

# Export of microRNAs and microRNA-protective protein by mammalian cells

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## ABSTRACT

The discovery of microRNAs (miRNAs) as a new class of regulators of gene expression has triggered an explosion of research activities, but has left many unanswered questions about how this regulation functions and how it is integrated with other regulatory mechanisms. A number of miRNAs have been found to be present in plasma and other body fluids of humans and mice in surprisingly high concentrations. This observation was unexpected in two respects: first, the fact that these molecules are present at all outside the cell at significant concentrations and second, that these molecules appear to be stable outside of the cell. In light of this it has been suggested that the biological function of miRNAs may also extend outside of the cell and mediate cell–cell communication. We report here that after serum deprivation several human cell lines tested promptly export a substantial amount of miRNAs into the culture medium and the export process is largely energy dependent. The exported miRNAs are found both within and outside of the 16.5 and 120 K centrifugation pellets which contain most of the known cell-derived vesicles, the microvesicles and exosomes. We have identified some candidate proteins involved in this system, and one of these proteins may also play a role in protecting extracellular miRNAs from degradation. Our results point to a hitherto unrecognized and uncharacterized miRNA trafficking system in mammalian cells that is consistent with the cell–cell communication hypothesis.

## INTRODUCTION

MicroRNAs (MiRNAs) are evolutionarily conserved, single-stranded non-coding RNA molecules of ~19–23 nt in length (1–3). It has been shown that miRNAs affect the

stability of messenger RNA (mRNA) and in some cases influence protein translation through partial sequence complementation with their interacting mRNA targets (4–6). It is believed that miRNA is involved in several important processes, such as development (7–9), viral infection (10,11) and oncogenesis (12–14). Recently, miRNAs have been found in extracellular space including plasma/serum, saliva and urine in significant concentrations (15–20).

The finding of stable extracellular miRNAs in plasma and other body fluid types raises the question of the function of such external miRNA. It suggests the possibility that they are involved in mediating cell–cell communication. This would imply that the miRNAs convey specific information, and therefore that only some cellular miRNAs are exported or released from cells in response to biological stimuli. If it exists, this miRNA export mechanism would likely be a universal phenomenon, affecting all cell types. The exporting cells would have to be able to (i) identify and select specific miRNAs for export in response to the state of the exporting cell, (ii) package and protect the miRNAs from the hostile extracellular environment and (iii) tag and transport these miRNA ‘packages’ to the extracellular space in a form suitable to be taken up by targeted cells. To our knowledge no such mechanism has yet been described or even hypothesized.

The existence of such a communication system was investigated using a mammalian cell culture model. The cells were abruptly switched to a serum-free medium, and the culture medium was collected to assess the extracellular miRNA spectrum and its dynamics in response to stress. Most of the miRNAs we tested appeared to be actively exported in a short pulse lasting about an hour after serum deprivation, followed by a very modest decline in concentration up to 48 h. Using a differential centrifugation approach to fractionate the medium, we found significant amounts of extracellular miRNAs that are external to known cell-derived vesicles such as microvesicles or exosomes. Exposing the cells to various chemical inhibitors, we showed that the exportation

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process is largely energy dependent. Furthermore, we found that cells release a significant number of RNA-binding proteins into the culture medium and one of them, nucleophosmin 1 (NPM1), can protect miRNA from degradation. We suggest that this protein may be involved in the above-hypothesized system and could play a role in the exportation, packaging and protection of extracellular miRNAs.

## MATERIALS AND METHODS

### Cell culture

The HepG2, A549, T98 and BSEA2B cells were obtained from The American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in the recommended medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C under 5% CO<sub>2</sub>. We determined that this strain of HepG2 does not express CYP2E1. The primary human pulmonary fibroblast cells were obtained from Sciencell Research Laboratories (Sciencell, Carlsbad, CA, USA) and grown in Fibroblast medium (Sciencell) on collagen I coated cell culture flasks (Becton Dickinson, Franklin Lakes, NJ). For serum depletion experiments, the cells were plated and grown for 24 h with 10% FBS containing media prior to switching to serum-free media. Serum-free medium contained the base medium (Invitrogen) with 100U/ml penicillin and 100µg/ml streptomycin. For rotenone, brefeldin A and 5-N, N-dimethyl amiloride treatments, the cell were inoculated and grown for 24h with 10% FBS prior to exposing the cells with different concentration of drugs for 30 min. Lactate dehydrogenase (LDH) levels were determined with the LDH-Cytotoxicity assay kit from Abcam (Abcam Inc., Cambridge, MA, USA) and ATP levels were determined with the ENLITEN ATP assay system (Promega, Madison, WI, USA) using manufacturer's protocol.

### RNA extraction

Total RNA including miRNA was isolated using miRNeasy kit (Qiagen, Germantown, MD, USA) with minor modifications that have been described earlier (21). In summary, 700 µl of QIAzol reagent was added to 300 µl of culture medium samples that were spun at 1000g for 10 min to remove floating cells or cell debris, followed by addition of 140 µl of chloroform. The samples were mixed vigorously for 15 s and centrifuged at 12000g for 15 min at 4°C to separate the aqueous and organic phases. After transferring the aqueous phase to a new collection tube, 1.5 volumes of ethanol were added and the samples were then applied to RNeasy mini column. Total RNA was eluted from the membrane with 30 µl of RNase-free water.

### Quantitative RT-PCR

The cDNA was generated using the miScript Reverse Transcription kit (Qiagen). In brief, ~100 ng of RNAs were polyadenylated by using poly (A) polymerase and

cDNA was generated with reverse transcriptase using a tag containing oligo-dT primers. The tag on oligo-dT primers served as universal primer in qPCR step. Human miScript Assay 384 set v10.1 (Qiagen) was used for real-time PCR analysis. To reduce pipetting error, the Matrix Hydra eDrop (Thermo Scientific, Hudson, NH, USA) was used to mix the cDNA sample and qPCR master reagent. The data was analyzed by SDS Enterprise Database 2.3 (Applied Biosystems, Foster City, CA, USA).

### Microarrays

MiRNA microarrays were performed using the manufacturer's (Agilent, Santa Clara, CA, USA) protocol as described earlier (21). Hundred nanograms of total RNA was dephosphorylated with calf intestinal alkaline phosphate, and denatured with heat in the presence of dimethyl sulfoxide. T4 RNA ligase added the Cyanine 3-cytidine biphosphate (pCp) to the dephosphorylated single stranded RNA. MicroBioSpin 6 columns (Bio-Rad, Hercules, CA, USA) were used to remove any unincorporated cyanine dye from the samples. The purified labeled miRNA probes were hybridized to human miRNA V2 oligo microarrays in a rotating hybridization oven at 10 r.p.m. for 20 h at 55°C. After hybridization, the arrays were washed in Agilent GE Wash Buffer 1 and 2 with Triton X-102. Then the array slides were dried immediately by a nitrogen stream and scanned at 5-um resolution by using a PerkinElmer ScanArray Express array scanner.

