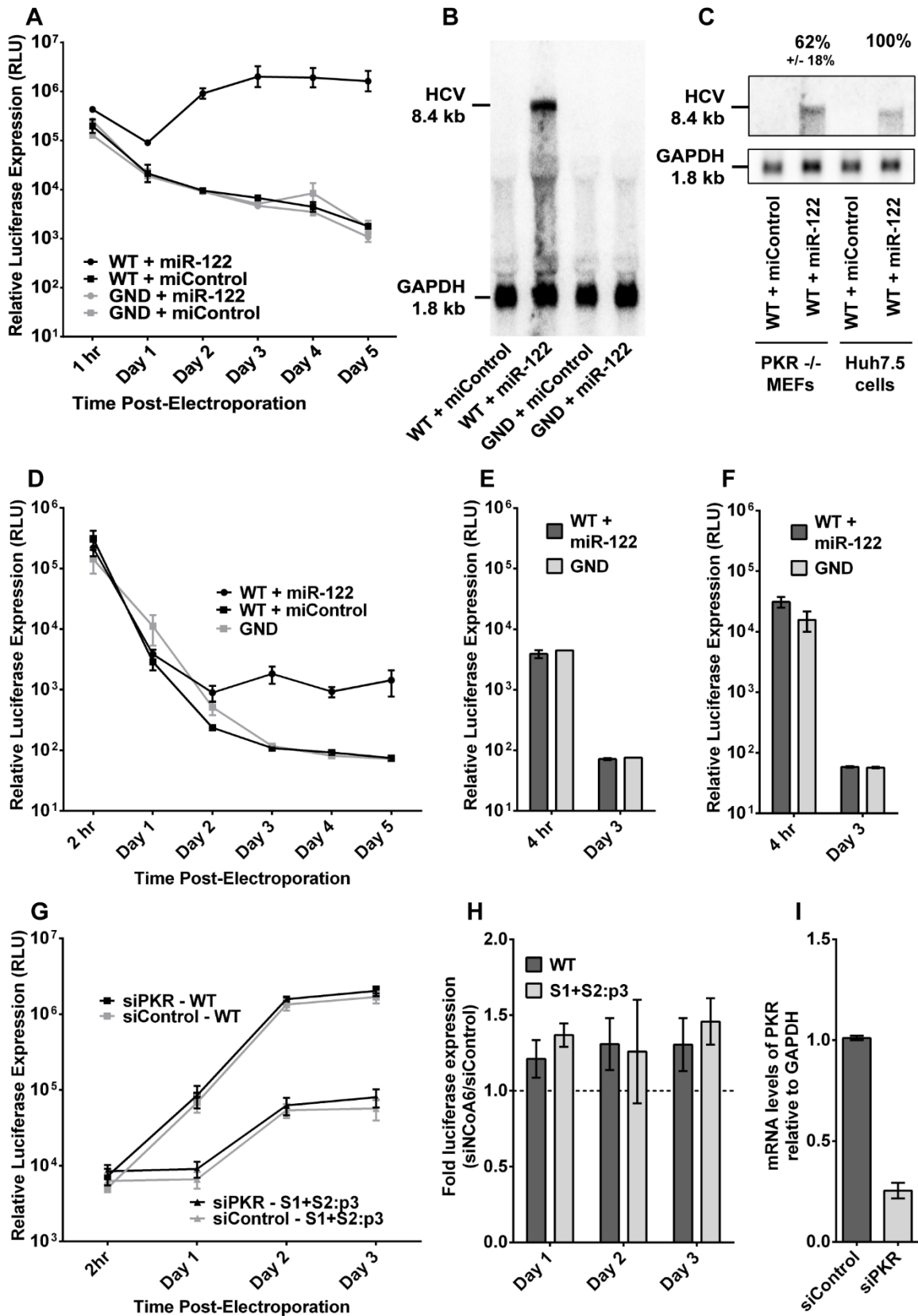
**Figure 3.1** Mouse cells with no known knockouts have varying permissiveness to HCV replication when supplemented with miR-122.

**A.** Wild-type MEFs obtained from Gregory Hannon were electroporated with either wild-type (WT) or replication-incompetent (GND) sub-genomic HCV RNA and the indicated miRNA (miControl, or miR-122), and luciferase expression was monitored at the indicated time points as a measure of HCV replication. **B.** Wild-type MEFs obtained from Per Antonson were treated as indicated in (A). Results from (A) and (B) are displayed as mean and standard error of the mean (SEM) of three or more independent experiments.

*3.3.2 PKR knockout MEFs are permissive to high levels of sub-genomic HCV RNA replication when supplemented with miR-122, but did not maintain their phenotype between passages and isolates.*

Protein kinase R (PKR) is a known anti-viral protein in mammalian cells (Dabo and Meurs, 2012), and PKR knockout MEFs were reported to support colony formation with selectable sub-genomic HCV more efficiently than wild-type MEFs (Chang et al., 2006; Vogt et al., 2013). Thus we hypothesized that PKR knockout MEFs would support efficient transient HCV replication if supplemented with miR-122. Upon electroporation of primary PKR knockout MEFs acquired from John Bell (Abraham et al., 1999) with sub-genomic HCV RNA and miR-122 (Figure 3.2A), we observed high levels of luciferase reporter expression, comparable to the levels achieved in Huh7.5 cells, the most common cell line used for HCV research. Northern blot analysis confirmed sub-genomic HCV RNA accumulation in these PKR knockout MEFs (Figure 3.2B), and levels were within 2-fold of those observed in Huh7.5 cells, which verified that luciferase expression accurately reflected HCV RNA accumulation (Figure 3.2C).

These data were generated from two replicate experiments with a single isolate of primary PKR MEFs that we now believe to have been different from other PKR MEFs we tested. When further replicates were attempted with other isolates of PKR MEFs, we were unsuccessful in achieving detectable replication (data not shown), including PKR knockout MEFs obtained from Robert Silverman, which had been shown previously to support HCV replicon colony formation (Yang et al., 1995). Moreover, primary MEFs (Figure 3.2D) that we isolated from PKR knockout mice could support only low levels of HCV replication in the presence of miR-122, and primary PKR knockout hepatocytes did not support detectable HCV replication at all (Figure 3.2E and 3.2F), despite efficient RNA transfection (based on luciferase expression 2 and 4 hours post-transfection) by both electroporation (Figure 3.2E) and transfection (Figure 3.2F). Since PKR MEFs are visually distinct from Huh 7.5 cells, the only other cell line in use in our lab at the time, and our PKR MEFs did not replicate HCV in the absence of miR-122 (Figure 3.2A, WT + miControl), we omit contamination of the MEF cell line with Huh 7.5 cells as a possible explanation for positive results shown in Figure 3.2A. Therefore we show that a single isolate of PKR knockout MEFs was highly permissive for transient HCV RNA replication when supplemented with miR-122.





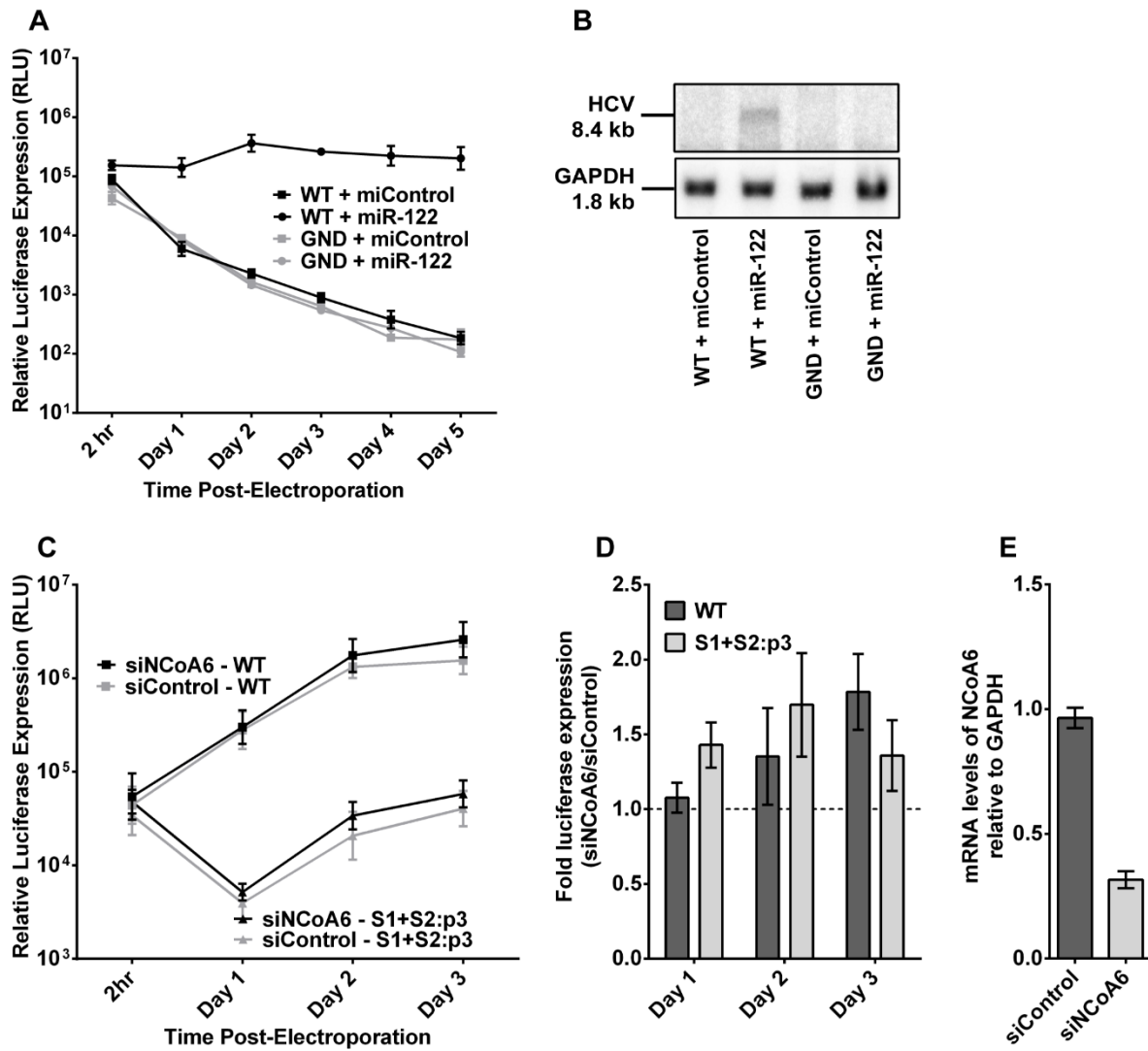
**Figure 3.2 PKR knockout MEFs are permissive to sub-genomic HCV RNA replication when supplemented with miR-122.**

**A.** PKR knockout MEFs were treated as in Figure 3.1, and sub-genomic HCV replication was monitored at the indicated time points via luciferase expression. Results are shown with SEM, and are an average of two independent experiments. **B.** RNA from (A) was isolated from PKR knockout MEFs three days post-electroporation and probed for HCV RNA accumulation by northern blot, with GAPDH as a loading control. **C.** RNA from (A) was compared with RNA from Huh7.5 cells via northern blot. Bands from two independent experiments were quantified by densitometry, and normalized to GAPDH. Percentage HCV RNA +/- standard deviation is presented relative to Huh7.5 cells. **D.** Primary MEFs isolated from PKR knockout mice were treated as in Figure 3.1. Results are shown with SEM and are an average of nine independent experiments. **E.** Primary hepatocytes were isolated from PKR knockout mice and electroporated with sub-genomic RNA and miR-122. Luciferase expression was evaluated four hours post-electroporation to assess RNA transfection efficiency and three days post-electroporation to detect sub-genomic RNA replication. Reporter activity above non-replicating (GND) levels was not detected. Results are an average of four experiments, shown with standard deviation. **F.** Primary hepatocytes isolated from PKR knockout mice were transfected via Lipofectamine 2000 with sub-genomic RNA and miR-122, and evaluated as in (E). Reporter activity above non-replicating (GND) levels was not detected. Results are an average of six experiments, shown with SEM. **G.** Huh7.5 cells were first electroporated with the indicated siRNAs (siControl or siPKR), three days before a second electroporation (Day 0) with the indicated sub-genomic HCV RNA (wild-type (WT) or S1+S2:p3) and a second dose of siRNA. Results are an average of six independent experiments, shown with SEM. Experiments were analyzed with a two-way repeated measures ANOVA, and differences with siRNA treatment were not found to be statistically significant. **H.** The effect of PKR knockdown on HCV replication in Huh7.5 cells was evaluated by examining the fold-change in replication over siControl-treated cells that is caused by siPKR treatment. Error is shown as SEM. **I.** PKR knockdown in Huh7.5 cells was confirmed by qRT-PCR, and is shown as an average of three experiments with SEM.

To determine the influence of PKR on cell permissiveness for HCV, we tested the effects of PKR knockdown in Huh7.5 cells. Huh7.5 cells were electroporated with siRNA to PKR (siPKR) or to a control sequence (siControl) and allowed three days for the knockdown to take effect. The cells were then electroporated again with the indicated siRNA, as well as wild-type (WT) sub-genomic HCV RNA or miR-122 binding site mutant (S1+S2:p3) sub-genomic RNA (Figure 3.2G). Because Huh7.5 cells already express miR-122, it was not added in this assay. The S1+S2:p3 RNA was tested because it is not responsive to miR-122, but is capable of replicating at low levels without miRNA supplementation, and so may be more sensitive to removal of anti-viral factors because the cell's machinery is not saturated. Both wild-type and S1+S2:p3 sub-genomic luciferase reporter expression was increased between 1.2 and 1.5-fold by knockdown of PKR (Figure 3.2H), but this increase was not significant when tested by two-way ANOVA (Figure 3.2G). Treatment of Huh7.5 cells with siPKR reduced PKR mRNA levels by 75% as measured by qRT-PCR (Figure 3.2I). Because knockdown of PKR in Huh7.5 cells has no significant impact on HCV RNA replication, we suggest that the permissive phenotype of the particular isolate of PKR knockout MEFs depicted in Figure 3.2A, B, and C may not be due to the genetic knockout, and may instead be due to other factors unique to that isolate.

### *3.3.3 NCoA6 knockout MEFs are permissive to sub-genomic HCV RNA replication upon supplementation with miR-122, but do not maintain their phenotype between passages.*

Nuclear co-activator 6 (NCoA6, also known as RAP250, TRBP, NRC, ASC2, and PTIP) is a member of the nuclear receptor co-activator family, and aids in activation of the liver X receptor (LXR $\alpha$ ) (Kim et al., 2003; Li et al., 2007; Mahajan and Samuels, 2005). This leads to transcription of genes involved in cholesterol metabolism and export, which may impact HCV replication and production of virus particles (Mahajan and Samuels, 2005). NCoA6 was also detected in a large-scale siRNA screen to negatively impact HCV replication, but is not associated with major innate immune deficiencies (Tai et al., 2009). We therefore acquired NCoA6 knockout MEFs from Per Antonson to test this knockout for permissiveness to HCV replication (Antonson et al., 2003). When electroporated with sub-genomic HCV and miR-122, these cells demonstrated levels of luciferase (Figure 3.3A) and HCV RNA (Figure 3.3B) that, while robust, were not as high as those detected in PKR knockout MEFs, nor in Huh7.5 cells (data not shown). The knockout is embryonic-lethal, so we were unable to acquire mice and generate hepatocytes to test for additional



**Figure 3.3** *NCoA6* knockout MEFs are permissive to sub-genomic HCV RNA replication upon supplementation with miR-122.

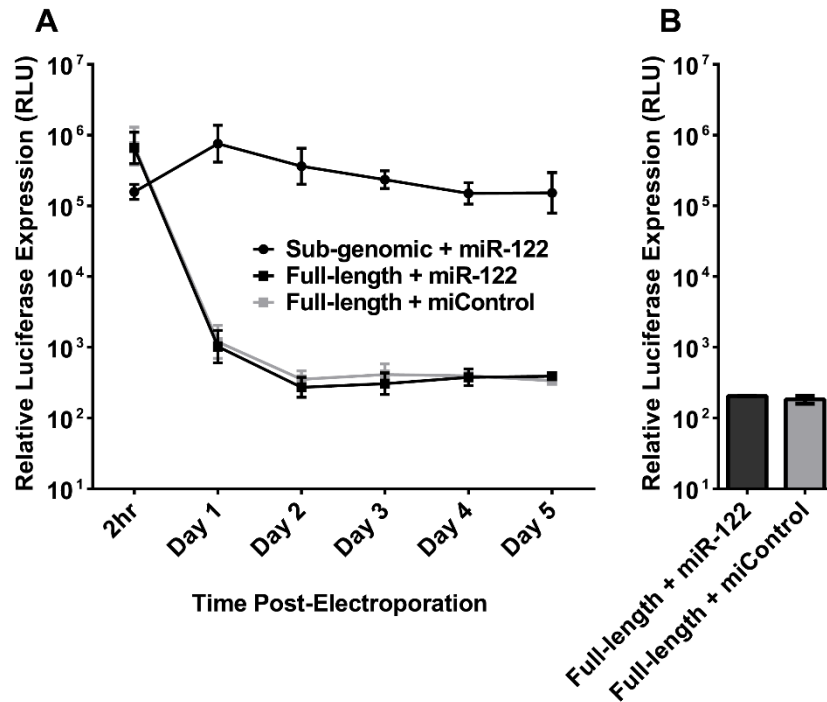
**A.** *NCoA6* knockout MEFs were treated as in Figure 3.1, and sub-genomic HCV replication was monitored at the indicated time points via luciferase expression. Results are an average of four experiments, shown with SEM. **B.** RNA from (A) was isolated from *NCoA6* knockout MEFs three days post-electroporation and evaluated for HCV RNA accumulation by northern blot, with GAPDH as a loading control. **C.** Huh7.5 cells were first electroporated with the indicated siRNAs (siControl or si*NCoA6*), three days before a second electroporation (Day 0) with the indicated sub-genomic HCV RNA (Wild-type (WT) or S1+S2:p3) and a second dose of siRNA. Replication was evaluated by luciferase expression. Results are an average of five independent experiments, shown with SEM. Experiments were analyzed with a two-way repeated measures ANOVA, and differences with siRNA treatment were not found to be statistically significant. **D.** The effect of *NCoA6* knockdown on HCV replication in Huh7.5 cells was evaluated by examining the fold-change in replication over siControl-treated cells that is caused by si*NCoA6* treatment, and are shown with SEM. **E.** *NCoA6* knockdown in Huh7.5 cells was confirmed by qRT-PCR in an average of three independent experiments, and is shown with SEM.

permissiveness (Antonson et al., 2003). In addition, the NCoA6 knockout MEFs displayed varying levels of permissiveness to HCV RNA replication, with no clear correlation between isolate, passage number, or experiment date; data shown here is from strongly-permissive cells, and we did eventually exhaust our stocks of permissive cells.

As we did for PKR, we also tested the NCoA6 knockdown phenotype in Huh7.5 cells via siRNA knockdown. Knockdown of NCoA6 increased luciferase expression from both miR-122-dependent (WT) and miR-122-independent (S1+S2:p3) sub-genomic replicons (Figure 3.3C) 1.4 to 1.8-fold at days 2 and 3 post-second electroporation (Figure 3.3D), but the effect of siNCoA6 treatment was not statistically significant as determined by a two-way ANOVA. siNCoA6 treatment of Huh7.5 cells resulted in a 68% reduction in NCoA6 mRNA levels in comparison to siControl treatment, relative to GAPDH mRNA (Figure 3.3E). As NCoA6 knockdown also has no significant impact on HCV RNA replication in Huh7.5 cells, we suggest that, as we found with the PKR knockout cells, the knockout of NCoA6 in the MEFs may not be responsible for the permissive phenotype of these cells.

#### *3.3.4 NCoA6 knockout MEFs are not permissive for full-length HCV RNA replication.*

To further investigate the possibility of using knockout MEFs as a model for HCV replication, we examined NCoA6 knockout cells' permissiveness for full-length HCV RNA replication. The cells were electroporated with J6/JFH-1(p7-Rluc2a), a full-length mono-cistronic HCV RNA construct that bears the virus' structural proteins and a Renilla luciferase reporter. Wild-type full-length (FL WT) HCV RNA was electroporated with either miControl or miR-122 (Figure 3.4A). Although sub-genomic HCV RNA, included as a positive control, showed the cells to be permissive for replication of HCV RNA, and a firefly luciferase mRNA was included to confirm consistent electroporation efficiency (data not shown), full-length HCV RNA did not replicate detectably when supplemented with miR-122. In addition, when supernatant was collected from the MEFs on Day 5 and transferred to susceptible Huh7.5 cells, no infectious virus was detected (Figure 3.4B). In our hands, luciferase expression in reporter virus infected cells is sensitive enough to detect a single HCV focus-forming unit (FFU; data not shown), indicating that the full-length RNA did not produce infectious virus in NCoA6 knockout MEFs. Therefore, NCoA6 cells, while competent for sub-genomic replication, do not permit replication of full-length HCV RNA or production of infectious virus particles.

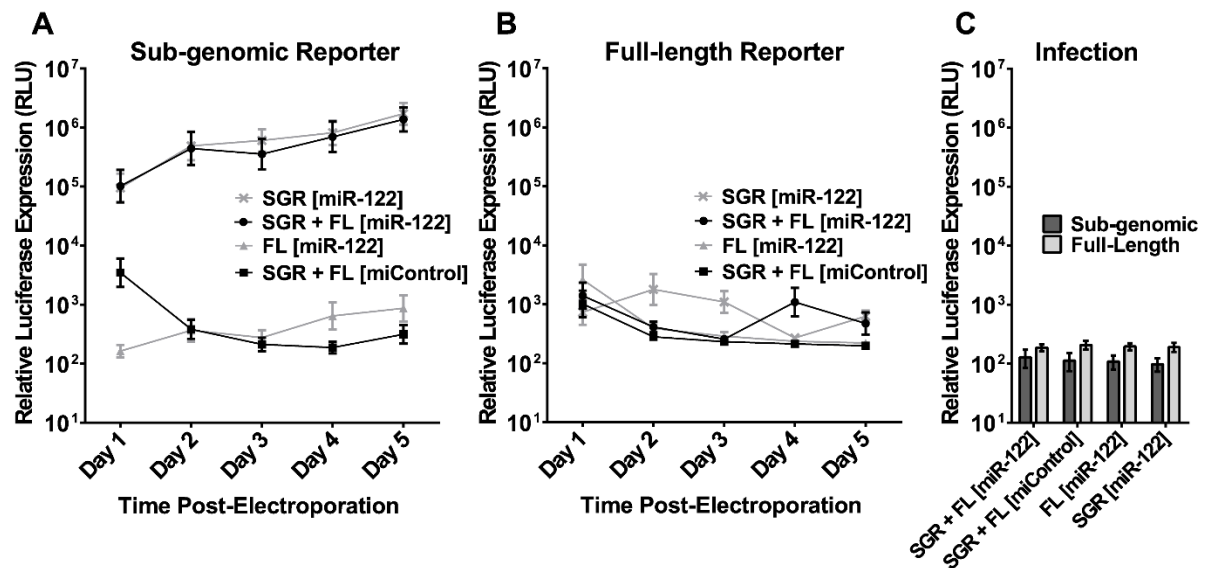


**Figure 3.4 NCoA6 knockout MEFs are not permissive for full-length HCV RNA replication.**

**A.** NCoA6 knockout MEFs were electroporated with full-length HCV RNA or sub-genomic HCV RNA and the indicated miRNA. Note that sub-genomic HCV RNA uses a firefly luciferase reporter, while full-length HCV RNA uses a Renilla luciferase reporter, and the relative light units measured (RLU) are not directly comparable. **B.** Supernatant from NCoA6 knockout MEFs electroporated with full-length HCV RNA was harvested five days post-electroporation and plated on Huh7.5 cells, which are permissive for HCV infection and replication without additional modification. Three days post-infection, the Huh7.5 cells were monitored for luciferase expression as an indication of replication. The luciferase levels indicated here are equivalent to background. Results in (A) and (B) are shown with SEM and are an average of three independent experiments.

### 3.3.5 Sub-genomic HCV RNA replication is unaffected by co-electroporated full-length RNA.

One explanation for the inability of full-length HCV RNA to replicate in NCoA6 MEFs is that the full length RNA, or structural proteins expressed by it, activate murine innate immune sensors in the mouse cells, leading to induction of an anti-viral response. To test this we co-electroporated NCoA6 knockout MEFs with sub-genomic and full-length HCV RNA simultaneously, with the expectation that if the full length HCV genomic RNA was inducing an antiviral response in the host cell, then replication of the co-electroporated SGR RNA will be negatively influenced by the presence of full length HCV RNA. Because electroporation results in a >95% efficiency of RNA transfection in MEFs (data not shown), we were confident that the majority of cells would receive both viral RNAs, as well as the indicated miRNA. The sub-genomic construct encodes firefly luciferase (Figure 3.5A), while the full-length construct encodes Renilla luciferase (Figure 3.5B); each luciferase utilizes a different substrate, and so their expression can be evaluated separately within the same sample. There was no indication of *trans*-inhibition of SGR replication by full-length HCV RNA, since replication of sub-genomic RNA was not impacted by addition of full-length RNA (SGR + FL [miR-122], Figure 3.5A), and replication levels were similar to those seen in cells electroporated with SGR RNA alone (SGR [miR-122], Figure 3.5A). As expected, no sub-genomic RNA reporter activity was identified in the sample that contained only full-length RNA (FL [miR-122]), and no sub-genomic replication was detected in cells given sub-genomic RNA without miR-122 (SGR [miControl]). This led us to conclude that the full-length RNA did not activate a *trans*-acting anti-viral response, nor cause any changes in the cell that could affect sub-genomic RNA replication. Due to the design of the assay, when firefly (sub-genomic) luciferase levels are very high, some signal will bleed through into the Renilla (full-length) measurement even when no full-length RNA is present (SGR [miR-122], Figure 3.5B). No detectable full-length replication was observed in any of the samples (Figure 3.5B), showing that sub-genomic RNA was also unable to *trans*-complement full-length replication. In addition, tissue culture supernatant from these experiments demonstrated no detectable infectivity in Huh7.5 cells (Figure 3.5C), showing that the full-length RNA also did not produce sufficient levels of structural protein to *trans*-package the sub-genomic HCV RNA (Pacini et al., 2009).



**Figure 3.5** *The inability of full-length HCV RNA to replicate in NCoA6 knockout MEFs cannot be complemented by sub-genomic HCV RNA, and sub-genomic RNA is not packaged into particles when co-electroporated with full-length HCV RNA.*

NCoA6 knockout MEFs were electroporated with sub-genomic and/or full-length HCV RNA as indicated, and either miControl or miR-122. **A.** Sub-genomic HCV RNA encodes a firefly luciferase reporter that was monitored simultaneously with **B.** the full-length HCV RNA Renilla luciferase reporter in these cells at the indicated time points. **C.** Supernatant from the cells in (A, B) was collected three days post-electroporation and plated on permissive Huh7.5 cells to test for infectivity. Three days post-infection, Huh7.5 cells were monitored for both firefly (dark grey, Full-length) and Renilla luciferase (light grey, Sub-genomic) reporter activity to detect either production of infectious particles containing trans-packaged sub-genomic HCV RNA or full-length HCV RNA respectively. Luciferase levels indicated here are equivalent to background. Results in (A), (B), and (C) are shown with SEM and are an average of five independent experiments.

### 3.4 Discussion

Here we demonstrate that supplementing non-permissive mouse cells with miR-122 can render them permissive to transient sub-genomic HCV RNA replication; we bypass the human entry factor requirements by electroporating cells with viral RNA, rather than infecting them. miR-122 is a liver-specific co-factor that is important for HCV RNA replication, and has thus been implicated in defining tissue tropism for the virus, and we confirm this as its addition renders murine embryonic fibroblasts permissive to HCV RNA replication. We identified wild-type and knockout (PKR and NCoA6) mouse embryonic fibroblasts that were permissive to detectable levels of HCV RNA replication when supplemented with miR-122. In general the gene knockouts increased the levels of HCV replication above that seen in wild-type MEFs and suggest that part of the permissive phenotype is mediated by the gene knockout as has been shown by others (Dorner et al., 2013a; Frentzen et al., 2013; Lin et al., 2010; Nandakumar et al., 2013; Vogt et al., 2013); however, we note that alternate isolates of wild-type and knockout MEFs were not permissive to HCV replication, and the permissiveness of each cell line further varied by passage and isolate. This variation was particularly observed with PKR knockout MEFs and hepatocytes from different isolates and multiple backgrounds (Figure 3.2), but was also observed with the wild-type and NCoA6 knockout MEFs through cell passage. In addition, when evaluating knockdown of either PKR or NCoA6 expression in the already-permissive Huh7.5 cells in the context of sub-genomic replication, we observed an insignificant pro-viral effect that we deemed unable to account for the permissiveness of the knockout in mouse cells, which leads us to suggest that at least part of the permissive phenotype of the knockout cell line is not directly due to the particular gene that is abolished. This, however, does not preclude the possibility that there are host and cell-type specific factors that may explain the lack of effect of knockdown in Huh7.5 cells. HCV, while adept at circumventing human host defense proteins such as PKR, could still be susceptible to murine PKR and this may be one of the reasons that the PKR knockout MEFs did originally permit high levels of replication. It is also possible that the absence of NCoA6 in non-hepatic cells (fibroblasts) had a more drastic effect than knockdown of NCoA6 in the liver-derived Huh7.5 cells, as modulation of lipids in liver-derived cells may be more nuanced or have more redundancies active than in fibroblasts; it is also possible that there is a species-specific effect of NCoA6 as we speculate with PKR. Ultimately, the loss of permissiveness indicates that there are factors other than the gene knockouts involved in permitting HCV replication in these cells.



Overall, we conclude that the permissiveness of a given MEF line may be influenced by both the targeted gene knockout, and genetic and/or epigenetic changes in the cell line that occur during transformation and/or immortalization. This is based on the varying permissiveness of different cell isolates and passages of the same knockout cell line, and our finding that the HCV permissive phenotype of an isolate of primary PKR knockout cells was not observed in other primary MEF isolates and hepatocytes. We concede that there may be some basic genetic differences in antiviral responses dependent upon the background of the mouse from which the MEFs were isolated, but the variability in permissiveness of immortalized MEFs over different passages suggests that this is not a key factor. Lohmann *et al.* noted that Huh7 cells themselves vary in permissiveness to HCV by up to 100-fold at different passages, so it is perhaps unsurprising to also observe this in other immortalized cell lines (Lohmann *et al.*, 2003). Thus, we suggest that future research with HCV in immortalized knockout cell lines be undertaken with caution and effort to confirm the effect of the knockout on HCV replication in primary cells, and to develop a means of characterizing and retaining the susceptible phenotype of the cultured cells.

Recently, Vogt *et al.* used drug selection to maintain sub-genomic and full-length HCV RNA replication in MEFs (Vogt *et al.*, 2013). They also concluded that the use of antiviral gene knockouts increased the ability to select for stable cells. Based on these results we speculate that in both our study and in theirs that a subset of cells in each MEF population support HCV replication, and that their use of drug selection reinforced maintenance of the susceptible subset while eliminating cells that lost permissiveness through passage. Thus it is possible that in our studies, cells that support HCV replication were lost from the cell population. Unlike our study however, in the Vogt report and other previous reports, miR-122 was not required for stable HCV colony selection (Chang *et al.*, 2008; Chang *et al.*, 2006; Lin *et al.*, 2010; Uprichard *et al.*, 2006; Vogt *et al.*, 2013; Zhu *et al.*, 2003). This suggests that perhaps the knockout cells supported sufficient replication independent from miR-122 for selection (or generation of viral mutants that do not require miR-122), or that the selected cells could express miR-122 or other pro-viral factors. More recently, the same group has demonstrated the complete virus life cycle in entry-factor transgenic mice, although these mice also benefit from the same immune gene knockouts identified in stably selected MEFs (Dorner *et al.*, 2013a; Vogt *et al.*, 2013). Two other recent publications have shown replication of HCV in MEFs, primary murine hepatocytes, and murine hepatoma cells from innate immune knockout mice in the absence of selection (Frentzen *et al.*, 2013; Nandakumar

et al., 2013); the wild-type and NCoA6 knockout cell lines we used here were immortalized through crisis, during which any number of changes may have occurred, and therefore we cannot directly compare them to the passage status and immortalization of the cell lines established by Frentzen *et al.* and Nandakumar *et al.* (personal communication).

