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MS2-TRAP (MS2-tagged RNA affinity purification): Tagging RNA to identify associated miRNAs

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Abstract

Cellular transcripts of all types, including coding messenger (m)RNAs and noncoding (nc)RNAs, are subject to extensive post-transcriptional regulation. Among the factors that elicit post-transcriptional control, microRNAs (miRNAs) have emerged as a major class of small regulatory RNAs. Since RNA-RNA interactions can be modeled computationally, several excellent programs have been developed to predict the interaction of miRNAs with target transcripts. However, many such predictions are not realized for different reasons, including absent or low-abundance expression of the miRNA in the cell, the existence of competing factors or conformational changes masking the microRNA site, and the possibility that target transcripts are not present in the prediction databases, as is the case for long ncRNAs. Here, we provide a systematic approach termed MS2-TRAP (tagged RNA affinity purification) for identifying miRNAs associated with a target transcript in the cellular context. We illustrate the use of this methodology by identifying microRNAs that associate with a long intergenic (l)ncRNA, based on the expression of the lincRNA tagged with MS2 RNA hairpins (*lincRNA-p21-MS2*) and the concomitant expression of a fusion protein recognizing the MS2 RNA hairpins, MS2-GST. After affinity pulldown of the ribonucleoprotein (RNP) complex comprising [MS2-GST / *lincRNA-p21-MS2*], the RNA in the pulldown material was isolated and reverse transcribed (RT). Subsequent assessment of the microRNAs present in the pulldown complex by using real-time quantitative (q)PCR analysis led to the identification of *bona fide* miRNAs that interact with and control the abundance of *lincRNA-p21*. We describe alternative designs and applications of this approach, and discuss its implications in deciphering post-transcriptional gene regulatory schemes.

1. INTRODUCTION

At the post-transcriptional level, gene expression is governed by two main classes of regulatory molecules: RNA-binding proteins (RBPs) and noncoding (nc)RNAs [1-3]. RBPs affect all facets of post-transcriptional gene regulation, including RNA splicing, maturation, transport, editing, decay, stabilization, storage, and translation [4-6]. Among ncRNAs, the best-studied subclass comprises microRNAs (miRNAs), which typically repress mRNA stability and translation [7-9]. The interaction of RBPs and miRNAs with a target RNA is dictated by the presence of *cis*-binding elements present in the RNA; some of these elements are universal [e.g., the 5' cap or the 3' poly(A) tail], but other elements are only found in

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select transcripts [e.g., internal ribosome entry sites (IRES), AU-rich elements (AREs), and specific miRNA sites] [10-12].

Computational approaches have been unable to predict accurately the formation of ribonucleoprotein (RNP) complexes, particularly for RNP interactions involving non-universal *cis*-elements. Therefore, a number of elegant experimental methods have been devised to identify RBP-mRNA interactions. These RNP associations are routinely studied using molecular biology techniques that rely on affinity interactions with antibodies and ligands [13-15].

However, the task of predicting miRNA-mRNA associations was theoretically easier, since RNA-RNA interactions could be modeled *in silico*. Accordingly, a number of algorithms have been developed for the purpose of predicting these associations [e.g., TargetScan, miRBase, miRanda, and PicTar; details at <http://www.exiqon.com/microrna-target-prediction>). However, since these algorithms vary in their use of sequence conservation, energetics, and structural parameters, their predictions overlap only partially. Further limitations of computational predictions are derived from 1) the difficulty in studying the ability to bind a target at endogenous miRNA concentrations and degrees of availability, since these depend on mRNA levels and/on competition with other miRNAs and RBPs, 2) the possibility that the endogenous target mRNA is folded in a specific conformation that hides the miRNA binding site or is otherwise inaccessible to the miRNA, 3) the existence of artifacts intrinsic to reporter analyses [16], and 4) the absence of complete collections of transcripts in the databases searched by the algorithm, as is the case for long noncoding (lnc)RNAs and microRNAs which are not presently known.

