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ABSTRACT

Antisense transcripts were initially identified as transcriptional noise, but have since been reported to play an important role in the quality control of miRNA functions. In this report, we tested the hypothesis that hnRNP-K regulates miRNA function via competitive endogenous RNAs, such as pseudogenes, long non-coding RNAs, and antisense transcripts. Based on analyses of RNA sequencing data, the knockdown of hnRNP-K decreased antisense PTOV1-AS1 transcript, which harbors five binding sites for miR-1207-5p. We identified *heme oxygenase-1 (HO-1)* mRNA as a novel target of miR-1207-5p by western blotting and Ago2 immunoprecipitation. The knockdown of hnRNP-K or PTOV1-AS1 suppressed **HO-1** expression by increasing the enrichment of *HO-1* mRNA in miR-1207-5p-mediated miRISC. Downregulation of HO-1 by a miR-1207-5p mimic or knockdown of hnRNP-K and PTOV1-AS1 inhibited proliferation and clonogenic ability. Taken together, our results demonstrate that hnRNP-K-regulated PTOV1-AS1 modulates HO-1 expression via miR-1207-5p.

INTRODUCTION

Approximately 40% of annotated transcripts in humans are transcribed in both directions, indicating that antisense transcription occurs at a significant proportion of the human genome (1, 2). Antisense transcripts were initially classified as transcriptional noise (3). However, they have recently been identified as functional non-coding RNA and influence almost all steps of gene regulation, including transcriptional and post-transcriptional gene regulation. For tight control of gene expression, antisense transcripts compete with microRNAs (miRNAs) for binding to target mRNA and thus act as miRNA sponges.

miRNAs, which are small non-coding RNAs, generally suppress the expression of target genes by binding to partially complementary sequences (miRNA response elements, MREs) in the 3' untranslated region (UTR) of target mRNA (4). The activity of miRNAs can be affected by competitive endogenous RNAs (ceRNAs), which harbor MREs (5, 6). ceRNAs play a regulatory role in the action of single or multiple miRNAs by influencing the accessibility of miRNAs in target mRNAs. Therefore, they act as miRNA quality controllers that govern the expression level of miRNAs, influencing the suppression of target mRNAs.

Heme oxygenase-1 (HO-1, encoded by *HMOX1*) is an inducible liver microsomal protein and plays an important role in the cytoprotective machinery. HO-1 is commonly induced by cellular stresses, including heavy metals, endotoxin, hypoxia, nitric oxide, and ultraviolet irradiation (7). In cancer cells, HO-1 is highly expressed and upregulated by anti-cancer agents, making cancer cells resistant to treatment (8). Overexpression of HO-1 also confers resistance to oxidative stress and increases angiogenic potential (9).

Since hnRNPK plays a critical role in the proliferation and metastasis of various cancer cells, we here searched for hnRNPK-regulated antisense transcripts and their target

miRNAs. We determined that hnRNPK positively regulates the level of PTOV1-AS1, which harbors five binding sites for miR-1207-5p. The knockdown of hnRNPK or PTOV1-AS1 increased the enrichment of *HO-1* mRNA in miR-1207-5p-mediated miRISC and thus suppressed the expression of HO-1. Decreased HO-1 expression by a miR-1207-5p mimic or PTOV1-AS1 siRNA inhibited proliferation and clonogenic ability.

RESULTS

Knockdown of hnRNPK decreases *PTOV1-AS1* expression

Through our efforts to search hnRNPK-regulated long non-coding RNAs (lncRNAs), we found that a significant numbers of antisense transcript were regulated by hnRNPK including *EMC3-AS1*, *FLVCR1-AS1*, *VIM-AS1*, *ZBED5-AS1*, and so on. Among them, we here focused on the functions of *PTOV1-AS1* because its sense transcript *PTOV1* is known to function as an oncogene. To validate the effect of hnRNPK on *PTOV1-AS1* expression, HeLa cells were transfected with control or hnRNPK siRNA. Cells transfected with specific hnRNPK siRNA showed efficient decrease of hnRNPK (Fig. 1A). The level of *PTOV1-AS1* was assessed by RT-qPCR and semi-quantitative PCR (Fig. 1B). As expected, hnRNPK-silenced cells showed decreased expression of *PTOV1-AS1*. To examine the localization of *PTOV1-AS1*, cellular fractionation was performed. The levels of α -tubulin and lamin B were checked to confirm cytoplasmic and nuclear fractions (Fig. 1C, left panel). We found that *PTOV1-AS1* was preferentially located in the cytoplasm and therefore speculated that it might function with posttranscriptional gene regulators such as miRNAs (Fig. 1C, right panel). Ribonucleoprotein immunoprecipitation (IP) with an hnRNPK antibody (Fig. 1D) indicated that hnRNPK is able to interact with *PTOV1-AS1* (Fig. 1E). These results suggest that hnRNPK influences the expression of its target genes via *PTOV1-AS1*.