Our findings that NCoA6 knockout MEFs do not support detectable transient replication of full-length HCV RNA also require consideration. We originally considered these cells as a control knockout cell line until we determined that replication was higher in these cells than in the wild-type MEFs; we then hypothesized that a MEFs cell line could be made to support HCV replication through knockout of a host gene that does not affect innate immunity. However, the finding that NCoA6 knockout MEFs were not permissive for full-length HCV RNA replication limits their usefulness as models. Why the cells could not replicate full length HCV genomic RNA remains to be determined, but elements of the structural gene region may be involved since Aly *et al.* noted a difference in permissiveness of mouse cell lines for HCV replication that varied by the structural gene region of the construct used (Aly et al., 2011). In our hands, the full-length RNA did not appear to affect co-electroporated sub-genomic replication, arguing that whatever prevents full-length replication in the cells does so in *cis* and is not, therefore, a dominant antiviral response triggered only by full-length HCV RNA. This is supported by others' data that mouse cells do not restrict replication of HCV when fused with permissive human liver cells (Frentzen et al., 2011). Thus, neither expression of the structural proteins, nor structural protein gene regions of the RNA, promotes an antiviral state in the cells, since neither protein nor RNA adversely affected replication of sub-genomic HCV RNA in the same cell. This suggests another, non-immune limiting factor present in this particular cell line – and perhaps others – that could be exploited in place of innate immune knockouts.

There are two major differences between the sub-genomic and full-length HCV RNAs that could affect replication in *cis*: the first is the structural protein coding region in the full-length RNA, while the second is the presence of the second (EMCV) IRES in the sub-genomic RNA construct. It is possible that the structural protein coding region contains *cis*-acting regulatory sequences that regulate HCV replication, and that a human specific host factor, absent from mouse cells, is required to activate this regulatory element. On the other hand, in the sub-genomic construct, the EMCV IRES drives expression of the non-structural proteins required for RNA replication instead of the HCV IRES. If the EMCV IRES drives translation more strongly than the

HCV IRES in mouse cells, then it may lead to more efficient expression of the viral non-structural proteins required for replication and this may overcome a host species barrier in the presence of miR-122. Finally, it may be a combination of these two factors, or the presence of the EMCV IRES may disrupt long-range *cis* regulatory elements in the non-structural genes in any host cell, permitting de-regulated replication of the sub-genomic RNA in any permissive environment.

We therefore suggest that there are additional factors that need consideration when attempting to develop non-human cell culture models for HCV; the immortalization and subsequent maintenance regime of the cells may affect their permissiveness in addition to any knockout background, particularly when attempting unselected HCV RNA replication. Furthermore, the viral construct may also be important when attempting to identify permissive cell lines as was seen in the work by Aly *et al.* (Aly et al., 2011). Future work with new viral isolates may also advance the search for new murine models of Hepatitis C virus.

### **3.5 *Materials and Methods***

#### *3.5.1 Ethics Statement.*

Mice were handled according to the guidelines provided by the Canadian Council on Animal Care and the University of Saskatchewan Policy on Care and Use of Animals in Research. Protocols for the collection of mouse cells were approved by the University of Saskatchewan's Animal Research Ethics Board (Surgical Procedures 19940211).

#### *3.5.2 Cell Culture.*

Cell lines and primary mouse cells are adherent, and were maintained in complete Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1% Penicillin/Streptomycin, and 1% non-essential amino acids as described in (Thibault et al., 2013). Huh7.5 cells were provided by Charles Rice (New York, USA) (Blight et al., 2002). Wild-type mouse embryonic fibroblasts (MEFs) were obtained from Gregory Hannon (Cold Spring Harbor, USA), Robert Silverman (Yang et al., 1995), and Per Antonson (Huddinge, Sweden). NCoA6 knockout MEFs (also known as RAP250 knockout MEFs) were also provided by Per Antonson (Antonson et al., 2003). PKR knockout MEFs and PKR knockout mice were provided by John Bell (Ottawa, Canada) (Abraham et al., 1999). We isolated primary PKR knockout MEFs from the mice as in (Lewis et al., 1992). Briefly, a 13 to 15-day pregnant mouse was euthanized with

isoflurane and embryos were extracted. Heads, appendages, and red matter were excised from the embryos, and the embryos were placed into a fresh dish. After chopping up the embryo tissue with scissors, it was incubated in trypsin for 1 hr at 37°C, before pelleting and plating in complete DMEM. PKR knockout hepatocytes were isolated as follows: a male 7 to 9 week-old PKR knockout mouse was euthanized with isoflurane, and the liver perfused from the vena cava with 25 mL 37°C Krebs Ringer with glucose and 0.1mM EDTA. The liver was then digested with collagenase (12.6mg) in 25mL Krebs Ringer with glucose and 150 µM CaCl<sub>2</sub> (Buffer 2). After digestion, the liver was removed from the body cavity and transferred to a petri dish, where it was punctured and resuspended in Buffer 2, and then filtered through a 70µM filter and washed twice with Buffer 2. Cells were then tested for viability with trypan blue, and, if greater than 80% viable, seeded at a density of 2.0 x 10<sup>5</sup> cells/well in collagen-coated 24-well dishes in complete DMEM.

### 3.5.3 Viral plasmids and RNA.

Plasmids pSGR JFH-1 Fluc WT and pSGR JFH-1 Fluc GND encode sub-genomic JFH-1-derived HCV replicons with a firefly luciferase reporter; the GND contains an inactivating mutation in the viral polymerase (Kato et al., 2003; Kato et al., 2005a). Plasmids pSGR S1+S2:p3 Fluc WT and pSGR S1+S2:p3 Fluc GND have C to G mutations at position 3 in both miR-122 seed binding sites in the HCV 5' UTR and were described in (Thibault et al., 2013). Plasmids pJ6/JFH-1(p7-Rluc2a) "FL WT" and pJ6/JFH-1(p7-Rluc2a) GNN "FL GNN" bear full-length viral sequences derived from the J6 (structural proteins) and JFH-1 (non-structural proteins) isolates of HCV, and a Renilla luciferase reporter, with the GNN having inactivating mutations in the viral polymerase (Jones et al., 2007a). Firefly luciferase control mRNA was transcribed from Luciferase T7 Control DNA plasmid (Promega; Nepean, ON, Canada), while Renilla luciferase control mRNA was transcribed from the pRL-TK plasmid (Promega). Plasmid templates for viral RNA and mRNA were prepared and *in vitro* transcribed with the MEGAScript T7 High Yield Transcription Kit and mMessage mMachine T7 Transcription Kit (Life Technologies; Burlington, ON, Canada), respectively, as described in (Thibault et al., 2013).

### 3.5.4 microRNAs and silencing RNAs.

miR-122: 5'-UGG AGU GUG ACA AUG GUG UUU GU-3' and miR-122\*: 5'-AAA CGC CAU UAU CAC ACU AAA UA-3', annealed. miControl: 5'- GAA GGU CAC UCA GCU AAU

CAC and miControl\*: 5'-GUG AUU AGC UGA CAG ACC UUC-3', annealed (Wilson et al., 2011). siPKR: 5'-GCG AGA AAC UAG ACA AAG U-3'. siNCoA6: 5'-CCA CAG AGC UGG ACA GUA AUU-3'. siControl: 5'- GAA GGU CAC UCA GCU AAU CAC dTTC-3' (Wilson et al., 2003). All small RNAs were synthesized by ThermoScientific Dharmacon (Lafayette, CO, USA).

### 3.5.5 Electroporation.

Cells were electroporated using 4mm cuvettes at infinite resistance, and were prepared and plated as described in (Thibault et al., 2013). Briefly, cells were trypsinized, washed twice with cold Dulbecco's PBS (D-PBS), then resuspended in D-PBS at a concentration of  $1.5 \times 10^7$  cells/mL, with 400 $\mu$ L ( $6.0 \times 10^6$ ) cells used per sample. Mouse cell lines were electroporated with 10 $\mu$ g viral RNA, 1 $\mu$ g control Fluc or Rluc mRNA as transfection control, and 60pmol miRNA. Huh7.5 cells were electroporated with 5 $\mu$ g viral RNA and 1 $\mu$ g Rluc mRNA as transfection control. When used for knockdown, Huh7.5 cells were first electroporated with 60pmol siRNA and incubated for three days; they were then electroporated again with 60pmol siRNA, along with 5 $\mu$ g viral RNA and 1 $\mu$ g Rluc mRNA as transfection control. Electroporation conditions for MEFs were 400V, 250 $\mu$ F; for PKR knockout hepatocytes were 220V, 950 $\mu$ F; and for Huh7.5 cells were 270V, 950 $\mu$ F. Cells were then resuspended in 4mL DMEM, and 500 $\mu$ L cells were plated in 6-well dishes for luciferase harvests, or 2mL cells were plated into 10cm dishes for RNA harvests.

### 3.5.6 Transfection.

Briefly, media was replaced with Pen/Strep-free C-DMEM on PKR knockout hepatocytes one day post-extraction. Lipofectamine 2000 reaction mixture was assembled according to recommended protocol, with 0.4  $\mu$ g viral RNA, 5.0 pmol miRNA, and 0.1  $\mu$ g mRNA assembled with 1.5  $\mu$ L Lipofectamine 2000 (approximately 3:1 ratio) in OptiMEM. Cells were transfected overnight, media was changed on Day 1 post-electroporation to C-DMEM, and luciferase was harvested as indicated.

### 3.5.7 Luciferase Assays.

Luciferase assays to monitor viral replication were carried out as described in (Thibault et al., 2013). Briefly, cells were harvested by scraping in 100 $\mu$ L of passive lysis buffer. 10 $\mu$ L of lysate

was assayed for light production in 50µL of the appropriate assay buffer (luciferase substrate) for the type of luciferase in the sample, according to the associated protocol, and using a GLOMAX luminometer (Promega).

#### *3.5.8 Infections.*

Naive Huh7.5 cells were plated at  $1.0 \times 10^5$  cells per well in a 6-well dish one day pre-infection. Supernatant from NCoA6 cells was collected at the indicated time, spun to pellet cell debris, and 2mL was plated on naive Huh7.5 cells. Huh7.5 cell extracts were harvested as above at three days post-infection and assayed for luciferase expression to detect HCV infection.

#### *3.5.9 RNA collection.*

Total RNA was collected from cells three days post-electroporation into 1mL Trizol (Life Technologies) and isolated according to the manufacturer's protocol.

#### *3.5.10 Northern blot.*

Northern blotting for HCV RNA was carried out as described in (Thibault et al., 2013).

#### *3.5.11 qRT-PCR.*

Total cellular RNA was reverse-transcribed using the iScript cDNA Synthesis Kit (BioRad; Mississauga, ON, Canada). qPCR reactions were carried out using the TaqMan kits Hs00169345\_m1 (EIF2AK2, PKR), Hs01052843\_m1 (NCoA6) and FAM-MGB 4352934-0803022 (GAPDH); samples were amplified in triplicate with 2X TaqMan Master Mix (Life Technologies). All data was analyzed with the CFX Manager Software (BioRad).

### **3.6 Acknowledgements**

We thank Dr. Charlie Rice, Dr. John Bell, Dr. Robert Silverman, and Dr. Per Antonson for reagents and cell lines. We also thank Adam Huys for valuable scientific discussion throughout the preparation of this manuscript.

#### **4.0 Alternative Cell Culture Model Systems for Growth of Hepatitis C Virus**

We had hoped that our work presented in Chapter 3 would allow us to make progress on the development of a mouse model for HCV propagation, but for several reasons decided to pursue alternative cell lines as models. Firstly, the murine cell lines described in Chapter 3 did not permit replication of the full-length virus, and hepatocytes isolated from the source mice were not permissive for HCV replication. This suggested to us that there were other impediments in our approach to developing cell lines or animals that could support the full HCV life cycle. Secondly, other researchers had recently developed an innate immune-deficient mouse model for Hepatitis C virus infection that supported virion entry, replication, and particle production (described in Chapter 1) (Dorner et al., 2013a; Vogt et al., 2013). In addition, simultaneously with the work described in Chapter 3, we were exploring alternative human hepatoma cell lines that may be rendered permissive for replication of Hepatitis C virus through supplementation with miR-122 and this seemed a path that would lead to interesting results in the laboratory focus.

The experiments in Chapter 5 were carried out by PAT with assistance from PD, with the exception of those in Figure 5.5, which were performed by AH. Experiments were conceived by PAT, JAW, and AH. PAT, AH, and JAW analyzed the data. PAT and JAW wrote the paper.

## **5.0 MicroRNA-122-dependent and -independent replication of Hepatitis C virus in Hep3B human hepatoma cells**

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Running Title: HCV Replication in Hep3B Cells

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## **5.1 Abstract**

The study of Hepatitis C virus (HCV) has benefitted from the use of the Huh7 cell culture system, but until recently there were no other widely-used alternatives to this cell line. Here we render another human hepatoma cell line, Hep3B, permissive to the complete virus life cycle by supplementation with the liver-specific microRNA miR-122, known to aid HCV RNA accumulation. When supplemented, Hep3B cells produce J6/JFH-1 virus titres indistinguishable from those produced by Huh7.5 cells. Interestingly, we were able to detect and characterize miR-122-independent replication of bi-cistronic replicons in Hep3B cells. Further, we show that Argonaute-2 (Ago2) is required for miR-122-dependent replication, but dispensable for miR-122-independent replication, confirming Ago2's role in mediating the activity of miR-122. Thus Hep3B cells are a model system for the study of HCV, and miR-122 independent replication is a model to identify proteins involved in the function of miR-122.

## **5.2 Introduction**

Hepatitis C is a blood-borne viral disease that naturally infects only humans and is prevalent worldwide. As of July 2012, the WHO estimates that 150 million people worldwide are infected with Hepatitis C virus (HCV) (WHO, 2012). HCV exposure leads to chronic liver infections in an estimated 70% of individuals, and can lead to the development of liver disease, steatosis, hepatocellular carcinoma, and other complications late in chronicity (Strader et al., 2004). Unfortunately, no preventative or therapeutic vaccine yet exists, and treatment options are limited. Current standard of care involves lengthy combination therapy with pegylated interferon- $\alpha$  (IFN $\alpha$ ) and ribavirin. Recent approval of two new protease inhibitors, telaprevir and boceprevir, has improved treatment outcomes and when one or the other is added to the cocktail, approximately 70% of genotype 1-infected patients (the most prevalent and difficult to treat) clear the infection (Myers et al., 2012). New direct-acting antiviral drugs currently under development should further improve the effectiveness of current treatment (Poordad and Dieterich, 2012).

A member of the Flaviviridae family, HCV is an enveloped virus with a 9.6kb single-stranded positive-sense RNA genome. The uncapped 5' un-translated region (UTR) of the viral genome bears an internal ribosomal entry site (IRES) that drives translation of the virus' single open reading frame as a polyprotein. The polyprotein is co- and post-translationally cleaved by cellular and viral proteases to produce the structural (core, E1, E2) and non-structural (p7, NS2, NS3, NS4a

and 4b, NS5a and 5b) viral proteins. Both the 5' and 3' UTRs have significant secondary RNA structure that is essential for viral translation and replication (Bartenschlager et al., 2004).

In 2005, Jopling *et al.* identified two binding sites for the liver-specific microRNA, miR-122, in the 5' UTR of HCV; miR-122 binding enhances accumulation of HCV RNA in infected cells (Jopling et al., 2008; Jopling et al., 2005). MicroRNA-122 comprises about 70% of the small RNAs in the mammalian liver, and modulates expression of mRNAs involved in cholesterol biosynthesis, proliferation, and cell differentiation (Esau et al., 2006; Lagos-Quintana et al., 2002; Lin et al., 2008; Norman and Sarnow, 2010).

miRNA expression is often dysregulated or abolished in cancer cells. Expression of miR-122 is typically reduced or lost in liver cancers, but is more likely to be maintained in cases of HCV-associated liver cancer (Coulouarn et al., 2009; Varnholt et al., 2008). Similarly, hepatocyte cell lines that were established from liver tumors also generally lack miR-122 expression. Huh7-derived cell lines are the only human liver cell lines known to have significant endogenous expression of miR-122, and these were also the first cell line to robustly support replication of HCV (Jopling et al., 2005). While expression of the miRNA is considerably lower in these cells than in primary human hepatocytes, other liver cancer cell lines such as HepG2 and Hep3B cells have undetectable levels of miR-122 and are also non-permissive for HCV replication (Varnholt et al., 2008). This information led to the hypothesis that miR-122 plays a role in the host cell tropism of HCV, and this hypothesis is supported by several reports. Supplementation with miR-122 of non-permissive mouse embryonic fibroblasts (MEF), HEK (human embryonic kidney), and HepG2 and Hep3B (human hepatoma) cells with miR-122 has rendered them permissive for transient replication of HCV RNAs derived from the JFH-1 isolate (Chang et al., 2008; Fukuhara et al., 2012; Kambara et al., 2012; Lin et al., 2010; Narbus et al., 2011). HepG2 and Hep3B cells were also capable of producing HCV particles. However, stable RNA replication of JFH-1-derived replicons has also been demonstrated in cells that do not express miR-122, such as murine embryonic fibroblasts (MEFs), through the use of selectable markers, suggesting that miR-122 is not essential for ongoing HCV replication (Chang et al., 2006; Uprichard et al., 2006).

The mechanism of action of miR-122 in the HCV life cycle is unknown. The activity of miR-122 in the replication of HCV is non-canonical, since it binds to the 5' end of the viral genome and requires annealing of nucleotides outside of the miR-122 seed sequence (Machlin et al., 2011; Shimakami et al., 2012b). MicroRNAs normally bind to the 3' ends of cellular mRNAs in a

sequence specific manner, within a complex with other proteins such as Argonaute-2 to form a RISC (RNA-induced silencing complex), and subsequently reduce mRNA translation and stability (Du and Zamore, 2005).

Recent evidence suggests that miR-122 binding to the HCV genome stabilizes the viral RNA (Shimakami et al., 2012b). However, miR-122 has also been reported to enhance translation and augment RNA accumulation (Henke et al., 2008; Jangra et al., 2010a, b; Jopling et al., 2005; Machlin et al., 2011; Roberts et al., 2011; Villanueva et al., 2010). It is possible that the primary function of miR-122 is to stabilize viral RNA, and that the observed influences of miR-122 on HCV translation and replication stem from miR-122 modulation of HCV RNA stability, but this remains to be confirmed. To further tease these activities apart, researchers have begun to examine the cellular factors involved in miR-122-mediated enhancement of HCV RNA accumulation, the most obvious of which are the proteins in the microRNA biogenesis pathway and proteins that comprise the RISC. Thus far, research has shown that Dicer and TRBP, proteins involved in the biogenesis of microRNAs, are required for processing of miR-122, and that Argonaute-2 (Ago2) is required for miR-122 stabilization of HCV RNA, for efficient HCV RNA accumulation, and for miR-122 stimulation of HCV translation (Machlin et al., 2011; Shimakami et al., 2012a; Shimakami et al., 2012b; Wilson et al., 2011; Zhang et al., 2012).

We, and others, have hypothesized that miR-122 is a limiting factor in HCV tissue tropism and cell culture models, and herein we have confirmed replication of the J6/JFH-1 viral RNA in Hep3B cells supplemented with miR-122, showing that these cells can produce virus particles to the same titres as Huh7.5 cells. Additionally, through use of Hep3B cells as a model system, we have identified and characterized miR-122-independent replication of two bi-cistronic HCV replicons, the sub-genomic JFH-1 replicon, and the full-genomic JFH-1 replicon. MicroRNA-122-independent replication of HCV provides a model system in which to investigate the roles of cellular proteins in miR-122 processes, compared to their roles in HCV replication that are independent of miR-122. As proof of principle we evaluated the role of Argonaute-2 (Ago2) in replication of the sub-genomic replicon with and without miR-122, and determined that Ago2 knockdown does not influence miR-122-independent replication, but has an effect on miR-122-dependent replication. This confirms that Ago2 is involved in the activity of miR-122 as it promotes HCV RNA accumulation.

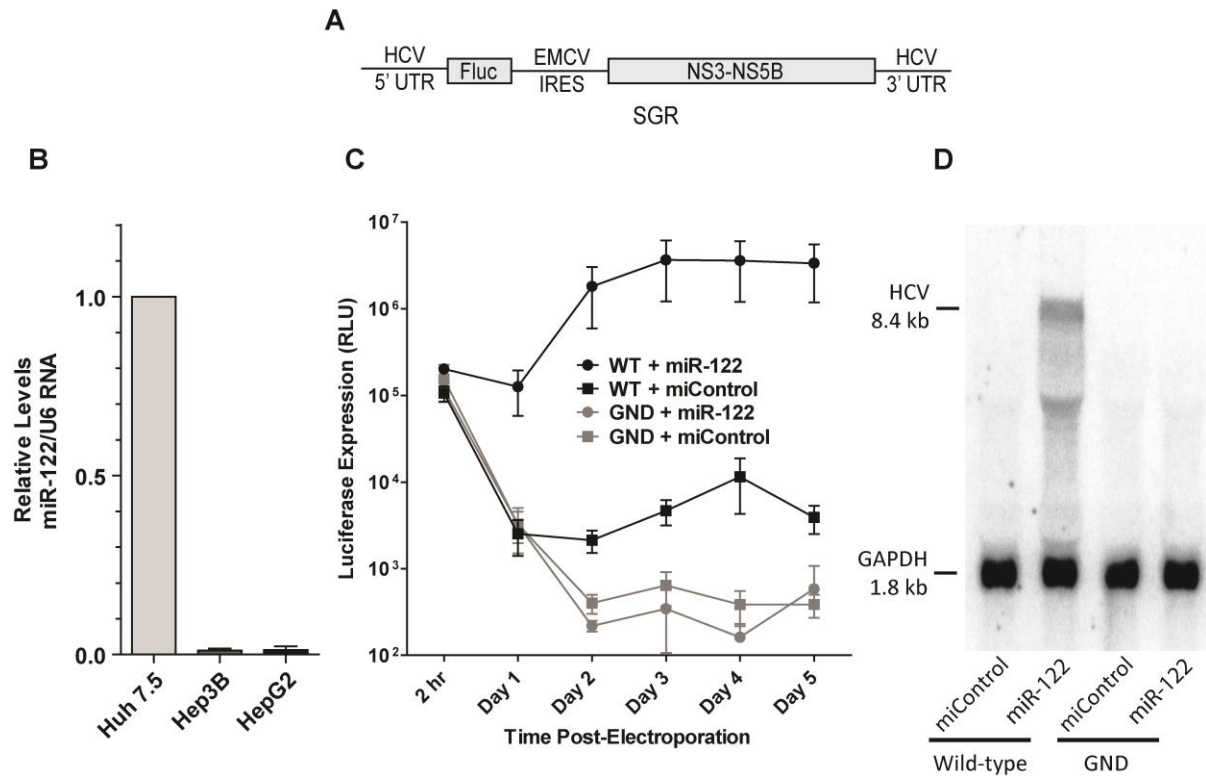
### 5.3 Results

#### 5.3.1 Supplementation with miR-122 renders Hep3B cells permissive to sub-genomic HCV RNA replication.

Many human hepatomas have lost the ability to express the liver specific miRNA, miR-122 (Coulouarn et al., 2009). Unlike commonly used human hepatoma cell lines such as Hep3B cells and HepG2 cells, Huh7-derived cells (such as Huh7.5) retained easily-detectable miR-122 expression after transformation (Figure 5.1B, (Jopling et al., 2005)), and are also permissive to Hepatitis C virus replication. Because of this, and because miR-122 has been shown to impact HCV translation and replication, we hypothesized that miR-122 is a limiting factor in some non-permissive cell lines, and that providing miR-122 will render them permissive for HCV replication and virion production. Electroporating Hep3B cells with synthetic mature miR-122 duplexed RNA permitted high levels of replication of the sub-genomic JFH-1 HCV replicon (SGR, depicted in Figure 5.1A), as measured by the firefly luciferase reporter gene in Figure 5.1C, and confirmed by northern blot analysis of viral RNA accumulation (Figure 5.1D). Replication-incompetent viral RNA (SGR GND) was used to establish basal levels of luciferase expression in the absence of replication. Both luciferase expression and accumulation of sub-genomic RNA in Hep3B cells were similar to that observed in Huh7.5 cells (data not shown). Transfection efficiency was determined by co-electroporation of a Renilla mRNA and analysis of Renilla luciferase expression levels two hours post-electroporation, and did not vary between samples (data not shown). Cell numbers were evaluated three days post-electroporation by WST-1 assay, and were consistent among samples in each experiment (data not shown).

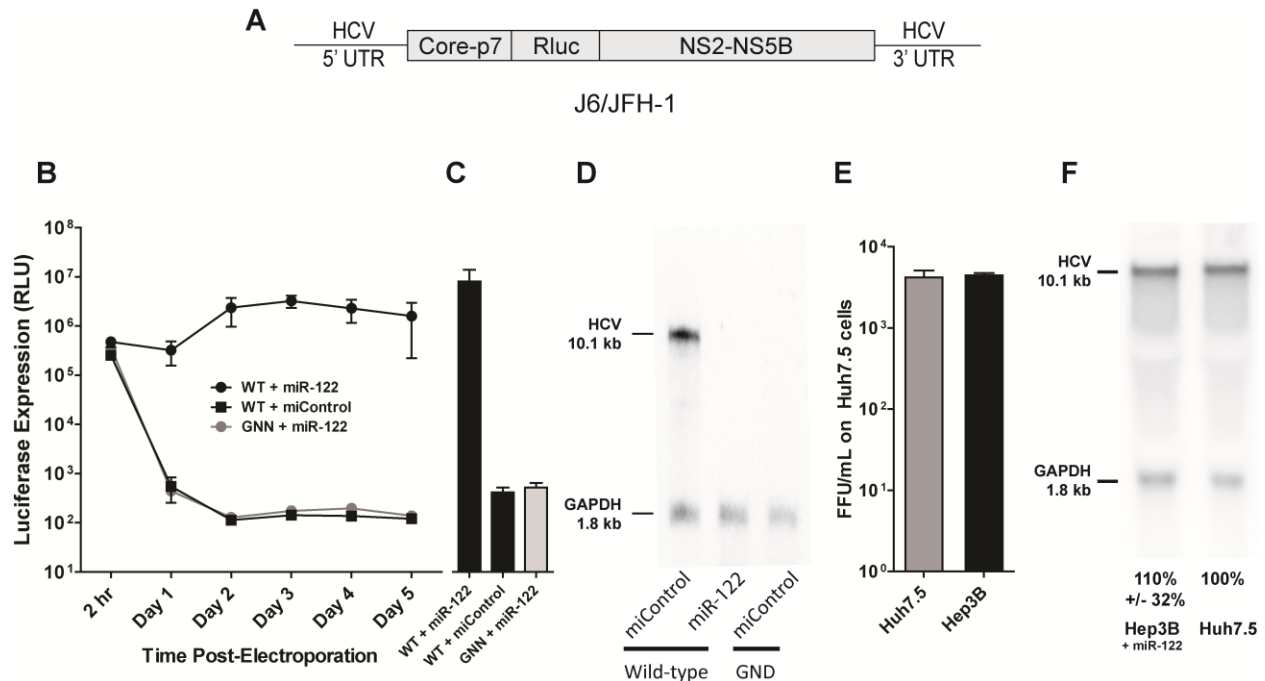
#### 5.3.2 Full-length HCV RNA can replicate and generate infectious virus particles in Hep3B cells.

Co-electroporation of J6/JFH-1 RNA (depicted in Figure 5.2A) and miR-122 also renders Hep3B cells permissive to replication of full-length HCV RNA (Figure 5.2B). Expression of the Renilla luciferase (Rluc) reporter confirmed replication of full length HCV RNA in Hep3B cells when co-electroporated with miR-122. In the absence of miR-122, the Rluc expression pattern overlapped that of the non-replicating GNN control (Figure 5.2B, compare WT + miControl and GNN + miR-122). Full-length HCV RNA accumulation by replication was confirmed by northern blot analysis (Figure 5.1D). Tissue culture supernatant was collected on day 3 post-electroporation



**Figure 5.1 Replication of sub-genomic (SGR) HCV in Hep3B cells.**

**A.** Components of “SGR,” the bi-cistronic sub-genomic firefly luciferase reporter replicon based on the JFH-1 isolate. The replicon lacks the structural genes required to form particles, but has all the necessary non-structural genes required for replication, along with the 5’ and 3’ un-translated regions (UTRs). The firefly luciferase reporter gene is driven by the HCV IRES, while viral protein expression is driven by an EMCV IRES. **B.** miR-122 levels in human hepatoma cell lines were evaluated by TaqMan qRT-PCR. Levels are determined relative to a housekeeping small RNA, RNU6B, for comparison between cell lines. Huh7.5 cells are derived from Huh7 cells; HepG2 and Hep3B cells are of separate lineages. **C.** Cells were electroporated with the indicated SGR replicon RNA (wild-type, WT; or replication-incompetent, GND), and miRNA. Firefly luciferase expression was evaluated at the indicated time points. RLU are a measure of light produced by the luciferase enzyme extracted from cell lysates. All data shown is the average of three or more independent experiments, unless otherwise indicated, and error bars represent standard error. **D.** Total cellular RNA from (C) was collected three days post-electroporation and evaluated by northern blot. The blot shown is representative of three experiments. GAPDH is shown as a loading control. HCV (8.4 kb) refers to the size of the SGR replicon genome.



**Figure 5.2 Replication of full-length (J6/JFH-1 Rluc) HCV in Hep3B cells.**

**A.** Representation of “J6/JFH-1 Rluc,” the mono-cistronic full-length J6/JFH-1 chimeric replicon, which bears all the viral genes, is capable of producing infectious particles in cell culture, and has a Renilla luciferase reporter gene. The HCV IRES drives translation of all the genes. **B.** Hep3B cells were electroporated with either wild-type (WT) or replication-incompetent (GNN) J6/JFH-1 Rluc RNA, and the indicated microRNA. Luciferase expression levels were measured at the indicated times post-electroporation. **C.** Day 3 supernatant from Hep3B cells in (B) was used to infect naïve Huh7.5 cells; luciferase expression in the Huh7.5 cells three days post-infection indicated the presence of infectious HCV virions in the supernatant. Luciferase expression levels are shown on the axis in part (B). **D.** Northern blot of J6/JFH-1 RNA in Hep3B cells 3 days post-electroporation, demonstrating HCV RNA replication in Hep3B cells supplemented with miR-122. HCV (10.1 kb) refers to the size of the full-length replicon genome. **E.** Supernatant from both Hep3B and Huh7.5 cells three days post-electroporation were titrated for HCV focus-forming units (FFU). **F.** Northern blot comparing levels of J6/JFH-1 RNA in Huh7.5 cells, and in Hep3B cells supplemented with miR-122, three days post-electroporation. Bands are quantified by densitometry, and normalized to GAPDH. Percentages are an average of seven independent experiments, +/- standard deviation, and are presented relative to Huh7.5 cells. In all other panels, the data are presented as the average of three independent experiments and the error bars represent standard error of the mean.