Here, we describe a method that can circumvent these limitations. The method is based on the addition of MS2 RNA hairpin loops to a target RNA of interest, followed by co-expression of the MS2-tagged RNA together with the protein MS2 (which recognizes the MS2 RNA elements) fused to an affinity tag, an approach that recapitulates some features of the 'ribotrap' method developed by Keene and colleagues [14] and . After purification of the MS2 RNP complex, the miRNAs present in the complex are identified. To illustrate the usefulness of this approach, we have tagged the mouse *lincRNA-p21* with MS2 hairpins and have co-expressed it in mouse embryonic fibroblasts (MEFs) along with the chimeric protein MS2-GST (glutathione S-transferase). After affinity purification using glutathione-SH beads, the microRNAs present in the RNP complex were identified by reverse transcription (RT) and real-time, quantitative (q)PCR. We discuss our findings and the variations on this methodology that could be used to identify a larger spectrum of regulatory miRNAs, other short and long ncRNAs, as well as RBPs.

2. DESCRIPTION OF THE METHOD

A handful of approaches to isolate ectopically expressed RNAs are available using specific RNA tags (other tags discussed in section 6). One of the most widely used tags, MS2, is a 19-nucleotide long viral (bacteriophage) RNA sequence present at the ribosomal binding site of the MS2 replicase mRNA, which folds into a hairpin loop structure. This hairpin loop is recognized with high specificity and affinity by the MS2 bacteriophage capsid RNA-binding protein MS2 (Kd of $3\text{-}300 \times 10^9$, depending on the stem loop sequence and the MS2 RBP variant [13, 17-19]). Expression of the RBP MS2 as a chimeric protein containing a peptide tag facilitates the isolation of the [MS2 RNA/MS2 protein] complex, together with other molecules present in the complex. MS2 has been used widely to tag RNA transcribed *in vitro* and *in vivo* for other applications [20-23]. The MS2 pulldown method consists of three basic steps:

2.1. Step 1

involves the construction of two plasmid vectors and their cotransfection into mammalian cells. The first plasmid expresses a chimeric RNA containing the test RNA of interest followed by several MS2 RNA hairpins (typically 12 or 24 tandem MS2 hairpin loops). It is generally advised to attach the MS2 sequences at the 3' end of the test RNA, but before the poly(A) tail, in order to avoid blocking translation or possibly translating the MS2 sequences. The control plasmid simply expresses MS2 hairpins without the test RNA of interest. The second plasmid expresses a chimeric protein comprising the MS2 capsid protein and an affinity tag. It is important to express more chimeric RNA than detecting chimeric protein in order to reduce non-specific binding. Here, we have expressed mouse *lincRNA-p21* tagged with MS2 hairpin loops (*lincRNA-p21-MS2*) as RNA of interest (*MS2* RNA was expressed in control parallel transfections), and the chimeric detection protein MS2-GST (**Figure 1**).

2.2. Step 2

involves harvesting the cultures 24-48 h after transfection of the plasmids, followed by cell lysis with a buffer that preserves the integrity of the native RNPs. The cell lysates are then mixed with the affinity reagent and allowed to bind, and the RNP complexes are separated and washed. Here, the affinity reagent is glutathione-SH (GSH) attached to beads; the GSH beads bind with high affinity to the GST component of the fusion protein (MS2-GST) and can effectively separate MS2 RNPs by centrifugation. The pellets recovered are then treated with DNase and proteinase, and the RNA is isolated for further analysis (**Figure 1**).

2.3. Step 3

is to identify the miRNAs present in the purified RNP complex. Among the different possible methods to characterize these microRNAs (discussed in **section 6**), here we have performed a targeted screen by reverse transcription (RT) and real-time quantitative (q)PCR analysis. The miRNAs specifically associated with mouse *lincRNA-p21* were identified by RT-qPCR-based detection of a subset of computationally predicted miRNAs.

3. EXPERIMENTAL RESULTS

We were interested in testing if the mouse *lincRNA-p21* associated with miRNAs. We first searched whether the mouse *lincRNA-p21* (whose sequence is known [24]) might be the putative target of miRNAs. Since current microRNA identification programs (e.g., TargetScan, miRBase, etc) do not search lncRNAs databases, we used the prediction programs RNA22 (IBM) and the Segal Laboratory program (Weizmann Institute, Israel) to identify mouse miRNAs which might putatively interact with *lincRNA-p21*. This analysis identified numerous putative miRNA target sites; 20 among them are shown in **Table 1**.

