RNAI-based gene therapy of hepatocellular carcinoma: targeting ABC transporters
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CELLULAR miRNAs miR-101, miR-135b, miR-199a/b, miR-296 REGULATE THE EXPRESSION OF ABCA1 AND ABCC1 TRANSPORTERS IN HEPATOCELLULAR CARCINOMA

Florie Borel, Ruiqi Han, Harald Petry, Sander JH van Deventer, Peter LM Jansen, Pavlina Konstantinova
ABSTRACT

ATP-binding cassette (ABC) transporters are drug efflux pumps responsible for the multidrug resistance phenotype causing hepatocellular carcinoma (HCC) treatment failure. Our previous data indicates that the expression of ABC transporters is regulated by cellular miRNAs in HCC patients. Here we demonstrate that miR-101 and miR-135b regulate ABCA1 expression, and miR-199a/b and miR-296 regulate ABCC1 expression. Luciferase reporter analyses revealed that the cellular miRNA have true targets in the 3’UTR of ABCA1 and ABCC1. We further verified the miRNA regulation of ABCA1 and ABCC1 by analysis of endogenous mRNA and protein expression. Moreover we show that miR-296 is up-regulated upon sorafenib treatment while ABCC1 is down-regulated which may indicate a direct post-transcriptional regulation mechanism. By potentially down-regulating ABCC1 via mir-296 up-regulation, this may be the mechanism by which sorafenib sensitizes tumor cells to doxorubicin treatment.

Authors’ affiliation

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INTRODUCTION

The expression of ATP-binding cassette (ABC) transporters is dysregulated in HCC prior to any chemotherapeutic treatment (1), suggesting that the selection of a multidrug resistant sub-population by chemotherapy may not be the only cause of ABC transporter genes overexpression in HCC. The regulation of ABC genes expression in HCC could be mediated by microRNAs (miRNAs), a family of small RNAs which is often dysregulated in cancer (2-4). Moreover, it was shown that miR-33 inhibits the expression of ABCA1 resulting in reduced cellular cholesterol efflux to ApoA1 acceptor (5).

miRNAs are ~22 nucleotide (nt) long endogenous, single-stranded, non-coding RNAs (6). miRNAs are loaded into the RNA-induced silencing complex (RISC) where further regulations will be undertaken. If the complementarity is perfect in the "seed region" (nt 2-7 from the 5’ end of the miRNA) between the miRNA and its target in the messenger RNA (mRNA), the mRNA will be cleaved by RISC and degraded; in case of imperfect complementarity, translation will be repressed (7-10). Specific miRNAs have been shown to be involved in various biological processes, including development, cellular proliferation, apoptosis, and oncogenesis (11, 12). The finding that individual miRNAs may target several hundred genes, and that a large percentage of mRNAs may be subject to regulation by miRNAs, further underscores the emerging importance of miRNA-mediated regulation (13, 14).

We previously identified 12 up-regulated ABC genes and 79 down-regulated miRNAs in HCC patient samples (1). Subsequently, we showed using luciferase reporters that miRNAs down-regulated the expression of 5 out of the 12 up-regulated ABC genes. Here we confirm in vitro that miR-101 and miR-135b regulate the expression levels of endogenous cholesterol transporter ABCA1 and miR-199a/b and miR-296 of multidrug resistance transporter ABCC1.

MATERIALS AND METHODS

miRNA target prediction

Softwares TargetScan (14) and PicTar (13) were used for ABCA1 and ABCC1 3’UTR target prediction of cellular miRNAs previously identified as down-regulated in HCC (1). Additionally, 3’UTR sequences were manually screened for miRNA seed-matching sequences.

Luciferase reporters and miRNA expression constructs

Cloning of wild-type and mutated luciferase reporters Luc-ABCA1, Luc-ABCC1, mLuc-ABCA1 and mLuc-ABCC1, and miRNA expression plasmids were described previously (1). Briefly, luciferase reporters were made by cloning of ABCA1 and ABCC1 3’UTR sequences behind the renilla luciferase gene in the psiCheck-2 vector (Promega, Madison, WI), and miRNA expression plasmids were made by cloning of the pri-miRNAs sequences in the pcDNA6.2 vector (Invitrogen, Carlsbad, CA).