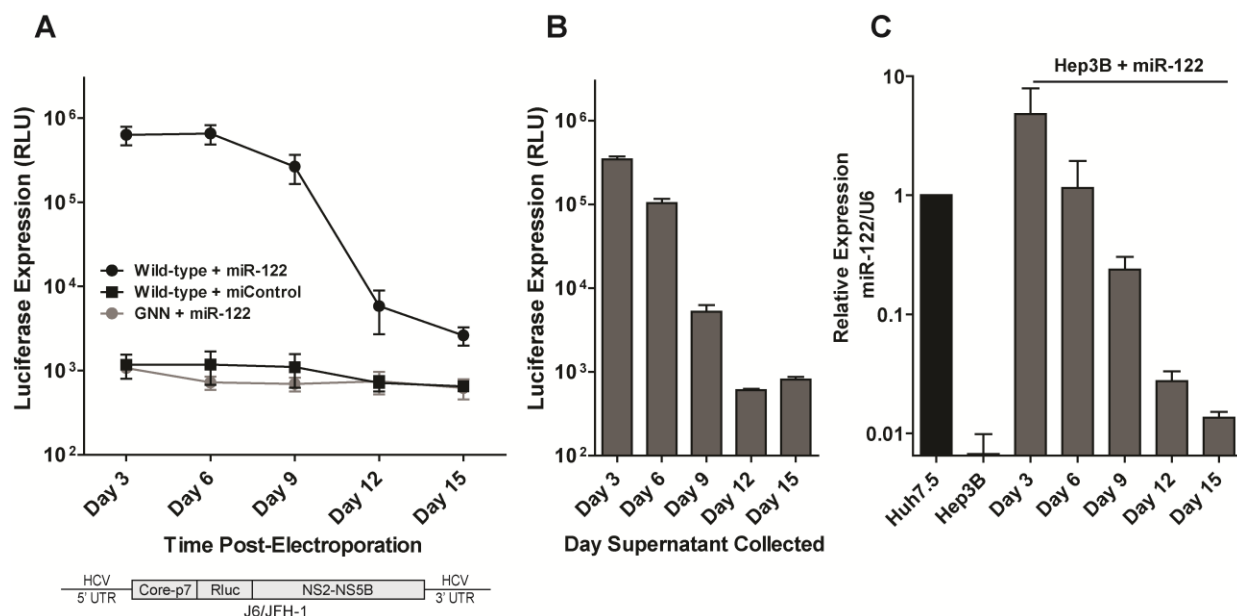
and analyzed for the presence of HCV particles. When the supernatant was used to infect naïve Huh7.5 cells, luciferase expression in the newly infected cells (Figure 5.2C) indicated the presence of infectious HCV particles in the supernatant from Hep3B cells supplemented with miR-122. Focus-forming assays showed that infectious virus titres in the supernatant from miR-122-supplemented Hep3B cells were similar to that from Huh7.5 cells (Figure 5.2E). Northern blot analysis of HCV RNA levels in miR-122 supplemented Hep3B cells compared with Huh7.5 cells (Figure 5.2F) indicates that at three days post-electroporation, Hep3B cells had similar levels of HCV RNA accumulation as that observed in Huh7.5 cells.

### *5.3.3 Replication levels and virus particle production coincide with miR-122 levels in Hep3B cells.*

To determine how long Hep3B cells can support full-length viral replication when supplemented with synthetic mature miR-122 duplex RNA, we monitored HCV replication via luciferase expression, until none was detectable. By 15 days post-electroporation, little to no replication or infectious virus particle production was apparent in Hep3B cells (Figure 5.3A, B). We evaluated miR-122 levels by qRT-PCR at the same time points and determined that HCV replication paralleled the levels of exogenously supplied miR-122 (Figure 5.3C). At Day 6, miR-122 levels were similar to that found in Huh7.5 cells, and by Day 9, miR-122 levels had dropped to 30% of miR-122 levels in Huh7.5 cells. The drop in miR-122 levels appears to correspond to the decline in HCV replication and particle production (as measured by luciferase). Thus, viral RNA replication and particle production coincide with miR-122 levels in Hep3B cells.

### *5.3.4 Hep3B cells supplemented with miR-122 are permissive for HCV infection.*

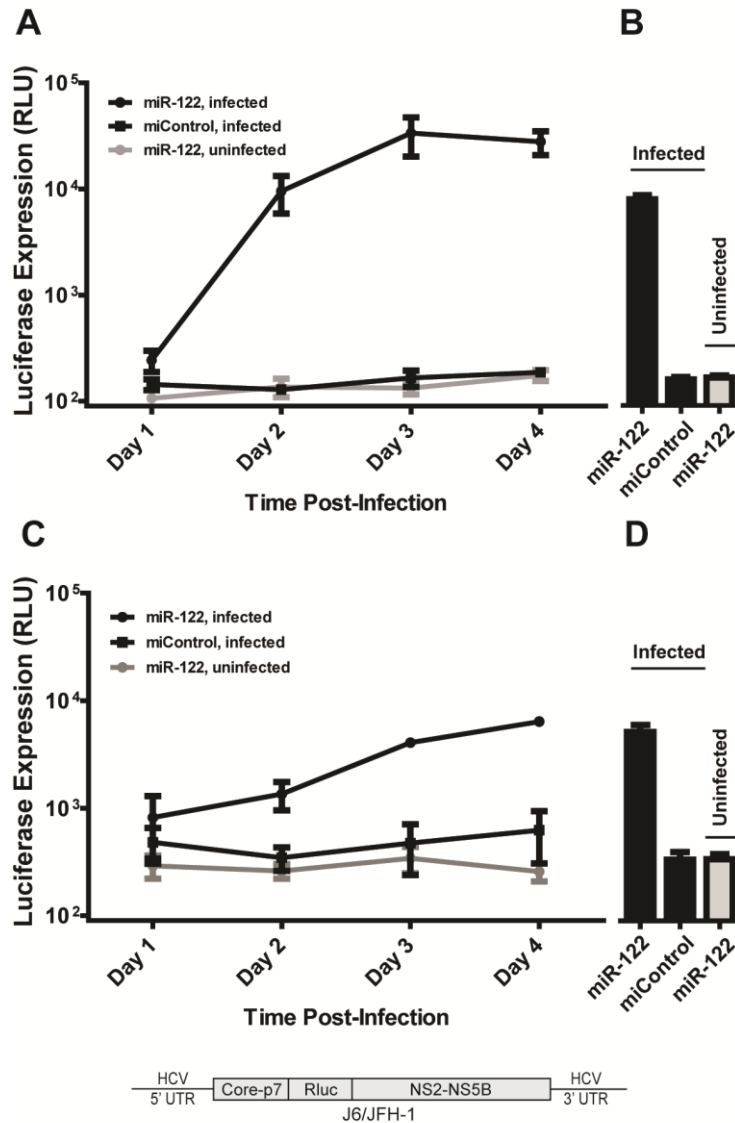
Finally, we examined whether Hep3B cells can also support virus entry, and thus recapitulate a full round of infection in Hep3B cells when supplemented with miR-122. One day before infection, Hep3B cells were transfected via Lipofectamine 2000 (Figure 5.4A) with either miR-122 or a control microRNA, and on day 0, were infected with J6/JFH-1 HCVcc (virus derived from Huh7.5 cells). Figure 5.4A shows luciferase expression from the infected cells at indicated time points post-infection. Hep3B cells supplemented with miR-122 show detectable luciferase expression post-infection. Furthermore, on Day 4 post-infection, supernatant from the infected cells was transferred to Huh7.5 cells for detection of infectious virus particle production. As can



**Figure 5.3 Long-term replication of full-length HCV RNA in Hep3B cells.**

**A.** Luciferase levels representing replication of J6/JFH-1 Rluc HCV RNA in Hep3B cells were evaluated at the indicated time points post-electroporation. **B.** Luciferase levels representing infectious HCV particles in supernatants collected from ‘wild-type + miR-122’ in (A). Supernatant was collected at each time point and used to infect naive Huh7.5 cells; three days later, luciferase levels were analyzed to measure approximate supernatant infectivity. **C.** Relative miR-122 levels in Huh7.5 cells, un-electroporated Hep3B cells, and Hep3B cells electroporated with miR-122 collected at the indicated times post-electroporation. miR-122 levels were determined by TaqMan qRT-PCR and normalized to RNU6B. Data represent the average of three independent experiments and bars represent standard error.





**Figure 5.4 Infection of Hep3B cells with full-length J6/JFH-1 HCVcc.**

**A.** Hep3B cells were transfected via Lipofectamine 2000 with the indicated miRNA on Day -1, and infected with HCVcc (J6/JFH-1 particles derived from Huh7.5 cells) or mock-infected with media on Day 0. Luciferase was analyzed at the indicated time points to detect infection and subsequent replication. **B.** Supernatant was collected from the infected Hep3B cells in (A) on Day 4, and was used to infect naïve Huh7.5 cell. Three days later, luciferase expression, in the same axis as in (A), was analyzed in the infected Huh7.5 cells to detect infection. Data are the average of three independent experiments and error bars represent standard error. **C.** Hep3B cells were electroporated with the indicated miRNA on Day -1, and infected with HCVcc (J6/JFH-1 particles derived from Huh7.5 cells) or mock-infected with media on Day 0. Luciferase was analyzed at the indicated time points to detect infection and subsequent replication. **D.** Supernatant was collected from the infected Hep3B cells in (C) on Day 4, and was used to infect naïve Huh7.5 cells. Three days later, luciferase expression, on the same axis as in (C), was analyzed in the infected Huh7.5 cells to detect infection. Data represent the average of three or more independent experiments and bars represent standard deviation.

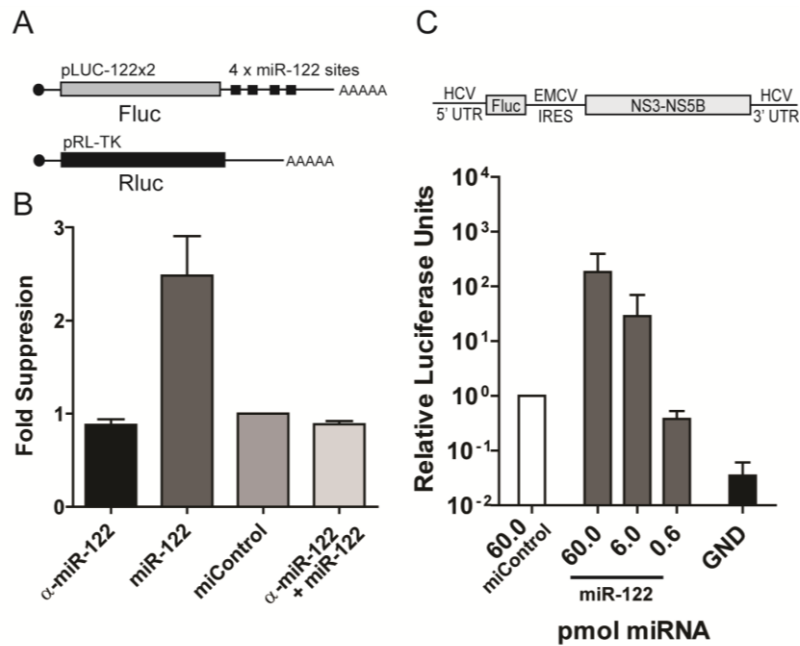
be seen in Figure 5.4B, Hep3B cells bearing miR-122 prior to infection can carry out the viral life cycle and produce infectious virions, but at lower levels compared to cells electroporated with viral RNA, which suggests inefficient HCV entry into miR-122 transfected Hep3B cells. To ensure that the original virus HCVcc used to infect the cells could not contaminate the virus supernatants collected on Day 4, the Hep3B cells were washed and media was changed the day following the initial infection. Failure to detect virus in supernatant from Hep3B cells electroporated with miControl (grey) confirmed that there was no contaminating HCVcc remaining. We also tested whether electroporating the cells with miR-122 on Day -1 prior to infection would improve their uptake of miR-122 and thus, potentially, improve infectability, but this was not seen (Figure 5.4C and D).

#### *5.3.5 Sub-genomic HCV RNA can replicate in Hep3B cells un-supplemented with miR-122.*

Interestingly, luciferase expression levels in Hep3B cells electroporated with HCV sub-genomic RNA (without miR-122) were greater than those of the replication incompetent GND control (Figure 5.1C, compare WT + miControl to GND + miControl). This suggested that SGR wild-type RNA was persisting longer than non-replicating RNA, even in the absence of miR-122 supplementation. RNA levels from SGR in the absence of miR-122 were not initially detected by northern blot (Figure 5.1D), but higher than background luciferase expression was consistently seen. In addition, luciferase levels increased after day 1 post-electroporation, indicating that SGR RNA replicated in Hep3B cells even without miR-122 supplementation. We ruled out the possibility that the miControl synthetic RNA impacted sub-genomic replication by electroporating viral RNA with no accompanying microRNA into Hep3B cells, and found similar luciferase production as when miControl was provided (data not shown). We hypothesized that HCV RNA replication could be due to either low levels of active miR-122 within Hep3B cells, or alternatively, that sub-genomic RNA can replicate in Hep3B cells independent of the activity of miR-122.

#### *5.3.6 Hep3B cells lack functional endogenous miR-122.*

To rule out possible contribution of endogenous miR-122 on un-supplemented SGR replication in Hep3B cells, we analyzed the levels and activity of miR-122 in Hep3B cells. Real-time PCR analysis of miR-122 levels in Hep3B cells had indicated that Hep3B cells do not express consistently detectable amounts of miR-122 (Figure 5.1B). To analyze for functional miR-122 in



**Figure 5.5 Hep3B cells lack functional miR-122.**

**A.** Reporters used in miRNA functional assays. *Top:* pLUC-122x2 encodes a firefly luciferase mRNA bearing miR-122 binding sites in its 3' UTR; binding of miR-122 to these sites suppresses translation of the firefly luciferase reporter. *Bottom:* pRL-TK encodes a Renilla luciferase mRNA to be used as a transfection control. **B.** Hep3B cells were electroporated with the indicated miRNA or  $\alpha$ -miR-122. Two days later, cells were transfected with two reporter plasmids that express the mRNAs shown in (B). One day post-transfection, cells were assayed for both luciferase reporters' expression levels, and data was normalized to Renilla luciferase levels. Data are the average of three independent experiments and are shown as fold suppression compared to miControl by each miRNA. Bars represent standard error. **C.** Hep3B cells were electroporated with SGR RNA and the indicated miRNA dilutions. Luciferase was measured three days post-electroporation, and dilutions are shown relative to 60.0 pmol miControl. miR-122 dilutions are 1:1 (60.0 pmol), 1:10 (6.0 pmol) and 1:100 (0.6 pmol). The GND mutant demonstrates non-replicating luciferase levels.

Hep3B cells we assayed for miR-122 suppression activity. In the assay, miR-122 suppression activity was monitored by using two plasmids (Figure 5.5A); one bears a firefly luciferase gene (Fluc) with HCV-derived miR-122 binding sites in the 3'UTR to measure miR-122 suppression, and the other bears a Renilla luciferase gene (Rluc) to control for transfection efficiency (Jopling et al., 2008; Machlin et al., 2011). If miR-122 is present in the co-electroporated cells, then expression of firefly luciferase will be reduced via the miRNA suppression pathway. If this is the case, then a miR-122 antagonist should relieve the suppression. When the reporter plasmids were electroporated alone or with the miR-122 antagonist, similar Fluc:Rluc ratios were observed, indicating no active miR-122 in Hep3B cells (Figure 5.5B). In control experiments, the miR-122 antagonist was confirmed to be active since electroporation of synthetic miR-122 enhanced suppression activity, and the miR-122 antagonist completely abolished this enhancement (Figure 5.5B, miR-122, and  $\alpha$ -miR-122 + miR-122). We also titrated amounts of miR-122 that can affect SGR by electroporating Hep3B cells with SGR RNA and ten-fold dilutions of miR-122 (Figure 5.5C). Addition of 60 pmol of miR-122 appeared to saturate the systems involved in replication, as addition of greater amounts of miR-122 (5X, 300 pmol, and 10X, 600 pmol) did not further increase replication (data not shown). A ten-fold dilution of miR-122 (6.0 pmol) led to approximately ten-fold less luciferase expression on Day 3 post-electroporation, and when we supplemented with 0.6 pmol (a hundred-fold dilution) of miR-122 we saw a reduction in replication to levels no different than miControl-supplemented SGR RNA. Thus, a hundred-fold dilution (0.6 pmol) of miR-122 is below the threshold level required to augment HCV RNA accumulation. This dilution of miR-122 is easily and consistently detectable by qRT-PCR (Figure 5.3C, and data not shown), and so we concluded that amounts of miR-122 not consistently detectable by qRT-PCR also do not affect HCV RNA accumulation, but we cannot rule out the existence of undetectable levels of miR-122 in Hep3B cells.

#### *5.3.7 HCV replication in Hep3B cells in the absence of miR-122 is not affected by a miR-122 antagonist.*

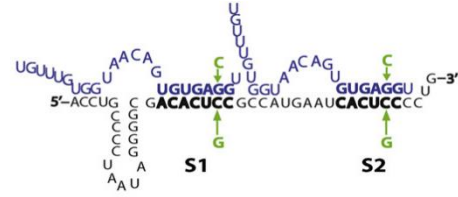
To confirm that miR-122 un-supplemented sub-genomic HCV RNA replication in Hep3B cells was not dependent on miR-122, we tested whether it was affected by miR-122 antagonism. In transient HCV replication assays, un-supplemented sub-genomic RNA replication was unaffected by the addition of the miR-122 antagonist (Figure 5.6B, compare  $\alpha$ -miR-122 to miControl and  $\alpha$ -

Con) or a mutant miRNA (miR-122/p3), while miR-122-supplemented sub-genomic RNA replication was reduced to un-supplemented levels by the antagonist (Figure 5.6B, compare miR-122 and miR-122 +  $\alpha$ -Con with miR-122 +  $\alpha$ -122). RNA harvested on day 3 following electroporation was evaluated by northern blot in Figure 5.6D, and both miR-122-dependent (miR-122) and miR-122-independent (miControl) HCV RNA accumulation was detectable upon overexposure of the blot. Thus, sub-genomic RNA can replicate independent of miR-122 to low levels in Hep3B cells.

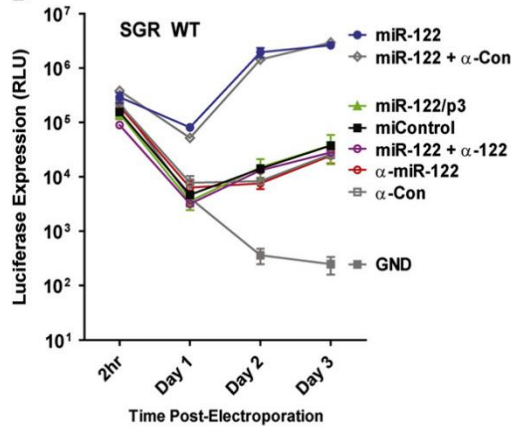
#### *5.3.8 Replication of p3 SGR RNA in Hep3B and Huh 7.5 cells is not affected by miR-122 or $\alpha$ -miR-122.*

If un-supplemented HCV replication in Hep3B cells is independent of miR-122, then sub-genomic constructs that cannot bind to endogenous miR-122 should also be capable of replication. To test this hypothesis we generated sub-genomic constructs having various point mutations in both miR-122 binding sites (S1 and S2) in the 5' UTR of the viral genome (Figure 5.6A). We and others have shown that point mutations in the miR-122 binding sites prevent interaction with miR-122, and miR-122-mediated enhancement of HCV replication, and that introducing complementary mutations into an exogenously-provided miR-122 can restore this interaction, and restore replication of HCV (Henke et al., 2008; Jangra et al., 2010b; Machlin et al., 2011; Wilson et al., 2011). We first tested for miR-122-independent replication of a 'p3-4' sub-genomic RNA. The p3-4 mutant bears mutations at positions 3 and 4 of both miR-122 binding sites in the 5' UTR and has been used by our lab and others (Jopling et al., 2005; Wilson et al., 2011). When we evaluated the p3-4 sub-genomic construct in Hep3B cells there was no indication of miR-122-independent replication (data not shown). However, even upon supplementation with a complementary miRNA, miR-122/p3-4, replication of the mutant could not be supplemented to wild-type levels (Wilson et al., 2011). Surmising that since these RNAs could not be fully complemented, they may be structurally flawed, we then evaluated two other constructs that each bore a single C to G point mutation in both miR-122 binding sites, one at position 5 of the miR-122 seed sequence (p5) and one at position 3 (p3). The p5 SGR construct did not demonstrate replication without microRNA supplementation (data not shown), but the p3 construct did (Figure 5.6C), and replicated in Hep3B cells to levels similar to that of the wild type construct (compare Figure 5.6B – miControl to Figure 5.6C – miControl). In addition, the p3 SGR was unresponsive

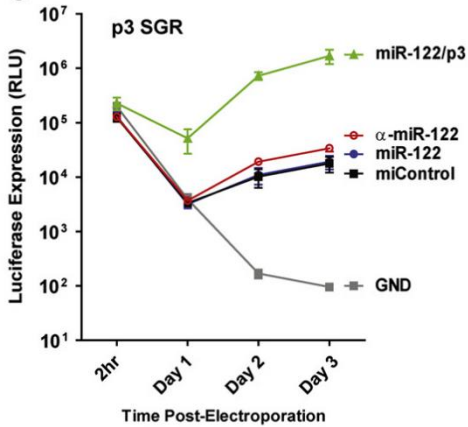
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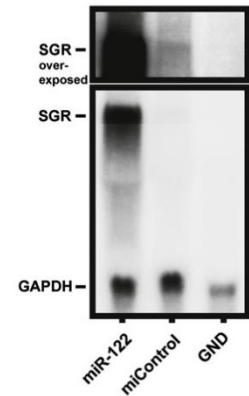
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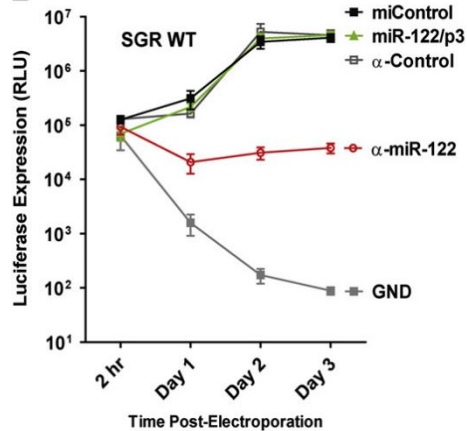
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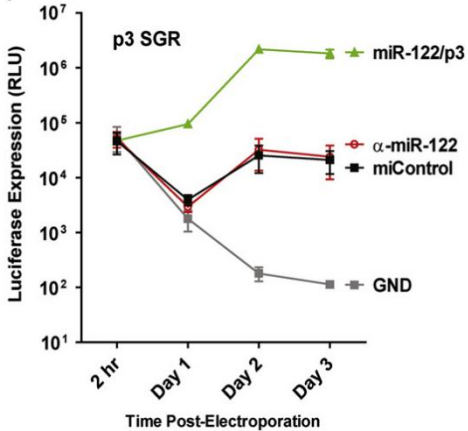
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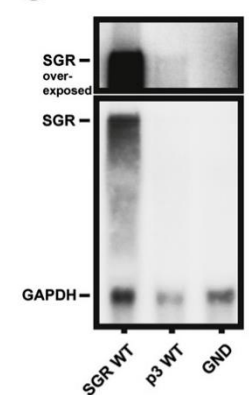
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F



G



**Figure 5.6 MicroRNA-122-independent replication of sub-genomic HCV.**

**A. Left:** Diagram of SGR construct. **Right:** Schematic of the miR-122 binding sites in the HCV 5' UTR. Blue letters show miR-122 and black letters are HCV RNA. Bold indicates miR-122 seed sequences, while other complementary sequences between HCV and miR-122 are shown as aligned bases. The green arrows and bases indicate the p3 mutations in both seed sequences on the HCV genome (C to G), and the complementary changes found in the microRNA miR-122/p3 (G to C). **B.** Hep3B cells were electroporated with SGR RNA and the indicated microRNAs and/or miRNA antagonists ( $\alpha$ -miR-122 and  $\alpha$ -Control). Luciferase was assayed at the indicated time points. GND is the replication-incompetent mutant of SGR as in Figure 5.1C. **C.** Hep3B cells were electroporated with p3 SGR RNA, which bears a mutation in position 3 of the miR-122 binding sites S1 and S2 as depicted in (A), and otherwise treated as in (B). **D.** RNA collected from Hep3B cells three days post-electroporation was evaluated by northern blot to detect miR-122-dependent (miR-122) and miR-122-independent (miControl) replication. The top panel is overexposed in order to detect miR-122-independent replication. **E.** Huh7.5 cells were electroporated as in (B). **F.**

Huh7.5 cells were electroporated as in (C). **G.** RNA was collected from Huh7.5 cells and evaluated as in (D). Luciferase data are the average of three independent experiments; bars represent standard error. Blots are representative of two independent experiments.

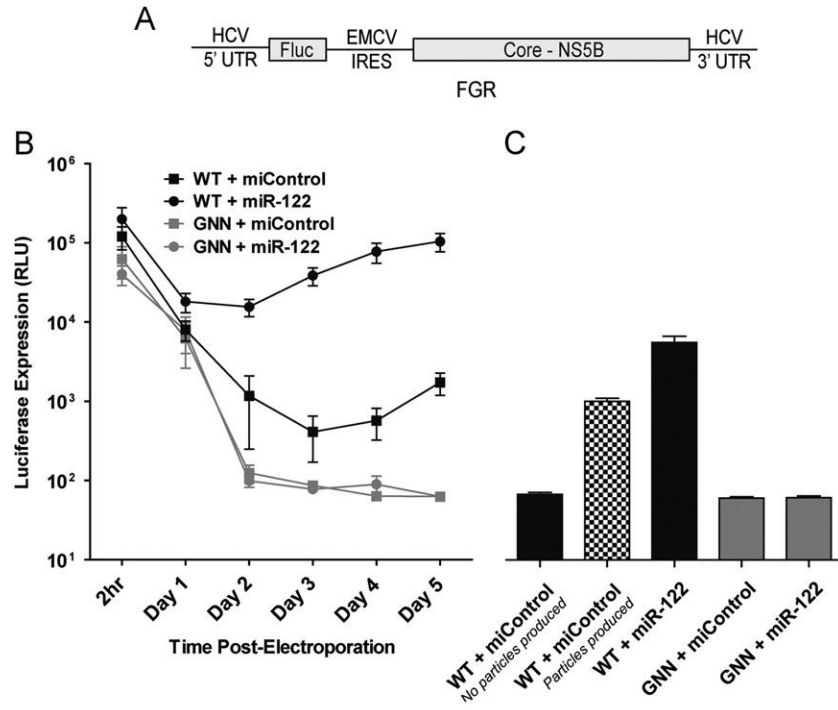
to exogenously provided miR-122 (Figure 5.6C, compare miR-122 to miControl), and miR-122 antagonist (Figure 5.6C, compare miControl to  $\alpha$ -miR-122;  $\alpha$ -Control also had no effect – data not shown) and could be complemented to near-wild-type levels by an exogenously provided miRNA, miR-122/p3, which bears a complementary sequence to the mutated construct (compare Figure 5.6B - miR-122 to Figure 5.6C - miR-122/p3). Because the mutated miRNA did not affect the wild-type replicon (Figure 5.6B, miR-122/p3), and because the mutated replicon was not impacted by wild-type miR-122 nor its antagonist (Figure 5.6C, miR-122 and  $\alpha$ -miR-122), we confirmed that the p3 mutation successfully abolished the effects of miR-122 binding on the replicon. This further confirms miR-122-independent replication of HCV SGR RNA.

To ensure that miR-122-independent replication is not specific to some factor present only in Hep3B cells, we also examined miR-122-independent replication of SGR RNA in Huh7.5 cells. By use of  $\alpha$ -miR-122 to antagonize the endogenous miR-122 in Huh7.5 cells, we were able to reduce replication of sub-genomic HCV RNA to levels similar to the miR-122-independent replication we observed in Hep3B cells (Figure 5.6E,  $\alpha$ -miR-122; compare with Figure 5.6B, miControl). To substantiate miR-122-independent replication in Huh 7.5 cells, we analyzed replication of the p3 SGR construct in Huh 7.5 cells (Figure 5.6F). In Huh 7.5 cells, p3 SGR replication was unaffected by the miR-122 antagonist (Figure 5.6F, compare miControl and  $\alpha$ -miR-122;  $\alpha$ -Control had no effect on p3 SGR replication – data not shown), confirming miR-122-independent replication. The p3 SGR construct still responded to the complementary microRNA miR-122/p3. Evaluating Day 3 RNA from these experiments by northern blot (Figure 5.6G), miR-122-dependent (“SGR WT” – SGR WT + miControl) and miR-122-independent (“p3 SGR” – p3 SGR + miControl) RNA accumulation can both be detected upon overexposure of the blot. Huh7 cells, the parent cells to the Huh7.5 line, also supported miR-122-independent replication of p3 SGR (data not shown). Thus, we have detected unselected miR-122-independent replication of sub-genomic HCV RNA in three cell lines, and have verified that a mutation in the miR-122 binding sites renders replication of the SGR construct independent of wild-type miR-122.

#### *5.3.9 miR-122-independent replication of a full length bi-cistronic replicon RNA.*

The miR-122-independent replication of HCV SGR RNA in Hep3B cells was not echoed by the J6/JFH-1 construct. Luciferase expression levels from full-length HCV RNA electroporated without miR-122 were indistinguishable from those of the non-replicative GNN control (compare





**Figure 5.7 Replication of the bi-cistronic full-genome replicon (FGR) in Hep3B cells.**

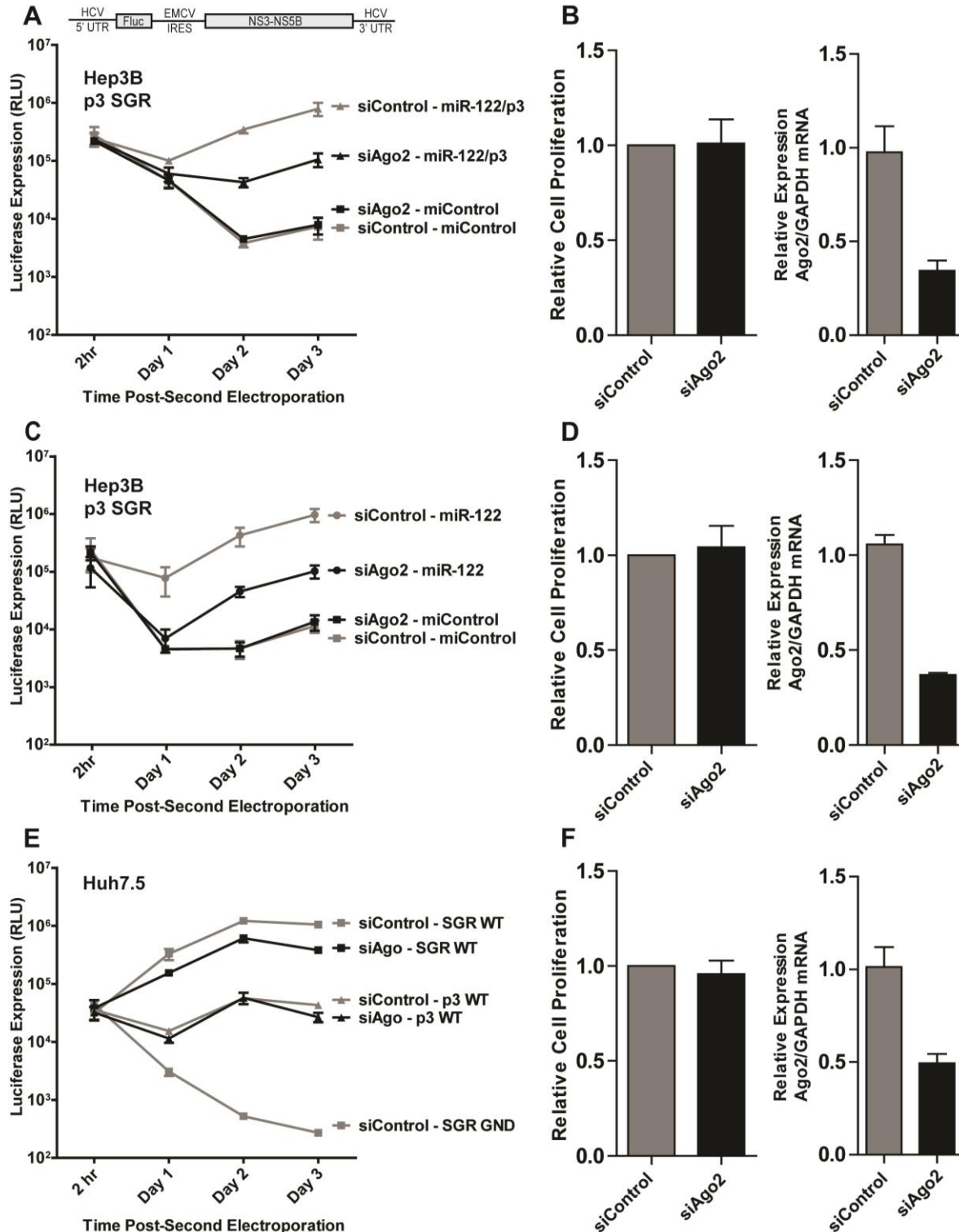
**A.** Diagram representation of “FGR,” the bi-cistronic full-genomic firefly luciferase replicon which contains structural genes that permit infectious particle production; it is otherwise identical to the SGR. **B.** Hep3B cells were electroporated with FGR wild-type (WT) or replication-incompetent (GNN) RNA and the indicated microRNA, and luciferase expression was measured at the indicated time points. **C.** Supernatants from cells in (B) were collected at Day 4 post-electroporation, and were used to infect Huh7.5 cells. Three days after infection, luciferase levels, on the same axis as in (B), were evaluated in the Huh7.5 cells as an indication of the presence of infectious virus particles produced from Hep3B cells. Note the first two bars are both supernatants derived from WT + miControl electroporations; the solid black bar is the subset of supernatants that showed no virion production, while the checkered bar is the subset of supernatants that showed detectable virion production. Data are the average of more than three independent experiments, and error bars represent standard error of the mean.

