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MicroRNA-122-dependent and -independent replication of Hepatitis C Virus in Hep3B human hepatoma cells

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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 di-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.

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 IFN-α 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 9.6 kb 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. (2005, 2008) 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. 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.,

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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; 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 (Ago2) 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, 2010b; 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 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, 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 titers 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 di-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,

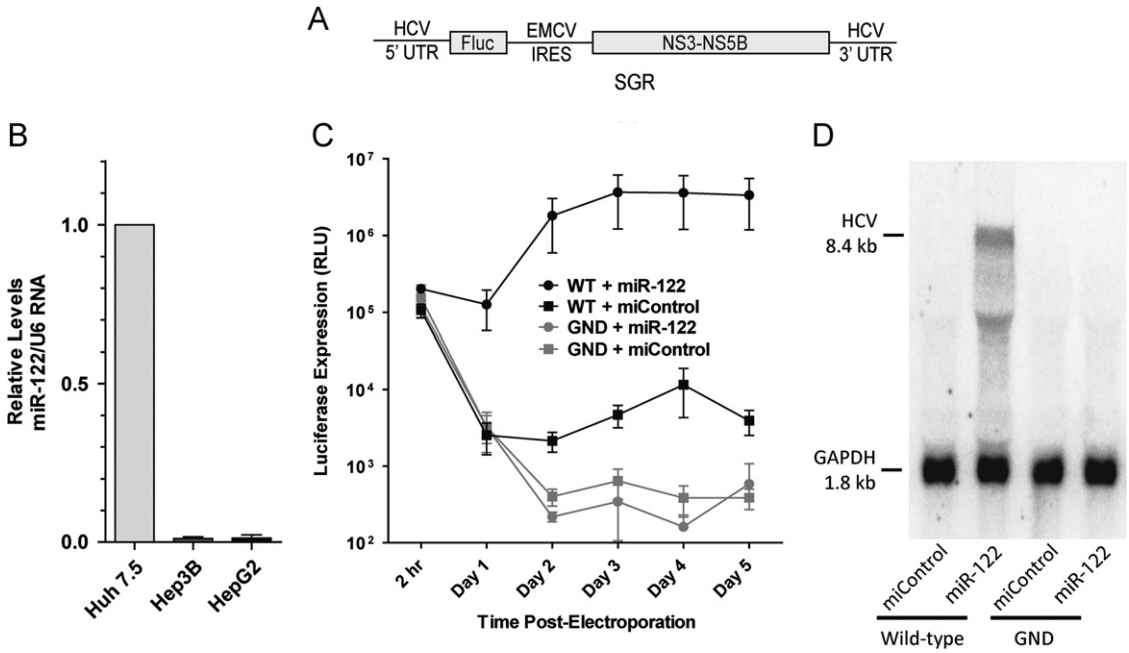

Fig. 1. Replication of sub-genomic (SGR) HCV in Hep3B cells. (A) Components of "SGR," the di-cistronic sub-genomic firefly luciferase reporter replicon based on the JFH-1 isolate. The replicon lacks the structural genes required to form particles, but has all the necessary non-structural genes required 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.

compared to their roles in HCV replication that are independent of miR-122. As proof of principle we evaluated the role of 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.

Results

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 levels of miR-122 expression after transformation (Fig. 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 Fig. 1A), as measured by the firefly luciferase reporter gene in Fig. 1C, and confirmed by northern blot analysis of viral RNA accumulation (Fig. 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).

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

Co-electroporation of J6/JFH-1 RNA (depicted in Fig. 2A) and miR-122 also renders Hep3B cells permissive to replication of full-length HCV RNA (Fig. 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 (Fig. 2B, compare WT+miControl and GNN+miR-122). Full-length HCV RNA accumulation by replication was confirmed by northern blot analysis (Fig. 2D). Tissue culture supernatant was collected on Day 3 post-electroporation 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 (Fig. 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 titers in the supernatant from miR-122-supplemented Hep3B cells were similar to that from Huh7.5 cells (Fig. 2E). Northern blot analysis of HCV RNA levels in miR-122 supplemented Hep3B cells compared with Huh7.5 cells (Fig. 2F) indicates that at three days post-electroporation, Hep3B cells had