Cell lines and transfection

Human embryonic kidney (HEK) 293T and human hepatocellular carcinoma HuH7 cell lines were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing 10% (v/v) fetal calf serum, 100U/ml penicillin and 100U/
ml streptomycin, at 37°C and 5% CO₂. Cells were plated in 6- or 96-well plates. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's instructions. For RT-qPCR analysis of ABC genes and miRNAs expression and for immunoblotting, 1.2µg miRNA expression plasmid or 125pmol miRNA inhibitor (Exiqon, Vedbaek, Denmark) were transfected in 6-well plates. For cell viability assays in HuH7 cells, 200ng miRNA expression plasmids or 4.2pmol miRNA inhibitors were transfected in 96-well plates. To assess the effect of sorafenib on ABCC1 gene and miRNAs expression, HuH7 were cultured for 7 days in 6-well plates with 2µl DMSO or 1mM sorafenib (S-8502, LC Laboratories, Woburn, MA).

**RNA isolation, RT-qPCR for ABC genes and for miRNAs**
For ABC genes and miRNA expression, cells were harvested 72-hr post-transfection with miRNA expression plasmid. Total RNA was isolated with Trizol (Invitrogen). For ABCA1 and ABCC1 expression, RT reactions were performed with the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA) using 900ng RNA template. RT-qPCR was performed using 4ng cDNA, 3µl 2x Fast SYBR Green Master Mix (Applied Biosystems) and 0.2µl of each primer (primers sequences, Table S1) and run on a 7500 RT-qPCR system (Applied Biosystems). For miRNA expression, RT reactions were performed with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) using 10ng RNA and 3µl miRNA-specific RT-stem-loop primer (Applied Biosystems) according to manufacturer's instructions. Taqman assay was done in 20µl using 9µl cDNA (5x diluted), 1µl miRNA-specific primer with FAM-labeled fluorogenic probe (Applied Biosystems) and 10µl Taqman 2x Universal PCR Master Mix (Applied Biosystems) and run in duplicates on a 7500 RT-qPCR system (Applied Biosystems).

**Luciferase assay**
HEK293T cells were transfected with 5ng luciferase reporter and 150ng miRNA expression plasmid or 4.2pmol miRNA inhibitor, and were assayed at 72-hr (miRNA) or 48-hr post-transfection (miRNA inhibitor). Firefly and renilla luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instructions, and the relative luciferase activity was calculated as the ratio between the renilla and firefly luciferase activities.

**Immunoblotting**
Cells were harvested 72-hr post-transfection with miRNA expression plasmid and homogenized in 10mM KCl, 1.5mM MgCl₂, 10mM Tris-Cl pH7.4, 0.5% SDS w/v, and one Complete Protease Inhibitor tablet (Roche Diagnostics, Basel, Switzerland) per 10ml buffer, completed by mechanical lysis through a 25G-needle. Protein concentration was determined using the QuickStart Bradford protein assay (Biorad, Hercules, CA). Antibody dilutions were as follows: 1:1,000 anti-ABCA1 (AB18180, Abcam, Cambridge, MA), 1:1,000 anti-ABCC1 (EB08734; Everest Biotech, Oxfordshire, UK), 1:10,000 anti-αTubulin (SC5286, Santa Cruz Biotechnologies, Santa Cruz, CA), 1:1,000 anti-Actin (4968, Cell Signaling Technology, Beverly, MA), 1:10,000 rabbit anti-mouse P0260 and rabbit anti-goat P0449 (Dako, Glostrup, Denmark). Antibody binding was detected by the Lumi-LightPLUS chemiluminescent detection kit (Roche Diagnostics, Basel, Switzerland).
Cell viability assay
HuH7 cells were transfected with miRNA expression plasmids, and 48-hr later, 0.2 or 0.5μl 1mM doxorubicin (44583, Sigma) or equal volume of DMSO (D2650, Sigma) was added. Viability of HuH7 cells was measured 24-hr later according to the manufacturer’s protocol (CellTiter-Glo Luminescent Cell Viability Assay, Promega).