### Fractionation of culture medium

The cells were grown as stated earlier. The serum free media were collected and centrifuged at 1000g for 10 min to remove cells. This supernatant (25 ml) was transferred to a new tube and spun at 16Kg for 60 min, the pellet microvesicles, were washed and resuspended in phosphate-buffered saline (PBS, 137mM NaCl, 2.7mM KCl, 10 mM Sodium Phosphate dibasic, 2mM Potassium Phosphate monobasic at pH of 7.4). The supernatant of the 16Kg spin was transferred to a new tube and further centrifuged at 120Kg for 60 min to pellet the exosome particles. The exosome depleted supernatant was then spun at 220Kg for 60 min. The final supernatant was concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA, USA) to a final volume of 0.5 ml. The pellets, microvesicles, exosomes, and 220Kg pellet were resuspended in 0.5ml PBS, so that the total volume of medium contributing to each was identical.

### Mass spectrometry sample preparation and data analysis

Cells were grown as above and the serum free media was concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore). The concentrated media was enzymatically digested with trypsin and desalted with C18 Ultramicrospin columns (The Nest Group, Southborough, MA, USA). After drying in a speedvac (Thermo Scientific, Waltham, MA, USA) the sample was re-suspended and run on Q-TOF Ultima API Mass Spectrometer (Waters, Bedford, MA, USA). The results

were analyzed using SEQUEST (v.27) against a human International Protein Index (IPI) database (v.3.38).

### miRNA protection assay and western blots

To test the ability of protecting miRNA from RNase by RNA-binding protein, synthetic mir-122 RNA (100 pmol) was mixed with different proteins, NPM1 (3 pmol), TGF- $\beta$  (4 pmol) or BSA (1.5 nmol) for 30 min followed by adding RNase A (7 nmol) for another 30 min of incubation at 37°C. The miRNA from each condition were then purified by miRNeasy kit and the miRNA levels were determined by qPCR. Control experiments including omitting RNase A, protein or miRNA were also included.

Concentrated medium and pellet samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were transferred to a nitrocellulose membrane (Bio-Rad). Human NPM1 was detected on the western blot blocked with 5% non-fat dry milk using an anti-NPM1 monoclonal antibody (Sigma, St Louis, MO, USA). Near-infrared IRDye 680 labeled secondary antibodies (Li-Cor, Lincoln, NE, USA) were used to visualize the NPM1 antibody. The membrane was scanned using the Odyssey infrared imaging system (Li-Cor).

## RESULTS AND DISCUSSION

To investigate the miRNA mediated cell–cell communication system, we tested whether cells in culture can still export specific miRNAs. It is not surprising that the different cell lines examined all release a significant population of extra-cellular miRNAs (Figure 1a) and they exhibit striking differences between the cellular and extracellular miRNA spectra (groups marked as ‘out’ or ‘in’ on the figure for HepG2). Validation and crosschecking of the array results was performed for HepG2 and A549 by qPCR on selected miRNAs, as indicated in Figure 1b. While there could be several possible explanations for this difference between cellular and extracellular miRNA population, including cell lysis and preferential stability inside or outside of the cell, these results are consistent with a specific packaging and exporting hypothesis for extracellular miRNAs. By measuring the levels of LDH in the medium at several time points, and examining the cells and culture media microscopically, we ruled out significant cell lysis during the experimental period, up to 48 h of culture with or without serum in the medium. Finding no detectable evidence of significant cell lysis and a difference between the intracellular and extracellular miRNA spectra, we concluded that the external miRNAs are most likely exported from intact cells by specific cellular mechanisms.

The elimination of serum from the culture medium provides a strong biological stimulus to cultured cells. When so stimulated, cells must alter their internal states significantly, leading to cell cycle arrest, and in some cases cell death, during extended periods of serum depletion (22–24). We hypothesized that cells could respond to this change of state, in part, by exporting miRNAs

immediately after deprivation of serum. To test this, we carefully characterized the miRNA spectra of two different well-established cell lines, A549 and HepG2, which were derived from different sources. HepG2 is from human hepatocellular carcinoma (25), and A549 is from lung epithelial carcinoma (26). The absence of bovine serum, and the miRNAs it contains, is a distinct advantage of using serum depletion to study extracellular miRNA as it removes a potential source of interference. There are 499 and 578 observable miRNA species in A549 cells and HepG2 cells, respectively. Among these, there are 476 miRNAs in common between the two cell lines. Fewer than 5% of the miRNAs observed in A549 cells are not observed in HepG2, and <18% of the miRNAs observed in HepG2 are not observed in A549. In the culture media, the percentages are similar (2% of the miRNAs observed from A549 are not observed in HepG2, and 15% of the miRNAs observed from HepG2 are not observed in A549). Based on the global miRNA profiling results, we selected a set of 30 miRNAs that showed good signals and consistent measurement profiles using qPCR to study in more detail.

In order to track the cellular responses carefully, we carried out a time series of measurements from just before serum deprivation (SD) to 48 h after SD. This revealed some dramatic differences in the profile of external miRNAs, not only among specific species, but also some differences between cell lines. If we compare the changes in levels of certain miRNAs inside and outside of the cells, before and after serum deprivation, the rate of export for most of the miRNAs examined increases dramatically during the first 2 h after SD and declines thereafter (Figure 2).

