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An integrated approach identifies IFN-regulated microRNAs and targeted mRNAs modulated by different HCV replicon clones

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Abstract

Background: Infections with hepatitis C virus (HCV) progress to chronic phase in 80% of patients. To date, the effect produced by HCV on the expression of microRNAs (miRs) involved in the interferon- β (IFN- β) antiviral pathway has not been explored in details. Thus, we compared the expression profile of 24 selected miRs in IFN- β -treated Huh-7 cells and in three different clones of Huh-7 cells carrying a self-replicating HCV RNA which express all viral proteins (HCV replicon system).

Methods: The expression profile of 24 selected miRs in IFN- β -treated Huh-7 cells and in HCV replicon 21-5 clone with respect to Huh-7 parental cells was analysed by real-time PCR. To exclude clone specific variations, the level of 16 out of 24 miRs, found to be modulated in 21-5 clone, was evaluated in two other HCV replicon clones, 22-6 and 21-7. Prediction of target genes of 3 miRs, confirmed in all HCV clones, was performed by means of miRgator program. The gene dataset obtained from microarray analysis of HCV clones was further used to validate target prediction.

Results: The expression profile revealed that 16 out of 24 miRs were modulated in HCV replicon clone 21-5. Analysis in HCV replicon clones 22-6 and 21-7 indicated that 3 out of 16 miRs, (miR-128a, miR-196a and miR-142-3p) were modulated in a concerted fashion in all three HCV clones. Microarray analysis revealed that 37 out of 1981 genes, predicted targets of the 3 miRs, showed an inverse expression relationship with the corresponding miR in HCV clones, as expected for true targets. Classification of the 37 genes by Panther System indicated that the dataset contains genes involved in biological processes that sustain HCV replication and/or in pathways potentially implicated in the control of antiviral response by HCV infection.

Conclusions: The present findings reveal that 3 IFN- β -regulated miRs and 37 genes, which are likely their functional targets, were commonly modulated by HCV in three replicon clones. The future use of miR inhibitors or mimics and/or siRNAs might be useful for the development of diagnostic and therapeutic strategies aimed at the recovering of protective innate responses in HCV infections.

Background

Infection with hepatitis C virus (HCV) represents the major cause of liver disease, affecting more than 170 million individuals worldwide. After a sub-clinical phase, greater than 80% of patients progress to persistent HCV

infection, the leading cause of chronic liver disease associated with cirrhosis and hepatocellular carcinoma [1,2].

In the last years, microarray technology provided a comprehensive analysis of alterations in gene expression induced by HCV and revealed important processes of virus-host interactions [3-7]. Interestingly, microarray studies indicated that HCV stimulates the endogenous Type I Interferon (IFN- α/β) pathway as suggested by activation of IFN-stimulated genes (ISGs) [8-15]. Recently, it has been proposed that also microRNAs (miRs), a class of small

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non-coding regulatory RNAs, are involved in the antiviral pathway induced by IFN- β treatment. The synthetic introduction of five IFN- β -induced miRs into HCV replicon cells may simulate the antiviral effect of IFN- β blocking HCV replication and infection. These five miRs (miR-196, miR-296, miR-351, miR-431 and miR-448) likely induced an antiviral state either through alteration of gene expression and/or directly targeting HCV RNA, as was demonstrated for two of them (miR-196 and miR-448) [16].

Although HCV activates the endogenous IFN- α/β pathway it conversely shows an impressive ability to induce persistent infections. Indeed, it is also clear that HCV has evolved several mechanisms to control the IFN antiviral response, inhibiting the pathway at different levels [17]. Recently, it has been suggested that an improper pre-activation of ISGs in the liver of HCV infected patients may hinder the antiviral response. The discovery of a genetic polymorphism in the interleukin 28B (IL28B) region on chromosome 19 of HCV patients depicted a more complex virus-host interaction. The IL28B non-CC variant has been associated with non-response to the IFN therapy and with lower rates of spontaneous clearance of HCV infection. The poor-response variant is also associated with higher intrahepatic expression level of ISGs [18-20].

A missing aspect in this scenario is the study of the effect produced by HCV on the expression of IFN- β -induced miRs. This is a relevant issue to understand how the virus can suppress the innate antiviral signaling and induce a persistent infection.

In a previous paper, we identified a common transcriptional response of Huh-7 cells to different clones of full-length HCV replicon [21]. Although a more advanced HCV cell culture models that release HCV viral particles has been developed [22-24], the replicon system has the advantage of taking into account the cellular gene expression variability of different HCV replicon cell clones. This approach allows searching for modulated genes shared by all clones, which are likely to be strictly needed for viral replication in different cellular contexts.