Figure 5.1C and 5.2A, WT + miControl to GNN or GND). Thus we hypothesized that miR-122-independent replication may occur because of the bi-cistronic nature of the sub-genomic replicon. Alternatively, the increased length of the J6/JFH-1 genome could have impeded miR-122-independent replication. In order to test both of these hypotheses, we evaluated the replication of the full-genomic replicon (FGR) in Hep3B cells. FGR is depicted in Figure 5.7A, and is a bi-cistronic replicon RNA similar to SGR in that it contains an EMCV IRES that drives translation of the viral proteins, but is considerably longer since it also encodes the entire HCV polyprotein, including the viral structural proteins. Thus, if the bi-cistronic nature of the SGR replicon facilitates miR-122-independent replication of viral RNA, then the FGR replicon should also replicate independently of exogenous miR-122. However, if the genome length of the J6/JFH-1 replicon is hindering miR-122-independent replication, FGR bears a longer sequence and should also exhibit no miR-122-independent replication.

Our data indicates that FGR replicons can replicate independent of miR-122 since when we electroporated FGR RNA with a control miRNA we saw higher luciferase expression than was observed with the replication incompetent mutant (Figure 5.7B, WT + miControl vs. GNN), particularly at later time points. However, luciferase levels were very low. Thus, this construct does not appear to replicate as efficiently as either the SGR or J6/JFH-1 constructs in Hep3B and Huh7.5 cells (compare Figure 5.7A to Figure 5.1C and 5.2A; and data not shown). Evaluation of supernatant from Day 4 post-electroporation in Figure 5.7C shows limited production of infectious virus particles from miR-122-supplemented cells. We periodically observed production of infectious virus particles from miR-122-independent replication of FGR RNA in Hep3B cells (checkered bar), but we do not see this in every experiment. Thus, we believe that miR-122 unsupplemented FGR replication in Hep3B cells can produce virus particles, but at very low levels. These results, however, suggest that the presence of a second IRES in the SGR and FGR replicons permits detectable replication of the viral RNA in the absence of miR-122.

#### *5.3.10 Argonaute-2 is not required for miR-122-independent replication of sub-genomic HCV RNA.*

We and others have shown that Argonaute-2 (Ago2), a key player in the microRNA suppression pathway, is required for the role that miR-122 plays in translation and stability of HCV RNA (Roberts et al., 2011; Shimakami et al., 2012a; Shimakami et al., 2012b; Wilson et al.,



**Figure 5.8 Ago2 is not required for miR-122-independent replication of sub-genomic HCV.**

**A.** Hep3B cells were initially electroporated with either siAgo2 or siControl on Day -3; on Day 0, cells were electroporated again with the same siRNA, p3 SGR, and the indicated microRNA, along with a control Renilla mRNA. Luciferase was assayed at the indicated time-points post-second electroporation (Day 0) to detect RNA replication. **B.** Argonaute-2 knockdown in Hep3B cells

with p3 SGR. *Left.* Relative Hep3B cell numbers were assayed via the biochemical WST-1 assay to confirm that siRNA treatment did not affect cell growth. *Right.* On Day 3 post-second-electroporation, RNA from all siControl-treated and all siAgo2-treated Hep3B samples was collected for qRT-PCR to confirm Ago2 knockdown. **C.** Hep3B cells were treated as in (A), but were electroporated with SGR WT and the indicated miRNA. **D.** Argonaute-2 knockdown in Hep3B cells with SGR WT, analyzed as in (B). **E.** Huh7.5 cells were treated as in (A); SGR RNA was used to represent miR-122-dependent replication, and p3 RNA was used to represent miR-122-independent replication. **F.** Argonaute-2 knockdown in Huh7.5 cells, analyzed as in (C). Data represent the average of three or more independent experiments and bars represent standard deviation.

2011). Ago2 has also been implicated in the mechanism of miR-122 augmentation of HCV RNA accumulation through the use of Ago2 siRNA knockdown, but these assays could not rule out the possibility that Ago2 affected HCV replication through indirect modulation of other cellular functions (Wilson et al., 2011). To test the hypothesis that Ago2 promotes HCV replication due to its role in mediating the activity of miR-122, we assessed the influence of Ago2 knockdown on miR-122-independent SGR replication in Hep3B and Huh 7.5 cells (Figure 5.8). In Hep3B cells, Ago2 knockdown had no effect on miR-122/p3-independent replication of the p3 SGR and the wild-type SGR (Figure 5.8A and 5.8C, compare siAgo2 – miControl to siControl – miControl). However, Ago2 knockdown severely hampered the ability of miR-122/p3 to enhance p3 SGR luciferase expression (Figure 5.8A, compare siAgo2 – miR-122/p3 to siControl – miR-122/p3, and Figure 5.8C, compare siAgo2 – miR-122 to siControl – miR-122). Thus, the influence of Ago2 knockdown on HCV replication is primarily via modulation of miR-122 activity. Evaluation of cell numbers confirmed that cell survival had no impact on our luciferase results, while we achieved 64% Ago2 mRNA knockdown as measured by qRT-PCR in both series of experiments (Figure 5.8B and 5.8D). We saw similar results in Huh7.5 cells. miR-122-independent replication of p3 SGR was unaffected by Ago2 knockdown (Figure 5.8E, compare siControl – p3 to siAgo2 – p3), and miR-122-dependent replication of wild-type SGR RNA was reduced by Ago2 knockdown (compare siControl – SGR to siAgo2 – SGR). That Ago2 knockdown had a less severe effect on miR-122 dependent replication in Huh 7.5 cells than in Hep3B cells may have been due to less efficient Ago2 knockdown, 51%, in these cells. Huh7.5 cell numbers were also not affected by multiple electroporation (Figure 5.8F). Thus, while Ago2 plays a critical role in miR-122-mediated enhancement of HCV RNA accumulation, we show here that its presence or absence does not appear to impact miR-122-independent replication of HCV, which suggests that the disruption of Ago2 activity outside of its role in supporting miR-122 does not significantly affect HCV replication following siRNA knockdown.

#### **5.4 Discussion**

We have demonstrated that Hep3B cells are a valuable model system for studying Hepatitis C virus. Hep3B cells can be rendered permissive for robust replication of sub-genomic (SGR), full-genomic (FGR), and full-length (J6/JFH-1) replicons by supplementation with the liver-specific microRNA, miR-122. Supplementation with miR-122 allows us to recapitulate the complete virus

life cycle in Hep3B cells, from infection to production of new infectious virus particles, although HCV entry was inefficient in our experiments. Because Hep3B cells lack endogenous miR-122, we were also able to identify miR-122-independent replication of sub-genomic (SGR) and full-genomic (FGR) bi-cistronic replicons. This enabled us to generate a sub-genomic mutant virus that can replicate independently of miR-122 in both Hep3B cells and Huh7-derived cells.

The liver-specific microRNA, miR-122, augments replication of HCV RNA in Huh7-derived cells, and has been used to enhance replication of stable HCV replicons in non-liver cells such as HEK 293s and mouse MEFs (Chang et al., 2008; Fukuhara et al., 2012; Jopling et al., 2005; Lin et al., 2010). This led to the development of our hypothesis that miR-122 could be used to permit transient, unselected replication of HCV in other non-permissive cells. In the last year, two other labs have also published data on human liver cell lines supplemented with miR-122 to render them permissive to HCV replication (Kambara et al., 2012; Narbus et al., 2011). In our hands, and in the lab of Y. Matsuura, Hep3B cells can support levels of HCV replication and virus particle production similar to that found in Huh7-derived cells with no selection or adaptation (Kambara et al., 2012). Additionally, HCV RNA accumulation (Figure 5.2F) and virion production (Figure 5.2E) in Hep3B cells were equivalent to that of Huh7.5 cells. However, contrary to a previous report in which Hep3B cells stably expressing miR-122 were highly infectable (Kambara et al., 2012), we observed that Hep3B cells transfected with miR-122 were much less permissive to HCV entry (Figure 5.4A). Ploss et al. and Sainz et al. found Hep3B cells to be equally as permissive as Huh7 cells for entry by using lentiviral particles pseudotyped with HCV envelope proteins (HCVpp) as well as HCVcc (Ploss et al., 2009; Sainz et al., 2012). Observations by Shimakami et al. agreed with this using Hep3B cells stably expressing miR-122, which leads us to suspect that transient means of providing miR-122 reduced the infectability of Hep3B cells. However, Hep3B infection efficiency was not improved by electroporation of miR-122 (Figure 5.4C and 5.4D), thus poor infection may have been due to harmful effects of transfection (Figure 5.4) and electroporation, or perhaps to a different characteristic of the Hep3B cells used in our laboratory.

High cellular levels of miR-122 coincide with efficient replication of the virus (as measured by luciferase) and production of infectious virus particles, and replication decreases as miR-122 levels decrease over time (Figure 5.3). This suggests that miR-122 supports the continuing high levels of HCV RNA during the virus life cycle. This data agrees with other reports which showed

that miR-122 antagonism reduces HCV RNA levels in stable HCV cell lines (Jopling et al., 2005; Lee et al., 2012), and in HCV infected chimpanzees (Lanford et al., 2010).

Interestingly, we have found that miR-122 is not essential for replication of bi-cistronic JFH-1 sub-genomic (Figure 5.1C, Figure 5.6) and full-length (Figure 5.7) replicons in Hep3B or Huh7.5 cells. By determining that Hep3B cells lack functional miR-122 (Figure 5.1B, 5.5, 5.6) we have shown that the basal replication of SGR and FGR in Figures 5.1, 5.6, and 5.7 occurs independently of miR-122, and have confirmed this through use of the mutant p3 SGR. Cells harbouring stably replicating HCV replicons have been established previously through antibiotic selection in cell lines that are not known to express miR-122, which indicated the possibility of miR-122-independent replication, but the mechanism remained a mystery (Ali et al., 2004; Date et al., 2004; Kato et al., 2005b; Uprichard et al., 2006). In these instances, low levels of miR-122 expression in the cells, or the evolution of adaptive mutations in the 5' UTR, had not been eliminated. Here, we have demonstrated transient miR-122-independent replication of HCV SGR RNA, confirming that miR-122 is not essential for replication of JFH-1 bi-cistronic constructs. Thus, HCV bi-cistronic replicons are capable of replicating independently of miR-122, while still being sensitive to supplementation with the microRNA.

We have validated the usefulness of a mutant miR-122 binding site replicon, the p3 SGR, to study miR-122-dependent and -independent HCV replication. The p3 SGR replicon replicates to levels similar to those of wild-type SGR in the absence of miR-122, and is completely unaffected by the addition or removal of miR-122 (Figure 5.6C and E). However, when supplemented with miR-122/p3, which bears the complementary mutation in the miRNA sequence, replication of the p3 SGR is restored to levels that are within three-fold of wild-type SGR in the presence of miR-122. By supplementing the p3 SGR RNA with exogenous miR-122 in Hep3B cells, and by using a miR-122 antagonist in Huh7.5 cells, we confirmed that it replicated entirely independent of miR-122. Our data also suggests that a sub-genomic HCV mutant with p3-4 mutations at both miR-122 binding sites exhibits impaired replication aside from lack of miR-122 binding (Wilson et al., 2011). HCV SGR RNA carrying the p3-4 mutation could not replicate independently of miR-122 (data not shown), and could not be complemented to within a log of wild-type through use of a complementary miR-122/p3-4 (Wilson et al., 2011). Interestingly, both p3 and p3-4 mutants have been shown to reach wild-type levels when complemented with the appropriate miRNAs in a sub-genomic genotype 1a H77 replicon by Jopling et al., leading us to suspect that deficiencies in the

p3-4 mutant may be particular to JFH-1 or genotype 2a viruses (Jopling et al., 2005). Thus the p3 mutation will be more useful than other miR-122 binding site mutants for studying replication of HCV JFH-1 in the presence and in the absence of miR-122, as it is fully completable in multiple genotypes of virus.

miR-122-independent replication is much less efficient than miR-122-dependent replication. Luciferase expression levels observed for miR-122-independent replication were approximately 100-fold lower than for miR-122 augmented replication. In time-course experiments to compare luciferase expression kinetics of miR-122-dependent and -independent replication, the 100-fold difference was apparent by 1 day post-electroporation (Figure 5.1C, Figure 5.6). However, after day 1, the rate of increase in luciferase expression of miR-122-dependent and miR-122-independent replication appears similar. Thus, we suspect that miR-122 is not essential for ongoing HCV RNA amplification per se, but appears to be required to establish and sustain high levels of HCV RNA inside the cell. This is consistent with the proposed role of miR-122 in promoting HCV RNA stability and suggests that a major role of miR-122 is to establish HCV replication at early stages in the life cycle (Machlin et al., 2011; Shimakami et al., 2012a; Villanueva et al., 2010). In addition, bi-cistronic, but not mono-cistronic HCV RNAs are capable of miR-122-independent replication. The SGR and FGR replicons are bi-cistronic constructs, with translation of the viral genes driven by an EMCV IRES and thus unaffected by miR-122. The HCV IRES only drives expression of the luciferase reporter genes, separating control of the replication machinery from normal HCV translation mechanisms. We propose that miR-122-independent replication relies on the presence of the EMCV IRES and possibly different translation regulation. It remains to be determined if full length JFH-1 is capable of miR-122 independent replication, and whether this phenomenon is relevant to the life cycle of HCV in infected patients. It is also unknown if bi-cistronic replicons from other HCV isolates and genotypes will also demonstrate capacity for miR-122-independent replication; however, we expect this may be difficult to detect in systems with replication levels lower than that of the JFH-1 isolate. However, miR-122 independent replication of JFH-1 SGR is a useful model to study the role of miR-122 in the life cycle of HCV in tissue culture.

Analysis of genes that influence miR-122-dependent but not miR-122-independent replication provides a new screening tool for the identification of genes involved in the activity of miR-122. In the past, Ago2 has been implicated in the mechanism of miR-122 augmentation of HCV



replication based on data showing that Ago2 knockdown reduces HCV RNA accumulation in infected cells (Wilson et al., 2011). However, an indirect effect of Ago2 knockdown on HCV replication due to modulation of other miRNA regulated pathways could not be excluded in these experiments. In addition, conflicting reports of a role for Ago2 in an experiment in which miR-122-induced augmentation of HCV RNA accumulation was measured suggest caution in interpreting data generated by using this method (Machlin et al., 2011; Wilson et al., 2011). Thus robust methods to confirm that Ago2 or other miRNA pathway proteins play a direct role in the mechanism miR-122 augmentation of HCV RNA accumulation were lacking. Using wild-type and p3 sub-genomic HCV replicons to analyse miR-122-dependent and -independent HCV replication in Hep3B and Huh 7.5 cells, we have shown that Ago2 knockdown does not affect miR-122-independent replication, verifying that the role of Ago2 in the HCV life cycle lies in primarily in the activity of miR-122 (Figure 5.8). Examination of replication of the SGR and FGR constructs in a miR-122-free system such as Hep3B cells will allow us to analyze whether other components of the miRNA pathway involved in miR-122-mediated enhancement of HCV stability and/or replication may also act on HCV independently of miR-122.

Ultimately, the development of a Hep3B-based cell line for studying the HCV life cycle will be useful for the field of HCV research. Nearly all HCV research is carried out in Huh7-derived cells, all of which will bear the same host genetics and polymorphisms, and the same or similar dysregulation of cellular pathways, potentially limiting the value of screens designed to discover virus-host interactions. Hep3B cells have already been useful in detecting and confirming miR-122-independent viral replication, and are an alternative cell line in which to identify or confirm other virus-host interactions.

## **5.5 *Materials and Methods***

### **5.5.1 *Cell Lines.***

Huh7.5 cells are a derivative cell line of Huh7 cells and were obtained from Charles Rice (Blight et al., 2002; Nakabayashi et al., 1982). Hep3B cells are a human hepatoma cell line (ATCC number HB-8064) containing an integrated Hepatitis B genome (Aden et al., 1979; Knowles et al., 1980). HepG2 cells (ATCC number HB-8065) are also a human hepatoma cell line (Aden et al., 1979). All cell lines were maintained as described previously (Wilson et al., 2011).

### 5.5.2 Plasmids and viral RNA.

Plasmids pSGR JFH-1 Fluc WT and pSGR JFH-1 Fluc GND bear sub-genomic JFH-1-derived replicons with a firefly luciferase reporter (Kato et al., 2005a). Plasmids pJ6/JFH-1 FL Rluc WT and pJ6/JFH-1 FL Rluc GNN bear full-length viral sequences derived from the J6 (structural proteins) and JFH-1 (non-structural proteins) isolates of HCV, and a Renilla luciferase reporter (Jones et al., 2007). Plasmids pFGR Fluc JFH-1 WT and pFGR JFH-1 Fluc GNN bear full-genomic bi-cistronic replicons of JFH-1, and a firefly luciferase reporter (Wakita et al., 2005). Plasmids pSGR p3 S1+S2 Fluc WT and pSGR p3 S1+S2 Fluc GND have C to G mutations at position 3 in the miR-122 seed binding sites S1 and S2 in the HCV 5' UTR. The mutations were generated within the plasmid pSGR Fluc WT by using the QuikChange Site-Directed Mutagenesis kit (Agilent Technologies; Mississauga, ON, Canada) and the following primers: Site 1: 5'-ATA GGG GCG ACA CTG CGC CAT GAA TCA CTG-3' and 5'-CAG TGA TTC ATG GCG CAG TGT CGC CCC TAT-3'. Site 2: 5'-CCA TGA ATC ACT GCC CTG TGA GGA AC-3' and 5'-GTT CCT CAC AGG GCA GTG ATT CAT GG-3'. "GNN" and "GND" mutants of each replicon bear the indicated inactivating mutations in the viral polymerase GDD motif. To make viral and sub-genomic RNA, all plasmid templates were linearized with XbaI and blunt-ended with mung bean nuclease (New England Biolabs; Pickering, ON, Canada), and then transcribed *in vitro* using the MEGAScript T7 High Yield Transcription Kit (Life Technologies; Burlington, ON, Canada) according to the accompanying protocol. Messenger RNA was transcribed *in vitro* using the mMessage mMachinE T7 Kit (Life Technologies) according to the accompanying protocol. Firefly luciferase mRNA was transcribed from the Luciferase T7 Control DNA plasmid (Promega; Nepean, ON, Canada), linearized using XmnI, while Renilla luciferase mRNA was transcribed from the pRL-TK plasmid (Promega), linearized using BglII. pFluc122x2 is a plasmid coding for firefly luciferase followed by two repeats of the HCV miR-122 binding sites in the 3' UTR of the reporter's mRNA (Machlin et al., 2011).

### 5.5.3 MicroRNAs and Anti-miR sequences.

miR-122: UGG AGU GUG ACA AUG GUG UUU GU and miR-122\*: AAA CGC CAU UAU CAC ACU AAA UA, annealed. miR-122/p3: UGC AGU GUG ACA AUG GUG UUU GU annealed to miR-122\*. miControl: UAA UCA CAG ACA AUG GUG UUU GU annealed to miR-122\*. The miRIDIAN microRNA Hairpin Inhibitor, human hsa-miR-122a (proprietary sequence)

was used to inhibit miR-122 ( $\alpha$ -miR-122), with miRIDIAN microRNA Hairpin Inhibitor Negative Control #1 (proprietary sequence) was used as a control ( $\alpha$ -Control). siControl: AAG ACA CUG AGA CAC CAA UUG AC (Wilson et al., 2003). siAgo2: CAG ACU CCC GUG UGU CCU ATT (Wilson et al., 2011). All short RNAs were synthesized by ThermoScientific Dharmacon (Lafayette, CO, USA).

#### *5.5.4 Electroporations.*

All electroporations were carried out according to Lohmann et al. (2001) with some modifications: each sample contained  $6.0 \times 10^6$  cells in 400  $\mu$ L Dulbecco's PBS for electroporation, cells were plated in culture media, and 500  $\mu$ L of cells were seeded in 6 cm dishes for each time point. Cells were electroporated using 4 mm cuvettes at infinite resistance; optimized for the BioRad GenePulser XCell (BioRad; Mississauga, ON, Canada), Hep3B cells were electroporated at 225 V and 950  $\mu$ F, while Huh7.5 cells were electroporated at 270 V and 950  $\mu$ F. Where indicated, samples were electroporated with 10  $\mu$ g viral RNA; 60 pmol microRNA and/or miR-122 antagonist; and 2  $\mu$ g messenger RNA coding for the luciferase reporter not found in the viral replicon. In dilution experiments, the indicated amount of microRNA was used instead. For Ago2 knockdown experiments,  $8.0 \times 10^6$  cells in 400  $\mu$ L of Dulbecco's PBS were first electroporated with 60 pmol of the indicated siRNA to create Ago2 knockdown cells; two samples were pooled and plated in 15 cm dishes to recover. Three days post-first-electroporation, cells were again prepared as above ( $8.0 \times 10^6$  cells in 400  $\mu$ L), and electroporated with 60 pmol of the same siRNA, plus viral RNA, microRNA, and mRNA as above. Cells were harvested at the indicated time points and indicated as time post-second electroporation. For 15-day experiments, cells were passaged 1/3 on Day 9 post-electroporation due to confluence.

#### *5.5.5 Transfections.*

For miR-122 suppression activity assays, Hep3B cells were electroporated with the indicated miRNA/miR-122 antagonist, and were plated in triplicate at  $2.5 \times 10^4$  cells per well in 24-well dishes. Two days post-electroporation, each sample was transfected with 50 ng pRL-TK control plasmid and 50 ng pFluc122x2 in 1  $\mu$ L Lipofectamine 2000 according to the suggested protocol (Life Technologies). Cell extracts were harvested and analyzed for luciferase expression one day post-transfection, to analyze miR-122 suppression activity. For miR-122 transfection to

supplement cells prior to HCV infection, Hep3B cells were plated at  $1.0 \times 10^5$  cells per well in 6-well dishes one day before transfection. Cells were transfected according to the Lipofectamine 2000 protocol with 100 pmol microRNA one day prior to infection.

#### *5.5.6 Infections.*

To evaluate HCV infectious virus production, naïve Huh7.5 cells were plated at  $1.0 \times 10^5$  cells per well in a 6-well dish one day pre-infection. Hep3B supernatant was collected at the indicated time, spun to pellet cell debris, and 2 mL was plated on the naïve Huh7.5 cells. Cell extracts were harvested as above three days post-infection and assayed for luciferase expression to monitor HCV infection. To evaluate Hep3B infectability, HCV infectious supernatant from Huh7.5 cells (described below, approximately  $10^4$  FFU/mL) was plated on Hep3B cells at the indicated time post-Hep3B-electroporation or transfection. 2 mL of supernatant was added to approximately  $1 \times 10^5$  Hep3B cells per well of a 6-well dish. Cell extracts were harvested at the indicated time points post-infection to detect infection of Hep3B cells by using luciferase assays.

#### *5.5.7 HCV Titration.*

HCV titre was evaluated by focus-forming assay. One day pre-infection, naïve Huh7.5 cells were plated on a chamber slide such that they would be 90% confluent in four days. Cells were infected on Day 0, and fixed with acetone three days post-infection. 1:200 mouse-anti-HCV Core (C7-50, Abcam; Cambridge, MA, USA) in 5% BSA was used to detect foci of infection, and 1:100 goat-anti-mouse IgG Alexa-fluor 488 (Life Technologies) in PBS was used for visualization. Results are reported as focus-forming units per mL of supernatant.

#### *5.5.8 Luciferase Assays.*

For replication assays, 500  $\mu$ L electroporated cells were plated with 4mL media in a 6cm dish and incubated for 1-5 days, and cell extracts were harvested in 100  $\mu$ L of the appropriate lysis buffer. For suppression assays, cells were plated as indicated for transfection, and were harvested in 100  $\mu$ L passive lysis buffer. The Dual Luciferase Assay Reporter Kit (Promega) was used for all samples analyzed for both Renilla and firefly luciferase activity. The Renilla Luciferase Assay Reporter System (Promega) was used for all samples analyzed solely for Renilla luciferase activity, and the Luciferase Assay System (Promega) was used for all samples analyzed solely for

firefly luciferase activity. Harvests and assays were carried out according to the kits' protocols; results were read in the GLOMAX luminometer (Promega) with a 2-second delay and 10-second reading.

#### 5.5.9 *Total RNA.*

Total RNA was harvested and isolated using Trizol reagent (Life Technologies) and the associated protocol. Untreated cells were plated at  $10^7$ - $10^8$  cells per 10 cm tissue culture dish and were harvested into 1 mL Trizol at least 24 hours later. Total RNA samples collected after HCV RNA electroporation were plated at  $1.5 \times 10^6$  cells (2 mL) on a 10 cm dish and harvested 3 days post-electroporation into 1 mL Trizol.

#### 5.5.10 *qRT-PCR.*

miRNA qRT-PCR: miRNAs were reverse-transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies) according to the provided protocol, with RT primers from the hsa-miR-122 (002245) and RNU6B (001093) TaqMan MicroRNA Assay kits (Life Technologies). miRNA qPCR reactions were assembled according to the TaqMan Small RNA Assay protocol with 2X TaqMan Master Mix (Life Technologies) and probes for hsa-miR-122 and RNU6B TaqMan MicroRNA Assay kits described above. Quantitative amplification was carried out with samples in triplicate in a 96-well plate in the CFX96 real-time PCR system (BioRad) according to kit protocol. Ago2 qRT-PCR: total cellular RNA was reverse-transcribed using the iScript cDNA Synthesis Kit (BioRad). qPCR reactions were carried out using the TaqMan kits Hs00293044\_m1 (Ago2) and FAM-MGB 4352934-0803022 (GAPDH); samples were amplified in triplicate as for miRNA qPCR. All data was analyzed with the CFX Manager Software (BioRad).

#### 5.5.11 *Northern blot.*

Protocol was carried out as described in (Wilson et al., 2011). Bands were imaged and quantified using a Molecular Imager (BioRad) and the QuantityOne software (BioRad).

#### *5.5.12 Relative cell numbers.*

20  $\mu$ L of cells immediately post-electroporation were plated in triplicate in a 96-well plate. Three days post-electroporation, WST-1 reagent (Roche Diagnostics; Laval, QC, Canada) was added to the cells according to the manufacturer's protocol. Colourimetric reactions based on mitochondrial activity were measured one to two hours post-treatment on a SpectraMax 340 PC384 Microplate Spectrophotometer (Sunnyvale, CA, USA). Cell numbers were based on a standard curve of ten-fold dilutions of the appropriate cell type.

#### *5.5.13 Data Analysis.*

All data is displayed as a mean of three or more independent experiments (except where indicated) and bars indicate standard error of the mean.

### ***5.6 Acknowledgements***

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## **6.0 miR-122-Independent Replication as a Tool for Understanding HCV and miR-122**

Having characterized and validated Hep3B cells and the p3 sub-genomic mutant HCV RNA as tools for understanding the role of miR-122 in the Hepatitis C virus life cycle (Chapter 5), we next set out to use these tools to better understand the mechanism of miR-122-mediated augmentation of HCV RNA accumulation. A recent publication provided evidence that one of the functions of miR-122 was to protect the viral RNA from degradation, mediated by the host cytoplasmic 5' to 3' RNA exonuclease, Xrn1 (Li et al., 2013d). We considered our miR-122-independent HCV replication system an ideal context in which to verify that miR-122 protects the viral RNA from Xrn1. Further, we considered this model system and its derivatives ideal to further characterize the mechanism of miR-122-mediated protection by understanding the role of binding at each miR-122 binding site on the viral 5' UTR.

The experiments in Chapter 7 were conceived by PAT, AH, JG, DP, and JAW, and were carried out primarily by PAT with assistance from JG and DP. PAT, AH, and JAW analyzed the data, and PAT and JAW wrote the paper.

**7.0 miR-122 binding Hepatitis C Virus at Site 2 plays a greater role in protecting the 5' end of the Hepatitis C Virus genome from Xrn1 during replication than binding at Site 1**

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Keywords: Hepatitis C Virus, model system, Hep3B, microRNA, miR-122, miR-122 binding, Xrn1, degradation, protection.

Running Title: Roles of miR-122 binding in HCV replication

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## **7.1 Abstract**

miR-122 is a liver-specific miRNA that plays a critical role in the HCV life cycle by binding to two sites (S1 and S2) on the 5' UTR of the viral RNA. It positively affects viral RNA stability, translation, and replication, but the mechanism for these functions is not well-understood. To unravel the role in the virus life cycle of miR-122 binding at each miR-122 binding site, we employed Hep3B cells, which do not express miR-122, miR-122 binding site mutant viral RNAs, and complementation with either miR-122 or a mutant miRNA that binds to the mutated sites. We found that miR-122 binding at either site alone increased replication equally, while binding at both sites had a moderately co-operative effect. miR-122 is known to protect HCV RNA from Xrn1, a cytoplasmic 5' to 3' exoribonuclease. Xrn1 depletion rescued miR-122-unbound full-length RNA replication to detectable levels, but not to miR-122-bound levels, confirming that miR-122 has additional functions beyond protection from Xrn1. In cells depleted of Xrn1, replication of S1-bound HCV RNA was greater than S2-bound, suggesting unequal contributions of each binding site to HCV replication when the need for protection from Xrn1 is reduced. miR-122 binding at S1 or S2 also increased translation, but when Xrn1 was knocked down, miR-122 binding at either site no longer had an effect, suggesting that the influence of miR-122 on HCV translation reflects protection from Xrn1 degradation. Our results show that both miR-122 binding sites contribute to protection of the viral RNA from Xrn1, but binding at S2 does so more than binding at S1. We also suggest then that miR-122 binding at S1 is more important for the additional function(s) of miR-122 in augmentation of viral RNA replication.

## **7.2 Introduction**

Hepatitis C virus is a hepatotropic virus that infects an estimated 150 million humans worldwide, a significant portion of whom do not know their status due to the largely asymptomatic nature of the infection (WHO, 2012). The virus is transmitted by blood to blood contact, and humans are the only known reservoir. Chronic infection occurs in approximately 70% of cases, and can lead to sequelae such as metabolic disease, steatosis, hepatocellular carcinoma, and decompensated liver disease late in infection (Strader et al., 2004).