PTOV1-AS1 functions as a molecular decoy for miR-1207-5p

Several prediction programs were used to identify miRNA recognition motifs in *PTOV1-AS1* transcripts. Since *PTOV1-AS1* has five binding sites for miR-1207-5p, we hypothesized that hnRNPK is able to regulate the functions of miR-1207-5p via *PTOV1-AS1* (Fig. 2A). First, we evaluated the enrichment of *PTOV1-AS1* in Ago2 IP (Fig. 2B). As shown

in Fig. 2C, *PTOV1-AS1* was highly enriched in Ago2 IP, indicating that it is closely associated with miRNA-induced silencing complex (miRISC). Therefore, we compared and analyzed predicted miR-1207-5p target genes (TargetScan7.0) and RNA sequencing data (Fig. 2D). In total, 97 miR-1207-5p target genes were downregulated in hnRNPK-silenced cells (Suppl. fig. S1). We also checked the levels of several genes in PTOV1-AS1-silenced cells and chose heme oxygenase-1 (HO-1) for further studies. Based on our results, it was hypothesized that hnRNPK regulates PTOV1-AS1, which acts as a decoy for miR-1207-5p. To evaluate our hypothesis, several points need to be confirmed. First, we checked whether hnRNPK directly influences miR-1207-5p expression. The expression level of miR-1207-5p in hnRNPK-silenced cells was almost identical to that in control cells (Fig. 2E). Next, the effect of PTOV1-AS1 silencing on the expression of hnRNPK and miR-1207-5p was examined. Neither hnRNPK nor miR-1207-5p was affected by PTOV1-AS1 expression (Fig. 2F and 2G, respectively).

The hnRNPK/PTOV1-AS1/miR-1207-5p axis is implicated in the expression of HO-1

Based on the above results, we found that hnRNPK regulates the level of PTOV1-AS1 which has five miR-1207-5p binding sites. Therefore, we investigated whether miR-1207-5p increases the enrichment of *PTOV1-AS1* in Ago2 IP (Fig. 3A). The interaction of PTOV1-AS1 with miRISC increased in response to the overexpression of miR-1207-5p. Moreover, miR-1207-5p suppressed the expression of HO-1 at the protein and mRNA levels (Fig. 3B), indicating that miR-1207-5p is a novel HO-1-targeting miRNA. Next, we examined the effect of hnRNPK silencing on HO-1 expression. The knockdown of hnRNPK increased the level of *HO-1* mRNA in Ago2 IP (Fig. 3C) and decreased the protein and mRNA expression of HO-1 (Fig. 3D). Similar to the results obtained for hnRNPK, we observed that PTOV1-AS1-silenced cells showed highly enriched *HO-1* mRNA in the Ago2

IP (Fig. 3E), and the knockdown of PTOV1-AS1 decreased the levels of HO-1 at the protein and mRNA levels as well (Fig. 3F). In the absence of hnRNP K and PTOV1-AS1, miR-1207-5p easily accesses the 3' UTR of *HO-1* mRNA, resulting in the downregulation of HO-1. Taken together, these results demonstrated that hnRNP K regulates the expression of HO-1 via the miR-1207-5p decoy PTOV1-AS1.

The hnRNP K/PTOV1-AS1/miR-1207-5p/HO-1 axis is closely associated with proliferation

Since HO-1 is closely associated with the proliferation of cancer cells, we investigated the role of the hnRNP K/PTOV1/miR-1207-5p/HO-1 axis in cell proliferation. Accordingly, cells were transfected with *hnRNP K* siRNA (Fig. 4A-B), *PTOV1-AS1* siRNA (Fig. 4C-D), pre-miR-1207-5p (Fig. 4E-F), or HO-1 siRNA (Fig. 4G-H). Viable cells were quantified by an MTS assay after incubation for 48 h. As expected, all transfected cells showed decreased proliferation as compared to that of control cells (Fig. 4A, C, E, and G). The clonogenic ability of cells indicates survival and proliferation. Therefore, we tested the clonogenic abilities of transfected cells by a colony forming assay. Similar to the results of the MTS assay, decreased colony numbers were observed for all transfected cells (Fig. 4B, D, F, and H).

In summary, our results demonstrate that the knockdown of hnRNP K potentiates the inhibitory function of miR-1207-5p by downregulating miR-1207-5p decoying transcripts, i.e., PTOV1-AS1, resulting in decreased HO-1 expression. Moreover, the downregulation of HO-1 inhibits proliferation and clonogenic ability.