On this basis, we used the replicon system to identify IFN-regulated miRs that are modulated by HCV RNA replication. In particular, we analyzed the expression profile of 24 selected miRs in IFN- β -treated Huh-7 cell line and in three cell clones carrying a full-length HCV replicon (clones 21-5, 22-6 and 21-7). Among the identified 16 miRs modulated in the 21-5 clone, 3 miRs showed concordant expression when analyzed in the two other HCV replicons. By a combined approach, based on bioinformatic prediction and microarray analysis, we also identified 37 genes, targeted by the 3 miRs, which are involved in pathways and biological processes potentially implicated in the control of antiviral response by HCV infection.

Results

Expression of IFN- β -regulated miRs in 21-5 HCV replicon cells and in IFN- β -treated Huh-7 cells

To determine the impact of HCV RNA replication and protein synthesis on IFN- β -regulated miRs, we compared the expression profile of selected miRs in 21-5 cells, harbouring a full-length HCV genome, and in IFN- β -treated Huh-7 cells with the Huh-7 parental cell line.

In particular, the list of assayed miRs includes (a) eight IFN- β -induced miRs, which displayed complementarity in their seed sequences with HCV RNA genome (miR-1, miR-30, miR-128, miR-196, miR-296, miR-351, miR-431, miR-448) [16], (b) two miRs reported as IFN- β -unresponsive miRs (miR-125 and miR-142) [16], (c) miR-122a that promotes HCV RNA replication [25] and (d) three miRs (miR-155, miR-146a and miR-146b) modulated in innate immune response in monocytes/macrophages [26,27]. Actually, the name of five of the above miRs (miR-30, miR-128, miR-196, miR-125 and miR-142) indicates miR families, not just individual mature miR species: thus, we analysed the level of each member of those families. Overall, the expression profile of 24 miRs in 21-5 and IFN- β -treated Huh-7 cell lines was analysed (Table 1). Three miRs (miR-351, miR-431 and miR-448) showed an expression level below the detection limit of the assay (Ct \geq 40), while five miRs were not differentially expressed in 21-5 cells (miR-125b, miR-30e-5p, miR-30e-3p, miR-30a-5p, miR-30d: fold-change values $<$ 1.2 or $>$ -1.2 in 21-5 cells). These eight miRs were not evaluated further.

The expression profile of the remaining 16 miRs revealed that they were modulated by IFN- β and/or HCV (Figure 1). In particular, concordant modulation (up- or down-regulation) of 9 miRs (miR-1, miR-30a-3p, miR-142-5p, miR-142-3p, miR-146a, miR-146b, miR-155, miR-128a and miR-296) was observed in IFN- β -treated Huh-7 cells and in 21-5 replicon cells (Figure 1, panel A). This result indicates that HCV replication can induce a miR signature as IFN- β treatment. Five miRs were modulated in 21-5 replicon cells only, as the level in IFN- β -treated Huh-7 cells was within the \pm 1.2 range set as background (Figure 1, panel B: miR-196b, miR-125a, miR-122a, miR-30c and miR-30b). Two miRs were modulated in an opposite manner in IFN- β -treated Huh-7 cells and in 21-5 replicon cells (Figure 1, panel C: miR-128b and miR-196a).

Identification of common miRs modulated in different HCV replicon clones

To exclude that the miR expression profile was peculiar of the 21-5 clone, we analyzed the expression level of the 16 miRs in two other HCV replicon clones, 22-6 and 21-7. The analysis revealed that 3 miRs showed concordant modulation in HCV clones as compared to Huh-7 cells

Table 1 Fold-change of 24 selected miRs in IFN β -treated Huh-7 and 21-5 cell lines vs. Huh-7

	Huh-7+ IFN β	21-5
miR-1	30, 13	22, 11
miR-30a-3p	4, 46	8, 79
miR-146a	1, 54	2, 73
miR-142-5p	2, 83	2, 20
miR-146b	2, 09	2, 11
miR-142-3p	2, 09	1, 74
miR-296	-1, 47	-1, 40
miR-155	-1, 69	-1, 61
miR-128a	-1, 47	-1, 88
miR-128b	-3, 30	2, 09
miR-196a	1, 31	-1, 50
miR-125a	1, 05	3, 43
miR-196b	1, 11	2, 92
miR-30c	-1, 12	1, 30
miR-30b	-1, 04	1, 28
miR-122a	1, 14	-1, 63
miR-125b	-1, 10	1, 19
miR-30e-5p	1, 14	1, 18
miR-30e-3p	-2, 12	1, 17
miR-30a-5p	-1, 38	1, 15
miR-30d	-1, 12	1, 02
miR-351	n.d.	n.d.
miR-431	n.d.	n.d.
miR-448	n.d.	n.d.

In bold: fold-change values > 1.2 or < -1.2

n.d.: not detected, i.e. miRs showing an expression level below the detection limit of the assay (Ct \geq 40)

(Figure 2). In particular, miR-128a and miR-196a were down-regulated while miR-142-3p was up-regulated in all HCV clones.