One of the major determinants of the virus' hepatotropism is its requirement for the liver-specific, liver-abundant microRNA, miR-122 (Thibault et al., 2013; Thibault and Wilson, 2014). miR-122 binds to two sites at the 5' end of the virus' positive sense RNA genome, and has been

shown to directly enhance viral RNA accumulation, since mutation of the miR-122 binding sites abolish RNA accumulation and provision of exogenous miR-122 sequences having compensatory mutations that restore binding reinstate RNA accumulation (Huys et al., 2013; Jangra et al., 2010b; Jopling et al., 2008; Machlin et al., 2011; Norman and Sarnow, 2010; Shimakami et al., 2012b; Thibault et al., 2013). We, and others have also ruled out any significant indirect influence of miR-122 on HCV in cell culture models (Jopling et al., 2008; Norman and Sarnow, 2010; Thibault et al., 2013). Argonaute-2, one of the key effector proteins in the microRNA pathway, is required for miR-122 to increase HCV replication, while several other proteins in the microRNA pathway and RNA-induced silencing complex (RISC) have been implicated in either biogenesis or the direct interaction of miR-122 with HCV (Huys et al., 2013; Roberts et al., 2011; Shimakami et al., 2012a; Wilson et al., 2011; Zhang et al., 2012). Although miR-122 uses canonical microRNA seed sequence binding and RISC components when interacting with the HCV genome, it also binds to HCV nucleotides outside the seed sequence, creating a double stranded RNA/protein structure that overhangs the very 5' end of the viral genome, and also interacts with the "spacer" sequence located between miR-122 binding sites S1 and S2 on the HCV 5' UTR (Machlin et al., 2011; Shimakami et al., 2012a).

While interaction between miR-122 and the HCV 5' UTR causes an increase in HCV RNA accumulation, evidence suggests that there are multiple mechanisms involved. miR-122 modestly increases translation of the viral RNA, but this has recently been attributed to simply increasing stability of the viral RNA, allowing more viral RNA to be translated (Henke et al., 2008; Jangra et al., 2010b; Roberts et al., 2011; Shimakami et al., 2012a). In particular, miR-122 has been implicated in shielding the 5' end of the viral RNA from degradation by Xrn1, the major cytoplasmic 5' to 3' exoribonuclease that normally functions to degrade decapped host mRNAs and other *Flavivirus* RNAs (Jones et al., 2010b; Li et al., 2013d; Nagarajan et al., 2013; Roby et al., 2014). Ultimately, though, the increase in translation and stability is considered insufficient to explain the increase in HCV RNA accumulation mediated by miR-122, and other mechanisms require exploration (Jangra et al., 2010b; Li et al., 2013d; Shimakami et al., 2012a).

Here we verify that miR-122 protects the 5' end of the viral genome from Xrn1, and that each of the miR-122 binding sites plays a role in this. We also show that knockdown of Xrn1 positively impacts translation, and that the function of miR-122 in promoting translation is primarily through protection from Xrn1. However, our results also indicate that protection from Xrn1 is not the only

function of miR-122 in the viral life cycle and that miR-122 must have additional functions. Finally, we found that binding of miR-122 at each site contributes equally to replication, while binding at both sites demonstrates a co-operative effect. However, in the absence of Xrn1, binding at S1 increased replication more than binding at S2, suggesting that S1 binding contributes more to the additional functions of miR-122 in HCV.

### **7.3 Results**

#### *7.3.1 Impact of Xrn1 on HCV RNA replication.*

##### *7.3.1.1 Xrn1 knockdown affects miR-122-unbound sub-genomic HCV replication more than miR-122-bound sub-genomic replication.*

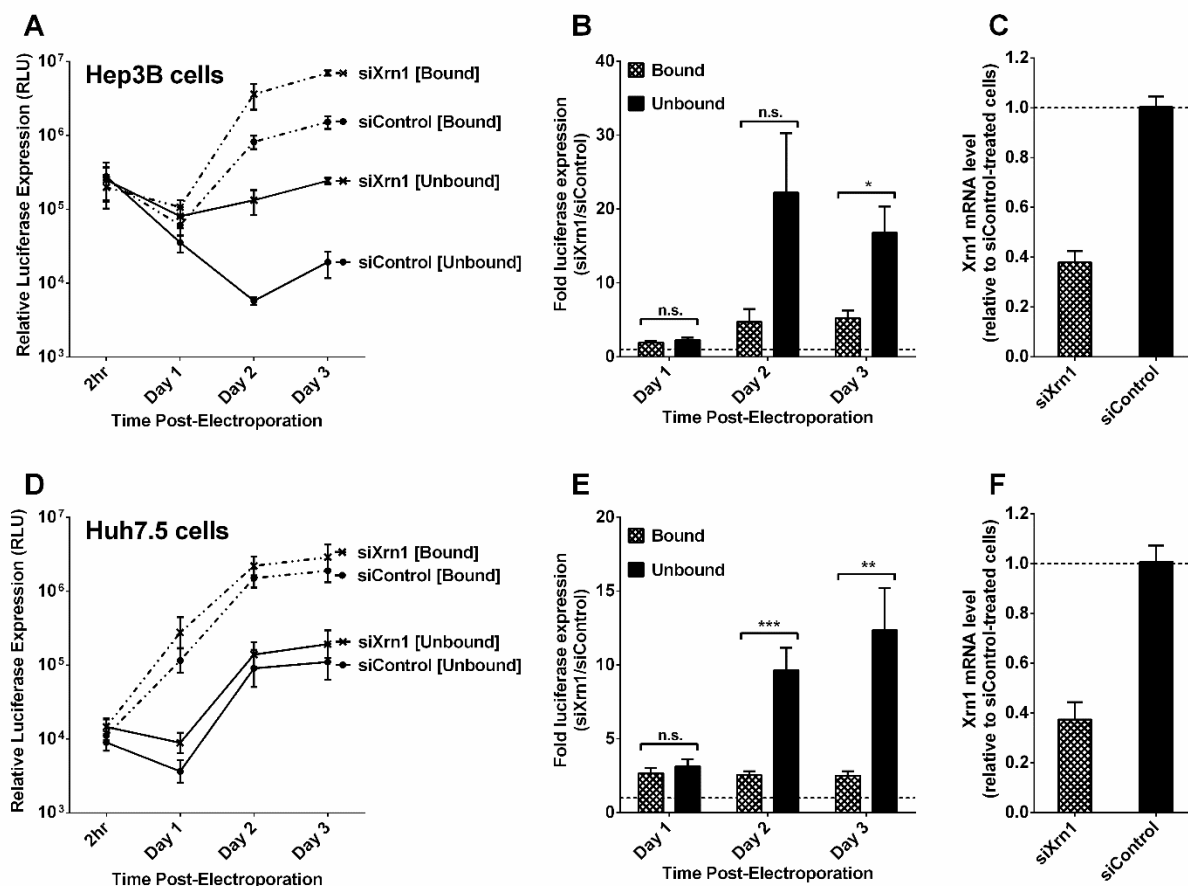
Xrn1, a host cytoplasmic 5' to 3' RNA exonuclease, has been shown to target the 5' end of HCV RNA for degradation; miR-122 has been implicated to have a role in protecting the viral RNA from Xrn1 (Li et al., 2013d). In spite of this relationship, in a previous report, Xrn1 knockdown did not restore any detectible replication of an HCV genome to which miR-122 binding had been abolished; this finding was deemed evidence that protection from Xrn1 was not the only function for miR-122 during the HCV life cycle. We believed that our previously-characterized system of miR-122-independent replication of HCV RNA would be ideal to further test whether knockdown of Xrn1 can restore or improve HCV replication in the absence of miR-122 binding and verify whether miR-122 plays a key role in abolishing the impact of Xrn1 on HCV RNA (Thibault et al., 2013). To test this, we used siRNAs to knock down Xrn1 in Hep3B cells, and after waiting for three days to allow knockdown to occur, we electroporated the cells again with siRNA and miRNA as indicated, along with transfection control Renilla luciferase reporter mRNA and sub-genomic viral RNA. We have previously shown that sub-genomic JFH-1 HCV RNA (SGR WT, a bi-cistronic construct that expresses a firefly luciferase reporter gene) can replicate at low levels without miR-122 binding to the 5' UTR, and that Hep3B cells are an ideal system to study miR-122-independent replication because they do not express detectable miR-122. We can therefore test miR-122-independent replication of wild-type HCV RNAs, instead of using constructs with miR-122 binding site mutations (Thibault et al., 2013). We hypothesized that if miR-122 functioned to protect the 5' end of the viral RNA from degradation by Xrn1, then abolition of Xrn1-mediated degradation of the viral RNA by Xrn1 knockdown should enhance miR-122-independent HCV replication. If protection from Xrn1 is the primary role for miR-122,

then Xrn1 knockdown should restore replication in the absence of miR-122 binding to levels similar to replication with miR-122 binding.

Our results (Figure 7.1A) show that while knockdown of Xrn1 increased miR-122-unbound replication levels by up to 22-fold (compare siControl [Unbound] to siXrn1 [Unbound]), it did not reach the same levels of replication as miR-122-bound replication (compare siXrn1 [Unbound] to siControl [Bound]). We verified that the siXrn1 treatment reduced Xrn1 mRNA by 62% in Hep3B cells (Figure 7.1C). We also ensured that knockdown of Xrn1 or other treatments did not affect Hep3B cell growth three days post-second electroporation (data not shown) and that transfection efficiency was similar for all samples as measured by Renilla luciferase expression from the control mRNA two hours post-second electroporation (data not shown). Thus Xrn1 knockdown can augment but cannot restore replication of miR-122-unbound HCV RNA, which suggests that protection from Xrn1 is not the only function for miR-122 in promoting HCV replication. Similar to the finding of Li *et al.* we observed that Xrn1 knockdown also enhanced replication levels of miR-122-bound HCV RNA, suggesting that if miR-122 protects the RNA from degradation by Xrn1, that protection is not complete (Li et al., 2013d).

If miR-122 functions to shield the viral RNA from Xrn1, then knockdown of Xrn1 would have a greater impact on HCV replication without miR-122 binding than with miR-122 binding. Conversely, if miR-122 did not play a role in shielding the viral RNA from Xrn1, then knockdown of Xrn1 would have the same impact on replication with and without miR-122 binding. To test this, we compared the effect of Xrn1 knockdown on miR-122-bound and miR-122-unbound replication (Figure 7.1B). We confirmed others' observations that knockdown of Xrn1 had a positive impact on viral replication when miR-122 was bound, increasing replication 5-fold by three days post-second electroporation. However, we also demonstrated that knockdown of Xrn1 had a greater impact on replication of miR-122-unbound SGR WT RNA and increased it by 22-fold on Day 2, and 16-fold on Day 3, considerably more than the impact of knockdown on miR-122-bound replication. Ultimately, this supports a model where miR-122 binding plays a role in protecting the HCV genome from Xrn1, but the protection is incomplete.

Huh7-derived cells are the most-commonly used cell lineage when studying Hepatitis C virus *in vitro*, and so we also verified our conclusions in Huh7.5 cells. However, because Huh7.5 cells express miR-122, in order to test miR-122-unbound replication we made use of a miR-122 binding site mutant sub-genomic RNA, SGR S1+S2:p3 (Thibault et al., 2013) that has already been shown



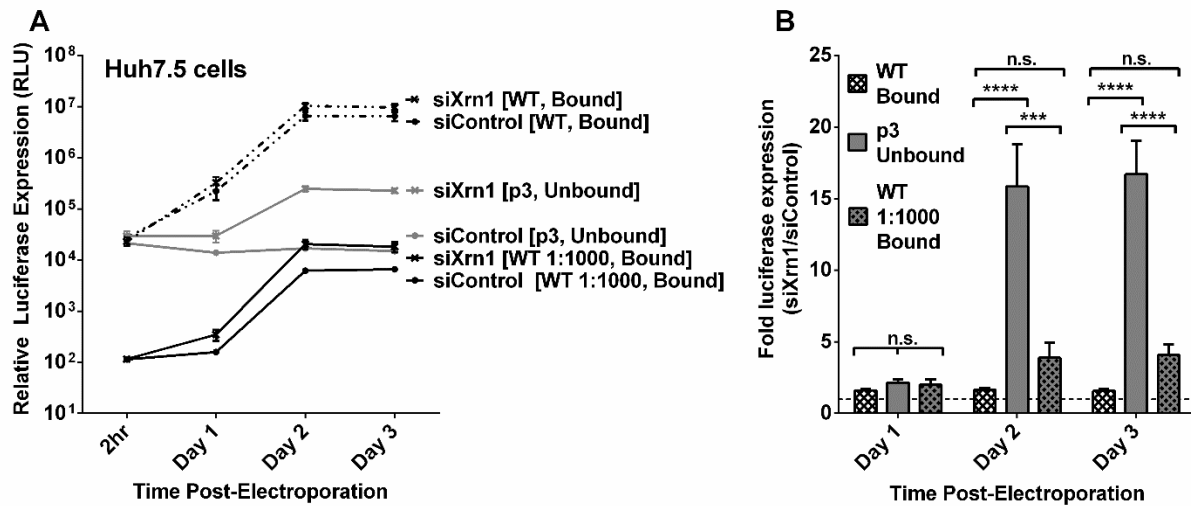
**Figure 7.1** Impact of *Xrn1* knockdown on miR-122-bound and miR-122-unbound rsub-genomic JFH-1 HCV replication in Hep3B cells.

**A.** Hep3B cells were electroporated with siXrn1 or siControl for pre-knockdown, and three days later (Day 0) cells were electroporated again with the indicated siRNA, wild-type sub-genomic HCV RNA (SGR) containing a firefly luciferase reporter gene, a Renilla messenger RNA to control for transfection efficiency, and miR-122 (Bound) or miControl (Unbound). Replication was measured by evaluating luciferase production at the indicated time points post-second electroporation. **B.** The effect of Xrn1 knockdown on miR-122-bound and miR-122-unbound sub-genomic HCV RNA replication was determined by measuring the fold increase in luciferase expression with siXrn1 treatment over luciferase expression with no knockdown (siControl), using luciferase data from (A) at the indicated time points. Significance was determined by paired parametric t-test. **C.** The effectiveness of siXrn1 at knocking down Xrn1 mRNA levels in Hep3B cells was determined by RT-qPCR of RNA collected three days post-second electroporation, shown here as fold mRNA reduction normalized to host GAPDH mRNA levels. **D.** Huh7.5 cells were treated as in (A), but no miRNA was added since Huh7.5 cells already express endogenous miR-122. “Bound” samples were electroporated with wild-type SGR RNA, while “Unbound” samples were electroporated with the miR-122 binding site-mutant SGR S1+S2:p3 RNA described in Thibault et. al. (Thibault et al., 2013), which does not respond to miR-122. **E.** The effect of Xrn1 knockdown on miR-122-bound and miR-122-unbound sub-genomic HCV RNA replication was determined as in (B). Significance was determined by unpaired parametric t-test. **F.** The effect of siXrn1 treatment on Xrn1 mRNA levels in Huh7.5 cells was evaluated as in (C).

to be unresponsive to miR-122. SGR S1+S2:p3 contains a C to G mutation at position 3 in both miR-122 binding sites (S1 and S2) that abolishes miR-122 binding, and replicates to similar levels as miR-122-unbound SGR WT. We again observed that knockdown of Xrn1 increased replication of both miR-122-bound and miR-122-unbound SGR (Figure 7.1D), that Xrn1 knockdown did not restore miR-122-unbound replication to miR-122-bound levels (Figure 7.1D), and that miR-122-unbound replication increased to a much greater degree (12-fold) than miR-122-bound replication (2.5-fold, Figure 7.1E). We also observed that siXrn1 treatment reduced Xrn1 mRNA levels by 63% in Huh7.5 cells (Figure 7.1F), Xrn1 knockdown did not affect Huh7.5 cell growth, and transfection efficiency was similar in all samples (data not shown). Thus, we show here that Xrn1 knockdown has a positive impact on HCV replication, and that it has a greater effect on HCV replication when miR-122 is not bound to the viral 5' UTR, suggesting a specific role for miR-122 in protecting the viral RNA from Xrn1.

#### *7.3.1.2 The greater impact of Xrn1 knockdown on miR-122-unbound sub-genomic HCV RNA replication is not due to resource limitations for miR-122-bound replication.*

To exclude the possibility that the difference in effects of Xrn1 knockdown on miR-122-dependent and miR-122-independent replication was impacted by the different levels of replication observed and the host cell's capacity to support HCV replication, we repeated our experiments using dilutions of viral RNA that would establish miR-122-bound replication levels similar to miR-122-unbound replication. To do this we electroporated Huh7.5 cells with 1000-fold less SGR WT RNA than S1+S2:p3 RNA (Figure 7.2A, compare siControl [p3, Unbound] to siControl [WT 1:1000]). In all cases, knockdown of Xrn1 still increased replication (Figure 7.2A). However, in both miR-122-bound samples (WT 1:1 and WT 1:1000, Figure 7.2B), Xrn1 knockdown increased replication by 2 to 4-fold with no significant difference between dilutions, while miR-122-unbound replication (p3 1:1) was increased 17-fold. Cell numbers were not affected by the differing amounts of viral replication, and transfection efficiency was likewise unaffected (data not shown). Thus, the effect of Xrn1 knockdown on miR-122-unbound replication is miR-122-specific, and is not an artefact of reaching the replication capacity of the host system.



**Figure 7.2** The greater impact of *Xrn1* knockdown on miR-122-unbound sub-genomic HCV RNA replication is not due to resource limitations for miR-122-bound replication.

**A.** Huh7.5 cells were electroporated as described in Figure 7.1D. “WT, Bound” samples used 5 $\mu$ g of wild-type SGR RNA, “p3, Unbound” samples used 5 $\mu$ g of mutant SGR S1+S2:p3 RNA, and “WT 1:1000” samples used 5ng of wild-type SGR RNA. **B.** The effect of *Xrn1* knockdown on bound, unbound, and diluted bound replication was evaluated as in Figure 7.1E. Significance was determined by paired parametric t-test.

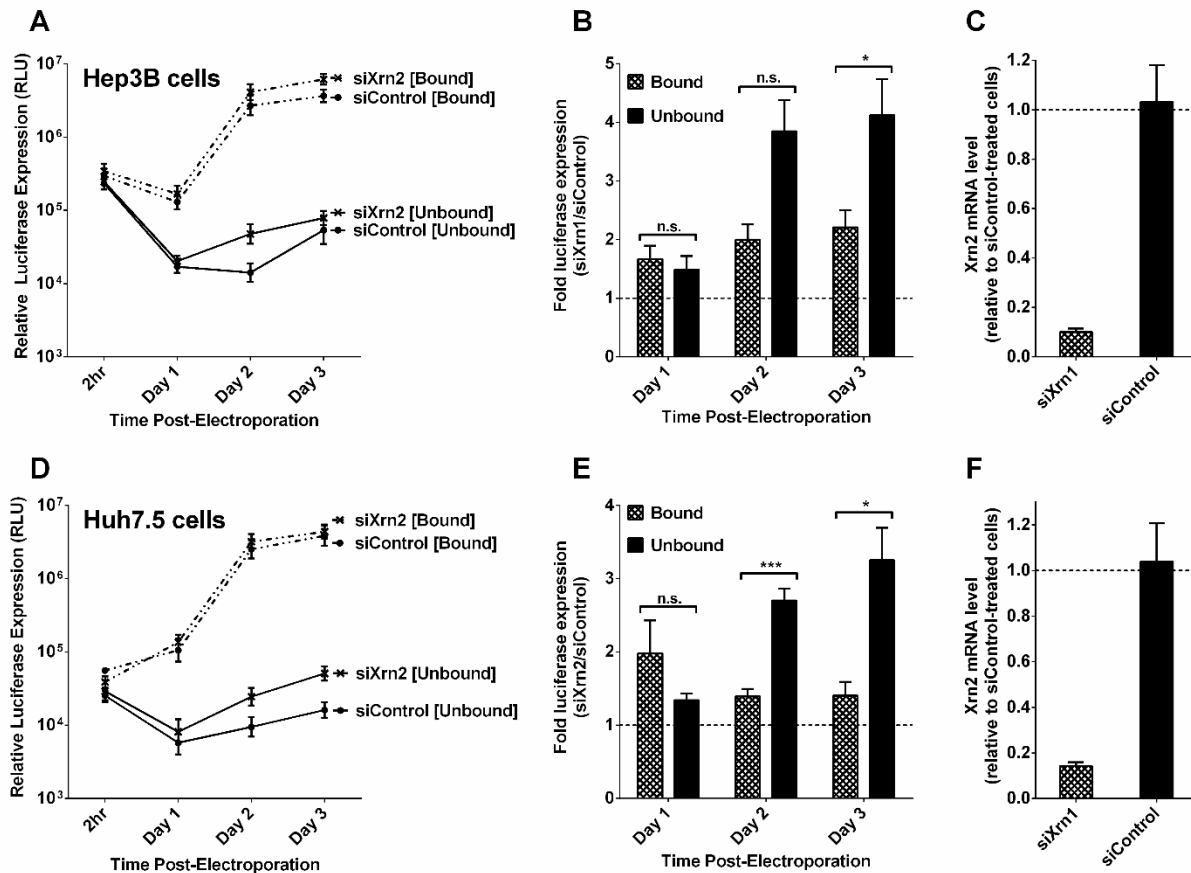
### *7.3.1.3 Knockdown of Xrn2 has a similar effect on both miR-122-unbound sub-genomic HCV replication and miR-122-bound sub-genomic HCV replication.*

Xrn2 is another major host 5' to 3' RNA exonuclease, but is normally found in the nucleus (Nagarajan et al., 2013). We hypothesized that since Xrn1 knockdown did not fully restore miR-122-unbound replication to miR-122-bound levels, miR-122 may also protect HCV RNA from Xrn2, and so we tested miR-122-bound and miR-122-unbound replication with and without Xrn2 knockdown as we had with Xrn1. In Hep3B cells, as expected, knockdown of Xrn2 led to increased replication of both miR-122-bound and miR-122-unbound SGR WT RNA (Figure 7.3A), but the difference in the impact of Xrn2 knockdown on bound (2-fold) and unbound (4-fold) replication (Figure 7.3B) was not as great as had been observed for Xrn1 knockdown in Figure 7.1. siRNA treatment achieved a 90% reduction in Xrn2 mRNA in Hep3B cells (Figure 7.3C) but did not affect Hep3B cell numbers, and transfection efficiency was similar among all samples (data not shown). When tested in Huh7.5 cells, the results were similar: knockdown of Xrn2 led to increased miR-122-bound and miR-122-unbound replication of SGR WT (Figure 7.3D), but the difference in increase between bound (1.4-fold) and unbound (3-fold) replication (Figure 7.3E) was again not as dramatic as it had been with Xrn1. As with Hep3B cells, siRNA treatment resulted in an 86% reduction in Xrn2 mRNA levels in Huh7.5 cells (Figure 7.3F), but did not affect Huh7.5 cell numbers, and translation was similar in all samples (data not shown). Because the effects of Xrn2 knockdown on miR-122 bound and miR-122-unbound replication were similar in both Hep3B and Huh7.5 cells, we conclude that Xrn2 may restrict HCV RNA replication to a small degree, and miR-122 may function to protect HCV from Xrn2, but its contribution to miR-122-mediated augmentation of the HCV life cycle is minimal.

### *7.3.1.4 Knockdown of Xrn1 enhances full-length viral RNA replication and permits detectable replication of full-length RNA in the absence of miR-122 binding.*

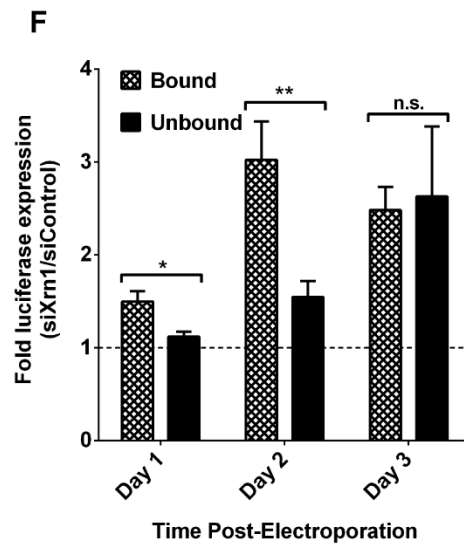
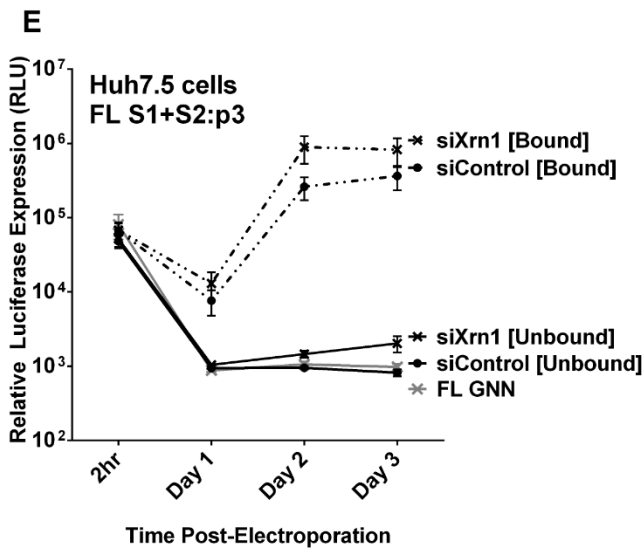
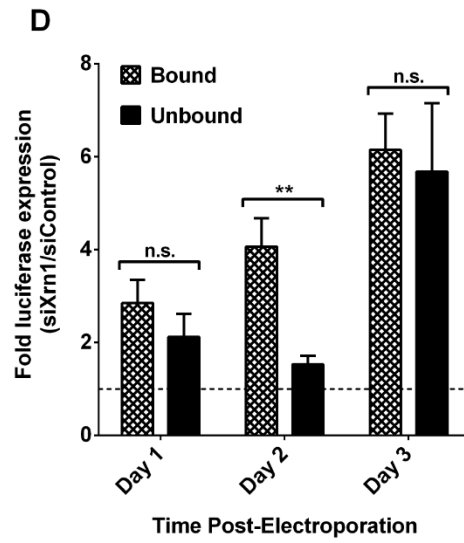
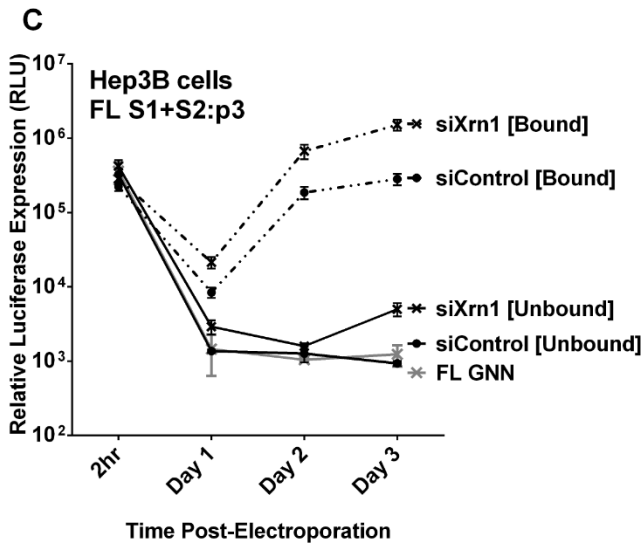
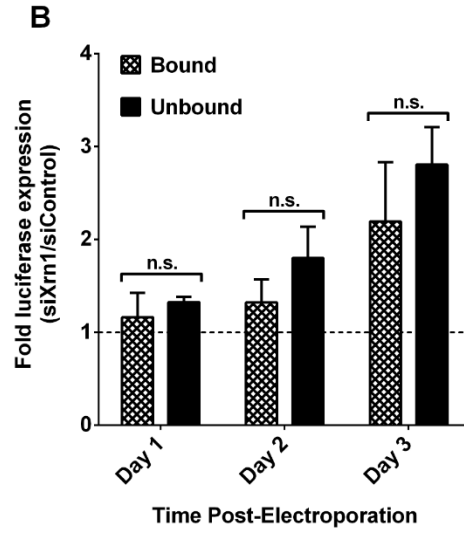
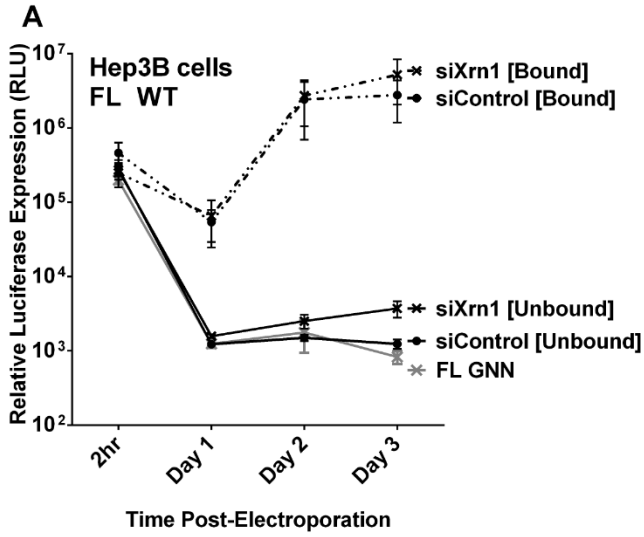
The JFH-1 sub-genomic replicon has been valuable in allowing us to work with HCV RNA replication in the absence of miR-122, but it lacks the structural proteins and coding regions of the virus, and also contains an additional IRES derived from the encephalomyocarditis virus that drives translation of the viral non-structural proteins. Thus, we next set out to test the effect of Xrn1 on replication of the full-length J6/JFH-1 mono-cistronic viral RNA (FL WT) with and without miR-122 binding. We had previously been unable to detect replication of this construct in





**Figure 7.3 Impact of Xrn2 knockdown on miR-122-bound and miR-122-unbound sub-genomic HCV replication.**

**A.** Hep3B cells were electroporated with siXrn2 or siControl for pre-knockdown, and three days later (Day 0) were electroporated again with the indicated siRNAs, miRNAs, mRNAs, and viral RNAs as described in Figure 7.1A. “Bound” samples were electroporated with miR-122, while “Unbound” samples were electroporated with miControl. **B.** The effect of Xrn2 knockdown on miR-122-bound and unbound replication was evaluated as in Figure 7.1B. Significance was determined by unpaired parametric t-test. **C.** The effectiveness of siXrn2 treatment on Xrn2 levels in Hep3B cells was evaluated by RT-qPCR as in Figure 7.1C. **D.** Huh7.5 cells were treated with siXrn2 as described in Figure 7.1D. “Bound” samples were electroporated with wild-type SGR RNA, while “Unbound” samples were electroporated with mutant SGR S1+S2:p3 viral RNA, which does not respond to miR-122. **E.** The effect of Xrn2 knockdown on miR-122-bound and unbound replication was evaluated as in Figure 7.1E. Significance was determined by paired parametric t-test. **F.** The effect of siXrn2 treatment on Xrn2 mRNA levels in Huh7.5 cells was evaluated as in Figure 7.1F.