RESULTS
miRNA target sites predictions in the 3’UTR of ABCA1 and ABCC1
For the 79 miRNAs previously identified as down-regulated in HCC (1), miRNA targets in ABCA1 and ABCC1 were predicted using algorithms PicTar and TargetScan. ABCA1 3’UTR is 3312-bp long and contains 3 putative miR-101 target sites, and 2 putative miR-135b target sites (Fig. 1A, upper panel). The close proximity of miR-101-2, miR-135b-2 and miR-101-3 target sites indicates functionality of ABCA1 3’UTR. In addition, the complementarity between the miRNAs and ABCA1 was high in this region, with multiple complementary bases outside of the seed region. Taking into account that in RNA G can pair with U, miR-101 binds via its 7-nt seed and via an additional 6, 7 and 9 bases complementary to the 3’UTR, for respectively miR-101-1, -2 and -3 target sites. miR-135b binds via its 7-nt seed and via 4 additional bases complementary to the 3’UTR (miR-135b-1) and via its 7-nt+A seed and 3 additional bases complementary to the 3’UTR (miR-135b-2). ABCC1 3’UTR is 1792-bp long and contains 1 miR-199a/b target site (7-nt seed + 6 additional bases), and 1 miR-296 target site (7-nt seed + 6 additional bases), (Fig. 1A, lower panel).

Validation of miR-101 and miR-135b targets in ABCA1 gene
In order to verify the predicted miRNA targets in ABCA1, a luciferase reporter was made that contained the 3’UTR of the ABCA1 gene (Luc-ABCA1). HEK293T cells were co-transfected with Luc-ABCA1 and miR-Scr, miR-101 or miR-135b. Seventy-two hours post-transfection firefly and renilla luciferase activities were measured and relative luciferase fluorescence (RLF) was calculated. RLF was reduced by more than 50% upon co-transfection of miR-101 or miR-135b with Luc-ABCA1 (Fig. 1B), indicating that the gene is a true target for both miRNAs. Next, we wanted to confirm that direct miRNA binding was responsible for the observed effect. All miRNA binding sites in the 3’UTR of Luc-ABCA1 were mutated to produce a mutated luciferase reporter (mLuc-ABCA1). Upon co-transfection of miR-101 or miR-135b with mLuc-ABCA1, inhibition of RLF was alleviated (Fig. 1B), hence confirming that the knock-down effect is a consequence of direct miRNA binding to ABCA1 target sequences. To further verify the miRNA targets in ABCA1, inhibitors of miR-101 and miR-135b were co-transfected with Luc-ABCA1. miRNA inhibitors bind to cellular miRNAs and therefore prevent their binding and inhibition of target gene expression, resulting in a reversal of the knock-down effect. 48-hr post-transfection firefly and renilla luciferase activities were measured and RLF was calculated. miR-101 and miR-135b sequestration lead to a significant increase in RLF (Fig. 1B), which shows that reducing endogenous miRNA levels alleviates the negative regulation exerted by miR-101 and miR-135b on Luc-ABCA1 and confirming those miRNAs as inhibitors of ABCA1 gene expression in HCC.
Figure 1. Prediction and validation of ABCA1 and ABCC1 as targets of respectively miR-101 and miR-135b, and miR-199a/b and miR-296. 

A. Schematic of ABCA1 and ABCC1 3’UTR with predicted targets of miR-101 and miR-135b, and miR-199a/b and miR-296, respectively. The 3’UTR of ABCA1 contains three miR-101 and two miR-135b target sites. The 3’ UTR of ABCC1 contains one miR-199a/b and one miR-296 target sites. The seed sequence is represented with bold characters, base-complementarity with grey highlighting, and G-U base-pairing with semi-colons.

B. Knock-down of Luc-ABCA1 by miR-101 and miR-135b. 

C. Knock-down of Luc-ABCC1 by miR-199a/b and miR-296. HEK293T cells were transfected with 5ng Luc-ABCA1, Luc-ABCC1, mLuc-ABCA1 or mLuc-ABCC1 reporter plasmid, 150ng miRNA expression plasmid or 4.2pmol miRNA inhibitor. mLuc-ABCA1 and mLuc-ABCC1 carry mutations in the miRNA binding sites. Luciferase assay was performed 72-hr post-transfection. Renilla luciferase was normalized with firefly luciferase (relative luciferase fluorescence, RLF), then RLF of each miRNA was normalized to RLF of miR-Scr which was set at 1.