Identification of candidate miR target genes

To predict target genes, which may be co-regulated by the 3 concordant miRs, we used miRGator, an on-line interface that uses multiple target prediction programs. It has been shown that, to date, no program is able to predict all experimentally confirmed target genes. Thus, to avoid as much as possible loss of putative target genes, relaxed options were used in miRGator [target genes predicted by at least one of the following programs: TargetScanS, miRanda and PicTar (4-way)]. After removal of multiple mRNAs corresponding to alternative mRNA transcripts from a single gene, individual gene lists were merged and a final list of 1981 total target genes was obtained, including genes controlled by at least one of the three miRs.

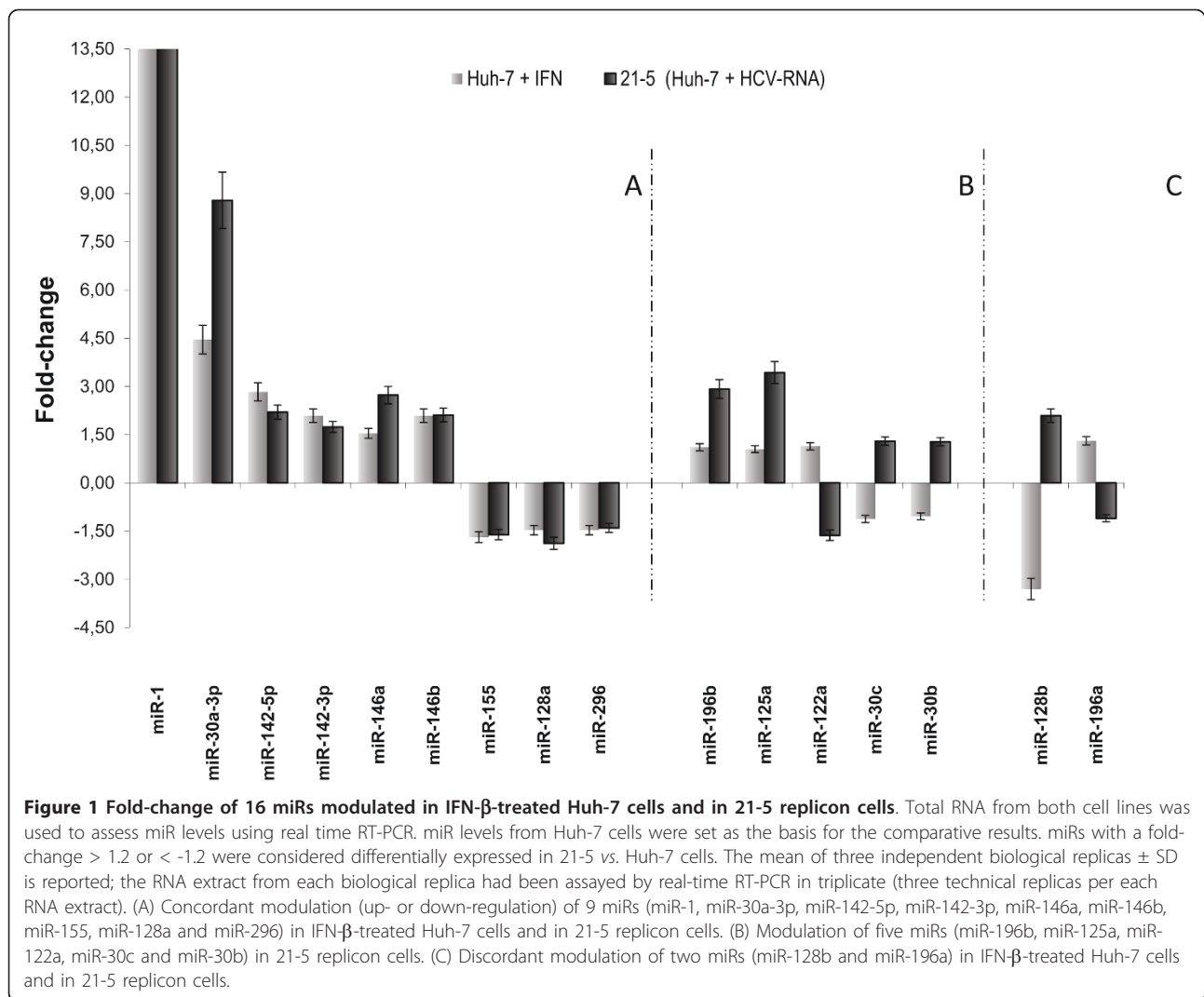
Identification of genes common to miR target list and HCV microarray dataset