**Figure 7.4 Knockdown of Xrn1 enhances full-length J6/JFH-1 viral RNA (FL WT) replication and permits detectable replication of full-length RNA in the absence of miR-122 binding.**

**A.** Hep3B cells were electroporated with siXrn1 as described in Figure 7.1A, but the wild-type full-length J6/JFH-1 viral RNA “FL WT” which encodes an in-frame Renilla luciferase reporter was used, and firefly messenger RNA was used as a transfection control. “Bound” samples were electroporated with miR-122, while “Unbound” samples were electroporated with miControl. **B.** The effect of Xrn1 knockdown on wild-type full-length HCV RNA replication was evaluated as described in Figure 7.1B. Significance was determined by unpaired parametric t-test. **C.** Hep3B cells were electroporated with siXrn1 as in Figure 7.1A, but replication was evaluated using the miR-122 binding site-mutant full-length J6/JFH-1 viral RNA “FL S1+S2:p3,” a full-length mimic of the SGR S1+S2:p3 mutant that does not respond to wild-type miR-122, but does respond to miR-122/p3, a miR-122 mimic bearing the G to C mutation at position 3 that matches the C to G mutation in the miR-122 binding sites on the viral RNA. “Bound” samples were electroporated with miR-122/p3, while “Unbound” samples were electroporated with miControl. **D.** The effect of siXrn1 on FL S1+S2:p3 miR-122-bound and unbound replication were evaluated as in Figure 7.1B. Significance was determined by unpaired parametric t-test. **E.** Huh7.5 cells were electroporated with siXrn1 as described in Figure 7.1D, but with full-length J6/JFH-1 viral RNA “FL S1+S2:p3,” which does not respond to miR-122 endogenous to Huh7.5 cells. “Bound” samples were electroporated with miR-122/p3 and “Unbound” samples were electroporated with miControl as in (C). **F.** The effect of Xrn1 knockdown on the miR-122 binding site mutant full length HCV RNA replication was evaluated as described for Figure 7.1E. Significance was determined by unpaired parametric t-test.

the absence of miR-122 binding, despite the construct expressing the highly sensitive Renilla luciferase as a reporter, since we could not detect luciferase expression above the background levels expressed from a polymerase-inactivated mutant, FL GNN (Thibault et al., 2013). We observed that knockdown of Xrn1 in Hep3B cells led to increased levels of replication of miR-122-bound FL WT RNA as expected (Figure 7.4A), but we also observed that Xrn1 knockdown allowed detectable replication of miR-122-unbound FL WT RNA, which had not been reported previously (compare siXrn1[Unbound] to GNN and siControl [Unbound]). Detectable replication continues at low levels for four and five days post-electroporation (data not shown), but does not increase further, perhaps due to cell confluence. This suggests to us that FL WT RNA can replicate in the absence of miR-122, but without knockdown of Xrn1, this replication is occurring at levels below our limit of detection as defined by luciferase expression from the GNN mutant.

When we quantified the effect of Xrn1 knockdown on miR-122-bound and unbound replication (Figure 7.4B) we noted that the impact of Xrn1 knockdown appeared to have no significant difference in increasing replication (2.5-fold and 3-fold). However, we speculate that the actual increase in unbound full-length viral replication due to Xrn1 is greater than our calculated values, since miR-122-unbound replication levels were equivalent to background (Figure 7.4B, compare siControl [Unbound] to GNN) and thus probably occurring below the limit of detection.

We also tested miR-122 binding site mutant full-length HCV RNA (FL S1+S2:p3) for the effect of Xrn1 on bound and unbound replication to confirm the phenotype using HCV RNAs having miR-122 point mutations (Figure 7.4C). As with SGR S1+S2:p3, FL S1+S2:p3 has two C to G mutations at position 3 in both miR-122 binding sites (S1 and S2), but is otherwise identical to FL WT. FL S1+S2:p3 replicates to similar levels as FL WT when complemented with miR-122/p3, a synthetic miR-122 that bears the G to C mutation at position 3 to complement the p3 mutation in the viral construct. Knockdown of Xrn1 increased both miR-122-bound and miR-122-unbound replication of FL S1+S2:p3 by 6-fold (Figure 7.4D), similar to what was observed with FL WT, and like we observed with FL WT, unbound FL S1+S2:p3 replication levels were undetectable in siControl treated cells (compare siControl [Unbound] to GNN), but Xrn1 knockdown increased replication of FL RNA, and permitted detectable, but low level, replication in the absence of miR-122 binding.

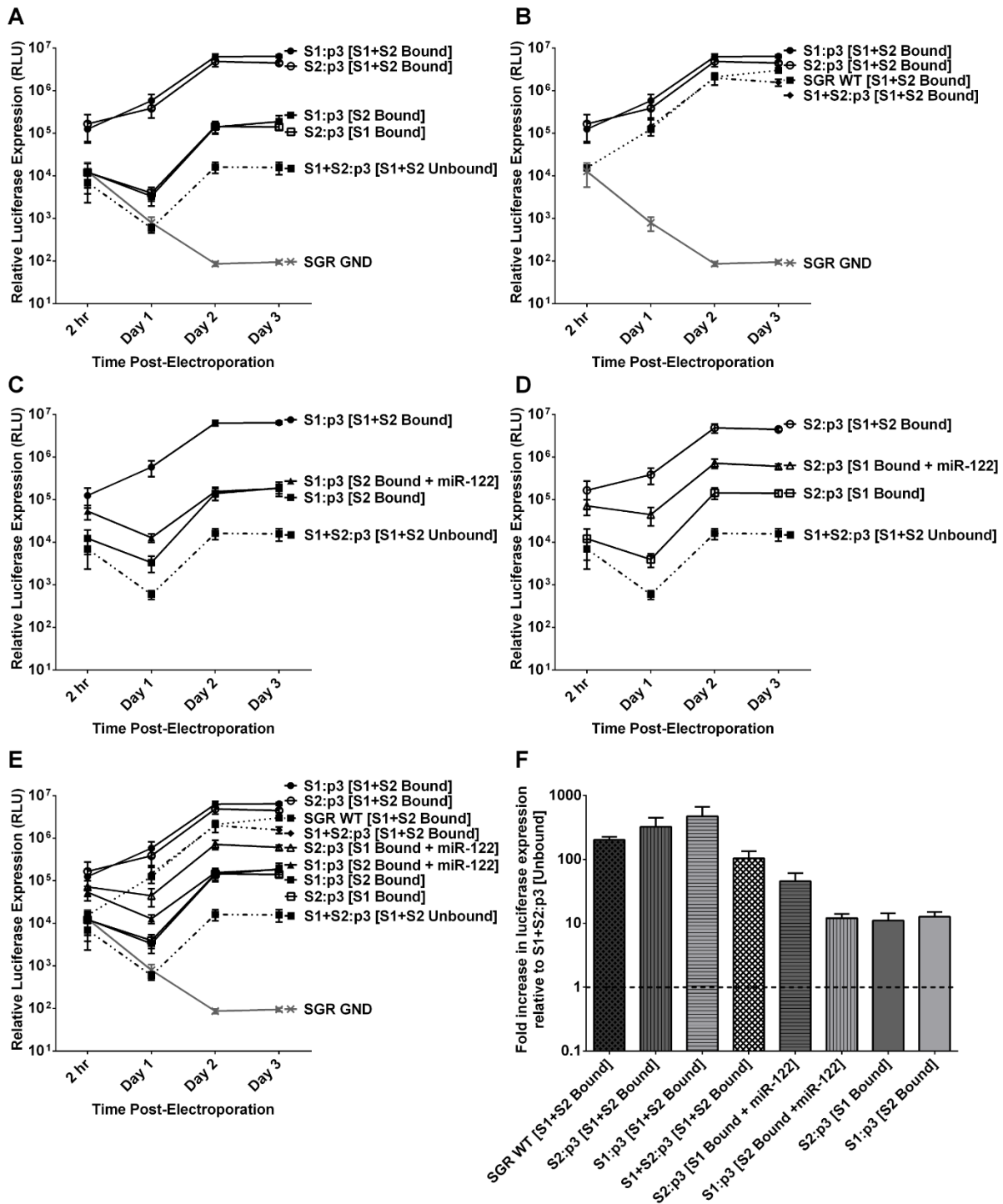
We also verified our findings in the more-commonly used Huh7.5 cell culture system, which required us to use the full-length miR-122 binding site mutant (FL S1+S2:p3) to test for the effect of Xrn1 on bound and unbound replication as was done in Hep3B cells. Like in Hep3B cells, we were unable to detect FL S1+S2:p3 replication without miR-122 binding in Huh7.5 cells (Figure 7.4E, compare siControl [Unbound] to GNN). Knockdown of Xrn1 led to a 3-fold increase in replication of bound FL S1+S2:p3 (Figure 7.4F), and also permitted replication of miR-122-unbound FL S1+S2:p3 (Figure 7.4E, compare siXrn1 [Unbound] to siControl [Unbound] or GNN). Similar to our observations in Hep3B cells, the effect of Xrn1 knockdown on miR122-unbound replication was not significantly different than its effect on miR-122-bound replication (Figure 7.4B and 7.4D), but as we noted previously, this comparison is not accurate because we do not have a true baseline for miR-122-unbound replication in the presence of Xrn1 since it is likely below the limit of detection. We show here that Xrn1 knockdown can also positively impact full-length HCV RNA replication, and can relieve Xrn1-mediated suppression of miR-122-unbound replication of the full-length construct such that it can be detected.

### *7.3.2 Roles of miR-122 binding at site S1 and S2 in HCV RNA accumulation.*

In the interest of further evaluating the role of miR-122 binding in HCV replication, we examined the possibility that each miR-122 binding site has a different function, or contributes to the effect of miR-122 differently. In order to evaluate this, we generated full length and sub-genomic constructs bearing the p3 mutation in either miR-122 binding site S1 or S2 (SGR S1:p3, SGR S2:p3, FL S1:p2, and FL S2:p3), meaning that the un-mutated site would still be able to use wild-type miR-122, but that the mutated site would require supplementation with the mutant miRNA, miR-122/p3.

#### *7.3.2.1 miR-122 binding at each site contributes equally to SGR RNA replication in Huh7.5 cells, and binding at both sites exerts a co-operative effect.*

To characterize the contribution of each site to sub-genomic HCV RNA replication, we examined replication of each mutant in the presence of miR-122 endogenous to Huh7.5 cells. When only S2 was occupied, SGR S1:p3 (Figure 7.5A, S1:p3 [S2 Bound]) replication levels were 11-fold higher than S1+S2:p3 [Unbound], where neither site was occupied (Figure 7.5F), and 36-fold lower than when both sites were occupied. Similarly, when only S1 was occupied, SGR S2:p3



**Figure 7.5** Replication of sub-genomic HCV RNA bearing p3 mutations in miR-122 binding sites S1 or S2 under various binding conditions in Huh7.5 cells.

**A-D.** Huh7.5 cells were electroporated with the indicated sub-genomic viral RNA and miRNA, along with a transfection control Renilla mRNA. SGR WT [S1+S2 Bound] was electroporated with miControl, since Huh7.5 cells express endogenous miR-122. SGR S1+S2:p3 was

electroporated with either miR-122/p3 [S1+S2 Bound] or miControl [S1+S2 Unbound]. SGR S1:p3 RNA contains a p3 mutation only in miR-122 binding site S1, leaving S2 intact; it was electroporated with miR-122/p3 [S1+S2 Bound], miControl [S2 Bound], or additional miR-122 [S2 Bound + miR-122]. SGR S2:p3 RNA contains the p3 mutation only in miR-122 binding site S2, leaving S1 intact. It was electroporated with miR-122/p3 [S1+S2 Bound], miControl [S1 Bound], or additional miR-122 [S1 Bound + miR-122]. SGR GND RNA contains an inactivating GDD to GND mutation in the viral polymerase, rendering it replication-incompetent. Viral replication was measured by evaluating firefly luciferase viral reporter expression at the indicated time points. **E.** The effect of binding at miR-122 sites S1 or S2 on replication are shown relative to replication of unbound sub-genomic viral RNA, “S1+S2:p3 [S1+S2 Unbound],” at three days post-electroporation.

replication levels were similar to those of SGR S1:p3 (Figure 7.5A) – 12-fold higher than S1+S2:p3 [S1+S2 Unbound] (Figure 7.5F) and 31-fold lower than S2:p3 [S1+S2 Bound]. Since binding at S1 increased replication by 12-fold, and binding at S2 increased replication by 11-fold, we would expect that binding at both sites would increase replication by a total of approximately 130-fold if the effects were merely multiplicative. However, binding at both sites increased replication by 200-fold over unbound replication (Figure 7.5F). Thus, each miR-122 binding site contributes equally to replication of HCV SGR RNA and binding to both sites has a moderately co-operative effect on replication over the binding at each individual site.

We tested SGR S1:p3 and SGR S2:p3 to confirm that their replication capacity had not been altered by the point mutations in any way other than in their ability to bind miR-122 (Figure 7.5B). This was confirmed by our finding that both SGR S1:p3 and SGR S2:p3 replicated to levels similar to the SGR WT when binding to the mutant site was restored through the addition of miR-122/p3. Similarly, replication of SGR S1+S2:p3 was also near wild type levels when miR-122 binding was restored by miR-122/p3. Thus, the S1 and S2 mutations did not affect the replication capacity of the construct in the presence of miR-122 binding.

#### *7.3.2.2 miR-122 binding to sub-genomic RNA is saturated at S2 but not S1 by endogenous miR-122 in Huh7.5 cells.*

We also supplemented the cells with synthetic miR-122 to analyse the impact of increased levels of miR-122 available for binding at each site. Supplementing Huh7.5 cells with additional synthetic miR-122 (which is able to bind at S2) did not further increase replication of SGR S1:p3 (Figure 7.5B, S1:p3 [S2 Bound + miR-122]), indicating that maximum replication capacity was achieved in Huh 7.5 cells with endogenous miR-122 levels. However, supplementing SGR S2:p3 with additional miR-122 (which is able to bind to S1) increased replication a further 4.6-fold (Figure 7.5C, S2:p3 [S1 Bound + miR-122]), indicating that the effect of miR-122 binding at S1 is not saturated in Huh 7.5 cells and greater levels of miR-122 binding enhances replication.

#### *7.3.2.3 In Huh7.5 cells, binding at S2 appears to be more important than binding at S1 for full-length HCV RNA replication.*

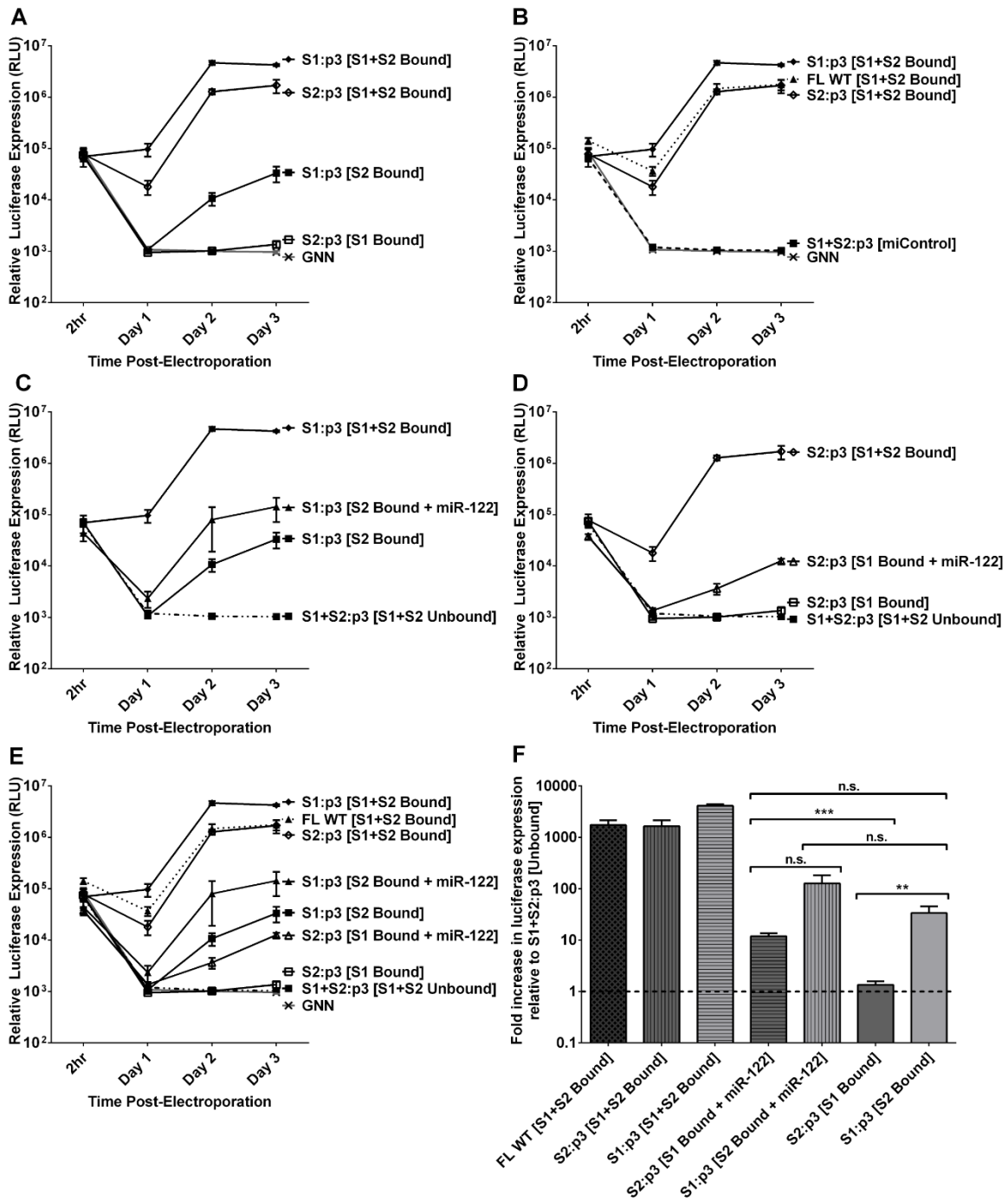
We chose to further examine the role of each miR-122 binding site on replication of the full-length viral RNA using full-length constructs bearing the p3 mutation in either S1 or S2 (FL S1:p2



and FL S2:p3), where the un-mutated site can still bind endogenous miR-122 present in Huh 7.5 cells, while the mutated site is unoccupied unless the experiment is supplemented with miR-122/p3. These constructs were tested in Huh 7.5 cells using conditions similar to those used to test SGR RNAs in Figure 7.5. Similar to the results seen using sub-genomic mutants, FL S1:p3 (where S2 is occupied by endogenous miR-122) showed intermediate replication (Figure 7.6A, S1:p3 [S1 Bound]). However, converse to the sub-genomic mutants, FL S2:p3 (where S1 is occupied by endogenous miR-122) did not demonstrate detectable replication (Figure 7.6A, compare S2:p3 [S1 Bound] to GNN). This suggests an unequal contribution to full-length replication by each miR-122 binding site. Replication of FL S2:p3 was restored to wild-type levels when both miR-122 binding sites were occupied (Figure 7.6B), as was replication of FL S1:p3, confirming that replication of the miR-122 binding site mutants was not impaired when both miR-122 binding sites were occupied. Since the FL S2:p3 mutant does not replicate at detectable levels when only S1 is bound, while the FL S1:p3 mutant does replicate at detectable levels when only S2 is bound, it appears that binding at S2 is more important for full-length HCV RNA replication in Huh7.5 cell than binding at S1.

#### *7.3.2.4 Additional miR-122 binding to full-length HCV RNA at S1 increases replication more than added miR-122 binding at S2 in Huh7.5 cells.*

Using the SGR RNA we found that S1 but not S2 responded to a greater abundance of miR-122 (Figure 7.5C and 7.5D). We also tested the effects of miR-122 supplementation on FL S1:p3 and FL S2:p3 and observed similar results. Both FL S1:p3 and S2:p3 responded to the addition of exogenous miR-122 (Figure 7.6C, S1:p3 [S2 Bound + miR-122] and Figure 7.6D, S2:p3 [S1 Bound + miR-122]; Figure 7.6F), but the response of FL S2:p3 was greater (8.8-fold increase, compare FL S2:p3 [S1 Bound] with FL S2:p3 [S1 Bound + miR-122]) and statistically significant, while the response of FL S1:p3 (3.8-fold increase, compare FL S1:p3 [S2 Bound] to FL S1:p3 [S2 Bound + miR-122]) was smaller and not significant, supporting our findings using the SGR that the response to miR-122 binding at S1 requires greater levels of miR-122 to become saturated. In fact, supplementation of S2:p3 with exogenous miR-122 was required for detectable levels of replication (Figure 7.6D, S2:p3 [S1 Bound + miR-122]), but it still did not replicate to the same level as S1:p3 [S2 Bound] (Figure 7.6E, 7.6F). Thus, S2 binding appears to be more important than S1 based on these results.



**Figure 7.6** Replication of full-length HCV RNA bearing p3 mutations in miR-122 binding sites S1 or S2 under various binding conditions in Huh7.5 cells.

**A-E.** Huh7.5 cells were electroporated with the indicated full-length viral RNA and miRNA, along with a transfection control firefly mRNA, as described in Figure 7.6. Viral replication was measured by evaluating Renilla luciferase viral reporter expression at the indicated time points. **F.** The effect of binding at miR-122 sites S1 or S2 on replication are shown relative to replication of unbound full-length viral RNA, “S1+S2:p3 [S1+S2 Unbound],” at three days post-electroporation. Significance for relevant comparisons was determined by unpaired parametric t-test.

### 7.3.2.5 *Binding at either S1 or S2 increases replication to the same degree within FL S1:p3 or FL S2:p3 RNA in Hep3B cells.*

Results from Huh 7.5 cells using S1:p3 and S2:p3 suggested that abolishing binding to S2 has a more potent effect on HCV RNA replication than binding to S1. However, we cannot exclude the possibility that the phenotype was due to the S2:p3 point mutation itself, and not simply due to S2 being unoccupied by miR-122. This cannot be tested in Huh7.5 cells because they express endogenous miR-122, but Hep3B cells, which do not express endogenous miR-122, are an ideal system to evaluate the contribution of each miR-122 binding site to replication of individual HCV RNA constructs, and thus can be used to identify possible impacts of the p3 mutations on each HCV RNA. We evaluated the relative contribution of binding at each site in FL S1:p3 (Figure 7.7A) and FL S2:p3 (Figure 7.7B) RNA in Hep3B cells by supplementing each construct with either miR-122 or miR-122/p3. For FL S1:p3 (Figure 7.7A), we compared the effect of binding at S1 (adding miR-122/p3) to the effect of binding at S2 (adding miR-122), relative to unbound (miControl) replication and S1+S2 bound (adding miR-122 + miR-122/p3) replication. Replication of S1:p3 was almost identical when only S1 or only S2 was bound (Figure 7.7A, S1:p3 [S1 Bound] and S1:p3 [S2 Bound]; Figure 7.7E). FL S2:p3 (Figure 7.7B) was evaluated the same way, but binding to S1 was assessed using miR-122, and binding to S2 was assessed using miR-122/p3; again, binding at either site impacted replication of the FL S2:p3 construct equally (compare Figure 7.7B, S2:p3 [S1 Bound] to S2:p3 [S2 Bound]; see Figure 7.7E). Thus, within a given construct, binding of miR-122 at either S1 or S2 has an equal impact on full-length HCV replication.

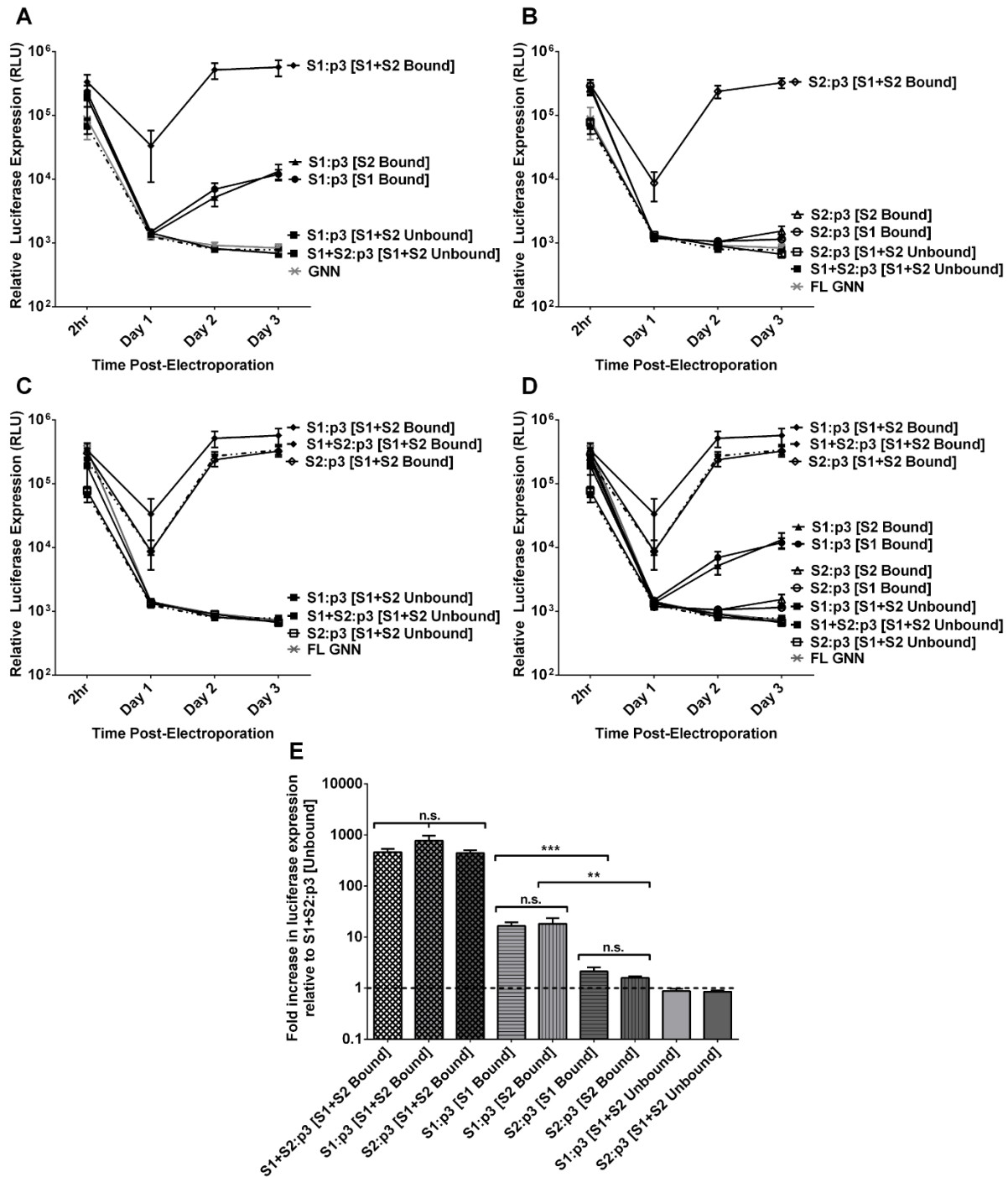
### 7.3.2.6 *FL S2:p3 replication is impaired in comparison to FL S1:p3 when only one site is occupied.*

Interestingly, replication of FL S2:p3 with only one site bound was significantly lower than replication of FL S1:p3 with only one site bound, regardless of which site was bound (compare Figure 7.7A S1:p3 [S1 Bound] or S1:p3 [S2 Bound] to Figure 7.7B, S2:p3 [S1 Bound] or S2:p3 [S2 Bound]; see also Figure 7.7E). In fact, replication of FL S2:p3 was near the lower limit of detection when only one site was bound (Figure 7.7B, compare S2:p3 [S1 Bound] and S2:p3 [S2 Bound] to S2:p3 [S1+S2 Unbound] and GNN). However, when both sites were bound, FL S2:p3 replicated at wild-type levels, as did FL S1:p3 (Figure 7.7C), as had been observed in Huh7.5 cells.

Thus, FL S2:p3 appears to have become severely dependent on miR-122 binding at both sites for replication. This construct therefore may be a useful model to study the influence of miR-122 on HCV by comparing replication of S2:p3 with wild type RNA, and identify the sequences that mediate the extreme dependence on miR-122. That the phenotype was only observed with the FL S2:p3 mutant and not with the SGR S2:p3 mutant suggests that this impairment may involve an interaction between the miR-122 binding sites and the structural coding region that is absent in the SGR constructs, or perhaps is influenced by the presence of a second IRES in the SGR construct. This also suggests that our observation that FL S2:p3 [S1 Bound] had lower replication levels in Huh7.5 cells (Figure 7.6A) was due to a phenotype of the mutant construct, rather than a function of binding at S1, and does not support our previous conclusion that S2 was more important for FL HCV RNA replication, and instead suggests that each miR-122 binding site has an equivalent contribution in magnitude to full-length HCV replication.

#### *7.3.2.7 miR-122 binding at both S1 and S2 together exerts a co-operative effect in increasing replication of full-length HCV RNA in Hep3B cells.*

The full-length S1 and S2 mutants also provide evidence that miR-122 binding at both sites co-operatively increases replication (Figure 7.7E). In FL S1:p3, binding at S1 increased replication 16.5-fold, and binding at S2 increased replication 18.3-fold over unbound “replication,” although it is important to note that we cannot detect unbound replication and so we are comparing this increase to the limit of detection of our luciferase assay (GNN). If the virus’ response to binding at both sites were multiplicative, S1+S2 binding would increase replication approximately 300-fold over unbound, but instead replication is increased 775-fold, demonstrating that S1 and S2 have a co-operative effect on binding. In FL S2:p3, the effect is much more drastic: S1 binding increased replication 2.1-fold, S2 binding increased replication 1.6-fold, and together they increased replication 440-fold, rather than 3.3-fold, which would be indicative of a multiplicative effect. A caveat to our interpretation is that in both instances, we cannot compare an increase in replication directly with miR-122-unbound replication of either construct, since – if it is occurring – it is at levels below the ability of our assay to detect.



**Figure 7.7** Replication of full-length HCV RNA bearing p3 mutations in miR-122 binding sites S1 or S2 under various binding conditions in Hep3B cells.