### The extracellular miRNAs derived from cultured cells are stable

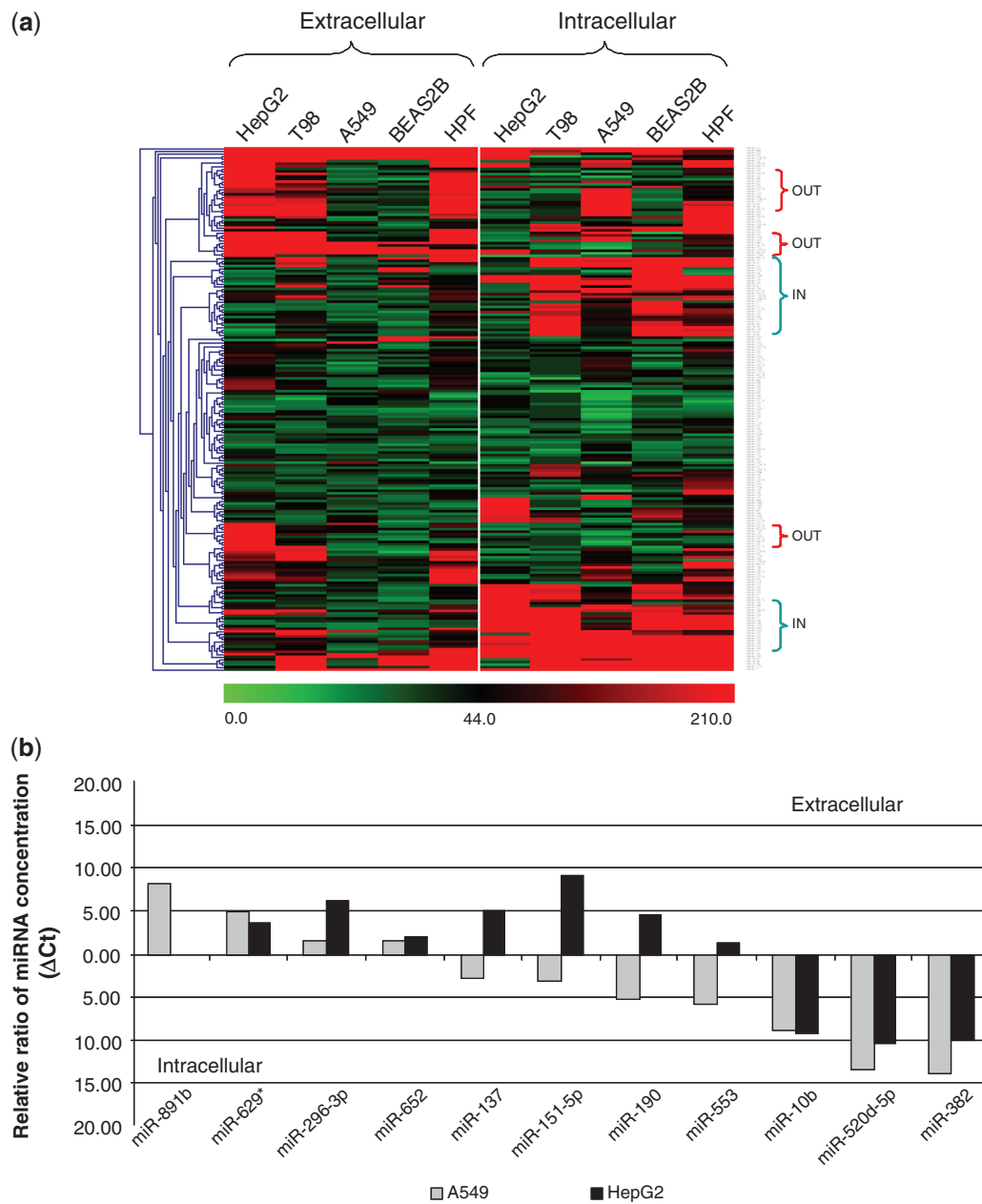
One observation from these data was that some miRNAs exhibited a modest decline in concentration in the medium after increasing strongly in the first few hours (Figure 2). To determine whether these miRNAs were specifically being degraded in the serum-free culture medium, we carried out extensive time course measurements on the decay of miRNAs in media from which the cells were removed. The data clearly show that different miRNAs decay at different rates (Figure 3a). The decay of miRNAs in the medium over many hours is readily detectable, but not severe for most miRNA species. The rate of decay can be measured by

$$m_i(t) = m_i(0)2^{-r_i t}$$

where  $m_i(t)$  is the level of miRNA ‘ $i$ ’ at time ‘ $t$ ’ and  $r_i$  is the decay rate for miRNA ‘ $i$ ’. The overall decay rate doesn’t appear to be >0.02/h (Supplemental Figure S1a).

### Is the exportation of miRNA immediately after serum deprivation from a pre-synthesized miRNA pool?

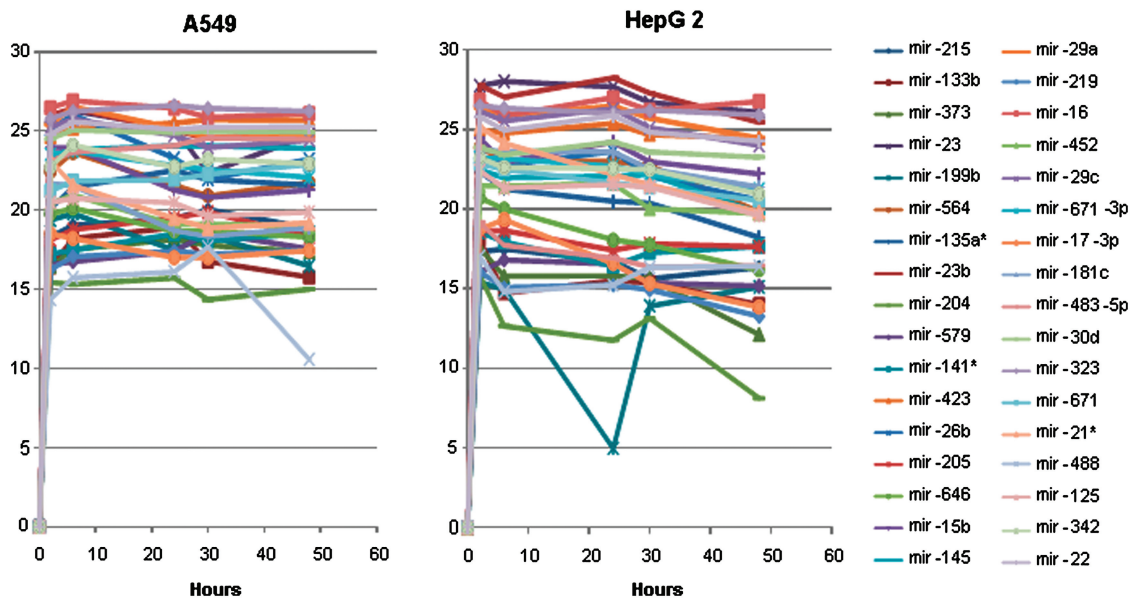
The time series data, taken together with the miRNA decay measurements, enables us to estimate the quantitative kinetics of the export of miRNA from cells after SD. Since the levels rise rapidly for approximately the first 2 h