In order to test whether *lincRNA-p21* associated with one or several of these miRNAs in the cellular context, we prepared plasmid plincRNA-p21-MS2 (using reagents kindly obtained from M. Huarte [24]), in order to express a chimeric RNA bearing *lincRNA-p21* followed at the 3' end by 24 copies of the MS2 hairpin. Plasmid plincRNA-p21-MS2 and plasmid pMS2-GST (generously provided by B.R. Cullen [25]), which expressed a fusion protein containing the MS2 coat protein and glutathione-S-transferase, were cotransfected into mouse embryonic fibroblasts (MEFs) using Lipofectamine 2000. Forty-eight hours later, cells were lysed and the resulting lysates were mixed with glutathione-SH (GSH) agarose beads. After pulldown and washes, we divided the beads: one half was saved for protein analysis, the other half for isolating RNA. For RNA analysis, the pulldown material was digested with DNase and proteinase and the RNA precipitated with ethanol for further analysis.

First, we examined the presence of known molecules in the *lincRNA-p21-MS2* pulldown. As anticipated, the pulldown was enriched in the *lincRNA-p21* transcript itself (**Figure 2A**), which was detected by RT-qPCR amplification of the target RNA (details in **section 4.4**). The *lincRNA-p21-MS2* pulldown followed by Western blot analysis of the proteins in the pellet revealed an enrichment in the interaction of this transcript with the RNA-binding proteins (RBPs) Ago2, and Rck, but not with the RBP hnRNP C; the abundant housekeeping protein Gapdh was detected at background levels in equal amount in all pulldown samples (not shown), indicating that input was even among the samples (**Figure 2B**).

We then sought to identify the levels of predicted target microRNAs. Currently two PCR-based miRNA detection methods are widely used. One method (from Taqman™) employs gene-specific antisense PCR primers for reverse transcription followed by real-time PCR with TaqMan™ probe [26]); this method is limited to the miRNAs for which TaqMan™ probes are available. The second method, from SBI QuantiMir, is based on the synthesis of Poly(A) tails using Poly(A) polymerase, followed by the use of oligo(dT) adaptors for the RT reaction. The resulting cDNA is used in PCR amplification reactions with primers complementary to the mature miRNA sequences along with the oligo(dT) universal primer; this method allows for easy and relatively inexpensive screening of essentially every miRNA (**Figure 3**).

Using the SBI method to detect several *lincRNA-p21* target miRNAs: let7b, let7c, let7i, miR130, and miR221. We also included a non-target miRNA, miR702. Our results showed that among the 5 predicted target miRNAs, only 4 miRNAs (let7b, let7c, miR130, and miR221) were enriched in the *lincRNA-p21-MS2* pulldown; let7i was not enriched in the pulldown, showing levels comparable to those seen for the nontarget miR702 (**Figure 4A**). The biological consequences of these interactions were studied by comparing the steady-state levels of endogenous *lincRNA-p21* when we overexpressed precursors (Pre-) of let7b, let7c, let7i, or control siRNA. As shown in **Figure 4B**, only overexpression of Pre-let7b or Pre-let7c, encoding the miRNAs that associated with *lincRNA-p21*, lowered significantly the abundance of endogenous *lincRNA-p21*, while overexpression of Pre-let7i (which was not associated with *lincRNA-p21*) did not affect the steady-state levels of *lincRNA-p21*. Taken together, our analysis provides proof-of-principle evidence that MS2-mediated pulldown of *lincRNA-p21* can help to identify interacting target miRNAs with functional roles upon *lincRNA-p21* expression.