Data are presented as average of 3-4 technical replicates ± SD. ****: P < 0.0001, **: P < 0.01.
Validation of miR-199a/b and miR-296 targets in ABCC1 gene

As described for ABCA1, Luc-ABCC1 reporter was designed to contain the 3’UTR of the ABCC1 gene. HEK293T cells were co-transfected with Luc-ABCC1 and miR-Scr, miR-199a, miR-199b or miR-296, and 72-hr post-transfection firefly and renilla luciferase activities were measured and RLF was calculated. RLF was reduced by more than 50% upon co-transfection of miR-199a, miR-199b or miR-296 with Luc-ABCC1 (Fig. 1C), indicating that it is a true target for both miRNAs. Mutating the ABCC1 target sites in Luc-ABCC1 (mLuc-ABCC1) resulted in a similar effect as for ABCA1, and inhibition of RLF was alleviated (Fig. 1C), hence confirming that the knock-down is a direct consequence of the miRNA binding. Inhibiting miR-199a/b or miR-296 expression with miRNA inhibitors did not lead to a significant increase in RLF (data not shown).

Endogenous ABC transporter genes and miRNAs expression in HEK293T and HuH7

As a next step, endogenous ABCA1 and ABCC1 knock-down by the previously described cellular miRNAs was determined. miRNAs regulate gene expression by causing mRNA degradation or translational repression. The effect of a given miRNA can therefore be assessed both at mRNA and protein levels. Initially, endogenous expression of ABCC1 and ABCA1 in HEK293T and HuH7 cells was determined by RT-qPCR. ABCC1 was expressed in both cell lines and ABCA1 was expressed in HuH7 only (Fig. 2A). Next, mature miRNA expression was quantified by RT-qPCR in HEK293T and HuH7 cells following plasmid transfection to determine if there was a significant increase in expression following miRNA transfection, indicating proper processing of the mature miRNA from the pri-miRNA expression plasmid. Cellular miRNAs predicted to target ABCA1 were detectable in non-transfected cells at Ct~30. Expression was significantly increased by 90 and 60000 in HEK293T and by 15 and 2000 in HuH7 upon transfection with miR-101 or miR-135b expression plasmid (Fig. 2B). Cellular miRNAs predicted to target ABCC1 were detectable in non-transfected cells at Ct~30-35, with the exception of miR-199a which was undetectable in both cell lines. Expression was significantly enhanced upon transfection with miR-199a/b or miR-296 expression plasmid (Fig. 2B). The endogenous expression of miR-199a/b and miR-296 is very low, and it would be difficult to artificially decrease it with miRNA inhibitors. This could be the reason why the previously described Luc-ABCC1 experiment using miRNA inhibitors was unsuccessful in alleviating miRNA inhibition.

Cellular miR-101 and miR-135b regulate endogenous ABCA1 expression in vitro

We previously showed that the 3’UTR of ABCA1 presents target sites for miR-101 and miR-135b. Cellular miRNAs regulate the expression of their targets either by causing degradation of the mRNA, or by repressing its translation. To determine the effect of these miRNAs on endogenous ABCA1 expression, HuH7 cells were transfected with 4μg miR-101, miR-135b, and miR-Scr expression plasmid or 125pmol of the respective miRNA inhibitor, and RNA and protein were isolated 72-hr post-transfection. ABCA1 expression was determined by RT-qPCR and significant differences in mRNA levels were measured between treatments. A negative correlation between miRNA and ABCA1 mRNA levels was observed. Increased miRNA expression reduced ABCA1 mRNA levels. On the contrary, miRNA inhibition increased ABCA1 mRNA levels (Fig. 3A). Next, ABCA1 protein
expression was determined by immunoblotting. Endogenous ABCA1 protein levels were reduced upon transfection with miR-101 or miR-135b expression plasmid (Fig. 3B). miR-135b transfection lead to the strongest effect throughout 3 biological replicates (data not shown).