**Figure 1.** Differential miRNA spectra between cultured cells and the medium. (a) Intra- and extracellular miRNA spectra for six different cell lines in culture as measured by miRNA arrays. The cell lines are as indicated (T98 is derived from human glioblastoma multiforma, BEAS2B is derived from lung bronchial epithelium and HPF are primary pulmonary fibroblast cells isolated from human lung tissue). Heat maps represent the levels of miRNA for the cell lines as indicated (red highest, green lowest). The brackets indicating 'in' or 'out' mark groups of miRNAs that show striking differences between the intra ('in') and extra ('out') cellular concentrations specifically for HepG2 cells. (b) qPCR measurements of selected miRNAs shown in 1a that illustrate different profiles for HepG2 (red) and A549 (blue). The histogram shows the external versus internal levels of selected miRNAs. The extracellular levels are indicated above, the intracellular below.

after SD and then remain roughly constant or decay somewhat, we conclude that for these miRNAs there is a strong pulse of miRNA exportation that rapidly subsides, accompanied by the steady, but relatively slow, decay of the miRNA outside the cell. Kinetic modeling shows that most of our miRNA export data is consistent with the scheme illustrated in Figure 3b. This kinetic profile, with a rapid rise in initial export rate after SD, raises the question of whether or not the miRNA exported is pre-synthesized and prepackaged.

To further investigate the early response from cells after SD, we measured the intracellular levels as well as the extracellular exported miRNA levels in the medium with a large set of miRNAs from both cell lines. We illustrate our general observations with some representative miRNAs in Figure 3c: an initial drop in cellular miRNA levels occurs simultaneously with a strong increase in miRNA levels in serum free culture media. The qPCR data shown here also indicates a recovery of intracellular miRNA levels after a few hours (2–6 h). This experimental



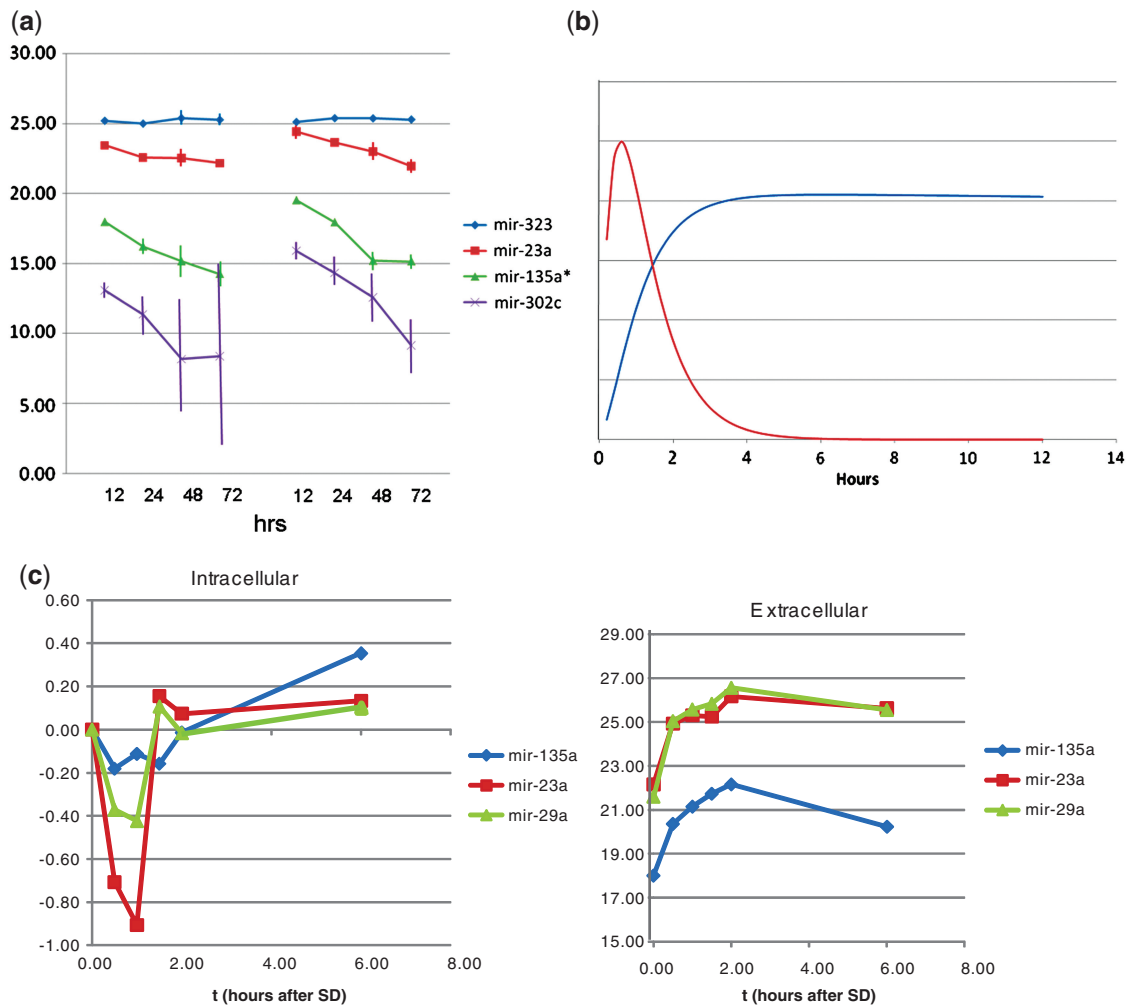
**Figure 2.** Time course of levels of miRNA exported into the medium. Samples were taken at various indicated time points after serum deprivation. The serum free medium contains no proteins or miRNAs. Most of the miRNA measured shown very similar behavior. The levels of 16 miRNAs measured by quantitative PCR from A549 (left frame) and HepG2 (right frame). The points are the means of three biological replicates. All vertical axes, the levels of miRNAs, are in  $\Delta C_t$ .

data fits perfectly with the kinetic model illustrated in Figure 3b. This data also allows us to obtain an expanded view of the behavior of the dynamical levels of miRNAs during the first few hours after SD, inside and outside of the cell, which illustrates the precise behavior expected from the kinetic model we propose (Supplemental Figure S1b). In addition, it seems that the levels of intracellular miRNA overshoot then tend to relax back to their original levels (Figure 3c). Even though we could not definitively rule out the possibility that the rapid changes in intra- and extracellular miRNA spectra we observed are due to some cell lysis, the data strongly suggests that most of the exported miRNAs are initially from a pre-synthesized pool since the substantial exportation of miRNA in the first hour is unlikely to come from newly transcribed and processed miRNA. The initial reduction in intracellular levels is consistent with this view. This observation raises an intriguing possibility of the existence of specific cellular compartment(s) to store pre-processed and prepackaged miRNA. This hypothesis will be carefully investigated in the future.