**A-D.** Hep3B cells were electroporated with the indicated full-length viral RNA and miRNA, along with a transfection control firefly mRNA. FL WT [S1+S2 Bound] was electroporated with miR-122, since Hep3B cells do not express endogenous miR-122. FL S1+S2:p3 was electroporated with either miR-122/p3 [S1+S2 Bound] or miControl [S1+S2 Unbound]. FL S1:p3 RNA contains a p3 mutation only in miR-122 binding site S1, leaving S2 intact; it was electroporated with miR-

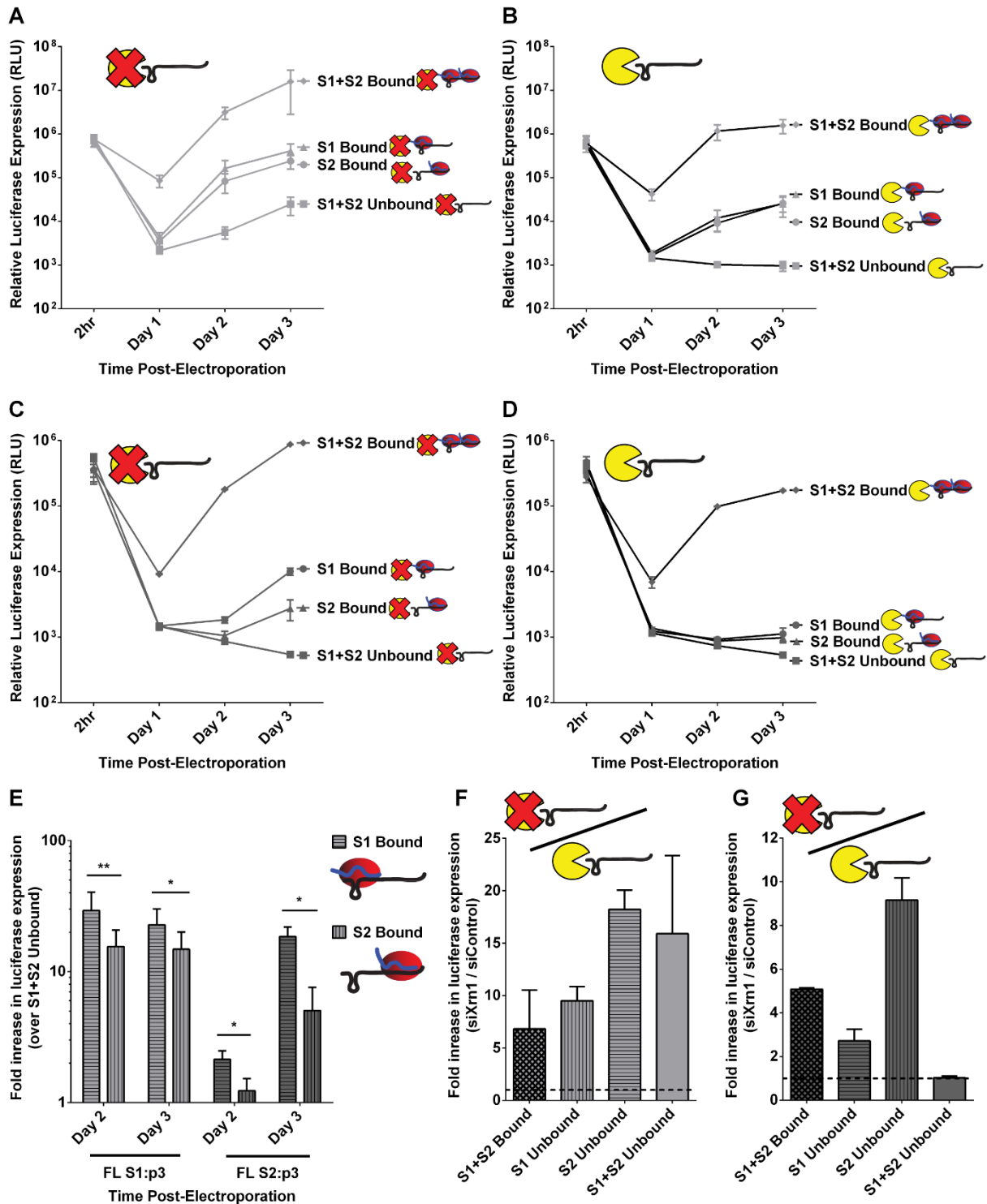
122/p3 [S1 Bound], miR-122 [S2 Bound], both miR-122/p3 and miR-122 [S1+S2 Bound], or miControl [S1+S2 Unbound]. FL S2:p3 RNA contains a p3 mutation only in miR-122 binding site S2, leaving S1 intact; it was electroporated with miR-122 [S1 Bound], miR-122/p3 [S2 Bound], both miR-122 and miR-122/p3 [S1+S2 Bound], or miControl [S1+S2 Unbound]. FL GNN RNA contains an inactivating GDD to GNN mutation in the viral polymerase, rendering it replication-incompetent. Viral replication was measured by evaluating Renilla luciferase viral reporter expression at the indicated time points. **E.** The effect of binding at miR-122 sites S1 or S2 on replication are shown relative to replication of unbound full-length viral RNA, “S1+S2:p3 [S1+S2 Unbound],” at three days post-electroporation. Significance for relevant groups was determined by unpaired parametric t-test.

#### *7.3.2.8 Binding at S1 increases FL RNA replication more than binding at S2 when Xrn1 is knocked down in Hep3B cells.*

We have confirmed that one of the functions of miR-122 binding at the HCV 5' UTR is to protect the viral RNA from the effects of Xrn1. Further, we have established that protection from degradation by Xrn1 is not the only role for miR-122. Thus, we propose that by evaluating the impact on viral replication of miR-122 binding to each binding site in the context of Xrn1 knockdown, we can determine their individual contributions to the Xrn1-independent miR-122 function. We compared the effect of binding at both sites, neither site, and either S1 or S2, in the context of Xrn1 knockdown on FL S1:p3 and FL S2:p3 replication (Figure 7.8A and 7.8C, respectively). Despite our earlier observation that under normal cellular conditions, S1 and S2 contribute to FL replication to an equal degree (Figure 7.7; see also Figure 7.8B and 7.8D), we observed that when Xrn1 was knocked down, S1 bound replication was significantly higher than S2 bound replication (Figure 7.8A and 7.8C, Figure 7.8E). Particularly, when Xrn1 was knocked down, binding at S1 increased replication 23-fold (FL S1:p3) and 18-fold (FL S2:p3) over unbound replication on Day 3, while binding at S2 only increased replication 15-fold (FL S1:p3) and 5.1-fold (FL S2:p3) over unbound replication on Day 3 (Figure 7.8E). This is in comparison to our previous observations with both SGR and FL constructs under normal (no knockdown) conditions, that binding at either S1 or S2 has equal impact on replication. These findings show that binding to S1 has a greater impact on the Xrn1-independent function(s) of miR-122, suggesting that S1 is more important for the Xrn1-independent functions of miR-122 binding, and by inference that binding at S2 may be more important in protection from Xrn1 during replication. In addition our data show that the Xrn1-independent role for miR-122 still functions co-operatively: with Xrn1 knockdown, binding at each site separately in FL S1:p3 increased replication by 18- and 23-fold, but binding at both sites increased replication 670-fold (data not shown), and binding to each site separately in FL S2:p3 increased replication 18-fold and 5.1-fold but together increased replication by 1620-fold (data not shown).

#### *7.3.2.9 Binding at S2 is more important for protection from Xrn1 than binding at S1, but both sites protect from Xrn1.*

We also used the data presented in Figure 7.8A-D to analyze the impact of Xrn1 on replication of HCV RNA when miR-122 occupied each individual binding site by calculating the increase in



**Figure 7.8** Binding at S1 increases replication of FL S1:p3 RNA more than binding at S2 when *Xrn1* is knocked down.

Hep3B cells were electroporated with **A.** siXrn1 or **B.** siControl for pre-knockdown, and three days later (Day 0) were electroporated again with the indicated siRNAs, miRNAs, a transfection

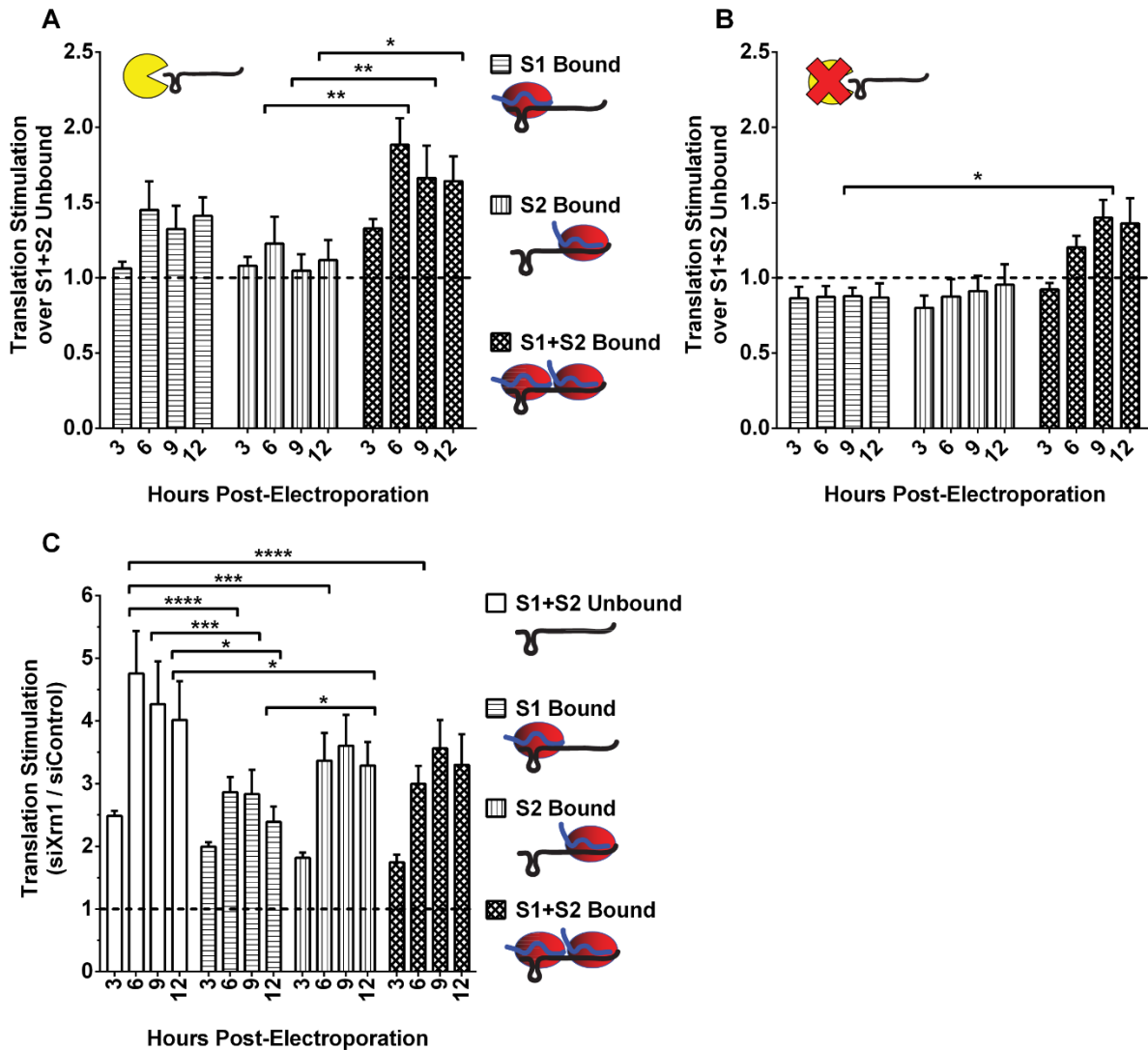


control firefly luciferase mRNA, and FL S1:p3 viral RNA. Viral RNA was supplemented with miR-122/p3 (S1 Bound), miR-122 (S2 Bound), miR-122/p3 and miR-122 (S1+S2 Bound), or miControl (S1+S2 Unbound), and replication was measured by evaluating reporter Renilla luciferase expression at the indicated time points. Results are an average of four independent experiments. **C.** siXrn1 and **D.** siControl-treated Hep3B cells were treated as in (A) and (B), respectively, but were electroporated with FL S2:p3 RNA. Results are an average of two independent experiments. **E.** The fold increase in replication of FL S1:p3 RNA (light grey, left) and FL S2:p3 RNA (dark grey, right) when either S1 or S2 is bound over unbound replication is shown in the context of siXrn1-treated cells. Significance was determined using paired parametric t-test. **F.** The effect of Xrn1 knockdown on FL S1:p3 with one, both, or neither binding site unoccupied is shown by comparing the fold increase in replication with Xrn1 knockdown from (A) to replication with siControl treatment from (B), no knockdown. The dotted line indicates 1-fold, or no increase in replication due to knockdown. Significance was tested using ordinary one-way ANOVA and Tukey's multiple comparisons post-test with pooled variance, and no group was found significantly different. **G.** The effect of Xrn1 knockdown on FL S2:p3 with one, both, or neither binding site unoccupied is shown by comparing the fold increase in replication with Xrn1 knockdown from (C) to replication with siControl treatment from (D), no knockdown. The dotted line indicates 1-fold, or no increase in replication due to knockdown. Significance was tested using repeated measures one-way ANOVA and Tukey's multiple comparisons post-test with individual variances, and no group was found significantly different.

replication when Xrn1 is knocked down compared to replication in the presence of Xrn1 (siXrn1/siControl) for each miR-122 binding condition: S1+S2 Bound, S1 Unbound, S2 Unbound, and S2+S2 Unbound (Figure 7.8F and 7.8G). We propose that the extent to which replication is enhanced by Xrn1 knockdown when a particular site is unprotected (unbound) is proportional to the contribution of that site to protection from Xrn1. For example, if a site is important for protection from Xrn1, when it is unbound, knockdown of Xrn1 will increase replication (relieve suppression) more than if that site is not important for protection from Xrn1. Our calculations showed that RNA without binding at S2 was more vulnerable to Xrn1 (Xrn1 knockdown had a greater enhancing effect) than when S1 was unbound, or when both sites were bound, although due to experimental variability these differences are not statistically significant. This indicates that binding at S2 serves to protect the viral RNA from Xrn1 more-so than binding at S1, and taken in conjunction with the results in Figures 7.5-7.7 and Figure 7.8D-F, S1 may play a different role in the viral life cycle. However, since Xrn1 knockdown also increased replication of RNA with miR-122 bound at both sites, this protection is not complete. Additionally, because Xrn1 knockdown could not rescue miR-122-unbound replication of FL S2:p3, we further conclude that the impairment of the S2:p3 construct when only one binding site is occupied is not related to susceptibility to Xrn1.

*7.3.2.10 miR-122 binding at either S1 or S2 does not impact FL S1:p3 RNA translation when Xrn1 is knocked down in Hep3B cells.*

One of the challenges in studying the role of miR-122 in the HCV life cycle is the difficulty in isolating effects on translation from effects on replication. Although we observe an impact of Xrn1 on replication, it is possible that it is instead actually affecting translation, and over the course of our three-day experiments this effect compounds to impact replication. Thus we chose to examine the effect of miR-122 binding in each combination in the context of Xrn1 knockdown, using FL S1:p3 GNN, which is incapable of replication. When cells were treated with siControl (Figure 7.9A), we observed that binding at S1+S2 increased translation over unbound translation up to 1.9-fold over the 12-hour time period, which is within range of others' observations (Henke et al., 2008; Huys et al., 2013; Jangra et al., 2010b; Shimakami et al., 2012a; Wilson et al., 2011). We also observed that binding at either S1 or S2 had a lesser, but still-discernible impact on translation,



**Figure 7.9** Knockdown of *Xrn1* increases FL S1:p3 RNA translation overall, but also reduces the contribution miR-122 binding makes to viral translation, particularly with binding at S1.

Hep3B cells were electroporated with siXrn1 or siControl for pre-knockdown. Three days later, cells were electroporated with FL S1:p3 GNN (replication-incompetent S1 miR-122 binding mutant) viral RNA, transfection control firefly mRNA, and the indicated miRNAs as described in Figure 7.7. Because the viral RNA is replication-incompetent, measuring luciferase at the indicated 3, 6, 9, and 12 hours post-second electroporation only measures viral translation. Viral translation was normalized to transfection control mRNA firefly luciferase expression levels measured at 3 hours post-second electroporation. The effect on translation of FL S1:p3 GNN viral RNA by miRNA binding at S1, S2, or both sites was compared to translation when neither site was bound in **A**. siControl-treated cells or **B**. siXrn1-treated cells, where the dotted line represents 1-fold or no increase in translation. **C**. Stimulation of translation by Xrn1 was determined by comparing normalized viral translation in siXrn1-treated cells to viral translation in siControl-treated cells. The dotted line indicates 1-fold, or no stimulation of viral translation. Significance for all figures was determined using two-way ANOVA with Tukey's multiple comparisons test. Only significant differences are indicated; all other comparisons were not significant.

and that there was no significant difference between the impact of S1 or S2 binding, suggesting that binding at each site plays an equivalent role in the effect of miR-122 on translation. However, when Xrn1 was knocked down (Figure 7.9B), binding at either S1 or S2 alone did not increase translation at all, and binding at both sites had a much weaker impact on translation (1.4-fold at most). This suggests that most, or all of the apparent impact of miR-122 on translation is due to protection from Xrn1; we suggest that the remaining impact of binding at both sites (S1+S2) when Xrn1 is knocked down is due to minimal amounts of Xrn1 remaining in the cell, rather than to a direct impact on translation independent of protection from Xrn1. Knockdown of Xrn1 increased overall viral RNA translation considerably (Figure 7.9C) and also positively impacted mRNA translation (data not shown). Particularly, knockdown of Xrn1 had a greater impact on maximal S1+S2 Unbound translation (4.8-fold) than it did on either S1 Bound (2.9-fold), S2 Bound (3.6-fold), or S1+S2 Bound (3.6-fold) translation, providing further evidence that binding at these sites protects from Xrn1.

#### **7.4 Discussion**

We have shown that miR-122 has a specific function in shielding the 5' un-translated region of the Hepatitis C virus RNA from the host RNA exonuclease Xrn1 (Figures 7.1, 7.2, and 7.4), but not Xrn2 (Figure 7.3). We have also demonstrated that replication of full-length HCV RNA in the absence of miR-122 can be detected following knockdown of Xrn1, and we speculate that full length HCV RNA replicates in the absence of miR-122 binding, but that this replication is too low to be detected using current methods (Figures 7.5 and 7.6), unless we first remove restriction by Xrn1 (Figures 7.4 and 7.8). Because knockdown of Xrn1 does not allow miR-122-un-supplemented replication to reach miR-122-supplemented levels, we conclude that miR-122 has additional functions in viral replication above that of protecting the viral RNA from Xrn1.

We quantified the relative contribution of miR-122 binding at each of the two 5' miR-122 binding sites to HCV replication, showing that each site contributes with equal magnitude to enhancing replication of the virus, and that binding at both sites increases replication cooperatively (Figures 7.5-7.7). We also evaluated the effect of miR-122 binding at either S1 or S2 in the context of Xrn1 knockdown to assess the impact of miR-122 on its Xrn1-independent role in full-length viral replication (Figure 7.8). We found that when Xrn1 was knocked down, binding at S1 caused a greater increase in replication than binding at S2; to analyze this differently,

knockdown of Xrn1 enhanced replication more when S1 was bound than when S2 was bound. Since Xrn1 is an HCV restriction factor, the greater the increase in replication observed by removal (knockdown) of Xrn1, the more Xrn1 was suppressing replication under those conditions. By inference, this suggests that binding at S2 more strongly protects from Xrn1 and binding to S1 more strongly affects Xrn1-independent functions of miR-122, but our observations also suggest that binding at both sites contributes to both Xrn1-dependent and Xrn1-independent functions.

In both sub-genomic and full-length RNAs, each miRNA binding site appeared to require a different amount of miR-122 to saturate the effects of binding. Providing greater amounts of miR-122 exogenously to Huh7.5 cells enhanced both sub-genomic (Figure 7.5D) and full-length (Figure 7.6D) RNA replication when binding at S1, suggesting that S1 binding is not saturated in Huh 7.5 cells. By contrast, exogenous addition of miR-122 for binding at S2 did not augment replication of sub-genomic RNA (Figure 7.5C), and increased full-length RNA replication only slightly (Figure 7.6C), suggesting S2 is already saturated in Huh 7.5 cells. This data may reflect the enhanced binding strength of miR-122 to S2 proposed by Mortimer *et al* (Mortimer and Doudna, 2013). This also has implications for many earlier explorations of the functions of miR-122, where Huh7.5 cells were supplemented with additional miR-122; these researchers may have been measuring only the effects of S1, since binding at S2 may have already been saturated (Cox *et al.*, 2013; Henke *et al.*, 2008; Huys *et al.*, 2013; Jangra *et al.*, 2010a, b; Shimakami *et al.*, 2012a).

When we evaluated translation, we found that under normal conditions, S1 and S2 binding contributed similarly to stimulation of HCV translation, and binding at both sites together increased translation more than either site alone (Figure 7.9A). However, when Xrn1 was knocked down, binding at S1 or S2 did not increase translation (Figure 7.9B), and binding at both sites together had only a small effect. This suggests that the effect of miR-122 on translation is mostly or solely due to protection from Xrn1.

Other researchers have also found that miR-122 functions to increase HCV translation (Henke *et al.*, 2008; Huys *et al.*, 2013; Jangra *et al.*, 2010b; Niepmann, 2009; Roberts *et al.*, 2011; Roberts *et al.*, 2014; Shimakami *et al.*, 2012a; Wilson *et al.*, 2011; Zhang *et al.*, 2012), and when the effect is compared to that of miR-122 on replication, it is most likely that miR-122 has a separate, additional role in replication (Jangra *et al.*, 2010b; Roberts *et al.*, 2014; Shimakami *et al.*, 2012a). More recently, researchers have been exploring whether the effect of miR-122 on viral translation is merely a stabilization of the viral RNA that leaves more copies available for translation (Conrad

et al., 2013; Li et al., 2013d; Mortimer and Doudna, 2013; Shimakami et al., 2012a; Shimakami et al., 2012b). In particular, Xrn1 has been implicated as the major host factor de-stabilizing and degrading the viral RNA (Jones et al., 2010b; Li et al., 2013d; Mortimer and Doudna, 2013), although not all screens involving Xrn1 knockdown show that it has an effect on HCV (Ariumi et al., 2011a; Jones et al., 2010b; Pager et al., 2013; Scheller et al., 2009). Our evidence (Figure 7.9) combined with others' suggests that the main impact that miR-122 has on translation is indeed due to protecting the viral RNA from degradation by Xrn1, leaving more copies of the RNA to be translated. Although we did observe an apparent slight increase in translation of viral RNA after Xrn1 knockdown when both miR-122 binding sites are occupied (Figure 7.9B), this could be due to the fact that we cannot eliminate 100% of Xrn1 through knockdown, and that miR-122 could be shielding the viral RNA from the small percentage of Xrn1 remaining in the cell. Interestingly, others show that the effect of miR-122 on stability does not actually require active replication or translation of the viral RNA (Mortimer and Doudna, 2013; Shimakami et al., 2012a).

We and others hypothesized that miR-122 binding to S1 creates a double stranded RNA structure that protects the 5' end of the viral RNA from degradation, and the specific impact of Xrn1 knockdown on miR-122-unbound replication supports this hypothesis (Li et al., 2013c; Li et al., 2013d). Our results support a role for protection from Xrn1 in miR-122 augmentation of HCV replication; we also found that binding of miR-122 at both sites contributes to protection from Xrn1, but Xrn1 knockdown impacts S1-unbound replication less than S2-unbound replication; this means that Xrn1 is better able to suppress replication when S2 is unprotected, than when S1 is unprotected. Thus we suggest that the mechanism by which miR-122 protects viral RNA from Xrn1 is not mediated by the overlap created by binding to S1, but that the miR-122/Ago2/RISC complex formed by binding at each site, and the concomitant protein/RNA structure protects the HCV RNA from Xrn1 (Machlin et al., 2011; Shimakami et al., 2012a; Shimakami et al., 2012b; Wilson et al., 2011). We further suggest that binding of miR-122/Ago2/RISC at S2 changes conformation of the viral RNA in such a way as to prevent Xrn1-mediated suppression of HCV replication.

Our observations that knockdown of Xrn1 did not eliminate the requirement for miR-122 on viral replication in any context tested (Figures 7.1, 7.2, 7.4, and 7.8) also supports the conclusion that protection from Xrn1 (and thus, increased stability) is not sufficient to account for the effect of miR-122 on HCV RNA accumulation, although we cannot rule out the possibility that

incomplete knockdown of Xrn1 could be responsible for the remaining difference (Jangra et al., 2010b; Roberts et al., 2014; Shimakami et al., 2012a). One possibility is that miR-122 protects the viral RNA from an as-yet unidentified pyrophosphatase that removes the 5' triphosphate (Li et al., 2013c; Wilson and Sagan, 2014). HCV requires a 5' triphosphate for efficient replication, but Xrn1 is much more effective against monophosphate 5' ends – such as those produced by de-capping of mRNAs – so degradation of the HCV genome by Xrn1 may require enzymes to generate a 5' monophosphate substrate on the 5' end of the viral RNA, and miR-122 may also protect the RNA from the putative pyrophosphatase (Garcia-Sastre and Evans, 2013; Li et al., 2013c; Li et al., 2013d). This may explain why Xrn1 knockdown alone is insufficient to restore replication to miR-122-bound levels in the absence of miR-122 (Li et al., 2013c; Li et al., 2013d; Nagarajan et al., 2013). Alternatively, miR-122 may have an additional function un-related to end-protection, such as in viral life cycle stages following translation like the switch from translation to replication, or aiding in initiation of replication. *In vitro* work suggests that miR-122 is not involved in elongation by the virus' RNA-dependent RNA polymerase, NS5b, but a role in initiation of replication has not been thoroughly examined, nor has a role in polymerase elongation been studied *in vivo* (Norman and Sarnow, 2010; Villanueva et al., 2010).

The relative contributions of miR-122 binding at S1 and S2 to the viral life cycle are not yet fully explained, as different researchers reach different conclusions, and all use Huh7-derived cell lines that express endogenous miR-122. The original discoverers of the two miR-122 binding sites in the 5' UTR first determined that binding at both sites was required for HCV RNA to accumulate to detectable levels; this was characterized using H77-derived virus constructs (genotype 1a) whose replication was detected solely by northern blot, and was verified by making mutations at positions p3, p3-4, and p6 of S1, S2, or both miR-122 binding sites, all of which abolished detectable replication unless the complementary microRNA was provided (Jopling et al., 2008; Jopling et al., 2005). Later analyses using more sensitive methods like detection of particle production showed that constructs with mutations in either S1 or S2 can replicate (to varying degrees) without compensatory miR-122 binding (Li et al., 2011a; Shimakami et al., 2012b).

Our results show that replication with either S1 bound or S2 bound occurs at similar levels, but others' results indicate that binding at S1 has an overall greater effect on replication (Jangra et al., 2010b; Li et al., 2011a; Shimakami et al., 2012b). Analysis of the roles of each miR-122 binding site by Li *et al.* using a JFH-1-derived genotype 2a virus concluded that binding at S2 was

unimportant for HCV replication, because when binding at S1 was abolished (either by insertion of a host hairpin RNA sequence, or by point mutations in the binding site), the construct did not respond to a miR-122 antagonist (Li et al., 2011a). However, both the hairpin insertion construct and the S1 binding site point mutants that failed to respond to the antagonist were severely impaired for replication at the time points indicated, suggesting that a failure to respond to the antagonist may have been due to an inability to detect a significant response due to poor overall replication. The authors of this paper separately made a point mutation in S2 and noted that replication was affected by the miR-122 antagonist to the same degree as if the mutation was not there; however, the mutation they used (p6, A to G) was already present in genotype 4, and could theoretically retain miR-122 binding via G-U base pairing. Structural data and *in vitro* analysis of miR-122 binding affinities also suggests that the binding at S2 is stronger and less-sensitive to binding site mutations (Mortimer and Doudna, 2013). Therefore, this mutation may not have abolished binding at S2, which would explain why the S2 mutant appeared to be as responsive to a miR-122 antagonist as the wild-type construct (Li et al., 2011a).

In experiments by the Lemon group measuring particle production and luciferase reporter levels, it was found that abolishing binding at S1 (S1:p6, A to U) had a greater impact on replication than abolishing binding at S2 (S2:p6, A to U) using a JFH-1-derived genotype 2a virus, and thus they suggest that binding at S1 is overall more important than binding at S2 (Jangra et al., 2010b; Shimakami et al., 2012b). In contrast, we found that binding at either S1 or S2 in full-length JFH-1-derived genotype 2a viruses (Figure 7.7) had an equal impact on replication when evaluated within the same construct but using the p3 C to G mutation instead of p6, A to U. We also observed differences between S1 and S2 binding mutants, but because we were able to use the Hep3B system that lacks endogenous miR-122, we were able to show that the differences were not because of miR-122 binding at those sites, but because of the point mutation itself; thus, the different findings may stem from differential effects of the p3 and p6 mutations. Interestingly, this group also found that supplementation of additional wild-type miR-122 (above that endogenous to Huh7.5 cells) had a greater impact on the S2:p6 mutant where it could bind to S1 than on S1:p6 where it could bind to S2, and support our findings that replication is more responsive to increased binding at S1 and relatively non-responsive to increased binding at S2 (Jangra et al., 2010b; Shimakami et al., 2012b).



We propose that the FL S2:p3 mutant is impaired for replication in some way that is only compensated for by miR-122 binding at both sites (Figure 7.6 and 7.7). Binding at just one site does not compensate for this impairment (Figure 7.7), nor does knockdown of Xrn1 (Figure 7.8C and 7.8B), and Xrn1 knockdown cannot rescue miR-122 unbound replication of FL S2:p3 to detectable levels (Figure 7.8C), but miR-122 binding at both sites reinstates wild-type levels of replication. Since the miR-122-unbound replication of the FL S1+S2:p3 mutant *is* rescuable by knockdown of Xrn1 (Figure 7.4), the severely impaired phenotype is only displayed when the RNA has the p3 mutation at S2 alone. In addition, since SGR S2:p3 does not demonstrate any impairment (Figure 7.5), we suggest that the alternative translation regulation or absence of the structural gene coding region in SGR can overcome this impairment. Overall the FL S2:p3 mutant appears to be severely dependent upon miR-122 for its replication, and characterization of this dependency – particularly through comparison of the RNA structures within the 5' UTR of S2:p3 and sequence-specific interactions with other *cis* viral RNA structures in the mutant with viral RNAs that do not exhibit the impaired phenotype such as wild-type, S2:p6, S1:p3, S1+S2:p3, and the SGR S2:p3 – may provide further insight into mechanisms of miR-122's interaction with the HCV life cycle.

Overall, our efforts add to the growing picture that the function of miR-122 in the Hepatitis C virus life cycle is nuanced and complicated. We find that miR-122 binding does protect the viral RNA from Xrn1 during replication and translation, but that it must have additional functions in the HCV life cycle. We also provide evidence to suggest that replication of full-length HCV RNA can occur in the absence of miR-122 binding since detectable replication can be rescued by relieving suppression by Xrn1. We further determine that binding of miR-122 at both S1 and S2 contribute equally to replication, and that their effects are co-operative when both sites are bound, but that when we remove the need to protect the viral RNA from Xrn1 (by Xrn1 knockdown), S1 binding increases replication more than S2 binding does, indicating that binding at each site contributes differentially to the various functions of miR-122. Finally, we have also validated the Hep3B cell culture system, which allows for identification of effects of point mutations in the miR-122 binding sites on virus replication that permit more accurate analysis of virus life-cycle processes in the presence and absence of miR-122 binding, and characterization of the roles of the different binding sites in the same HCV RNA construct. Further characterization of the effects of specific binding-site point mutations may also shed light on the various functions of miR-122. By

exploring the functions of miR-122 on the Hepatitis C virus life cycle in this system, we have provided insight into both others' results with respect to the roles of miR-122 binding to the HCV genome and future avenues of exploration to further understand the mechanisms of miR-122's interaction with Hepatitis C virus.

## **7.5 Materials and Methods**

### **7.5.1 Cell Lines.**

Huh7.5 and Hep3B cells were grown and maintained as described in Thibault *et al.* (Thibault *et al.*, 2013).

### **7.5.2 Plasmids and Viral RNA.**

Plasmids pSGR JFH-1 Fluc WT and pSGR JFH-1 Fluc GND contain bi-cistronic JFH-1-derived sub-genomic replicons with a firefly luciferase reporter; the GDD to GND mutation renders the viral polymerase non-functional (Kato *et al.*, 2005a). pSGR JFH-1 S1:p3 Fluc WT, pSGR JFH-1 S2:p3 Fluc WT, and pSGR JFH-1 S1+S2:p3 Fluc WT contain C to G mutations at position 3 of miR-122 binding site 1, site 2, or sites 1 and 2, respectively, generated as described in Thibault *et al.* (Thibault *et al.*, 2013). Plasmids pJ6/JFH-1 FL Rluc WT and pJ6/JFH-1 FL Rluc GNN contain mono-cistronic chimeric replicons with J6-derived structural proteins and JFH-1-derived un-translated regions and non-structural proteins, along with an in-frame Renilla luciferase reporter; the GDD to GNN mutation renders the polymerase non-functional (Jones *et al.*, 2007a). pJ6/JFH-1 FL S1:p3 Rluc WT, pJ6/JFH-1 FL S2:p3 Rluc WT, and pJ6/JFH-1 FL S1+S2:p3 Rluc WT contain C to G mutations as described for the concomitant S1:p3, S2:p3, and S1+S2:p3 SGR mutants. These were generated by replacing the 177bp EcoRI to AgeI fragment from J6/JFH-1 with the fragment from the appropriate miR-122-binding site mutant SGR plasmid. Plasmids pT7 luciferase (containing firefly luciferase, Promega; Nepean, ON, Canada) and pRL-TK (containing Renilla luciferase, Promega) were used as templates for production of mRNA. Viral RNA and mRNA were *in vitro* transcribed from these plasmids as described in Thibault *et al.* (Thibault *et al.*, 2013).