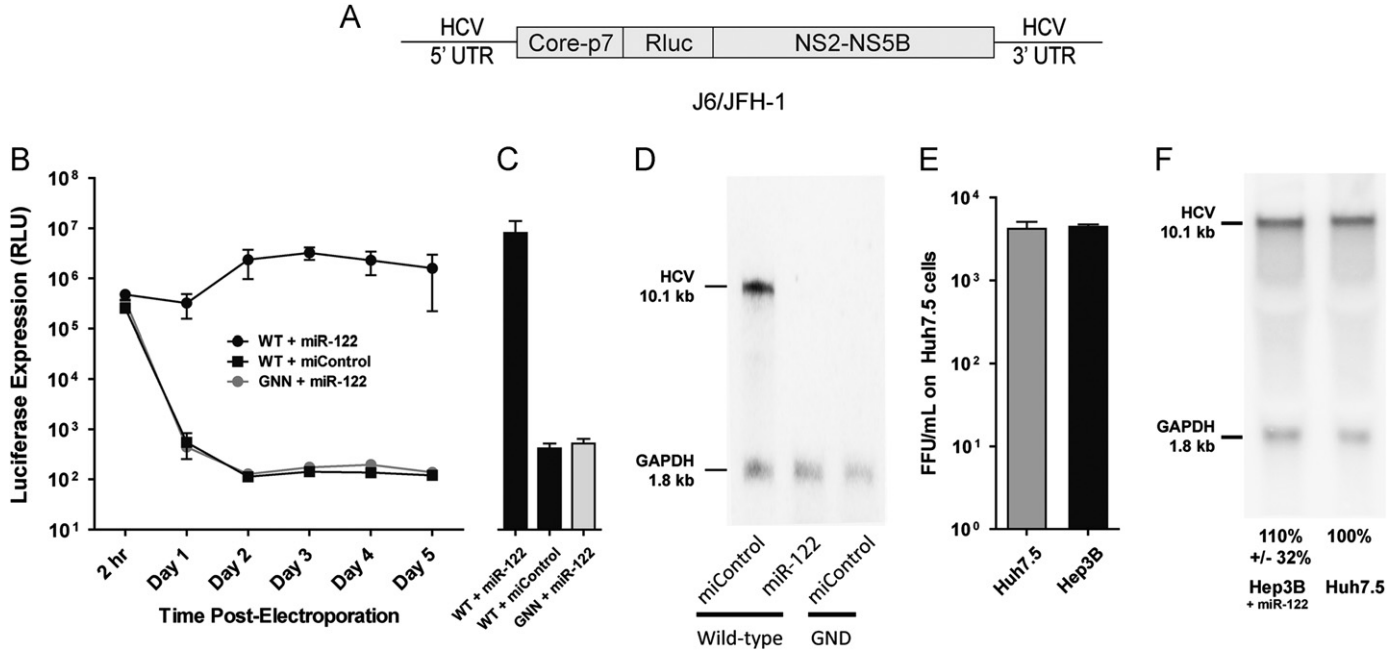

Fig. 2. Replication of full-length (J6/JFH-1 Rluc) HCV in Hep3B cells. (A) Representation of "J6/JFH-1 Rluc," the mono-cistronic full-length J6/JFH-1 chimeric replicon, which bears all the viral genes, is capable of producing infectious particles in cell culture, and has a Renilla luciferase reporter 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.