#### Significant fraction of extracellular miRNAs resides outside of previously described vesicles

The observations of miRNA outside of cells have focused on known cell-derived vesicles, like microvesicles and exosomes (17–19). In the serum deprivation system used here, we were able to examine the question of how the exported miRNA are contained or packaged in more detail. The absence of interfering bovine miRNAs, proteins, and other bovine serum derived biological products in the serum-free medium enables quantitative measurements that are more accurate. Using well-developed differential-centrifugation-based methods

for microvesicle and exosome isolation (27,28), we fractionated the media isolated from the cultures 2 h after SD (the time point which has the highest level of extracellular miRNA based on our kinetic study, Figure 3), into four fractions (Figure 4a). The microvesicle (16.5 K pellet), exosome (120 K pellet), ‘hard spin’ pellet (this is the pellet of a final 220 Kg spin for 1 h), and supernatant fractions were then assayed for the presence of the miRNA species. The results of these measurements (with three biological replicates—repeated experiments) were surprising in several respects (Figure 4b and c). First, counter to the expectation that miRNAs are confined to previously described cell-derived vesicles; significant concentrations of miRNAs are in the final supernatant fraction. The second surprise is that many, but not all, of the miRNAs are also found both in the vesicles and the final supernatant. The patterns of distribution showed a strong diversity of profiles, falling into three to four distinct patterns (Figure 4b for A549 and 4c for HepG2). There are several miRNAs that are found in almost equal proportions in all four fractions, while several others are almost completely absent from all but the final supernatant fraction. This observation strongly counters the idea that the presence of miRNA in the final supernatant is due to disruption of cell-derived vesicles during fractionation procedures. The data also suggest that many of the vesicles remain intact, as evidenced by the high levels of some miRNAs seen in this fraction. Therefore, the absence of some of the miRNAs, present in the supernatant, in the vesicle fraction cannot be explained simply by ruptured vesicles during sample processing. Thus, while the measurements from all of these fractions demonstrate some experimental noise, it is clear that the dramatic quantitative differences represent real



**Figure 3.** Decay of miRNAs in the medium. The cells in culture were serum deprived for 2h, then the medium was removed and incubated under the same conditions for varying periods of time. Specific miRNA levels were measured (three biological replicates) with quantitative PCR on each sample. (a) The time course measurements of four selected miRNAs with standard derivations showing diverse profiles. (b) Model kinetics for the export response of the majority of the miRNAs considered here, showing the rate of export as a function of time (in red) and the accumulated level of extracellular miRNA as a function of time (in blue). (c) the intracellular response in HepG2 cells of three selected miRNAs.

qualitative differences between miRNA distributions. These observations of miRNA distribution demonstrate that the miRNA export system in the cells must somehow be able to target specific miRNAs to specific extracellular structures, but does not exclusively channel specific miRNAs to a given fraction. There are, however, some miRNAs that are found in the supernatant almost to the exclusion of all other fractions (miR-219 in A549 culture medium, Figure 4b, for example). Another significant observation from this data is that even though we showed that the two cell lines studied here export a similar spectrum of miRNAs (Figure 1b) the distributions of miRNAs from the two cell lines demonstrate clear differences, as shown in Figure 4b and c. For example, miR-145 is present at very low levels in HepG2 derived microvesicles (Figure 4c), while the same miRNA is present at significant levels in both A549 derived microvesicles and exosomes (Figure 4b). On the other hand, there are some miRNAs, notably those that are almost uniformly distributed among the fractions, with

the same distribution no matter which cell-line is the source. These observations certainly indicate the degree of complexity of this export system that will bear further investigation.

#### Intracellular ATP levels affect the exportation of miRNA

To further investigate the miRNA export process, we treated the HepG2 cells with different concentration of rotenone, a respiratory chain inhibitor, to reduce the cellular ATP level (29), brefeldin A (BFA), an inhibitor of protein secretion through the interference with the Golgi apparatus (30), and 5-N, N-dimethyl amiloride (DMA), an inhibitor of exosome secretion through the inhibition of  $H^+/Na^+$  and  $Na^+/Ca^{2+}$  exchangers (31), for 30 min prior to SD. As expected, the cellular ATP level showed a significant decrease in rotenone treated cells while the LDH levels in media showed no significant changes in all three treatments. We examined the levels of select miRNAs with qPCR in both cells and culture

media and found that the level of miRNAs inside the cells showed no significant changes in all the treatments. However, the levels of most of the extracellular miRNAs examined displayed a dose dependent decrease with rotenone treatment only (Supplemental Figure S2). This finding suggests that: (i) most of the miRNA exporting process is an energy dependent, active transport process, and (ii) during the time frame examined, the export of miRNA is not affected in any significant way either by BFA, independent of the Golgi apparatus, and DMA, an inhibitor of exosome formation. It is interesting to note that the extracellular levels of the mir-671-3P and mir-943 are not affected by a decrease in cellular ATP level, suggesting that the export pathway is complex and probably involves multiple branches.

#### A number of RNA-binding proteins were released into the medium by the cells

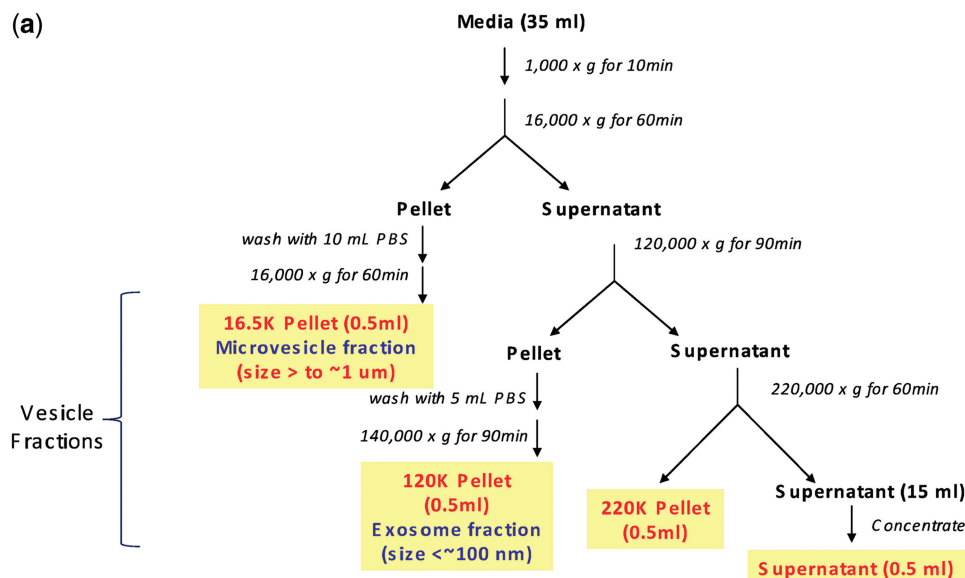
To examine the protein content of the exported miRNA complexes, we collected and concentrated the primary human fibroblast medium 2 h after SD. The proteins were digested with trypsin and then profiled by mass spectrometry. A total of 197 proteins that are represented by more than one peptide were identified. Of these, 12 were known RNA-binding proteins (Table 1). Besides several ribosomal proteins, it is notable that there was a substantial level of a known nucleolar RNA-binding protein, nucleophosmin 1 (NPM1), in the medium. Nucleophosmin 1 is implicated in the nuclear export of the ribosome (32,33). There was also a significant amount of nucleolin, a known NPM1 interacting protein (32,34), in the medium. Since NPM1 is known to be located primarily in the nucleolus and involved