The observation of an inverse relationship between levels of miRs and levels of their target mRNAs, due to

mRNA degradation of target genes, provides opportunities for validation of predicted targets using microarray profiling [28]. On this basis, to determine the candidate target genes directly regulated by miR-128a, miR-196a and miR-142-3p, we overlapped two datasets: the list of 1981 total target genes predicted for the 3 miRs (dataset 1) and a microarray dataset including 676 genes (725 probes) modulated in all HCV clones as compared to Huh-7 cells (dataset 2) reported in our previous study [21]. As shown in Figure 3, 83 genes were common to both datasets indicating that target genes of the 3 miRs account for 12, 3% (83 out of 676) of the differentially expressed genes detected in all three HCV clones. The list of 83 genes, including relevant informations, is provided in Additional file 1, Table S1. As levels of most miRs and their target mRNAs exhibit an inverse expression relationship, we used gene expression profiling data to identify functional targets and validate target prediction, as previously reported [29]. By using this approach we found that 37 out of 83 (44, 5%) of the predicted target genes showed an expression level inversely correlated with that of the corresponding miR suggesting that, at least for these genes, a direct connection to miR regulation may be suggested. A complete list of the 37 genes (22 up-regulated and 15 down-regulated) and the corresponding 3 miRs is provided in Table 2. The list includes symbol, name, probe ID, fold-change (FC) and p-value of the genes all obtained from microarray dataset. In addition, a functional description of the genes by Panther Protein Classification System is enclosed [30]. Overlapping the 37 genes with the dataset of genes resulting from microarray analysis of Huh-7.5 cells infected with HCV genotype 2a chimeric virus J6/JFH [31] revealed 4 common genes (RND3, LTB4DH, RIOK3 and CHAF1B) which showed the same direction of regulation (i.e. up-regulated or down-regulated genes) in both HCV clones and J6/JFH microarray datasets supporting the biological relevance of these genes in HCV replicative cycle. Finally, to explore the involvement of the identified genes in HCV response to endogenous IFN, we also overlapped the list of 37 genes with the dataset of 1996 human genes annotated in the INTERFEROME database [32]. As shown in Table 2, four genes (HNMT, XPO1, PMPCB and HMGB1) were identified as Interferon Regulated Genes (IRGs).

Biological functions of the miR target genes

To classify genes into biological categories, we analyzed the Gene Ontology annotations of the 37 common genes with the Panther Protein Classification System [30]. As shown in Table 3, Panther System found several functional categories that were significantly enriched in this gene set compared to the entire NCBI reference list of human genome. We considered, as potentially

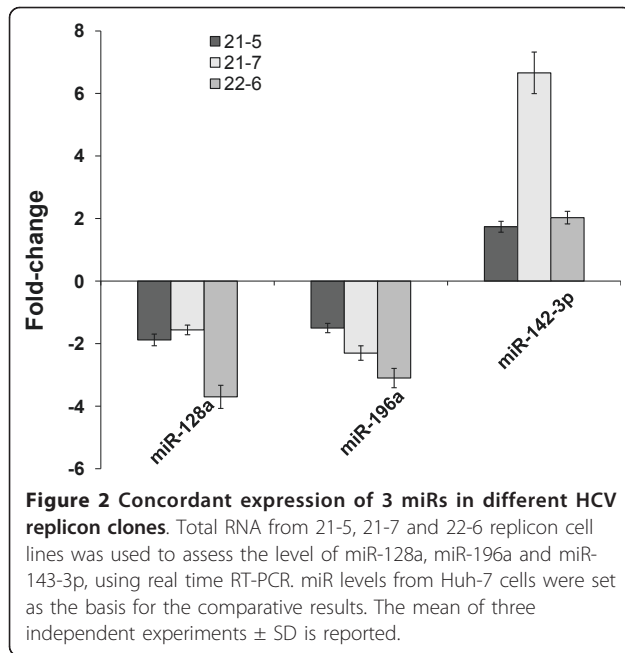


interesting, only categories showing a p-value ≤ 0.05, as determined by the binomial statistic [33]. The 37 genes of the dataset were significantly classified by the Panther system in 6 biological processes (i.e., processes in which genes participate) and 3 molecular functions (i.e., biological functions of gene products). Compared with the NCBI reference list of human genome, this dataset showed a larger proportion of genes encoding proteins involved in chromatin binding and architecture, organelle organization, intracellular transport and neurotransmitter secretion (Table 3). In addition, genes associated with catalytic activity, enzyme regulator activity and chromatin binding were represented much more abundantly in the dataset. Interestingly, genes involved in the Ubiquitin proteasome pathway were also present in the dataset (Table 3). Additional file 2, Table S2 reports the complete list of the genes that are responsible for statistical enrichment of each category shown in Table 3