4. DETAILED PROTOCOL

4.1. Step 1: Cell culture, transfection, and lysate preparation

1. Prepare 1×10^6 mouse embryonic fibroblast (MEF) per 60-mm culture dish, cultured in DMEM (Invitrogen) supplemented with 10% (v/v) Fetal Bovine Serum (Hyclone) and antibiotics.
2. Cotransfect 2 μ g of plasmids pMS2-*lincRNA-p21* or pMS2, together with 1 μ g of pMS2-GST diluted in 150 μ l of OPTIMEM (Invitrogen), mixed with 5 μ l of lipofectamine 2000 (Invitrogen) diluted with 150 μ l OPTIMEM.
3. After incubating for 30 min at room temperature, add transfection mix to MEF cultures that were previously washed with phosphate-buffered saline (PBS) twice and supplemented with OPTIMEM.
4. Six hours after incubation at 37°C in 5% CO₂ incubator, wash MEFs twice with PBS, and add DMEM with 10% FBS and no antibiotics.
5. Forty-eight hours later, wash cells twice with PBS and lyse in 300 μ l lysis buffer containing 20 mM Tris-HCl at pH 7.5, 100 mM KCl, 5 mM MgCl₂, 0.5% NP-40,

protease inhibitors (Roche), RNase inhibitor (Fermentas), and 10 mM DTT for 10 min on ice.

6. After collecting the cell lysates by scraping, centrifuge lysates at $10,000 \times g$ for 15 min at 4°C.
7. Collect clear supernatants and measure protein concentration using the Bradford assay.
8. Use 1000 μ l of lysate (at 2 μ g/ μ l) for pulldown assay.

4.2. Step 2: Pulldown assay and RNA purification

1. Wash GSH agarose beads (GE Healthcare) with ice-cold PBS twice and resuspend with equal volume of PBS to make a 50% slurry.
2. Incubate supernatants with 50 μ l of GSH agarose bead slurry for 3 hours at 4 °C.
3. After centrifugation at 4,500 rpm for 1 min at 4 °C, wash beads twice with NT2 buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM MgCl₂ and 0.05% NP-40).
4. Incubate the resulting pellets with 20 units of RNase-free DNase I in 100 μ l reaction volume for 15 min at 37°C.
5. Add 700 μ l of NT2 buffer and centrifuge at 4,500 rpm for 1 min at 4 °C.
6. (for RNA analysis only) Incubate pellets with 0.1% SDS and 0.5 mg/ml Proteinase K for 15 min at 55 °C.
7. Collect supernatant from centrifugation at $10,000 \times g$ at 4 °C for 5 min.
8. Add 500 μ l of RNase-free water and 500 μ l of acidic phenol (Ambion) and vortex for 5 min.
9. Centrifuge at $10,000 g$, 4°C for 20 min and collect supernatants.
10. Mix 400 μ l of supernatants with 860 μ l of 100% ethanol, 40 μ l of 3 M sodium acetate, and 3 μ l of glycoblue.
11. After incubation at -20 °C for 2 hours, centrifuge at $10,000 \times g$ at 4°C for 20 min.
12. Wash the resulting RNA pellets with 500 μ l of 70% ethanol and centrifuge at $10,000 \times g$ at 4°C for 10 min.
13. Dry the RNA pellets and resuspend in 10 μ l of RNase-free water.

4.3. Step 3: detection of miRNAs

1. Mix 5 μ l of RNAs from MS2 pulldown with 2 μ l of 5 $\times$ Poly(A) buffer, 1 μ l of 25 mM MnCl₂, 1.5 μ l of 5 mM ATP and 0.5 μ l Poly(A) polymerase (System Biosciences QuantiMir kit).
2. Incubate for 30 min at 37 °C
3. Add 0.5 μ l of Oligo dT adaptor (QuantiMir kit)
4. Incubate for 5 min at 60 °C.
5. Cool at room temperature for 2 min
6. Add 4 μ l of 5 $\times$ RT buffer, 2 μ l of dNTP mix, 1.5 μ l of 0.1 M DTT, 1.5 μ l of RNase-free water, and 1 μ l of reverse transcriptase (QuantiMir kit).

7. Incubate for 60 min at 42 °C, heat for 10 min at 95 °C.
8. Mix 2.5 µl of cDNAs with 2.5 µl of miRNA specific primers [2.5 µM (**Table 2**)], universal primer (10 µM provided by QuantiMir Kit), and SYBR green master mix.
9. Quantify by PCR (Applied Biosystems 7300 Instrument).