ribosomal RNA processing, it is surprising to observe it in significant levels outside the cell. Using an antibody to nucleophosmin 1, we identified the presence of NPM1 protein in the culture media from other cell lines.

#### Nucleophosmin binds miRNA and may play a role in protecting miRNA from degradation

The vast majority of the NPM1 protein is present in the supernatant fraction (as defined in Figure 4a), and is undetectable by western blot in either vesicle fraction (Figure 5a). The distribution of the NPM1 protein parallels the distribution of miRNAs—by far the largest concentration is found in the supernatant (Figure 5b). The presence of a RNA-binding protein in this fraction, where most of the miRNAs are found, suggests this protein may bind miRNAs. We carried out a number of experiments, including gel shifts and immunoprecipitations that confirm the binding of nucleophosmin to miRNAs.

To see whether NPM1 is involved in the unexpected stability of the extracellular miRNA, we tested its ability to protect miRNA from RNase. The NPM1 protein was incubated with synthetic miR-122 RNA prior to RNaseA challenge. The RNA was then isolated and assessed by qPCR. The result showed that NPM1 alone can fully protect this miRNA from RNaseA digestion (Figure 5c). It has been suggested that NPM1 may be involved in shuttling RNAs and ribosomal proteins to the cytosol (32,33,35), and in a recent report, the NPM1 protein also been found outside the cell (36). This shuttling mechanism may be relevant to a possible role in miRNA export. Our findings collectively are highly suggestive



**Figure 4.** miRNA levels in fractionated serum-free culture medium for A549 and HepG2. The medium, 2 h after serum deprivation, was fractionated by differential centrifugation into four fractions, microvesicles (size range: 100–1000 nm), exosomes (size range: 30–100 nm), one higher g spin pellet (220K for 1 h), and the remaining supernatant. The final supernatant were also resuspended in 0.5 ml of PBS and 200  $\mu$ l of the concentrated media were used for miRNA analysis. Pellets were also resuspended in 0.5 ml of PBS and 200  $\mu$ l of the solution were used for miRNA isolation. (a) Experimental design for fractionation of medium. The levels of miRNA distribution from A549 (b) and HepG2 (c) were grouped into distinct distribution profiles.

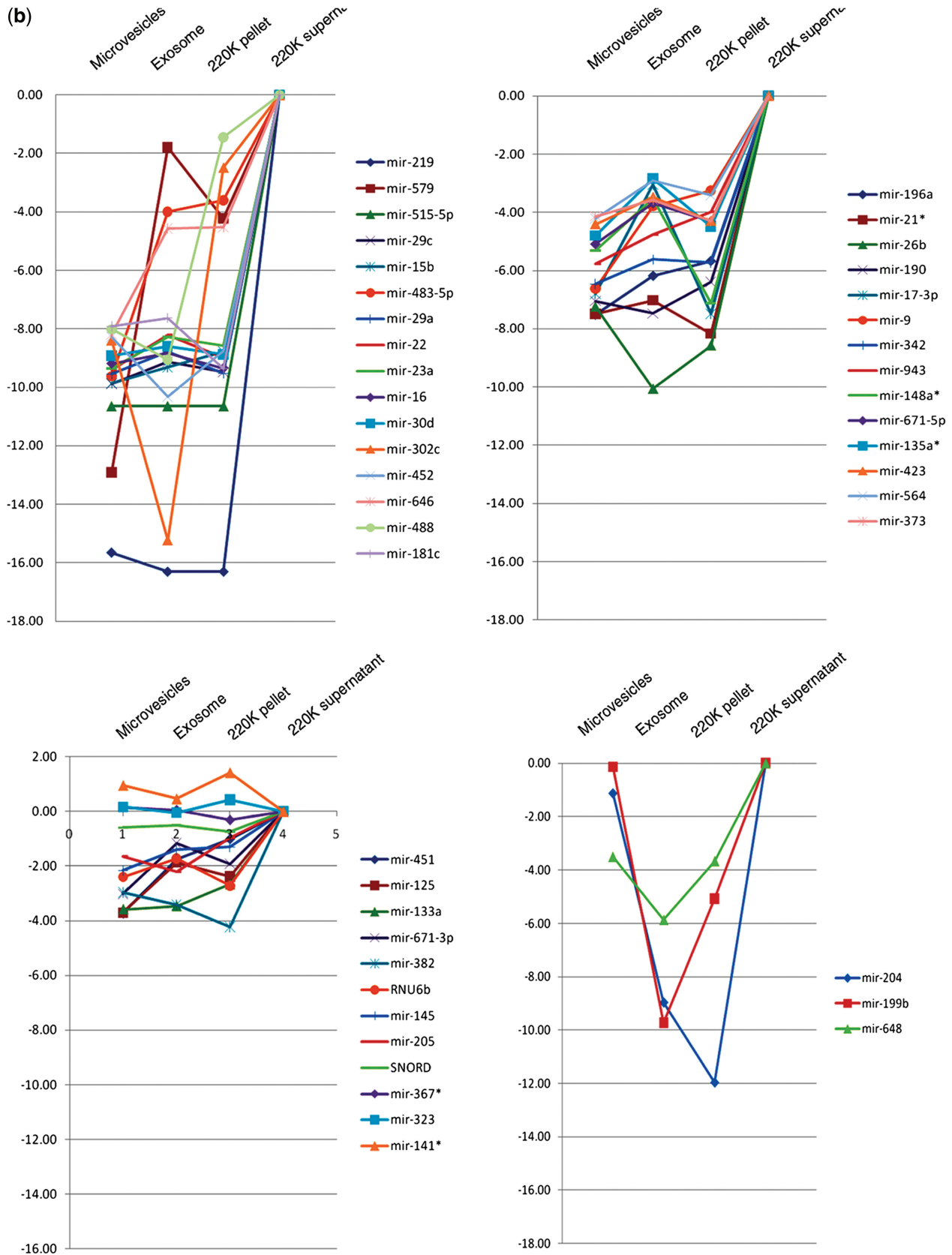


Figure 4. Continued.