**Cellular miR-199a, miR199b and miR-296 regulate endogenous ABCC1 expression in vitro**

We previously showed on luciferase reporters that the 3’UTR of ABCC1 presents target sites for miR-199a, miR-199b and miR-296. To determine the effect of these miRNAs on endogenous ABCC1 expression, HEK293T cells were transfected with 4μg miR-199a, miR-199b, miR-296 and miR-Scr expression plasmid, and RNA and protein were isolated 72-hr post-transfection. ABCC1 expression was determined by RT-qPCR, but no significant

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**Figure 2. miRNAs and ABC genes expression levels in HEK293T and HuH7 cells.**

A. Endogenous ABCA1 and ABCC1 gene expression in HEK293T and HuH7. Expression of actin was set at 1. Data are represented as average of three technical replicates ± SD. B. miRNA expression levels in wild-type (wt) and transfected (tfx) HEK293T and HuH7 cells. Cells were transfected with the corresponding miRNA expression plasmid and expression was measured 72-hr later. Data are represented as average of two technical replicates.

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**Figure 3. Cellular miR-101 and miR-135b regulate endogenous ABCA1 in vitro.**

A. Endogenous ABCA1 mRNA levels upon transfection of HuH7 cells with 1.2μg miRNA expression plasmid or 125pmol miRNA inhibitor. ABCA1 gene expression was normalized with actin, and miR-Scr was set at 1. **: P-value < 0.01. B. Levels of endogenous ABCA1 protein levels upon transfection of HuH7 cells with 1.2μg of miR-101 and miR-135b expression plasmid.
differences in mRNA levels were measured between treatments (data not shown). Knock-down of ABCC1 protein expression by miR-199a/b and miR-296 was determined by immunoblotting. Endogenous ABCC1 protein levels were reduced upon transfection with miR-199a, miR-199b or miR-296 expression plasmid (Fig. 4A). miR-199a transfection lead to the strongest effect throughout 3 biological replicates (data not shown). Subsequently, because ABCC1 effluxes the cytotoxic drug doxorubicin, we determined if decreasing ABCC1 levels by transfecting HuH7 cells with miRNA expression plasmid would decrease cell viability. HuH7 cells were transfected with 20 and 200ng miRNA expression plasmid, increasing dose of doxorubicin was added to the cells 48-hr later, and cell viability was measured after 24-hr. Surprisingly, no significant differences in cell viability were observed between transfections with 20ng (Fig. 4B) or 200ng miRNA (data not shown).

**miR-296 up-regulation in response to sorafenib treatment may down-regulate ABCC1**

To date sorafenib is the only drug proved to be efficient for treatment of HCC. Having previously shown that ABCC1 is up-regulated in untreated HCC (1), we postulated that this up-regulation might be a hallmark of HCC rather than a consequence of chemotherapeutic treatment. Though there is limited literature on sorafenib transport, it should be noted that up to now sorafenib was not shown to be a substrate for ABCC1. However, we hypothesized that sorafenib may change cellular miRNA expression levels, resulting in altered target gene regulation. Since ABCC1 is targeted by miR-199a/b and miR-296, we determined the expression of those miRNAs and of ABCC1 in HuH7 cells cultured on sorafenib. RNA

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**Figure 4. Cellular miR-199a/b and miR-296 regulate endogenous ABCC1 in vitro.**

A. Endogenous ABCC1 protein knock-down upon transfection of HuH7 cells with 1.2µg of miR-199a/b and miR-296 expression plasmid. B. Cell viability of HuH7 cells in response to doxorubicin treatment upon transfection with miRNA expression plasmid. Cells were transfected with 200ng miRNA expression plasmid, doxorubicin was added 48-hr later, and cell viability was measured after 24-hr. C. Expression of ABCC1 and miR-296 in HuH7 cells treated with 1mM sorafenib.
was isolated from cells cultured with 1 mM sorafenib for 1 week and the expression of ABCA1 and ABCC1 was determined by RT-qPCR. ABCA1 expression was not affected by sorafenib treatment; however, ABCC1 was significantly down-regulated (Fig. 4C). Next, expression of cellular miRNAs predicted to target ABCC1 was subsequently determined in the same samples. Interestingly, miR-296 was up-regulated 1.5 times while miR-199a/b expression didn't change. That could be due to the extremely low expression levels in Huh7 cells (Fig. 4D). This finding indicates that sorafenib may chemosensitize HuH7 cells via up-regulation of cellular miR-296 that subsequently down-regulates ABCC1 by a post-transcriptional silencing mechanism.