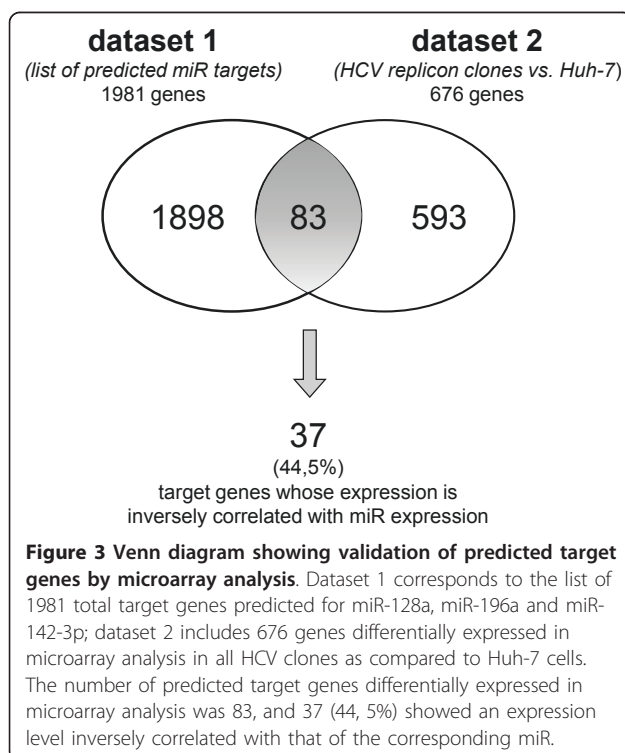
Discussion

In the present study we analyzed the effect of HCV replication on the expression of selected miRNAs involved in the IFN-pathway. In particular, we identified 3 miRNAs that are equally modulated by HCV in three HCV replicon clones and by IFN treatment. Moreover, we also identified 37 out of 83 predicted target genes, differentially expressed in HCV replicon cells, which are most likely functional targets of these 3 miRNAs: in fact they showed an inverse expression relationship with the level of the 3 miRNAs, as described for true targets. These genes could be implicated in regulation of the host response to HCV.

About one half predicted targets did not show the expected inverse expression relationship with miR level, but this result is not surprising. First, computational prediction by miRgator was based on predictions from the widely used programs miRanda, TargetScanS and



PicTar (see Materials and Methods). To date, these programs, as well as any other available prediction program, still have a very high false-positive rate, estimated to be up to 40% [34,35]: i.e. up to 40% genes predicted to be targeted by a miR are not, actually, true targets, and they will not show any inverse relationship. Second, each mRNA is usually targeted by multiple miRNAs and, in



addition, each mRNA has its own set of modulating miRNAs. Whether or not a change in the level of a single miR can prevail on the effect of the other miRNAs simultaneously targeting the same mRNA in physiological conditions is, at present, poorly understood. However, it is possible in some cases it will produce no effect and, thus, even a true target will not show the expected inverse relationship.

The recent discovery of the roles of miRNAs in many human diseases suggests that studies exploring the relationship between HCV and miRNAs/mRNA may provide new insights into host cell response to HCV infection. Importantly, this approach also gives the opportunity to identify viral mechanisms that control the antiviral defense. Recently, it has been demonstrated that five IFN- β -modulated miRNAs (miR-196, miR-296, miR-351, miR-431 and miR-448) showed significant effect on HCV replication and at least two of them (miR-196 and miR-448) are directly targeting the HCV genomic RNA [16]. Moreover, it seems that level of miR-122 is inversely correlated with the antiviral defense [16,36,37].

Our expression analysis revealed that miR-196a was down-regulated in all three HCV replicon clones. Thus, at least for this component of the pathway, it seems that modulation of IFN-miRNAs may be altered by HCV in replicon cells. Interestingly, this miR targets the HCV RNA [16], thus, down-regulation of miR-196a may indirectly influence viral replication also by up-regulation of specific target genes. Accordingly, 11 genes, controlled by miR-196a, showed by microarray analysis an inverse expression relationship suggesting that they can be likely considered functional targets of miR-196a. Moreover, gene ontology analysis of the 11 genes highlights that some of them are really involved in pathways such as, extracellular matrix constitution (LAM4), oxidative stress (HADH2, LTB4DH) and cytoskeletal network (CENPE), which are relevant for HCV RNA replication [7,38,39].

As for the IFN- β -regulated miR-296, miR-351, miR-431, miR-448 and miR-122 (16) our data indicate that their expression in different HCV replicon clones is either not concordant (miR-296, and miR-122a) or not detected (miR-351, miR-431 and miR-448). In particular, miR-122a was down-regulated in 21-5 and 21-7 clones but its level was not modified in clone 22-6 (data not shown) while miR-296 was down-regulated in 21-5 clone and up-regulated in clone 22-6 and 21-7, respectively (data not shown). Moreover, miR 351, miR-431 and miR-448 were not detected in all clones examined supporting, at least for miR-448, what found in human biopsies where miR-448 is totally absent [37].