4.4. Validation of proteins and RNA present in MS2 pulldown

1. Forty-eight hours after transfecting MEFs with the pMS2 constructs, prepare whole-cell lysates in RIPA buffer [10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM EDTA, 0.1% (w/v) SDS and 1 mM DTT].
2. After pulldown, add SDS sample buffer to one half of the beads for western blot analysis.
3. After SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transfer samples onto PVDF membranes (Invitrogen iBlot Stack) and probe membranes with primary antibodies recognizing Ago2 (Abcam), hnRNP C1/C2, Rck or GST (Santa Cruz Biotechnology).
4. Detect primary antibody signals using HRP-conjugated secondary antibodies (GE Healthcare) and detect using chemiluminescence (Pierce).
5. For RNA analysis, treat the other half of the beads with DNase (as above).
6. Perform RT and qPCR using gene-specific primers CCTGTCCACTCGCTTTC and GGAAGTGGAGACGGAATGTC (**Table 2**) and SYBR green master mix (Kapa Biosystems) in an Applied Biosystems 7300 instrument.

4.5. Validation of the effect of miRNAs on lincRNA-p21 abundance

1. After transfecting MEFs with lipofectamine 2000 as explained above, transfect control (Ctrl) siRNA (UUCUCCGAACGUGUCACG UdTdT), Pre-let-7b, Pre-let-7c or Pre-let-7i (Ambion) at 10 nM final concentration with lipofectamine 2000.
2. Forty-eight h later, wash cells with PBS twice, add 1 ml of Trizol (Invitrogen) and 200 µl chloroform.
3. Vortex for 3 min, centrifuge samples at 10,000 × *g* for 20 min at 4 °C and mix the supernatant with 100% ethanol and 3M sodium acetate as above.
4. Use 0.5 µg of RNA for RT followed by qPCR using specific primers (**Table 2**) and SYBR green master mix (Kapa Biosystems) and an Applied Biosystems 7300 instrument.

5. TROUBLESHOOTING

Unable to detect enrichment in chimeric *RNA-MS2* compared with *MS2* alone

Generally, this problem is solved by adding molar excess of the plasmid expressing the tagged chimeric RNA (plincRNA-p21-MS2 in this case) relative to the plasmid expressing the chimeric detection protein (pMS2-GST in this case). This modification decreases non-specific binding of cellular RNAs to the MS2-GST fusion protein. To meet the specific demands of each cell system, additional adjustments of amount of plasmid and times of collection after transfection are likely needed.

Low specificity

Frequent solutions to this problem are to lower the time of incubation with the GST beads to less than 6 hours, and/or reduce the amount of beads or lysate used.

Unable to validate microRNA effects

If miRNAs found associated with the MS2-tagged RNA cannot be validated using reporter constructs, it is advisable to lower the concentration of reporter target RNA.

Inadequate positioning the MS2 tags

Depending on the application of the method, placing the MS2 tags towards the 3' end of the lincRNA may be inadequate, perhaps interfering with the binding of RBPs. If this is the case, MS2 tags can be inserted towards the 5' segment of the tagged RNA. Care should be taken to avoid the introduction of translation start sites by this strategy. It is also important to keep in mind that tagging at 3' positions may not yield identical interacting molecules as tagging at 5' positions.

Background amplification of residual DNA

In some instances, plasmid DNA remaining in the sample may produce background amplification. To test if this is a problem, the authors must always include RT-minus PCR amplification reactions (which would specifically detect contaminating plasmid DNA). To eliminate the problem, the investigator can perform longer incubations with RNase-free DNase, can increase the concentration of the DNase in the reaction, and can perform sequential DNase digestions.

Identifying siRNAs by PCR

Since we use PCR primers to amplify small RNAs, it is possible that the same primer pairs also amplify the longer target RNAs. Even though this may not be a problem with miRNAs (since there are mismatches), with siRNAs the primer sequences are fully complementary, so it may lead to unwanted amplification. Nonetheless, it should be possible to distinguish the amplified small-RNA product from the long-RNA product.

6. ADAPTATIONS OF THIS METHODOLOGY

6.1. RNA tags

Besides MS2, the tag used in this report, other natural RNA tags can be added to an RNA of interest, including the boxB sequence, which is recognized by the bacteriophage protein λ N; artificial RNA tags have also been developed, including the D8 Sephadex RNA motif (recognized by Sephadex) and the S1 Streptavidin RNA motif (recognized by streptavidin) [27]. These tags have different advantages and limitations and can be chosen depending on the molecular application, the type of detection required, the cell types studied, and other considerations.