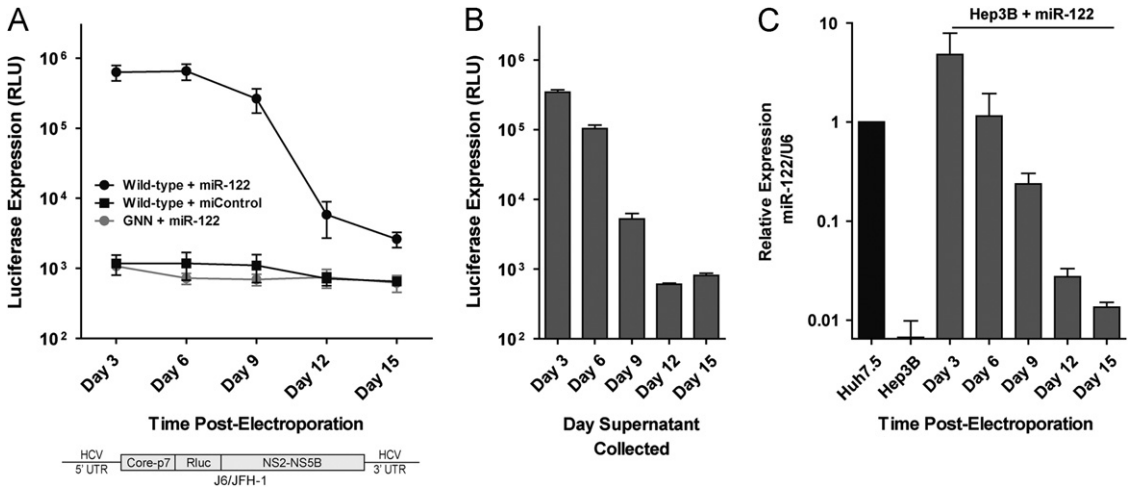

Fig. 3. Long-term replication of full-length HCV RNA in Hep3B cells. (A) Luciferase levels representing replication of J6/JFH-1 Rluc HCV RNA in Hep3B cells were evaluated at the indicated time points post-electroporation. (B) Luciferase levels representing infectious HCV particles in supernatants collected from 'wild-type+miR-122' in (A). Supernatant was collected at each time point and used to infect naive Huh7.5 cells; three days after infection, 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.

similar levels of HCV RNA accumulation as that observed in Huh7.5 cells.

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 (Fig. 3A and 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 (Fig. 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.

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 when supplemented with miR-122. One day before infection, were transfected via Lipofectamine 2000 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). Fig. 4A show luciferase expression from the infected cells at the 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 be seen in Fig. 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

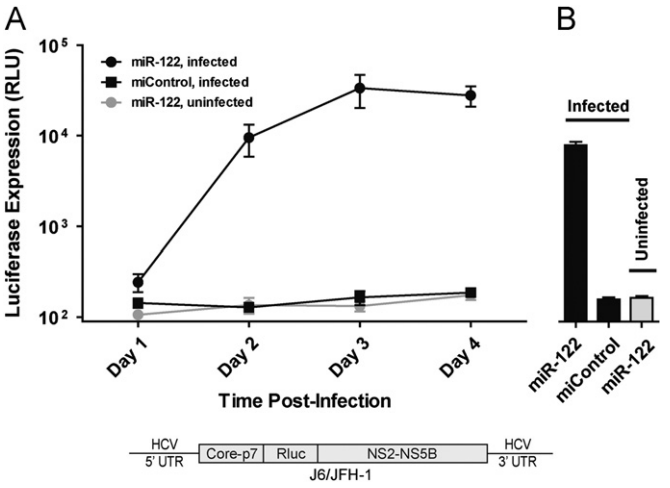

Fig. 4. Infection of Hep3B cells with full-length J6/JFH-1 HCVcc. (A) Hep3B cells were transfected via Lipofectamine 2000 with the indicated miRNA one day before being infected with HCVcc (J6/JFH-1 particles derived from Huh7.5 cells) or mock-infected with media on Day 0. Luciferase was analyzed at the 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.

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.

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 (Fig. 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 (Fig. 1D), but above-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.

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 (Fig. 1B). To analyze for functional miR-122 in Hep3B cells we assayed for miR-122 suppression activity. In the assay, miR-122 suppression activity was monitored by using two plasmids (Fig. 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 (α-miR-122), similar Fluc:Rluc ratios were observed, indicating no active miR-122 in Hep3B cells (Fig. 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 (Fig. 5B, miR-122, and α-miR-122+miR-122).

We also titrated amounts of miR-122 that can affect SGR by electroporating Hep3B cells with SGR RNA and 10-fold dilutions of miR-122 (Fig. 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 10-fold dilution of miR-122 (6.0 pmol) led to approximately 10-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 (Fig. 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, although we cannot rule out the existence of undetectable levels of miR-122 in Hep3B cells.