DISCUSSION

miRNAs are closely associated with multiple oncogenic processes, including proliferation, migration, invasion, and angiogenesis. In general, miRNAs induce the decay or translational suppression of target mRNAs via MREs in their 3' UTRs. The 3' UTRs of protein-coding transcripts are closely associated with cancer progression by post-transcriptional gene regulation. Alteration of the 3' UTR may influence not only the stability or translation of its own transcripts in *cis*, but also miRNA functions by sharing MREs in *trans* (10). Recently, ceRNAs have been identified as a new class of functional RNAs that act as miRNA sponges (11). ceRNAs, including several non-coding RNAs, such as pseudogenes, antisense long ncRNAs (lncRNAs), and circular RNAs, compete with target mRNAs to provide MREs to corresponding miRNAs, suggesting that they are important for the **quantity** control of miRNAs. For example, antisense transcripts for the β -APP-cleaving enzyme 1 gene (*BACE1*) hinder the miR-485-5p binding site of its sense transcript, which consequently inhibits miRNA decay and the translational suppression of *BACE1* (12). HOTAIR (HOX antisense intergenic RNA) is a well-studied antisense lncRNA transcribed from the *HOHC* locus from an intergenic position and in an antisense direction to the flanking *HOXC11* and *HOXC12* loci (13). It plays a functional role in oncogenic processes, influencing proliferation, mobility, invasion, and metastasis.

Prostate tumor overexpressed gene 1 (*PTOV1*) is overexpressed in the early and late stages of prostate cancer (14). *PTOV1* was initially identified in prostate cancer, but it has recently been reported to enhance the population of cancer stem cells via the activation of Wnt/ β -catenin signaling in breast cancer (15). *PTOV1* is also a key player in tumor progression and is associated with a poor prognosis in laryngeal squamous cell carcinoma, urothelial carcinoma, and ovarian cancer (16-18). In addition to the oncogenic roles of its

sense transcripts, our findings strongly suggest that antisense *PTOV1-AS1* is also involved in cancer proliferation via miR-1207-5p. We found that *PTOV1-AS1* is able to bind to and inhibit the function of miR-1207-5p. The knockdown of *PTOV1-AS1* did not influence the levels of hnRNPK and miR-1207-5p, but decreased the expression of HO-1 by enhancing the enrichment of its mRNA in miRISC.

HO-1 is highly expressed in various types of cancer, and its expression level is positively correlated with cancer progression and poor prognosis in patients (19). It is also highly expressed in tumor-associated macrophages, suggesting that HO-1 plays an important role in cancer microenvironments (20). Since cancer cells have high metabolic rates for rapid proliferation, they exhibit extremely high oxidative stress. Low expression of HO-1 exacerbates reactive oxygen species-induced DNA damage and accelerates the initiation of carcinogenesis. In addition, high levels of HO-1 are associated with rapid proliferation, invasiveness, and drug resistance (21-26). In response to various stimuli, such as growth factors and cytokines released from stromal cells, cancer cells express high levels of HO-1 via the activation of several transcriptional factors, including Nrf2, NF- κ B, and AP2 (27). In addition to transcriptional regulation, the expression level of HO-1 is also governed by a post-transcriptional mechanism. Suppression of HO-1 by miR-378 was reported to inhibit the proliferation and metastasis of lung carcinoma (28). In renal cell carcinoma, the downregulation of HO-1 by miR-200c makes cancer cells more susceptible to chemotherapeutic agents (24). In this report, we identified a novel HO-1-targeting miRNA, miR-1207-5p, involved in the inhibition of the proliferation and clonogenicity of HeLa cells. We also found that hnRNPK-regulated *PTOV1-AS1* acts as a molecular decoy for miR-1207-5p.

MATERIALS AND METHODS

Cell culture and transfection

Cervical carcinoma HeLa cells were cultured at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (GIBCO-BRL, Grand Island, NY, USA). For transient transfection of siRNA or miRNA, cells were plated at appropriate confluency and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. For hnRNPK silencing, a mixture of two different siRNAs targeting the coding region or 3' UTR was used. To reduce the level of PTOV1-AS1, specific siRNA was designed and synthesized (Bioneer, Daejeon, South Korea). The sequences of these siRNAs are provided in Supplementary table 1.

Western blot analysis

For western blot analyses, cells were rinsed with ice-cold phosphate-buffered saline (PBS) and lysed using radioimmunoprecipitation (RIPA) buffer containing protease inhibitors and phosphatase inhibitors (Roche, Basel, Switzerland) (10). Cell lysates were clarified by centrifugation, and the protein concentration was quantified using Bradford reagent (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of cell lysate were separated by SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After blocking with 5% skim milk (BD Biosciences, San Jose, CA, USA) for 1 hour at room temperature, membranes were incubated with the indicated primary antibodies overnight at 4°C, washed with Tris-buffered saline with

Tween 20 (TBST), and then incubated with the appropriate secondary antibodies for 1 hour at room temperature. Antibodies used for this study are provided in Supplementary table 2.

Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and used as a template to synthesize complementary DNA (cDNA) using the SuperScript III First-Strand Synthesis System (Invitrogen). The mRNA level was quantified by RT-qPCR (ABI Prism 7900) using power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The primers used in this study are provided in Supplementary table 3. The level of miRNA was assessed using miR-1207-5p-specific TaqMan primer (Applied Biosystems).

Ribonucleoprotein immunoprecipitation (RNP-IP)

For RNP-IP, Dynabeads® Protein G (Thermo Fisher Scientific, Rockford, IL, USA) were coated with control IgG (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) or hnRNP K antibody (Abcam, Cambridge, UK). For Ago2 IP, beads were coated with an Ago2 antibody (Sigma, St. Louis, MO, USA). Cytoplasmic lysates were prepared using PEB lysis buffer containing protease inhibitor, phosphatase inhibitor, and RNaseOUT (Invitrogen). Equal amounts of lysates were incubated with antibody-coated Dynabeads for 4 hours at 4°C. hnRNP K-IP materials were washed several times with PEB buffer and treated with DNase I (Ambion, Austin, TX, USA) and protease K (Bioneer, Daejeon, South Korea). The RNA was isolated with acid phenol (Ambion) and precipitated with absolute ethanol overnight at -20°C. The mRNA expression level was determined by RT-qPCR, as described above.

Subcellular fractionation

For isolation of the cytosolic and nuclear fraction, cells were harvested and lysed with digitonin-containing RSB buffer. After centrifugation at $2,000 \times g$ for 10 min, the supernatant was transferred to a new tube (Cytosolic extracts). The remaining pellet was washed five times with RSB buffer and lysed with RIAP buffer. Nuclear extracts were isolated by centrifugation at 13,200 rpm for 20 min.

Determination of cell proliferation and clonogenicity

Transfected cells were resuspended in 96-well plates (1×10^4 cells per well), and cells were then stained after 48 hours with MTS solution containing PMS according to the MTS Assay Kit protocol (Promega, Madison, WI, USA). To determine clonogenic ability, a colony-forming assay was performed. Briefly, transfected cells were seeded in triplicate in 6-well plates (3×10^2 cells per well) and incubated at 37°C and 5% CO_2 in complete medium for 2 wks. After incubation, cells were washed in cold PBS and fixed with 4% paraformaldehyde (Biosesang, Gyeonggi, South Korea) for 5 min, then washed 2 times in cold PBS. Fixed cells were stained with 0.2% crystal violet for 1 hour and washed 3 times in cold PBS, and then stained colonies were counted.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

The authors declare no competing financial interests

FIGURE LEGENDS

Figure 1. Knockdown of hnRNPK decreases the expression of PTOV1-AS1. **A-B.** To verify RNA sequencing data, HeLa cells were transfected with control (CTRL) or hnRNPK siRNA. The level of hnRNPK was assessed by western blotting (A) and the level of *PTOV1-AS1* was determined by RT-qPCR and semi-quantitative PCR (B: left and right panel, respectively). **C.** Cellular fractionation was performed as described in the Materials and Methods section. The levels of α -tubulin and lamin B were checked as cytoplasmic and nuclear markers, respectively. Localization of *PTOV1-AS1* was determined by RT-qPCR. **D-E.** Interaction of PTOV1-AS1 with hnRNPK was assessed by RNP IP as described in the Materials and Methods section. To verify efficient hnRNPK IP, western blotting was performed with IP materials (D). The level of *PTOV1-AS1* in the hnRNPK IP materials was determined by RT-qPCR and semi-quantitative PCR (E: left and right panel, respectively).

Figure 2. PTOV1-AS1 acts as an endogenous putative decoy for miR-1207-5p. **A.** Schematic diagram shows that *PTOV1-AS1* has five binding sites for miR-1207-5p. **B-C.** To check whether *PTOV1-AS1* is associated with miRNA-mediated RISC, Ago IP was performed (B) and the level of *PTOV1-AS1* was assessed by RT-qPCR (C). **D.** To identify common genes regulated by hnRNPK and miR-1207-5p, we compared and analyzed gene lists. In total, 97 genes were identified as putative target genes of PTOV1-AS1. **E.** To rule out the possibility that hnRNPK regulates miRNA biogenesis, the level of miR-1207-5p was determined by RT-qPCR in hnRNPK-silenced cells. **F-G.** HeLa cells were transfected with control (CTRL) or PTOV1-AS1 siRNA and the levels of hnRNPK and miR-1207-5p were determined by RT-qPCR.

Figure 3. The hnRNPK/PTOV1-AS1/miR-1207-5p axis is implicated in the expression of HO-1. To examine the effects of miR-1207-5p, hnRNPK, and PTOV1-AS1 on HO-1 expression, HeLa cells were transfected with miR-1207-5p mimic (A, B), siRNA targeting hnRNPK (C, D), or PTOV1-AS1 (E, F). The enrichment of *HO-1* mRNA in Ago2 IP was assessed by RT-qPCR in transfected cells (A, C, E). The protein and mRNA expression levels of HO-1 were determined by western blotting and RT-qPCR, respectively (B, D, F). Antibodies and primers used for these experiments are provided in Supplementary table 2 and 3, respectively.