(c)

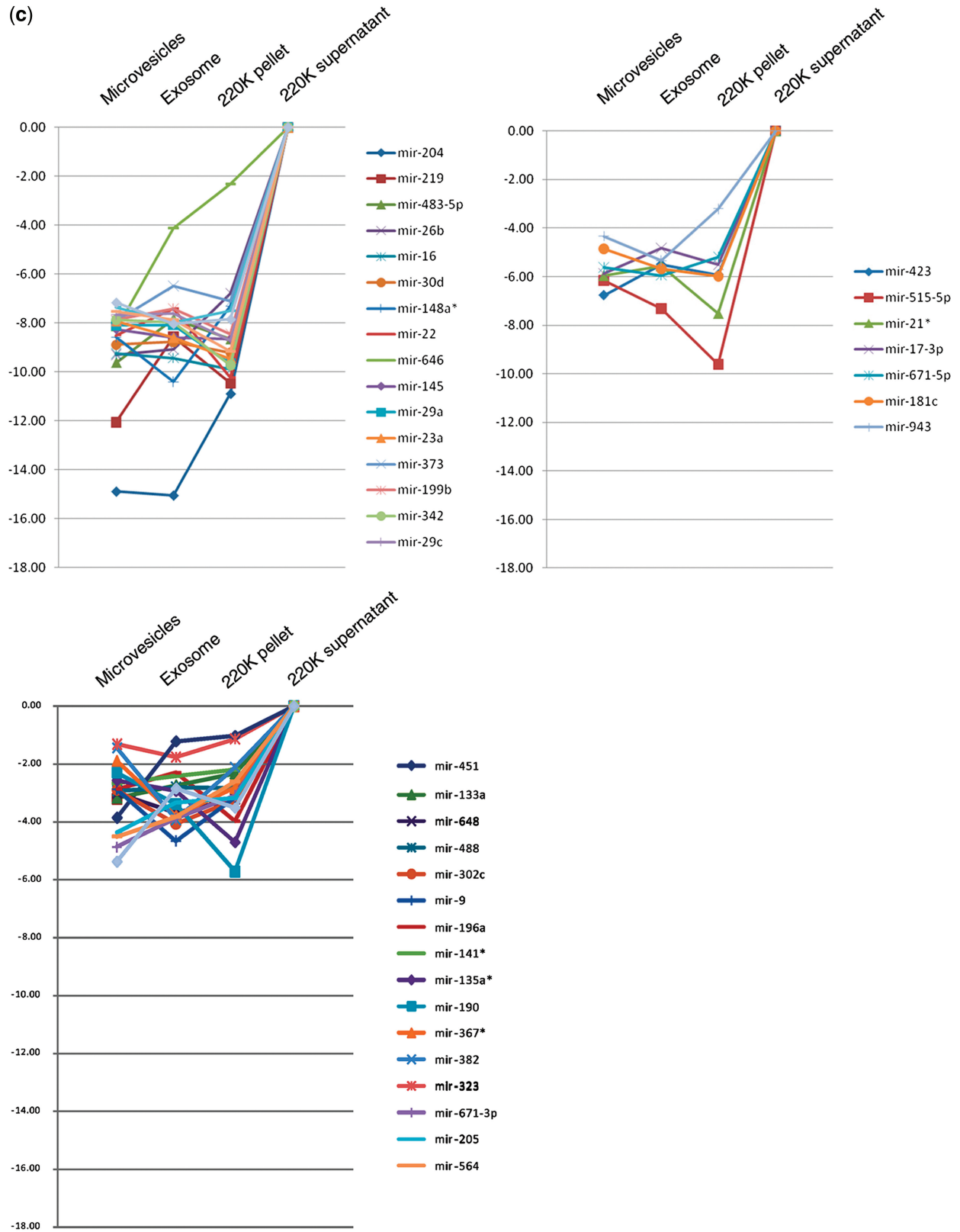


Figure 4. Continued.

**Table 1.** List of all of the known RNA-binding proteins which we observed with two or more peptide fragments in the medium (2 h after SD)