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

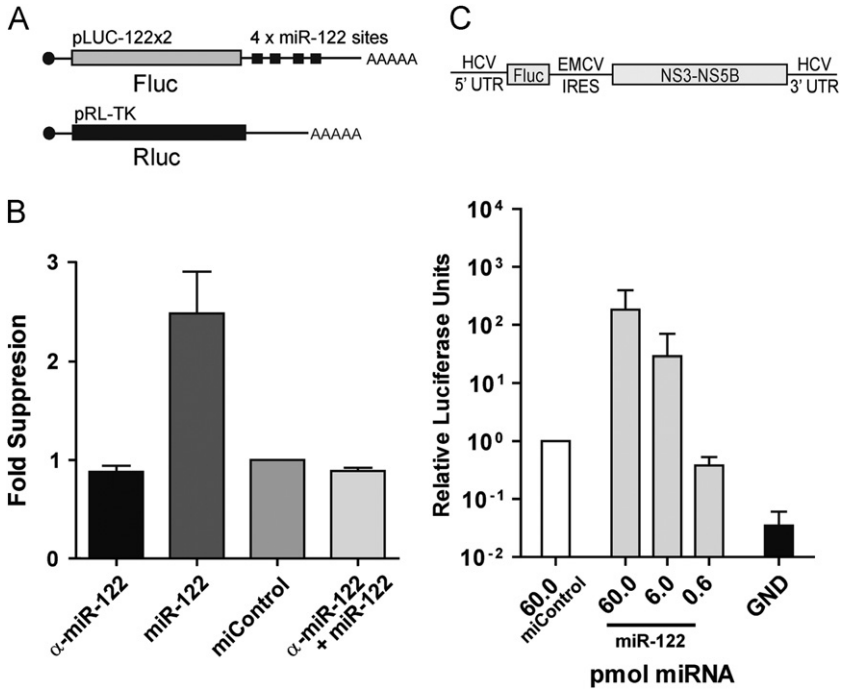

Fig. 5. Hep3B cells lack functional miR-122. (A) Reporters used in miRNA functional assays. *Top*: pLUC-122 × 2 encodes a firefly luciferase mRNA bearing miR-122 binding sites in its 3′ UTR; binding of miR-122 to these sites suppresses translation of the firefly luciferase reporter. *Bottom*: pRL-TK encodes a Renilla luciferase mRNA to be used as a transfection control. (B) Hep3B cells were electroporated with the indicated miRNA or α-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.