Figure 4. HO-1 is involved in cell proliferation and clonogenicity. HeLa cells were transfected with hnRNPK siRNA (A, B), PTOV1-AS1 siRNA (C, D), pre-miR-1207-5p (E, F), or HO-1 siRNA (G, H). After 24 h post-transfection, cells were resuspended into 96-well plates for the MTS assay (A, C, E, and G) or in 6-well plates for the colony forming assay (B, D, F, and H). Cell proliferation and clonogenicity were determined as described in the Materials and Methods section.

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Shin et al. Figure 1

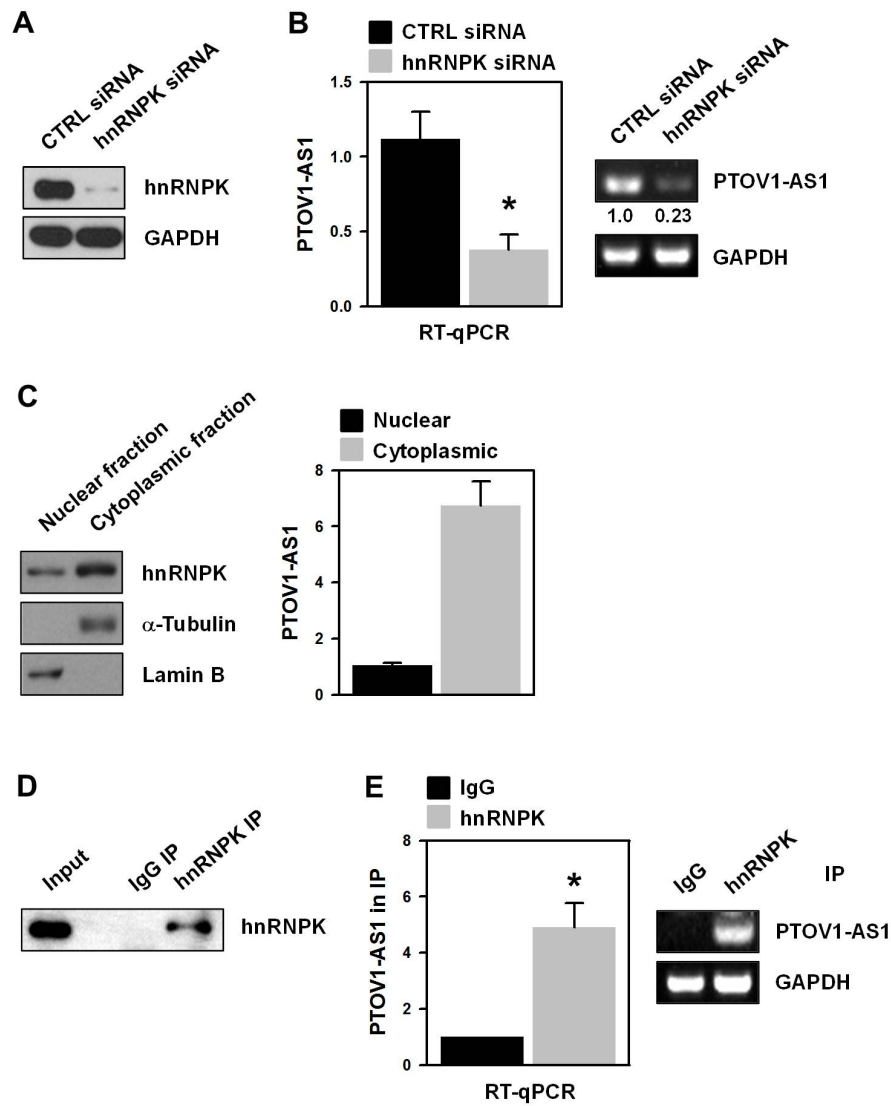