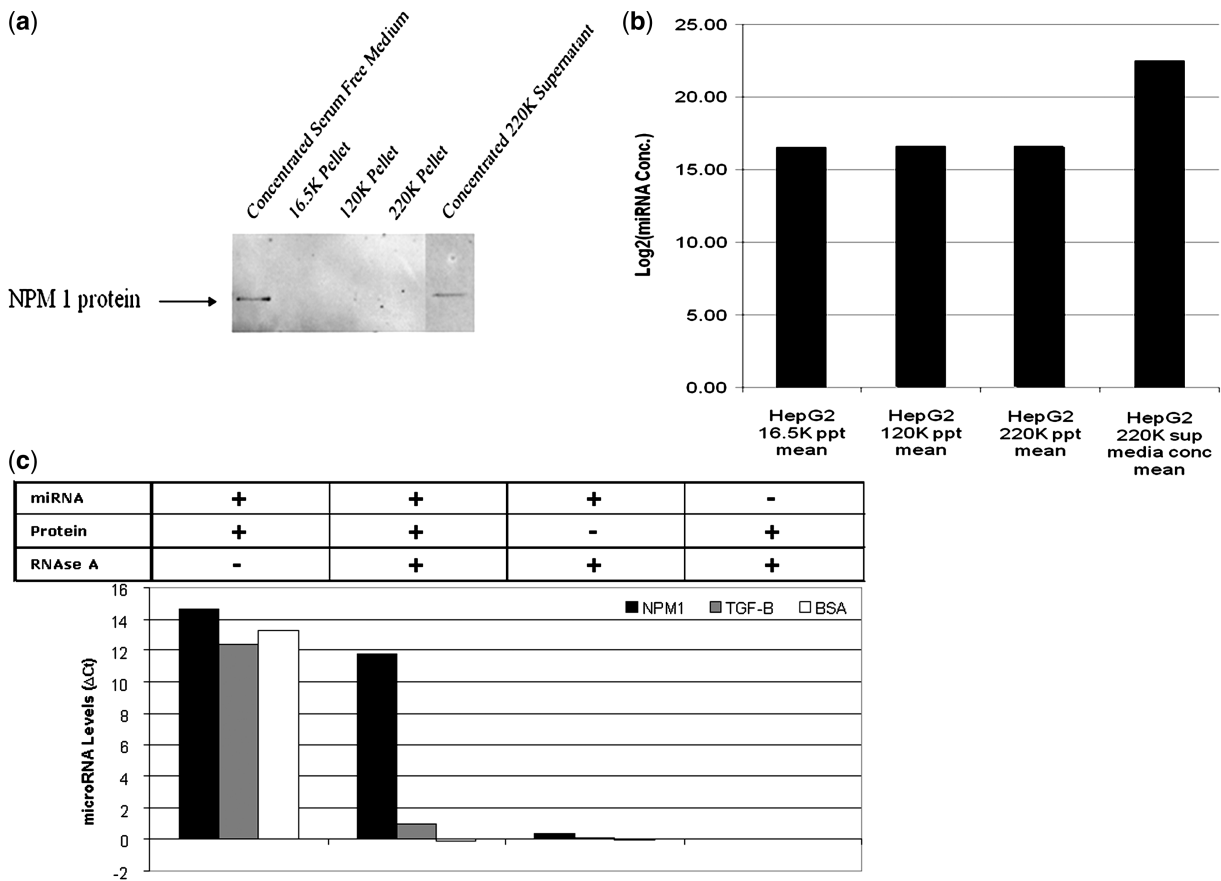
Gene symbol	Number of peptides observed	Gene name
HNRNPA2B1	2	Heterogeneous nuclear ribonucleoprotein a2/b1
HNRPAB	3	Heterogeneous nuclear ribonucleoprotein a/b
ILF2	2	Interleukin enhancer binding factor 2, 45 kda
NCL	7	Nucleolin
NPM1	4	Nucleophosmin (nucleolar phosphoprotein b23, numatrin)
RPL10A	2	Ribosomal protein l10a
RPL5	2	Ribosomal protein l5
RPLP1	6	Ribosomal protein, large, p1
RPS12	2	Ribosomal protein s12
RPS19	2	Ribosomal protein s19
SNRPG	2	Small nuclear ribonucleoprotein polypeptide g
TROVE2	2	Trove domain family, member 2

that the nucleophosmin 1 is involved both in some part of the miRNA exporting process and in protecting the external miRNAs outside the cell.

To see if NPM1 is specific in its protective effect on miRNAs, we tested a number of other RNA-binding proteins for their protective effect under the same conditions. These included nucleolin (NCL), nucleophosmin 2 (NPM2), nucleophosmin 3 (NPM3), eukaryotic translation initiation factor 2C (EIF2c2), Musashi homolog 1 (MSI1), and ELAVL protein family 3 (ELAVL3), none of which had any significant protective effect on our synthetic miRNA, miR-122. However, we could not rule out the possibility of a sequence preference on these RNA-binding proteins. More synthetic miRNA are needed to answer the question of whether other RNA-binding proteins can also bind and protect miRNA.

**Cells may have an array of miRNA packaging and transportation systems**

We have demonstrated that miRNAs are actively exported from cells. This export process appears to be a short pulse



**Figure 5.** Protein exported upon serum deprivation. (a) NPM1 protein is observed in western analysis of the concentrated medium from HepG2 cells. This protein is exported into the medium, but is not found in any fraction except the supernatant (as defined in legend to Figure 4) as shown by westerns on the fractions. (b) The relative concentrations of all miRNAs measured in this work were averaged for each fraction to obtain a relative miRNA levels in each fraction. These averages for HepG2 cells are shown in the histogram using a scale of log to base 2. (c) NPM1 protein protects miRNA from RNase degradation. Synthetic mir-122 RNA (100 pmol) was mixed with different proteins, NPM1 (black bars, 3 pmol), TGF-β (gray bars, 4 pmol) or BSA (open bars, 1.5 nmol) for 30 min followed by adding RNase A (7 nmol) for another 30 min of incubation at 37°C. The miRNA levels were determined by qPCR. Control experiments: omitting RNase A, protein, or miRNA were also included, as indicated on top of the graph. The scale is Ct of relative mir-122 levels.

lasting about 1 h immediately after serum deprivation. The dynamic nature of this miRNA export system has not been reported before, to our knowledge, and strongly suggests both the existence of a complex response system involving miRNA transportation in the cell and the possible involvement of miRNA in cell–cell communication. Our results from differential centrifugation and chemical inhibitor experiments also suggest that extracellular vesicle-associated miRNAs represent only a part of the extracellular miRNA distribution, at least for our cell culture conditions. A significant fraction of extracellular miRNA is, in fact, not within the known cell-derived vesicles. It is yet unclear how they are packaged outside these vesicles. This leads us to speculate that there may be at least two pathways for the packaging and export of miRNAs, exosome/microvesicle mediated and ‘other particle’ mediated processes. We were surprised to find a number of reportedly intracellular proteins in the culture media shortly after serum depletion. At least one of these associated RNA-binding proteins, NPM1, may play a role in the packaging, and perhaps the export, of extracellular miRNAs. Its role in miRNA packaging/exporting is currently being investigated.

The hypothesis of miRNA-dependent cell–cell communication based on the export of miRNA seems to be the most satisfying explanation for the existence of extracellular miRNA. There has been circumstantial evidence in favor of this interpretation, but we provide here direct evidence that demonstrates the export system in action in response to serum deprivation, characterizes some of properties of extracellular miRNA packaging, and supports the miRNA mediated cell–cell communication hypothesis. Our biological observations indicate an important role for miRNAs, and enable future investigations of this potential communication system, unknown until now.

There are important pieces of the puzzle still missing. We have no direct evidence yet that miRNA containing complexes are taken up by other cells, although vesicles containing miRNA are reported to be taken up by cells (17). We expect that miRNA outside of vesicles, complexed with proteins, could be targeted to specific cell surface receptors, and are currently investigating this important hypothesis. Our results raise significant questions about miRNA-mediated cell-to-cell communication, and enable the characterization of such a system. The elucidation of a novel and perhaps extensive, biological information transduction system between cells will certainly be of the utmost importance in understanding many biological processes in mammalian systems including development, stress response, and tissue renewal.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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