Interestingly, we observed that two miRNAs, miR-142-3p and miR-128a, were up-regulated and down-regulated respectively by IFN- β treatment of Huh-7 cells and in HCV replicon-expressing cells. Although modulation of

Table 2 Reverse regulatory association of 3 differentially expressed miRNAs and their 37 predicted target genes in HCV replicon clones vs. Huh-7

	Gene_Symbol	Gene_name	ProbeID	Fold-change	p-value	Biological process and Molecular function
Down-regulated miR						
miR-128a	CAMTA1	calmodulin binding transcription activator 1	164871	1.426	0.045	Signal transduction/Calcium mediated signaling
miR-128a	CAST	calpastatin	222724	1.788	0.002	Cysteine protease inhibitor Select regulatory molecule
miR-128a	CORO1C	coronin, actin binding protein, 1C	107201	1.402	0.027	Cytoskeletal protein Actin binding cytoskeletal protein
miR-128a	PARK7	Parkinson disease (autosomal recessive, early onset) 7	136848	1.529	0.003	Nucleic acid binding
miR-128a	REPS1	RALBP1 associated Eps domain containing 1	147893	1.352	0.002	Intracellular protein traffic/Endocytosis
miR-128a	RND3 [#]	Rho family GTPase 3	129829	2.161	0.041	G-protein Small GTPase Select regulatory molecule
miR-128a	SAA4	serum amyloid A4, constitutive	214434	1.880	0.029	Apolipoprotein;Defense/immunity protein
miR-128a	TMCC1	transmembrane and coiled-coil domain family 1	112169	1.369	0.031	unclassified
miR-128a	TRAPP4	trafficking protein particle complex 4	205369	1.502	0.012	Membrane traffic protein
miR-128a	UBE2E3	ubiquitin-conjugating enzyme E2E 3 (UBC4/5 homolog, yeast)	201108	1.505	0.030	Ligase Other ligase
miR-128a	VAMP8	vesicle-associated membrane protein 8 (endobrevin)	226943	2.586	0.011	SNARE protein Membrane traffic protein
miR-196a	ARMET	arginine-rich, mutated in early stage tumors	144765	1.816	0.029	unclassified
miR-196a	HADH2	hydroxyacyl-Coenzyme A dehydrogenase, type II	142475	1.421	0.031	Oxidoreductase Reductase Dehydrogenase;Oxidoreductase
miR-196a	HIST1H2BD	histone 1, H2bd	148940	2.656	0.020	DNA binding
miR-196a	HNMT [▲]	histamine N-methyltransferase	227509	1.423	0.039	unclassified IRG [▲]
miR-196a	LAMA4	laminin, alpha 4	101059	2.660	0.033	Extracellular matrix Extracellular matrix linker protein
miR-196a	LTB4DH [#]	leukotriene B4 12-hydroxydehydrogenase	210882	1.961	0.001	Oxidoreductase Reductase Dehydrogenase;Oxidoreductase
miR-196a	PI3	peptidase inhibitor 3, skin-derived (SKALP)	128827	2.558	0.018	Proteolysis Protein metabolism and modification
miR-196a	RIOK3 [#]	RIO kinase 3 (yeast)	142169	3.226	0.018	Protein metabolism and modification Protein phosphorylation
miR-196a	RRAGA	Ras-related GTP binding A	105497	1.243	0.023	G-protein Small GTPase Select regulatory molecule
miR-196a	ZMYND11	zinc finger, MYND domain containing 11	161540	1.665	0.036	Transcription factor Transcription cofactor
miR-128a/miR-196a	CENPE [§]	centromere protein E, 312kDa	165425	1.436	0.018	Structural constituent of cytoskeleton
Up-regulated miR						
miR-142-3p	ZCCHC14	zinc finger, CCHC domain containing 14	200292	0.759	0.035	unclassified
miR-142-3p	XPO1 [▲]	exportin 1 (CRM1 homolog, yeast)	105030	0.748	0.002	Intracellular protein transport IRG [▲]
miR-142-3p	ATAD2	ATPase family, AAA domain containing 2	114584	0.745	0.007	Hydrolase activity
miR-142-3p	TRIM33	tripartite motif-containing 33	134526	0.714	0.004	Transcription cofactor;Nucleic acid binding Transcription factor
miR-142-3p	RAB1A	RAB1A, member RAS oncogene family	122650	0.704	0.045	G-protein Small GTPase Select regulatory molecule
miR-142-3p	TFG	TRK-fused gene	210281	0.686	0.026	unclassified
miR-142-3p	PMPCB [▲]	peptidase (mitochondrial processing) beta	210046	0.684	0.015	Oxidoreductase activity IRG [▲]
miR-142-3p	ZNF226	zinc finger protein 226	178824	0.682	0.043	Zinc finger transcription factor
miR-142-3p	ANKHD1	ankyrin repeat and KH domain containing 1	224628	0.658	0.006	unclassified
miR-142-3p	CXADR	coxsackie virus and adenovirus receptor	108284	0.620	0.025	Other receptor Receptor