6.2. Detection of associated RNA

In addition to testing associated miRNAs chosen 'a priori' because they are predicted computationally (as done in this report and in [28-31]), a number of other detection methods can be employed. A more global search to identify associated microRNAs can be carried out using microRNA microarray analysis, which is fast, reliable, and relatively low-cost. However, the most systematic approach to identifying comprehensively the associated microRNAs – and indeed all small RNAs (piRNA, tiRNA, snoRNA, siRNA, etc) – is by deep sequencing (RNAseq) of all associated RNAs. Evidently, if the RNAseq analysis includes long RNAs, this approach would also identify interacting mRNA, rRNA, and other lincRNAs, although associations among long RNAs are not well characterized at present.

6.3. Interaction with RBPs

If the interaction of RBPs with the tagged RNA is stable, the ribonucleoprotein immunoprecipitation (RIP) assay [32] is well suited for analysis of the associated RBPs. If the RBP-RNA interactions are transient, RIP analysis may not be sufficient [33, 34] and crosslinking of the RNP complexes (using methodologies reviewed recently in [35]) before pulldown may be necessary. Native or crosslinked, the systematic identification of RBPs interacting with a tagged RNA can be carried out by scaling up the pulldown assay and performing various proteomic approaches [e.g. by Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF) or by Tandem mass spectroscopy (MS)].

6.4. Localization of tagged RNA

If tracking the subcellular localization of the tagged RNA is of interest, the chimeric detection protein (MS2-GST) may be substituted for a fluorescent chimeric protein (e.g., MS2-GFP or MS2-YFP [36-38]) which can be tracked in the cytoplasm of yeast and mammalian cells. The subcellular localization can be studied by using live microscopy; it can also be studied in fixed-cell preparations, should it be necessary to study the colocalization of the fluorescent RNP with cellular proteins that need immunofluorescent detection (as described in [39]).

7. CONCLUDING REMARKS

As our understanding of post-transcriptional gene regulation increases, so does our realization that this process is startlingly complex and versatile. RNA-binding factors critically contribute to this rich regulation and hence there is escalating interest in elucidating their identity and influence on gene expression. The method described here, termed MS2-TRAP (MS2-tagged RNA affinity purification) permits the analysis of factors associated with RNAs in the context of the intact cell. Although the test RNA studied here is a lncRNA and the miRNAs associated with it, other RNA-RNA and RNA-protein interactions can be investigated using a similar approach. Besides its usefulness to characterize the transport and localization of RNPs, MS2-TRAP can be used to detect RNA complexes with biochemical readouts that assess systematically the composition of RNPs – i.e., the proteins and RNAs that interact with an RNA of interest. In light of the ever-expanding universe of RNA actions, we anticipate that the uses of MS2-TRAP will grow rapidly in the years ahead.

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Figure 1. Schematic of affinity purification and detection of MS2-tagged RNA

Methodology discussed in this report. **STEP 1**, plasmids pMS2 [expressing control *MS2* RNA, consisting of tandem 24 MS2 hairpins (red)], pMS2-GST [expressing a fusion protein that contains the MS2 RNA-recognizing portion (MS2, black), a region (GST, glutathione S-transferase, yellow) that recognizes the affinity purification reagent glutathione-SH (GSH), and a nuclear localization signal (NLS)], and pMS2-lincRNA-p21 [expressing the test RNA of interest (gray) tagged with 24 MS2 hairpins (*lincRNA-p21-MS2*)], were transfected into mouse embryonic fibroblasts (MEFs) for expression of the encoded protein and RNAs. Control cells **(1)** were transfected with plasmids to express control RNA (*MS2* RNA) and

reporter protein (MS2-GST), while test cells (2) were transfected with plasmids to express the experimental RNA of interest (*lincRNA-p21-MS2* RNA) and the reporter protein (MS2-GST). **STEP 2**, after formation of the RNP complexes [*MS2* RNA/MS2-GST in cells (1), and *lincRNA-p21-MS2* RNA/MS2-GST in cells (2)], cells were lysed and the complexes were affinity-purified by using GSH agarose beads. **STEP 3**, RNA was isolated from the complexes formed in cells (1) and (2) and RT-qPCR reaction was employed to detect associated microRNAs. The relative abundance of microRNAs in (2) relative to (1) was used to quantify the interaction of these miRNAs with the *lincRNA-p21* transcript.