### 7.5.3 *MicroRNAs and siRNAs.*

miR-122, miR-122/p3, and miControl are described previously (Thibault et al., 2013). siXrn1 (s29015: 5' – GAG AGU AUA UUG ACU AUG Att–), siXrn2 (s22412: 5' – GGA AAG UUG UGC AGU CGU Att – 3'), siControl (5' – GAA GGU CAC UCA GCU AAU CAC ttc – 3'). All small RNAs were synthesized by ThermoScientific Dharmacon (Lafayette, CO, USA).

### 7.5.4 *Electroporation of Hep3B and Huh7.5 cells.*

Electroporations were carried out as described in Thibault *et al.* (Thibault et al., 2013). Both Hep3B and Huh7.5 cells were electroporated using the following conditions: 225V, 950 $\mu$ F, 4mm, and  $\infty\Omega$ .

### 7.5.5 *Transient HCV Replication Assays Without Knockdown.*

On Day 0,  $6.0 \times 10^6$  cells in 400 $\mu$ L Dulbecco's PBS were electroporated with 1 $\mu$ g mRNA, 60pmol miRNA, and 5 $\mu$ g (Huh7.5) or 10 $\mu$ g (Hep3B) *in vitro*-transcribed viral RNA. After electroporation, cells were resuspended in 4mL cell culture media. 500 $\mu$ L of cells were incubated in microfuge tubes at 37°C for 2 hours, then harvested for luciferase analysis. For all other luciferase analysis, 500 $\mu$ L cells per well were plated in 6-well dishes and incubated at 37°C to be harvested at the indicated time points. 2mL cells were plated in a 4mm tissue culture dish to be collected for RNA analysis three days post-electroporation.

### 7.5.6 *Transient HCV Replication Assays With Pre-Knockdown.*

Three days before Day 0,  $6.0 \times 10^6$  cells in 400 $\mu$ L Dulbecco's PBS were electroporated with 60pmol siRNA for pre-knockdown, and were plated in 15cm tissue culture dishes, two cuvettes per dish. siRNA-treated cells were incubated for three days at 37°C. On Day 0, cells were collected from the dishes, prepared as described for electroporation (two cuvettes' worth from each dish), and then electroporated and plated as described above.

### 7.5.7 *HCV Translation Assays With Pre-Knockdown.*

Cells were electroporated for pre-knockdown as above. Three days later, cells were prepared as above and electroporated again with 5 $\mu$ g viral RNA, 1 $\mu$ g mRNA, and 60pmol miRNA, and recovered in 4mL culture media. 500 $\mu$ L of cells were incubated at 37°C in microfuge tubes for

three hours before collecting for luciferase analysis; 500 $\mu$ L cells per well were plated in 6-well dishes for the six, nine, and twelve-hour luciferase samples. 2mL of cells per well were plated in 6-well dishes for total RNA collection at three hours post-electroporation.

#### 7.5.8 *Luciferase Assays.*

Each sample was harvested for luciferase by first washing with Dulbecco's PBS, and then scraping into 100 $\mu$ L 1X Passive Lysis Buffer (Promega). Samples were assayed using the Dual Luciferase Assay Kit, Luciferase Assay Kit, or Renilla Luciferase Assay Kit (Promega) according to the manufacturer's protocol, and using the GLOMAX luminometer (Promega) with a 2-second delay and 10-second reading.

#### 7.5.9 *Total RNA Extraction.*

Each sample for RNA analysis was washed with Dulbecco's PBS and collected into 500 $\mu$ L Trizol (Life Technologies; Burlington, ON, Canada). RNA was isolated by following the manufacturer's protocol.

#### 7.5.10 *RTq-PCR.*

1  $\mu$ g of total cellular RNA was reverse-transcribed using the iScript cDNA Synthesis Kit (BioRad; Mississauga, ON, Canada). qPCR reactions were carried out using the TaqMan kits (Life Technologies) FAM-MGB Hs00404871\_m1 (Xrn1), FAM-MGB Hs01071302\_m1 (Xrn2), and FAM-MGB 4352934-0803022 (GAPDH); samples were amplified in triplicate in a 96-well plate in the CFX96 real-time PCR system according to kit protocol (BioRad). All data was analyzed with the CFX Manager 2.0 Software (BioRad).

#### 7.5.11 *Data Analysis.*

All experiments are shown as an average of at least three independent experiments (unless otherwise noted) with error bars indicating standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism 6.0.4; statistical significance was determined by the tests indicated in the figure legend. \*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$ ; \*\*\*\*  $p \leq 0.0001$ ; n.s. = not significant ( $p > 0.05$ ).

## 8.0 Conclusions and Discussion

### 8.1 General Conclusions

- Supplementation of miR-122 to non-permissive cells can render them permissive to HCV RNA replication
- Mouse embryonic fibroblasts can permit sub-genomic HCV RNA replication
- miR-122 is not sufficient to overcome some barriers to HCV replication in transformed and immortalized cells
- Passage and culture conditions can affect cell culture permissiveness to HCV RNA replication
- Replication of full-length, mono-cistronic HCV RNA is subject to greater constraints than replication of sub-genomic, bi-cistronic HCV RNA
- Hep3B human hepatoma cells lack only miR-122 to be able to support the entire HCV life cycle
- Hep3B cells produce infectious virus to the same titers as the specialized Huh7.5 cells, but did not undergo adaptation to generate their permissiveness
- Sub-genomic bi-cistronic HCV RNA can replicate in the absence of miR-122 binding
- C-to-G mutations at position p3 in both miR-122 binding sites on the viral 5' UTR abolish miR-122 binding, but do not affect the miR-122-independent replication of sub-genomic HCV RNA, nor do they affect miR-122-dependent replication when the sites are bound by the matching mutant miRNA, miR-122/p3
- miR-122-independent replication is also independent of the requirement for Argonaute-2
- Binding of miR-122 at both sites (S1 and S2) in the 5' UTR contribute equally in magnitude to sub-genomic and full-length HCV RNA replication
- Binding of miR-122 at both sites exerts a co-operative effect on HCV RNA replication, rather than merely a multiplicative effect
- Full-length HCV RNA bearing a p3 C-to-G mutation at miR-122 binding site S2 is impaired for replication when only one (or neither) miR-122 binding site is occupied, but replicates at wild-type levels when both sites are occupied
- miR-122 functions to protect the 5' end of HCV RNA from the host cytoplasmic 5' to 3' RNA exonuclease, Xrn1, but also has additional functions in the HCV life cycle

- Binding of miR-122 at site S2 functions to protect HCV RNA from Xrn1 during replication more than binding at site S1
- Binding of miR-122 at site S1 is important for an alternate function of miR-122, and binding at S2 may also contribute to these alternate functions
- The effect of miR-122 on translation is mostly or entirely accounted for by protection from Xrn1

## 8.2 Discussion and Future Directions

Our results in Chapters 3 and 5 with both mouse embryonic fibroblasts and human hepatoma cells show that miR-122 is a major determinant of tissue tropism for Hepatitis C virus, since supplementation of a given cell line with miR-122 was sufficient to permit replication of viral RNA. This idea has interesting implications for the development of culture systems for the newly-discovered non-primate *Hepaciviruses*, as well as for understanding their origins, since all the sequences isolated thus far show putative miR-122 binding sites at S1, but lack a putative S2, while GBV-B has been shown to use miR-122 binding at two sites much like HCV (Burbelo et al., 2012; Drexler et al., 2013; Kapoor et al., 2011; Kapoor et al., 2013; Lauck et al., 2013; Lyons et al., 2012; Sagan et al., 2013). However, particularly with mouse cells, we noted that merely supplementing with miR-122 was not necessarily sufficient to permit RNA replication, as not all mouse cell lines supplemented with miR-122 were permissive, and some cell lines lost their permissiveness over time despite addition of miR-122. It is not surprising that more than just miR-122 is required to permit HCV RNA replication in cell culture models; we found that MEFs bearing a knockout for the host antiviral sensor PKR, as well as MEFs bearing a knockout for the liver-X receptor-interacting protein NCoA6, permitted replication of viral RNA better than wild-type MEFs, suggesting that the knockouts also aided in rendering the cells permissive to HCV. However, these cells later lost permissiveness, and when we tested knockdown of these factors in the already-permissive Huh7.5 cell line, we observed no significant increase in viral RNA replication, which calls into question whether it was the knockouts, or another unique property of each cell line acquired through transformation, which rendered them permissive in the first place. It is still possible that HCV already has mechanisms to prevent restriction by human PKR and NCoA6 such that knockdown of these in Huh7.5 cells had no effect, while removing these restriction factors in a mouse setting was beneficial to the virus, but since the wild-type MEFs also

lost permissiveness for replication, we were unable to test knockdown of the murine factors directly to verify the roles of PKR and NCoA6 in mouse cells.

Others have shown that cell culture conditions and passage of well-established cell lines can change their properties, so it is not surprising that less-characterized and “fresher” cell lines also changed in permissiveness to HCV over time and through passage (Calles et al., 2006; Jensen and Norrild, 2000; Lohmann et al., 2003; Richards et al., 2010; Siissalo et al., 2007). The PKR knockout cell line appeared to have gone through crisis when we first started working with them, which may have involved a genetic or epigenetic change that rendered them highly permissive for sub-genomic HCV RNA replication; however, as the cell line was not immortalized, the cells soon became senescent, and other isolates from the same source never experienced the same crisis. It is also useful to note at this point that primary hepatocytes isolated from the PKR knockout mice were not permissive for RNA replication, regardless of supplementation with miR-122, but primary hepatocytes do not grow in culture, and others have shown that HCV replication benefits from a growing and dividing host cell; thus, it is again not certain whether the PKR knockout would have benefited HCV RNA replication (Nelson and Tang, 2006). The wild-type and NCoA6 cells were already immortalized, and were permissive to low and intermediate levels of replication, respectively; it is possible that some event in the lab triggered their loss of permissiveness, but since this occurred at vastly different points in time for the two cell lines, we propose that it was unrelated epigenetic changes that affected the ability of the two cell lines to support sub-genomic HCV RNA replication.

Based on our findings, acquiring other currently-permissive mouse cell lines and monitoring them for changing permissiveness could lead to identification of murine-specific HCV restriction factors such as the ones that we hypothesize affected our murine cell lines. Some of these – particularly any with no known involvement in innate or adaptive immunity – would be candidates for development of knockout mouse model systems. While the most recent mouse model actually achieves infection in the mouse liver, as opposed to a human liver tissue xenograft, it is still impaired for interferon signaling since it is a Stat1<sup>-/-</sup> mouse and is therefore not ideal for vaccine and immunopathogenesis studies (Dorner et al., 2013a). Developing a similar mouse in a non-immunity-related restriction factor knockout background would finally permit an immunocompetent mouse model of infection that could be used for vaccine development and other studies that involve innate and adaptive immunity.

We observed that in both mouse cells and Hep3B cells, sub-genomic HCV RNA behaved differently than full-length HCV RNA. In mouse cells, only sub-genomic HCV RNA would replicate when supplemented with miR-122; full-length HCV RNA would not. In Hep3B cells, we were able to easily detect miR-122-independent replication of sub-genomic HCV RNA, but could not detect miR-122-independent replication of full-length HCV RNA unless we first knocked down the host restriction factor Xrn1. This suggests that there is some additional requirement for or restriction on replication of the full-length genome that is not present for the sub-genomic RNA. There are a plethora of differences between these two constructs: the sub-genomic construct lacks the viral structural proteins and their coding regions, and also has an additional IRES – which adds both secondary RNA structure and a separation of translation from replication – while the full-length construct has an additional protein (Renilla luciferase) within the viral polyprotein. We have begun work in characterizing the differences between these two viral RNA constructs, with the aim of discovering what permits easily-detectable miR-122-independent replication of sub-genomic HCV RNA. Preliminary results using a series of mono-cistronic constructs sequentially deleted for structural and scaffolding proteins (data not shown) suggest that the presence of the p7 coding region may be the key difference between sub-genomic and full-length miR-122-independent replication. Because co-electroporation experiments of full-length and sub-genomic HCV RNA together in Hep3B cells (data not shown) show no impairment of miR-122-independent sub-genomic replication when full-length RNA is also translating and replicating, we do not believe it is the p7 protein itself. However, the full-genomic replicon used in Chapter 5 is bi-cistronic, and does express the p7 protein (thus containing the p7 coding region), yet it does show miR-122-independent replication. We hypothesize that the p7 coding region is involved in some regulatory long-range RNA-RNA interactions with other portions of the genome – which may in turn be regulated by miR-122 – and these interactions are impaired by the second IRES present in the full-genomic replicon.

It is also possible that the difference between replication of sub-genomic and full-length RNA in mouse cells and in Hep3B cells occurs through two separate mechanisms, particularly since we did not detect miR-122-independent replication of the sub-genomic replicon even in the highly-permissive PKR knockout MEFs. In mouse cells, the impairment of the full-length HCV RNA may be due to restriction factor binding in the structural coding region, rather than viral *cis*-RNA-RNA interactions; inability to detect miR-122-independent replication may have been a function



of higher levels of Xrn1 or a similar ribonuclease from which miR-122 functions to protect the viral RNA. Since much of the experiments described above involve characterization of requirements for miR-122, it is likely that identification of the driving force behind the observed differences between sub-genomic and full-length replication will also provide further insight into the mechanisms of miR-122-mediated enhancement of HCV RNA accumulation, such as regulation of potential RNA-RNA interactions that affect the virus life cycle. As we have shown, there is much work remaining to understand the role of miR-122 in the HCV life cycle.

At the time that miR-122 was first identified as a major requirement for the Hepatitis C virus life cycle, JFH-1-derived full-length constructs were replacing sub-genomic replicons of various origins in the literature, since the full-length constructs were both more biologically relevant and more useful for studying all the stages of the viral life cycle (Vieyres et al., 2013). The sub-genomic replicon based from the high-capacity JFH-1 isolate was not studied in detail by many researchers in the field in favour of the full-length genome, including those attempting to develop new cell culture and animal models for the virus. Due to the poor replication capacity of the other sub-genomic replicons at the time, it is likely that miR-122-independent replication would only have been detectable with the JFH-1 sub-genomic replicon (Vieyres et al., 2013). Additionally, while others had shown selectable replication of various isolates in cells unlikely to express miR-122, most of this work had been done before the finding that miR-122 was a requirement for HCV replication, and so the absence of miR-122 in these cell lines had never been established (Ali et al., 2004; Chang et al., 2006; Kato et al., 2005b; Uprichard et al., 2006; Zhu et al., 2003). Thus, it was not surprising that others did not identify the capability of the sub-genomic replicon to replicate at low levels in the absence of miR-122 without selective pressure.

The value in having a system where replication occurs completely independent of miR-122 is clear, since it is a key control in any experiment done to understand the role of miR-122. Researchers have made use of miR-122 antagonists such as that described in Chapter 5, or have added more miR-122 to systems that already express miR-122. However, the antagonist may not reach 100% of the cells that have viral RNA, or may not sequester 100% of the miR-122 in the cells it does reach, and thus cannot be used to achieve a miR-122-free system in Huh7-derived cells, which are still the cell culture model most used for HCV. Addition of miR-122 to a system like the Huh7.5 cell line comes with its own potential pitfalls as well, since the dynamic range of both miR-122 availability and HCV replication is considerably reduced. For example, using the

electroporation conditions we have established in our lab, addition of five or ten-fold *more* miR-122 to Huh 7.5 cells had only a limited impact on HCV RNA replication (two- to four-fold increase, data not shown), indicating that the amount of miR-122 available in Huh7.5 cells or typically provided exogenously is nearly saturating, and either the cells or the virus cannot support much higher levels of replication than are observed under normal conditions. By contrast, addition of miR-122 to Hep3B cells covers replication ranging from conditions with no miR-122 to saturating amounts of miR-122, and we determined that replication increased in proportion to amount of miR-122 added, such that a 1/10 dilution of miR-122 resulted in an approximate 1/10 reduction in replication over a 10,000-fold range. Thus, the practice of adding more miR-122 to Huh 7.5 cells to attempt to observe requirement for a particular host factor (or restriction by a particular host factor) in mediating the effect of miR-122 may not yield relevant results (Cox et al., 2013; Henke et al., 2008; Huys et al., 2013; Jangra et al., 2010a, b; Shimakami et al., 2012a; Wilson et al., 2011). We also observed that binding of miR-122 at site S2 is saturated or nearly-saturated under typical conditions; combined with our observation that each miR-122 binding site contributes more to different functions of the miRNA, experiments where additional miR-122 is added to systems that already contain nearly-saturating levels of miR-122 are likely to only evaluate the effect of miR-122 on functions associated with site S1, which does not appear to be saturated under normal conditions in our experiments. Thus, Hep3B cells provide a valuable miR-122-null background in which to study the mechanisms of action of miR-122.

Finally, researchers have circumvented the requirement for specific binding of miR-122 by mutating single nucleotides in the miR-122 binding sites to abolish binding, and supplementing with a mutant miRNA that is based on miR-122 but bears the complementary point mutation(s) to restore binding (Jopling et al., 2008; Jopling et al., 2005). Particularly, this experiment and others like it have been used to demonstrate a direct interaction between the miRNA and the viral RNA and rule out any systematic effects of miR-122 on the cell that may also benefit the virus (Huys et al., 2013; Jangra et al., 2010b; Jopling et al., 2006; Jopling et al., 2008; Li et al., 2013d; Machlin et al., 2011; Norman and Sarnow, 2010; Shimakami et al., 2012a; Shimakami et al., 2012b; Thibault et al., 2013; Wilson et al., 2011; Zhang et al., 2012). However, in many cases when binding at just one site is abolished this way, and in almost every case when binding at both sites is abolished this way, replication of the viral RNA is undetectable without reinstating binding with

the mutant miRNA, which again highlights the previous difficulty of studying replication of HCV RNA without miR-122 binding.

In our lab, we have used our miR-122-independent system to validate the requirement for miR-122 (or lack thereof) for particular host proteins to impact the virus' life cycle. Particularly, we have shown that while knockdown of Ago2 (the major effector protein in the miRNA and siRNA silencing pathways) significantly impacts miR-122-dependent replication of HCV, it has no impact on miR-122-independent replication. This confirms our and others' hypotheses that not only does miR-122 require Ago2 to positively modulate HCV RNA accumulation, but Ago2 does not impact HCV RNA replication in any other way than mediating the interaction between HCV RNA and miR-122/RISC (Conrad et al., 2013; Shimakami et al., 2012a; Wilson et al., 2011). We have also shown the converse: DDX6, a protein downstream in the miRNA pathway, is responsible for enhancing some aspect of HCV RNA replication; while others have hypothesized that it is involved in miR-122-dependent modulation of HCV RNA accumulation, we determined that DDX6 has the same impact on both miR-122-dependent and miR-122-independent replication, which shows that DDX6 enhances HCV RNA replication in some way that is entirely independent of miR-122 (Ariumi et al., 2011a; Huys et al., 2013; Jangra et al., 2010a).

We have used a combination of our initial miR-122-independent system (sub-genomic replication in Hep3B cells, where there is no endogenous miR-122) and the system we adapted for use in Huh7.5 cells (C to G mutations at position 3 of the miR-122 binding sites) to better characterize the functions of miR-122 in the HCV life cycle. The key to these systems was the finding that the p3 mutation did not impair miR-122-independent replication of sub-genomic RNA; we also explored a p5 mutation, but found that this would not permit detectable miR-122-independent replication (data not shown). We then used this system to evaluate the role of miR-122 in protecting HCV from Xrn1, a host cytoplasmic RNA exonuclease. Li *et al.* provided evidence that HCV RNA was vulnerable to degradation by Xrn1, and that miR-122 had the same effect on HCV RNA stability as knockdown of Xrn1, suggesting that miR-122 functioned partially to protect HCV RNA from degradation by Xrn1 (Li et al., 2013d). We used our system to show that when miR-122 binding was abolished, knockdown of Xrn1 increased replication of sub-genomic RNA much more than when miR-122 was present; thus, in the absence of miR-122, HCV RNA is more vulnerable to Xrn1 than when miR-122 is present, confirming the conclusions of Li *et al.* (Li et al., 2013d).

Li *et al.* also concluded that miR-122 must play additional roles in the HCV life cycle, because knockdown of Xrn1 did not fully complement the effect of adding miR-122 to the cells, and because knockdown of Xrn1 did not rescue replication of full-length HCV RNA without miR-122 binding (Li *et al.*, 2013d). Because we observed no impairment of the sub-genomic replicon (with or without miR-122) with the p3 mutations, we generated a full-length replicon that bore p3 C to G mutations in its 5' UTR miR-122 binding sites and tested the effect of Xrn1 knockdown on this construct. Contrary to what Li *et al.* found, we observed that knockdown of Xrn1 could permit detectable replication of miR-122-unbound full-length HCV RNA, either wild-type (in Hep3B cells) or p3 binding mutants (in Huh7.5 and Hep3B cells). Nonetheless, in either sub-genomic or full-length HCV RNA constructs, knockdown of Xrn1 did not restore miR-122-independent replication to miR-122-dependent levels, indicating that miR-122 has additional functions that further increase replication.

In order to explore the potential additional functions of miR-122 in the HCV life cycle, we wished to determine what contribution each miR-122 binding site made to HCV replication. We initially hypothesized that miR-122 binding at the most 5' site (S1) on the viral UTR would better protect from Xrn1, since it would better shield the 5' tri-phosphate end from attack by Xrn1. We further hypothesized that if this were the case, then binding at S2 must serve a different function in the HCV life cycle. To test this, we first determined the relative overall contribution of each miR-122 binding site to the HCV life cycle by generating mutant sub-genomic and full-length constructs with the p3 mutation only in site S1 or only in S2 and testing their replication. We found that all mutants replicated to wild-type levels when both miR-122 binding sites were occupied (by endogenous miR-122 in Huh7.5 cells, exogenous miR-122 in Hep3B cells, and a mutant miR-122 “miR-122/p3” that matches the mutated binding sites in both cell lines). We concluded that each miR-122 binding site contributes to an equal magnitude to increasing HCV RNA accumulation, since the sub-genomic S1:p3 and S2:p3 mutants replicated to the same levels when only one site was occupied in Huh7.5 cells, while in Hep3B cells, the full-length S1:p3 mutant demonstrated identical levels of replication when either S1 or S2 was occupied, as did the full-length S2:p3 mutant. We also found that binding at both sites co-operatively impacted replication.

We then used the full-length S1:p3 and S2:p3 mutants to test the effect of Xrn1 knockdown on each binding site, as well as to evaluate the impact of binding at each site on replication when the need for protection from Xrn1 was abolished. Our results demonstrated that miR-122 binding at

site S2 protected more from Xrn1 than binding at S1, although binding at S1 did also aid in protection from Xrn1. Because each site contributes equally in magnitude, but S2 protects more from Xrn1, binding at S1 must contribute more to an additional function of miR-122. However, these results are contrary to our hypothesis and a simplistic end-protection model of shielding HCV RNA from Xrn1. If binding at S2 is more important for protection from Xrn1 than binding at S1, then either the 5' UTR and miR-122 binding assume a structure that is not yet understood, or the mechanism of Xrn1-mediated suppression of HCV RNA is not entirely through direct degradation of the viral RNA from the 5'-most nucleotide. Either of these possibilities is supported by our results when we evaluated the impact of each miR-122 binding site on viral translation with or without Xrn1 knockdown; both sites contribute equally to increasing translation under normal (no knockdown) conditions, but when Xrn1 is knocked down, binding at either site has no further impact on translation. This suggests that the only impact miR-122 had on viral translation was in fact stabilization of the viral RNA by protecting it from Xrn1, and because each site contributed equally to translation, each site therefore contributed equally to protection from Xrn1 during translation. Thus, either the viral RNA assumes a different conformation during translation only that allows either binding site to contribute equally to end-protection, or Xrn1 negatively impacts the viral RNA in some other way than the destabilization and degradation of the viral RNA measured in the course of a translation assay.

Finally, the question remains: what are the other functions of miR-122, and how does each binding site singly or together contribute to these functions? miR-122 was originally hypothesized to enhance translation of the viral RNA by changing the structure of the IRES to increase its affinity for translation initiation factors, but this mechanism was never shown *in vivo*, and the effects of miR-122 on translation have now been attributed to increasing the stability of the viral RNA, which allows more RNA to be translated (Conrad et al., 2013; Diaz-Toledano et al., 2009; Li et al., 2013d; Shimakami et al., 2012a). miR-122 does function to protect the viral RNA from Xrn1, albeit perhaps not by the simple end-protection model first hypothesized. Others have also hypothesized that miR-122 must also protect the 5' UTR from phosphatase activity: Xrn1 strongly prefers a mono-phosphate substrate to the tri-phosphate substrate found on the HCV 5' UTR, and so it is possible that a phosphatase first "attacks" the viral 5' tri-phosphate and removes the pyrophosphate, thus leaving the RNA unable to replicate (HCV requires a 5' tri-phosphate to replicate) and vulnerable to Xrn1 (Garcia-Sastre and Evans, 2013; Li et al., 2013c; Wilson and

Sagan, 2014). This again leans heavily on a 5′ end-protection model, where miR-122 binding at S1 shields the very 5′ nucleotide of the viral RNA from this attack, but this has not yet been tested, as candidate phosphatases and pyrophosphatases have yet to be identified. Alternatively, miR-122 binding at the 5′ end of the viral RNA may protect it from detection by such 5′ tri-phosphate innate immune sensors as RIG-I, MDA5, IFIT-1, or IFIT-5 (Li et al., 2013c; Wilson and Sagan, 2014). We have tested this possibility by co-electroporating miR-122-bound and miR-122-unbound HCV RNA into cells to see if any innate immune signaling or response occurs that could limit HCV RNA replication, and we observed no effect on miR-122-bound replication in the presence of miR-122-unbound replication (unpublished data). We also could not detect increased transcripts of IFN- $\beta$  or TNF- $\alpha$ , downstream products of innate immune signaling, when miR-122-unbound replication was occurring (unpublished data). Together, this suggests that 5′ end-protection from detection to prevent an innate immune response is not a function of miR-122 in enhancing HCV RNA replication.

miR-122 has been implicated in regulating the switch between different stages of the virus' life cycle. *In vitro*, miR-122 has been shown to change the conformation of the 5′ UTR and IRES to a structure more available to translation initiation factors as discussed above, and was thus hypothesized to re-direct newly-produced HCV RNA from replication back to translation, or to promote translation of the viral RNA when it first enters the cell (Diaz-Toledano et al., 2009; Wilson and Sagan, 2014). However, the proposed structural changes occurred when single-stranded miR-122 guide strand was provided, even though ss-miR-122 does not function *in vivo*, and the interactions involved structural coding regions that aren't present in a sub-genomic replicon, even though sub-genomic replicons respond to miR-122 in the same way that full-length constructs do. Although the proposed mechanism is thus unlikely, the possibility of miR-122 functioning as a switch is still plausible. Rather than promoting translation – which, as we and others have shown, is not a direct function of miR-122 (Huys et al., 2013; Li et al., 2013d; Shimakami et al., 2012a) – miR-122 binding or dissociation may act as the switch to send viral RNA from translation into replication; alternatively, it may be a switch to keep viral RNA in replication, where dissociation sends the viral RNA to be packaged. Studying this may require quantifying replication complexes and comparing to viral translation and/or particle production; the mechanism of this regulation would then also involve a complicated interaction between viral and host proteins and the miR-122/RISC/viral RNA complex. Other imaging and intracellular

tracking of miR-122 and viral RNA may also provide insight into the dynamics of miR-122/RISC interaction with the viral RNA, whether it is transient or stable, and at what points in the virus life cycle the interaction(s) occur(s).

miR-122 binding could also be involved in direct initiation of replication. While there is no evidence for miR-122 modulating the elongation activity of the viral polymerase, researchers have been unable to study the initiation of the polymerase in a system that accurately reflects the *in vivo* conditions (Norman and Sarnow, 2010; Villanueva et al., 2010). HCV NS5b is thought to initiate replication of the viral RNA without the use of a primer, but this cannot be achieved *in vitro*, even with replication complexes isolated from infected cells; perhaps miR-122 and other RISC components are key to this step, or perhaps other viral proteins in the replication complex (Luo et al., 2000; Zhong et al., 2000). miR-122 and the RISC may be responsible for permitting replication without priming, either by acting as a protein/RNA primer, or by circularizing the viral RNA to permit replication as other *Flaviviridae* family members do (Filomatori et al., 2006; Khromykh et al., 2001; Villordo and Gamarnik, 2009). As yet, no one has been able to detect miR-122 in replication complexes, but this may not be surprising if miR-122 is only involved in the initial assembly and initiation of the replication complex and not in elongation, nor secondary initiation of later strand replication (Villanueva et al., 2010). It is also important to note that until recently, no one was able to show replication in the absence of miR-122, either.

While hypothesizing about potential host and viral proteins involved in the additional functions of miR-122 may lead to discovery of these additional functions, it may also be beneficial to approach the question from a different angle. We have already identified Xrn1 as a host restriction factor from which miR-122 functions to protect HCV RNA; thus, later assays to determine other roles for miR-122 should occur in the context of Xrn1 knockdown or, if possible, Xrn1 knockout. We have also shown that binding at S2 is important for protection from Xrn1; in a simplistic model, eliminating the effects of binding at S2 and only examining the functions of miR-122 binding at S1 may also be useful to specifically target the process in question. Finally, the specific impairment of our full-length S2:p3 construct is intriguing and may itself be a valuable tool. This impairment is unique to the full-length construct, which suggests that a property of the sub-genomic construct compensates for this impairment, or that a long-range RNA-RNA interaction between the 5' UTR and the structural protein coding region is initiated by the S2:p3 mutation, or that a detrimental long-range RNA-RNA interaction caused by the mutation is disrupted by the size or structure of

the EMCV IRES present in the sub-genomic replicon. However, this impairment does not appear to be present in the FL S1+S2:p3 mutant – within our ability to test it – since knockdown of Xrn1 rescues the double-mutant but not the S2:p3 mutant, suggesting that generating the matching mutation in S1 somehow compensates for whatever has been disrupted by the p3 mutation in S2. When both binding sites are occupied, the S2:p3 mutant does not appear impaired for replication. Taken together, the impaired phenotype of the full-length S2:p3 mutant and exploring the possible mechanisms – structural or otherwise – for this impairment has the potential to offer considerable insight into processes regulated by miR-122 binding and their mechanisms, and may serve to generate hypotheses for the various functions of miR-122, as well as explaining the co-operative effect of binding at both sites over the lesser effect of binding at only one or the other site. Particularly, it would be interesting to create selectable full-length mono-cistronic replicons bearing either the S1:p3 or S2:p3 mutation, and select for compensatory mutations in the presence (Huh7.5 cells) and absence (Hep3B cells) of miR-122; comparison of the mutations achieved in the two constructs, and the two different conditions, may provide leads on structural changes and interactions within the viral RNA that are mediated by the miR-122 binding sites and miR-122 binding, together or separately.

More broadly, exploring other HCV isolates for their dependence on miR-122 and their ability to accommodate binding site mutations like p3, p3-4, p5, and p6 has the potential to validate – or invalidate – the findings we have made regarding dependence on miR-122 binding at specific sites for specific functions, and provide further avenues of exploration. Manipulating viral constructs to determine the mechanism for the differences between the full-length and sub-genomic JFH-1-derived constructs and their interactions with miR-122 and binding site mutations has great potential to offer insight into how miR-122 affects HCV replication; preliminary data discussed above suggests that the presence of the p7 coding region may be a contributing factor in this difference, and this requires further exploration.

Throughout the course of this work, we have developed model systems for the study of the Hepatitis C virus, and more particularly, for the study of the role of miR-122 in the virus' life cycle. We have validated the miR-122-independent system for its usefulness in identifying and characterizing host components involved in the functions of miR-122 on the virus life cycle, and we have used it to further characterize the mechanisms for one of the functions of miR-122. Further, we have generated a system that offers several approaches to understanding other



functions of miR-122, and the future findings from this system have a wide variety of implications for the study of the unique interaction between Hepatitis C virus and miR-122, as well as the potential interactions between the recently-discovered non-primate *Hepaciviruses* and miR-122.

## 9.0 References

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