Fig. 1 Figure 1

Shin et al. Figure 2

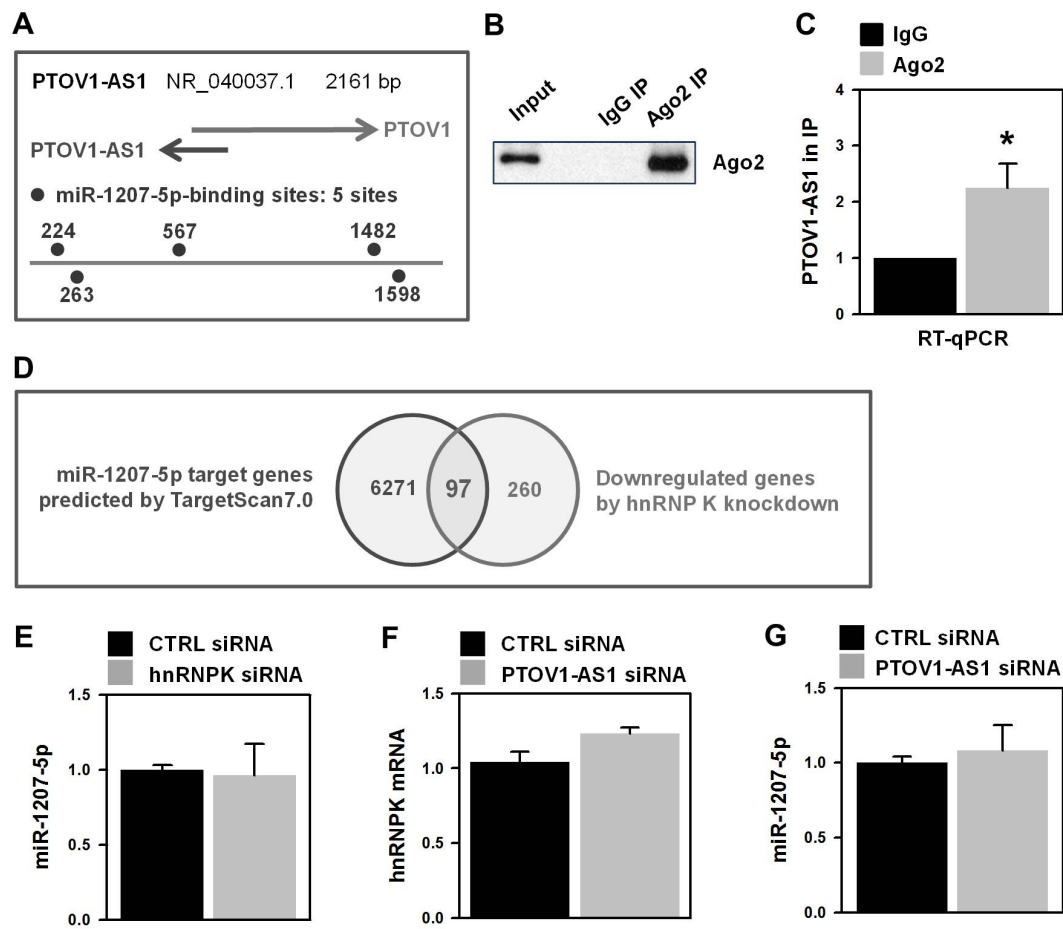


Fig. 2 Figure 2

Shin et al. Figure 3

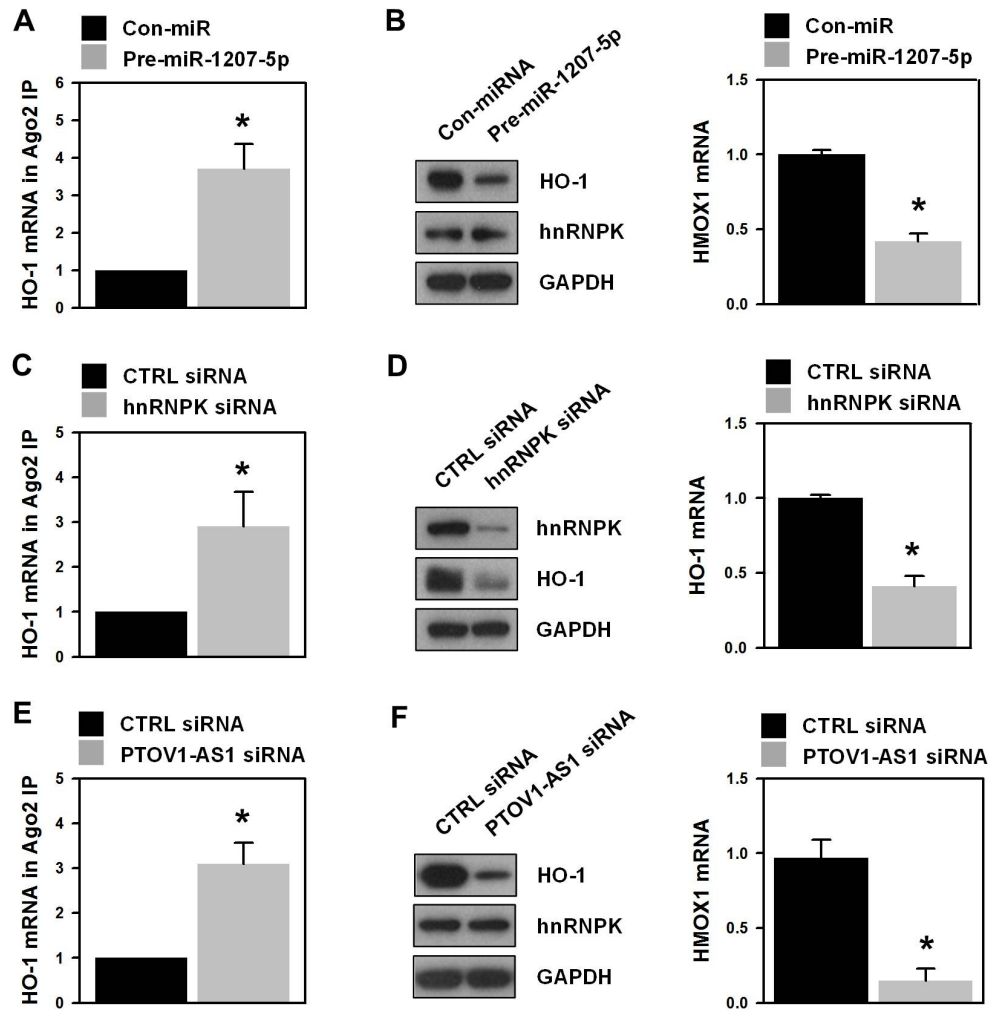


Fig. 3 Figure 3

Shin et al. Figure 4

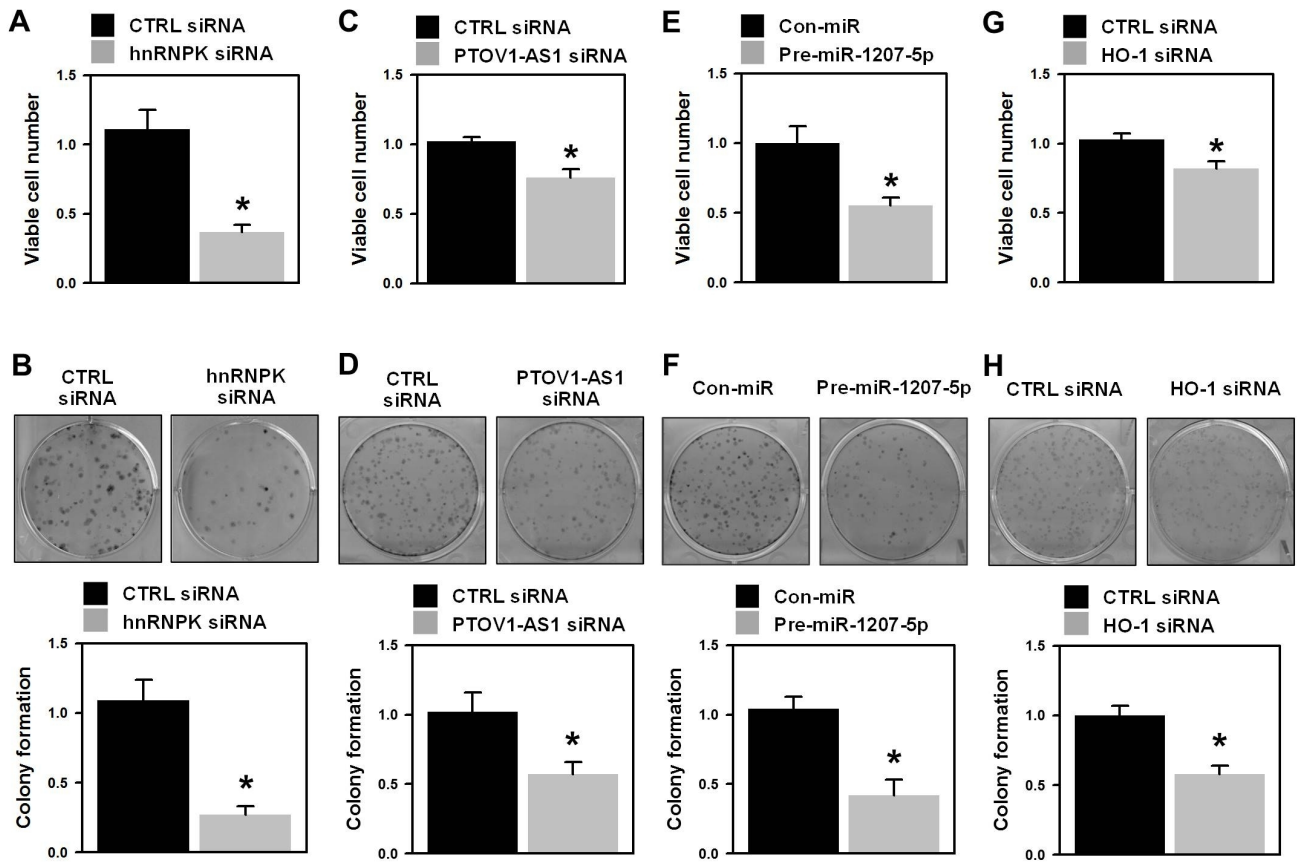
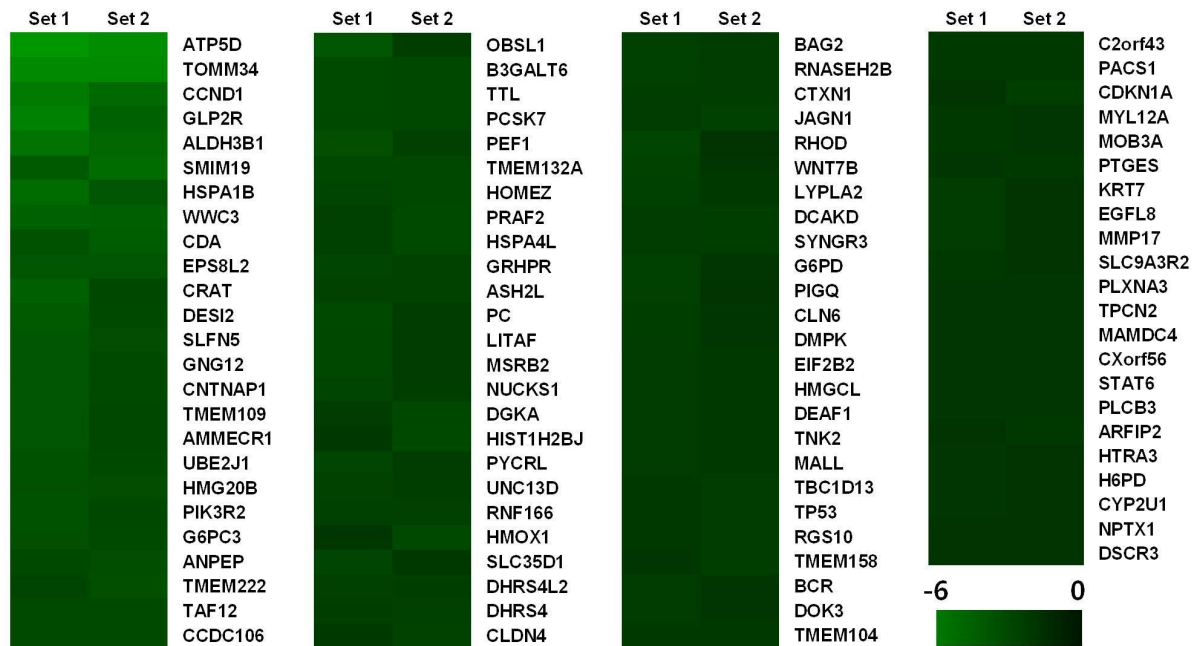