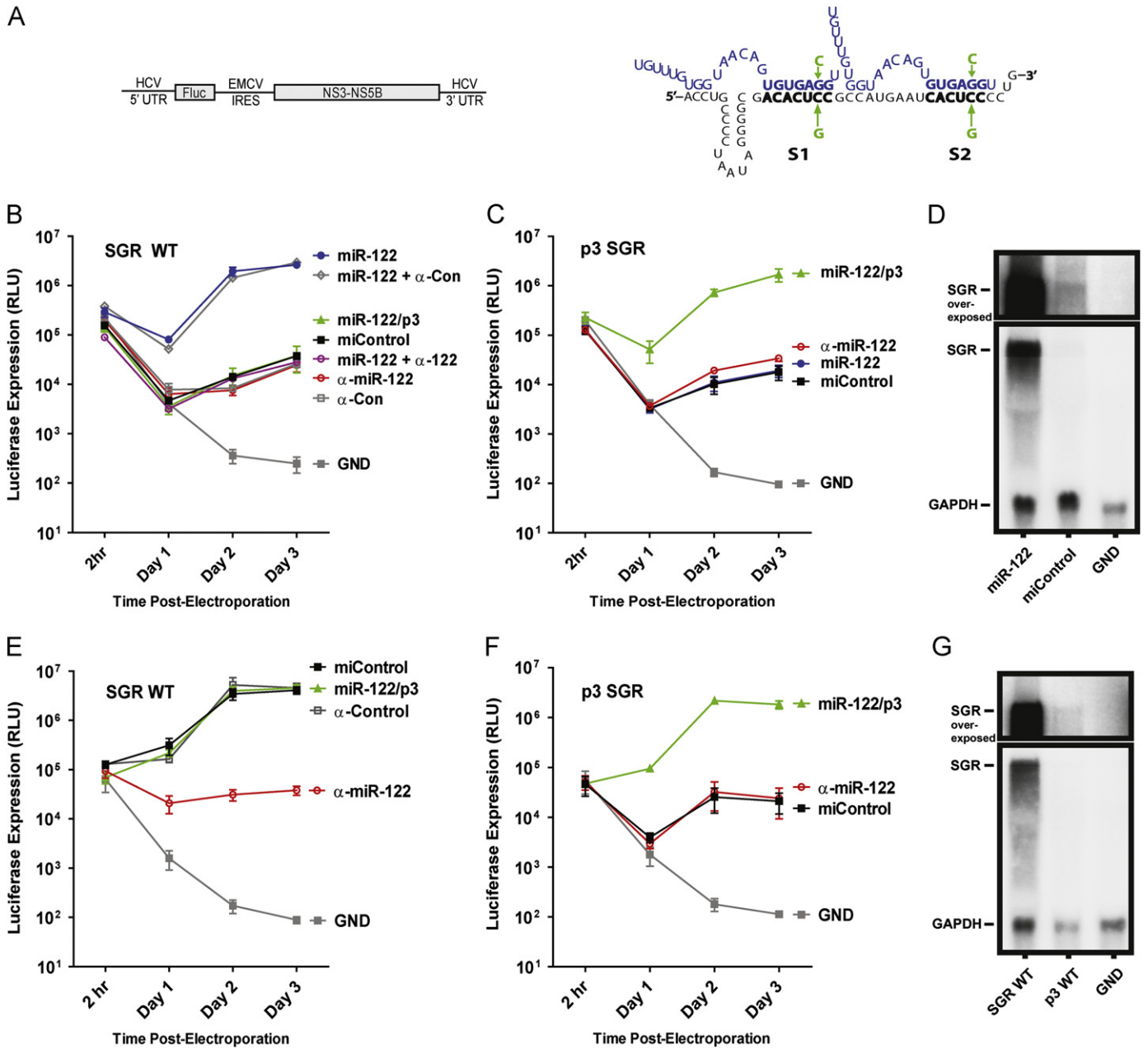

Fig. 6. MicroRNA-122-independent replication of sub-genomic HCV. (A) *Left*: Diagram of SGR construct. *Right*: Schematic of the miR-122 binding sites in the HCV 5′ UTR. Blue letters show miR-122 and black letters are HCV RNA. Bold indicates miR-122 seed sequences, while other complementary sequences 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 (α-miR-122 and α-Control). Luciferase was assayed at the indicated time points. GND is the replication-incompetent mutant of SGR as in Fig. 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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

transient HCV replication assays, un-supplemented sub-genomic RNA replication was unaffected by the addition of the miR-122 antagonist (Fig. 6B, compare α-miR-122 to miControl and α-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 (Fig. 6B, compare miR-122 and miR-122+α-Con with miR-122+α-122). RNA harvested on Day 3 following electroporation was evaluated by northern blot in Fig. 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.

Replication of p3 SGR RNA in Hep3B and Huh7.5 cells is not affected by miR-122 or α-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 (Fig. 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 'p34' sub-genomic RNA. The p34 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 p34 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/p34, 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 (Fig. 6C), and replicated in Hep3B cells to levels similar to that of the wild-type construct (compare Fig. 6B – miControl to Fig. 6C – miControl). In addition, the p3 SGR was unresponsive to exogenously provided miR-122 (Fig. 6C, compare miR-122 to miControl), and miR-122 antagonist (Fig. 6C, compare miControl to α-miR-122; α-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 Fig. 6B – miR-122 to Fig. 6C – miR-122/p3). Because the mutated miRNA did not affect the wild-type replicon (Fig. 6B, miR-122/p3), and because the mutated replicon was not impacted by wild-type miR-122 nor its antagonist (Fig. 6C, miR-122 and α-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 α-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 (Fig. 6E, α-miR-122; compare with Fig. 6B, miControl). To substantiate miR-122-independent replication in Huh7.5 cells, we analyzed replication of the p3 SGR construct in Huh7.5 cells (Fig. 6F). In Huh7.5 cells, p3 SGR replication was unaffected by the miR-122 antagonist (Fig. 6F, compare miControl and α-miR-122; α-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 (Fig. 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