Figure 2. Detection of RNA and protein components of the MS2 complexes

The levels of protein and RNA present in the MS2 RNP complex were studied after pulldown of MS2 complexes in the cultures transfected as in **Figure 1**. **(A)** The relative enrichment of *lincRNA-p21* RNA in the GSH beads was measured by RT-qPCR; as anticipated, *lincRNA-p21* RNA is enriched in the pulldown beads of transfection group (2) relative to those of transfection group (1); the levels of *gapdh* mRNA in each reaction were used to normalize sample input. **(B)** The levels of RBPs (Ago2, Rck, hnRNP C, and MS2-GST) in the MS2 pulldown material (*left*) and lysate (*right*) were detected by Western blot analysis.

Figure 3. SBI QuantiMir miRNA detection method

RNA prepared after MS2 pulldown is extended at the 3' end by addition of a poly(A) tail by using the enzyme poly(A) polymerase. After hybridization of the adaptor, first-strand synthesis is performed by reverse transcriptase (RT) and real-time quantitative (q)PCR analysis is carried out using a microRNA-specific primer (Table 2) and a universal primer.

Figure 4. *lincRNA*-p21-associated miRNAs

(A) In the pulldown materials prepared from the MEF transfection groups (2) *lincRNA*-p21-*MS2* and (1) *MS2* (Figure 1), the levels of predicted target miRNAs let7b, let7c, let7i, miR130, miR221, as well as non-target miR702 were measured by RT-qPCR analysis, performed as explained in Figure 3. The data represent the enrichment in miRNA levels in RNA from group (2) relative to RNA from group (1). (B) MEFs were transfected with the pre-miRNAs shown; 48 h later, the levels of endogenous mouse *lincRNA*-p21 in each transfection group [(2) vs (1)] were evaluated, normalized to the levels of *gapdh* mRNA, and represented as percentage of the values in transfection group (1). Data are the means and S.D. from three independent experiments; *P* values are shown

Table 1
Partial list of mouse miRNAs predicted to interact with mouse *lincRNA-p21*

The miRNAs (*left* column) and the sites on the *lincRNA-p21* where the miRNAs putatively interact (first and last nucleotides, *middle* and *right* columns, respectively) are listed. The list includes one negative control miRNA (miR702) which is not predicted to interact with *lincRNA-p21*.

miRNA	start	end
let7a-1/b/c/i	652	673
let7a-1/b/c/i	565	586
let7b	621	642
let7b	1044	1065
let7c	618	639
let7d	643	664
let7d	2922	2943
let7d	1462	1483
let7d	2033	2054
let7d	1418	1439
let7d	2184	2205
let7d	1407	1428
mir130b	1042	1063
mir143	88	107
mir206	728	748
mir22	92	113
mir22	641	662
mir221	2020	2042
mir320a	94	115
mir660	634	655
miR702	-	-

Table 2
Primers used for qPCR amplification

Table includes primers employed for the detection of microRNAs tested in this report, as well as forward (F) and reverse (R) primers to amplify *lincRNA-p21* RNA and *gapdh* mRNA.

Primer	Sequence
let7b	TGAGGTAGTAGGTTGTGTGGTT
let7c-1	TGAGGTAGTAGGTTGTATGGTT
let7i	TGAGGTAGTAGTTGTGCTGTT
miR130a	GCTCTTTTCACATTGTGCTACT
miR221	ACCTGGCATAACAATGTAGATTCTGT
miR702	TGCCCACCCTTTACCCCGCTC
U6	TGGCCCCTGCGCAAGGATG
lincRNA-p21 F	CCTGTCCACTCGCTTTC
lincRNA-p21 R	GGAACTGGAGACGGAATGTC
GAPDH F	GGGAAATTCAACGGCACAGT
GAPDH R	AGATGGTGATGGGCTTCCC