**DISCUSSION**

miRNA dysregulation has been clearly linked to cancer and HCC, but the molecular mechanisms by which miRNAs modulate tumorigenesis are poorly described. In this study we focused on miR-101, miR-135b, miR-199a/b and miR-296 and evaluated their effect on their targets ABCA1 and ABCC1. Decreased expression of miR-101 (1, 15, 16) and miR-199a/b in HCC has been repeatedly shown (1, 15-20).

Bioinformatics prediction that ABCA1 is a target of cellular miR-101 and miR-135b, with respectively three and two targets in the 3'UTR of ABCA1, was confirmed in the current study on luciferase reporters, on endogenous mRNA levels, and endogenous protein levels.

ABCA1 is a cholesterol transporter whose expression was previously shown to be regulated by a cellular miRNA. miR-33a represses ABCA1 expression, thereby reducing cholesterol efflux to ApoA1 (5). Our data indicate that miR-101 and miR-135b, together with miR-33a and miR-122 (21), belong to the small group of cellular miRNAs involved in cholesterol metabolism. To clearly prove this relationship, radiolabeled cholesterol efflux study would provide a definitive functional validation of the miRNA regulation. For this, hepatocytes or macrophages should be used, which was not feasible in our current research project.

Bioinformatics prediction that ABCC1 is a target of cellular miR-199a/b and miR-296 was confirmed on luciferase reporters in the current study and on endogenous protein levels. miRNAs can regulate gene expression via two mechanisms, either mRNA degradation or translational repression. This could explain why no differences were observed on endogenous mRNA levels, yet endogenous protein levels were reduced upon miRNA expression plasmid transfection. ABCC1 gene is highly relevant to HCC, e.g. increase in ABCC1 expression has been associated with a more aggressive HCC phenotype (22), and ABCC1 transports clinically relevant drugs for HCC such as doxorubicin (23). However, we were unable to show any differences in viability of miRNA-transfected HuH7 cells upon doxorubicin treatment. When one ABC gene is down-regulated, its function may be taken over by other member(s) of the family. The absence of a measurable effect on cell viability may be due to functional overlapping of ABC transporters. In this case, doxorubicin is a substrate not only for ABCC1 but for several other ABC transporters including ABCB1 (24), ABCB2 (24) and ABCB10 (25). If doxorubicin efflux is taken over by other transporter(s), it would mask the effect of ABCC1 knock-down. Assessing a functional effect of miRNA-mediated endogenous ABCC1 knock-down may hence require to also inhibit and/or down-regulate other ABC transporters with overlapping transport capacities.

Another clinically relevant drug for HCC treatment is sorafenib. Sorafenib treatment reduces ABCC1 expression levels *in vitro*, as it was shown in this study and previously for
several ABC transporters (26). Here we demonstrated that ABCC1 expression is regulated by cellular miR-199a/b and miR-296. In addition we showed that in sorafenib-treated cells, miR-296 is up-regulated, which may cause ABCC1 down-regulation. This finding, may indicate a novel involvement of miR-296 regulation of ABCC1 in the multidrug resistance phenotype in HCC. Further research in primary hepatocytes and in murine models of HCC will characterize this interesting potential mechanism of sensitizing cells to chemotherapy by sorafenib-induced miRNA regulation of gene expression. Further work is warranted to evaluate the effect of modulating miRNA expression, using gene therapy vectors encoding the cellular miRNAs in an animal model of multidrug resistant HCC. Artificially increasing miR-199a/b and miR-296 levels in HCC patients may reduce ABCC1 levels, and may improve chemotherapy effectiveness and/or render HCC tumors less aggressive.

Table S1. List of primers used in this study.

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REFERENCES

14. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines,