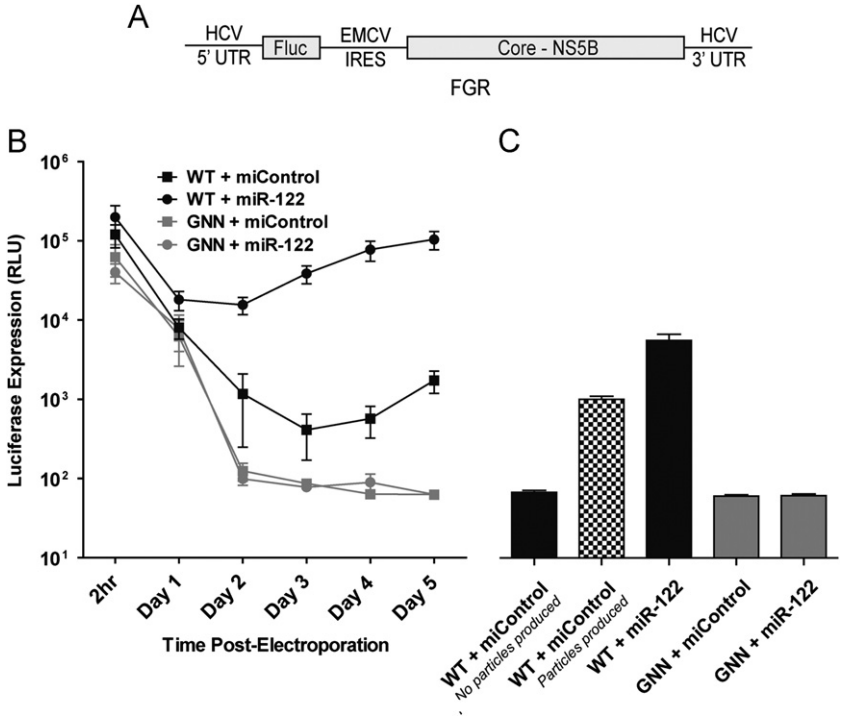

Fig. 7. Replication of the di-cistronic full-genome replicon (FGR) in Hep3B cells. (A) Diagram representation of "FGR," the di-cistronic full-genomic firefly luciferase replicon which contains structural genes that permit infectious particle production; it is otherwise identical to the SGR. (B) Hep3B cells were electroporated with FGR wild-type (WT) or replication-incompetent (GNN) FGR 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.

sites renders replication of the SGR construct independent of wild-type miR-122.

miR-122-independent replication of a full length di-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 Figs. 1C and 2A, WT+miControl to GNN or GND). Thus we hypothesized that miR-122-independent replication may occur because of the di-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 Fig. 7A, and is a di-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 di-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 (Fig. 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 Fig. 7A with Figs. 1C and 2A; and data not shown). Evaluation of supernatant from Day 4 post-electroporation in Fig. 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 un-supplemented 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.

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, 2012b; Wilson et al., 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 Huh7.5 cells (Fig. 8). In Hep3B cells, Ago2 knockdown had no effect on miR-122/p3-independent replication of the p3 SGR and the wild-type SGR (Fig. 8A and B,