Table 2 Reverse regulatory association of 3 differentially expressed miRNAs and their 37 predicted target genes in HCV replicon clones vs. Huh-7 (Continued)

miR-142-3p	CHAF1B [#]	chromatin assembly factor 1, subunit B (p60)	174351	0.586	0.017	Nucleic acid binding Chromatin/chromatin-binding protein
miR-142-3p	HMGB1 [^]	high-mobility group box 1	151074	0.586	0.011	Proinflammatory cytokine IRG [^]
miR-142-3p	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	109165	0.551	0.000	unclassified
miR-142-3p	COG4	component of oligomeric golgi complex 4	151150	0.394	0.042	Other RNA-binding protein Nucleic acid binding
miR-142-3p	USH1C	Usher syndrome 1C (autosomal recessive, severe)	195953	0.367	0.016	Cytoskeletal protein

^s Gene targeted by two down-regulated miRs

[^] Interferon regulated gene (IRG) according to the INTERFEROME database

[#] Gene showing same direction of regulation (up or down) in microarray analysis of HCV genotype 2a chimeric virus J6/JFH infection

Table 3 Panther classification of Biological processes, Molecular functions and Pathways significantly enriched in the set of 37 genes

	Observed Genes [*]	Expected Genes ^{**}	p-value ^{***}
BIOLOGICAL PROCESS			
Cellular process			
Establishment or maintenance of chromatin architecture	4	0.55	2.12E-03
Cellular component organization			
Organelle organization	4	0.59	2.85E-03
Transport			
Intracellular protein transport	9	2.98	2.16E-03
Vesicle-mediated transport	7	2.10	4.26E-03
Endocytosis	4	1.04	1.96E-02
System process			
Neurotransmitter secretion	3	0.63	2.45E-02
MOLECULAR FUNCTION			
Catalytic activity			
GTPase activity	3	0.52	1.53E-02
Enzyme regulator activity			
Peptidase inhibitor activity	2	0.32	4.08E-02
Binding			
Chromatin binding	2	0.34	4.59E-02
PATHWAYS			
Ubiquitin proteasome pathway	2	0.13	7.19E-03

^{*} Number of genes, in the dataset of 37, that map to the indicated Panther classification categories

^{**} Expected number of genes, in the dataset of 37, based on the NCBI reference list of human genome

^{***} p-value as determined by the binomial statistic. A cutoff of 0.05 has been applied

these 2 miRs by IFN- β has never been described before, it is consistently observed in all clones suggesting that it may be part of the endogenous IFN response to HCV. The IFN pathway is a highly regulated process and several controls has been evolved to activate and turn off this pathway. In this scenario, the temporal modulation of specific miRs seems to represent one of the control elements [40]. It is important to note that this process cannot properly occur in cells sustaining HCV replication. In this case a chronic up or down-regulation of IFN-miRs, likely induced by the virus, may negatively affect the control of the pathway finally improving the efficacy of the antiviral effectors. It would be interesting to investigate whether the experimental use of miR inhibitors (for miR-142-3p) or miR mimics (for miR-128a) could influence the control of the endogenous IFN system.

Among the 37 predicted target genes showing an inverse expression relationship with the 3 miRs, four genes were identified as Interferon Regulated Genes (IRGs) according to the INTERFEROME database [32]. One of these genes (HNMT) is a predicted target gene of miR-196a while the other three (XPO1, PMPCB and HMGB1) are all targeted by miR-142-3p. Importantly, in autoimmune diseases the high mobility group box 1

(HMGB1) protein was identified as a component of immune complex-containing DNA or RNA, which may act as endogenous IFN- β inducer [41]. Down-regulation of HMGB1 gene (fold change 0, 58; p-value 0, 01) in all HCV replicon clones suggests that it might contribute to impair the activation of the IFN signaling. Currently, the role of these four IRGs in the IFN response to HCV replication is unknown. Thus, unraveling their contribution to the regulation of the IFN response may reveal new mechanisms of viral persistence.

Gene Ontology annotations of the 37 common genes also revealed the presence of two genes, UBE2E3 and ATAD2 targets of miR-128a and miR-142-3p respectively, which are involved in the Ubiquitin proteasome pathway. The contribution of this pathway to HCV subversion of the IFN response has never been investigated. This is a quite interesting issue as several viruses use the ubiquitin proteasome system to destabilize proteins, such as IRF3 and STAT proteins, that are important for transcription of Interferon and Interferon-stimulated genes [42].

In attempt to validate our data, we found that 4 out of 37 genes, targeted by the 3 miRs, were also modulated, in a concerted fashion, in HCV genotype 2a chimeric virus J6/JFH microarray datasets [31] supporting the biological relevance of our results. In addition, 6 genes

(4 up-regulated: CEACAM1, DDIT3, SCARB2, ZFAND2A; and 2 down-regulated: C9orf19 and RPL5) selected from the HCV clones microarray dataset were found to be modulated in a same way in liver biopsies of patients showing non-CC IL28B polymorphism [20]. This polymorphism is not a good predictor of response to IFN therapy and it is also associated with higher level of ISG expression in the liver and propension to chronicity. So, it can be speculated that modulation of IFN signature as mediated by common miRs in replicon cells, can mirror de-regulation of the IFN signaling proposed for such patients.