Fig. 4 Figure 4

Shin et al. Supplementary figure S1



Shin et al. Supplementary tables

Supplementary table 1. siRNA sequences

| Gene | Brand | Sequence |
|--------------|----------|---------------------------------|
| PTOV1-AS1 | Bioneer | 5'-CGUAGCUCUUACUAUGCUU(dTdT)-3' |
| HMOX1 | Bioneer | 5'-CAAAAAGAUUGCCCAGAAA(dTdT)-3 |
| hnRNPK/CDS | ST Pharm | 5'-GCAUAAAGAUCAUCCUUGA(dTdT)-3' |
| hnRNPK/3'UTR | ST Pharm | 5'-CCAACAUCCUCUGCUUCA(dTdT)-3' |

Supplementary table 2. Antibodies

| Protein | Brand | Cat | MW |
|---------------------------|------------|-------------|-------|
| HMOX1 | Bethyl | A303-662A-T | 32 kD |
| hnRNPK (D-6) | Santa Cruz | sc-28380 | 65 kD |
| hnRNPK (ChIP Grade) | Abcam | ab70492 | 65 kD |
| GFP (B34) | Santa Cruz | sc-73556 | 27 kD |
| Ago2 | Sigma | SAB4200085 | 85 kD |
| GAPDH (6C5) | Abcam | ab8245 | 37 kD |
| α -Tubulin (6A204) | Santa Cruz | sc-69969 | 55 kD |
| Lamin B (C-20) | Santa Cruz | sc-6216 | 62 kD |

Supplementary table 3. Primer sequences

| Gene | Forward Primer | Reverse Primer |
|-----------|----------------------|-----------------------|
| hnRNPK | GGCAGTGATTGGAAAAGGAG | CACTGCTGTCTGGGACTGAA |
| HMOX1 | ACATCTATGTGGCCCTGGAG | GTGCAGCTCTTCTGGGAAGT |
| PTOV1-AS1 | CTGTGGCTTTTGCTGATT | GTCGTTGCATCCATCATCAG |
| GAPDH | TGCACCACCAACTGCTTAGC | GGCATGGACTGTGGTCATGAG |
| 18S | AACCCGTTGAACCCATT | CCATCCAATCGGTAGTAGCG |