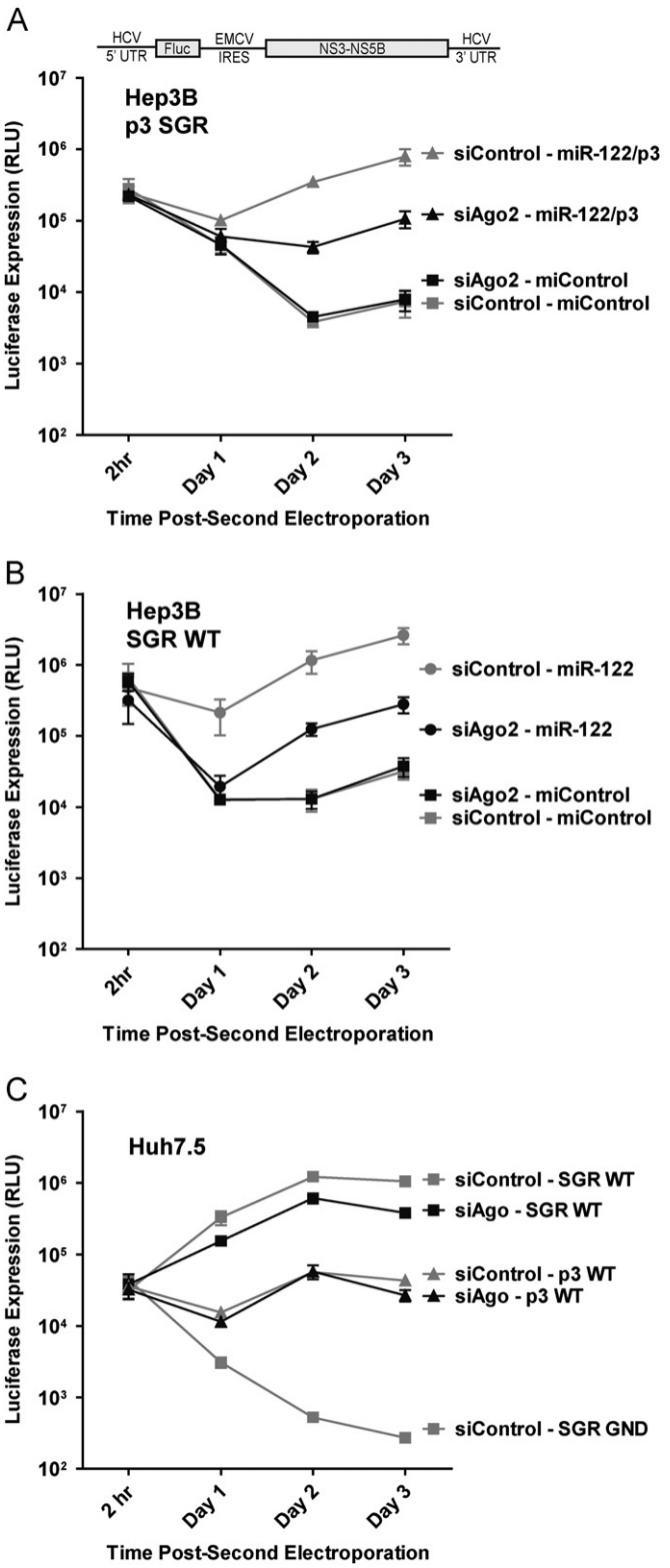

Fig. 8. Ago2 is not required for miR-122-independent replication of sub-genomic HCV. (A) Hep3B cells were initially electroporated with either siAgo2 or siControl on Day −3; on Day 0, cells were electroporated again with the same siRNA, p3 SGR, and the indicated microRNA, along with a control Renilla mRNA. Luciferase was assayed at the indicated time-points post-second electroporation (Day 0) to detect RNA replication. (B) Hep3B cells were treated as in (A), but were electroporated with SGR WT and the indicated miRNA. (C) 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. Data represent the average of three or more independent experiments and bars represent standard deviation.

compare siAgo2 – miControl to siControl – miControl). However, Ago2 knockdown severely hampered the ability of miR-122/p3 to enhance p3 SGR luciferase expression (Fig. 8A, compare siAgo2 – miR-122/p3 to siControl – miR-122/p3, and Fig. 8B, 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 (Supplementary Fig. S2A and B). We saw similar results in Huh7.5 cells. MicroRNA-122-independent replication of p3 SGR was unaffected by Ago2 knockdown (Fig. 8C, 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 Huh7.5 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 (Supplementary Fig. S2C). 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.

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) di-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 293 cells 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 (Fig. 2F) and virion production (Fig. 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 (Fig. 4A). Ploss et al. (2009) and Sainz et al. (2012) 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. 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. We also observed that, Hep3B infection efficiency was not improved by electroporation of miR-122 (Supplementary Fig. S1); thus poor infection may have been due to harmful effects of transfection (Fig. 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 (Fig. 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 di-cistronic JFH-1 sub-genomic (Figs. 1C and 6) and full-length (Fig. 7) replicons in Hep3B or Huh7.5 cells. By determining that Hep3B cells lack functional miR-122 (Figs. 1B, 5, and 6) we have shown that the basal replication of SGR and FGR in Figs. 1, 6, and 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 di-cistronic constructs. Thus, HCV di-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 (Fig. 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 p34 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 p34 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/p34 (Wilson et al., 2011). Interestingly, both p3 and p34 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 p34 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 complementable in multiple HCV genotype.