Conclusions

In the present study we used the HCV replicon system to identify IFN-regulated miRs that are modulated by HCV RNA replication. By a combined approach, based on Real-Time PCR, bioinformatic prediction and microarray analysis, we identified 3 IFN- β -regulated miRs and 37 genes, which are likely their functional targets, commonly modulated by HCV in three replicon clones. Gene ontology classified the 37 genes into functional categories potentially implicated in the control of antiviral response by HCV infection. The future design of siRNAs directed against some of these genes and the use of miRs and antimiRs may provide an experimental background for the development of therapeutic strategies aimed at the recovering of protective innate responses in HCV infections.

Methods

Cell lines

The Huh-7 cells carrying the Sfl HCV full-length replicon (genotype 1b) were obtained from Dr. R. Bartenschlager. The 21-5, 21-7 and 22-6 clones are cell lines that stably replicates the HCV replicon and were passaged as described [43-45]. HCV replicon cells were cultured in complete DMEM supplemented with 10% FCS, antibiotics, 1 \times non-essential amino acids, and 250 μ g/ml (21-5, 21-7) and 500 μ g/ml (22-6) G418. Huh-7 cells were stimulated with 100 UI/ml IFN- β for 16 h.

Quantitation of miRNAs

Total RNA was extracted from 1 \times 10⁶ cells using miR-Neasy mini kit (QIAGEN) according to manufacturer's instructions and quantified by Bioanalyzer 2100 (Agilent Technologies). TaqMan[®] MicroRNA Assays were used to quantitate miRs according to manufacturer's instructions (Applied Biosystems, Foster City, CA). A single TaqMan MicroRNA assay is used for each miR. All necessary primers and TaqMan probes are provided by the manufacturer with each assay, but details about sequence of primers and probes are not available (patented). Each TaqMan MicroRNA assay includes: (a)

a "looped" primer, specific for each miR, for the reverse transcription step (carried out with the TaqMan[®] MicroRNA Reverse Transcription Kit) and (b) a pair of "conventional" primers for amplification as well as a fluorescently labeled TaqMan probe for detection for the Real-Time amplification step (carried out with the TaqMan Universal PCR Master Mix).

In brief, 5 ng total RNA was reverse transcribed in 7.5 μ l reaction volume containing 50 nM looped miR-specific primer, 1 \times RT buffer, 0.25 mM each dNTPs, 3.33 U/ μ l MultiScribe[™] reverse transcriptase and 0.25 U/ μ l RNase inhibitor. The reactions were incubated in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) in a 96-well plate for 30 min at 16°C, 30 min at 42°C, followed by 5 min at 85°C, and then held at 4°C. Reverse transcription products were diluted three times with nuclease-free water prior to setting up PCR reactions. Each microRNA Real-Time PCR (10 μ l volume) was carried out in triplicate, and each 10 μ l reaction mixture included 2 μ l of diluted reverse transcription reaction product, 5 μ l of 2X TaqMan[®] Universal PCR Master Mix, 1X assay mix (including TaqMan[®] probe and forward and reverse primers). The reactions were incubated in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) in 96-well plates at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Fold induction was calculated by 2^{- Δ Ct} method [46] using the level of Huh-7 cell line as a calibrator.

Prediction of genes targeted by modulated miRs

Putative gene targets of miRs found to be modulated in HCV clones were predicted by means of the miRGator program (available at <http://genome.ewha.ac.kr/miRGator/miRNAexpression.html>) that allows to combine gene predictions by TargetScanS, miRanda and PicTar softwares. To avoid loss of potential targets, a relaxed option was selected (retrieval of genes predicted by at least one program), so as to obtain for each miR a gene list as wide as possible.

Gene network pathway analysis

Gene Ontology (GO) annotations were analyzed with the Panther Protein Classification System [30] to identify functional annotations that were significantly enriched in this gene set compared to the entire human genome. Gene lists modulated by HCV were mapped onto biological pathways that were significantly represented.

Additional material

Additional file 1: Table S1. List of 83 genes resulting from overlapping (a) predicted target genes of miR-128a or miR-196a or miR-143-3p and (b) genes experimentally showing an expression change in HCV replicon clones vs. parental Hu-h7 cells by microarray analysis [21].

Additional file 2: Table S2. Individual genes in each of the classification groups found to be significantly enriched in the set of 37 genes by Panther.

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Authors' contributions

RB had been involved in conception and design of the study, data analysis and interpretation and revised the paper critically; CM and PT carried out real-time PCR assays, most of microarray experiments and contributed to data interpretation; ET and ES carried out cell culture experiments; AC and UV participated in data analysis; AB contributed to the revision of the paper critically for important intellectual content; ARC had been involved in conception and design of the study, data analysis and interpretation and drafting the manuscript. All the authors had given final approval of the version to be published.

Competing interests

The authors declare that they have no competing interests.

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