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 one day post-electroporation (Figs. 1C and 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 viral life cycle (Machlin et al., 2011; Shimakami et al., 2012a; Villanueva et al., 2010). In addition, di-cistronic, but not mono-cistronic HCV RNAs are capable of miR-122-independent replication. The SGR and FGR replicons are di-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 conclude 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 di-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 Huh7.5 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 (Fig. 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.

Materials and methods

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).

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 di-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 A: 5′–ATAGGGGCGACACTGCGCCATGAATCACTG-3′ and 5′–CAGTGATTCATGGCGCAGTGTCGCCCCTAT-3′. Site B: 5′–CCATGAATCACTGCCCTGTGAGGAAC-3′ and 5′–GTTCCTCACAGGGCAGTGATTCATGG-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. pFluc122 × 2 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).

MicroRNAs and anti-miR sequences: miR-122: UGGAGUGUGACAAUGGUGUUUGU and miR-122*: AAACGCCAUUAUCACACUAAAUA, annealed. miR-122/p3: UGCAGUGUGACAAUGGUGUUUGU annealed to miR-122*. miControl: UAAUCACAGACAAUGGUGUUUGU annealed to miR-122*. The miRIDIAN microRNA Hairpin Inhibitor, human hsa-miR-122a (proprietary sequence) was used to inhibit miR-122 (α-miR-122), with miRIDIAN microRNA Hairpin Inhibitor Negative Control #1 (proprietary sequence) was used as a control (α-Control). siControl: AAGACACUGAGACACCAAUUGAC (Wilson et al., 2003). siAgo2: CAGACUCCCGUGUGUCCUATT (Wilson et al., 2011). All short RNAs were synthesized by ThermoScientific Dharmacon (Lafayette, CO, USA).

Electroporations: All electroporations were carried out according to Lohmann et al. (2001) with some modifications: each sample contained 6.0×10^6 cells in 400 μL Dulbecco's PBS for electroporation, cells were plated in culture media, and 500 μL 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; Missisauga, ON, Canada), Hep3B cells were electroporated at 225 V and 950 uF, while Huh7.5 cells were electroporated at 270 V and 950 uF. Where indicated, samples were electroporated with 10 μg viral RNA; 60 pmoles microRNA and/or miR-122 antagonist; and 2 μg 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, 8.0×10^6 cells in 400 μL of Dulbecco's PBS were first electroporated with 60 pmoles 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 (8.0×10^6 cells in 400 μL), and electroporated with 60 pmoles 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.

Transfections: For miR-122 suppression activity assays, Hep3B cells were electroporated with the indicated miRNA/miR-122 antagonist, and were plated in triplicate at 2.5×10^5 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 pFluc122 × 2 in 1 μL 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 1.0×10^5 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.

Infections: To evaluate HCV infectious titers, naive Huh7.5 cells were plated at 1.0×10^5 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 naive 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 infectibility, 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. Supernatant (2 mL) was added to approximately 1×10^5 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.

HCV titration: HCV titer was evaluated by focus-forming assay. One day pre-infection, naive 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.

Luciferase assays: For replication assays, 500 μL electroporated cells were plated with 4 mL media in a 6 cm dish and incubated for 1–5 days, and cell extracts were harvested in 100 μL of the appropriate lysis buffer. For suppression assays, cells were plated as indicated for transfection, and were harvested in 100 μL 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.

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 h later. Total RNA samples collected after HCV RNA electroporation were plated at 1.5×10^6 cells (2 mL) on a 10 cm dish and harvested 3 days post-electroporation into 1 mL Trizol.

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).

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).

Relative cell numbers: 20 μL 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 h post-treatment on a SpectraMax 340 PC^{384} Microplate Spectrophotometer (Sunnyvale, CA, USA). Cell numbers were based on a standard curve of ten-fold dilutions of the appropriate cell type.

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2012.11.007.

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