MicroRNAs Transfer from Human Macrophages to Hepato-Carcinoma Cells and Inhibit Proliferation

Anne Aucher, Dominika Rudnicka and Daniel M. Davis

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Recent research has indicated a new mode of intercellular communication facilitated by the movement of RNA between cells. There is evidence that RNA can transfer between cells in a multitude of ways, including in complex with proteins or lipids or in vesicles, including apoptotic bodies and exosomes. However, there remains little understanding of the function of nucleic acid transfer between human cells. In this article, we report that human macrophages transfer microRNAs (miRNAs) to hepatocarcinoma cells (HCCs) in a manner that required intercellular contact and involved gap junctions. Two specific miRNAs transferred efficiently between these cells—miR-142 and miR-223—and both were endogenously expressed in macrophages and not in HCCs. Transfer of these miRNAs influenced posttranscriptional regulation of proteins in HCCs, including decreased expression of reporter proteins and endogenously expressed stathmin-1 and insulin-like growth factor-1 receptor. Importantly, transfer of miRNAs from macrophages functionally inhibited proliferation of these cancerous cells. Thus, these data led us to propose that intercellular transfer of miRNA from immune cells could serve as a new defense against unwanted cell proliferation or tumor growth. The Journal of Immunology, 2013, 191: 6250–6260.

MicroRNAs Transfer from Human Macrophages to Hepato-Carcinoma Cells and Inhibit Proliferation

Anne Aucher,* Dominika Rudnicka,* and Daniel M. Davis*†

MicroRNAs (miRNAs) that can influence neighboring monocytes. In this article, we report that endogenous miRNAs can transfer from macrophages to hepatocarcinoma cells (HCCs), including some that can directly inhibit their proliferation. Thus, alongside production of soluble proteins such as cytokines, and cell-mediated cytotoxicity, the secretion of miRNAs may be considered a third type of immune cell effector function.

Materials and Methods

Cells

PBMCs were isolated from lymphocyte cones or fresh blood by density gradient centrifugation (Ficoll-Paque Plus; Amersham Pharmacia Biotech). All blood donors were healthy and gave informed consent for their blood to be used (ethics approved by The National Research Ethics Service). Human macrophages were derived as described (18). In brief, PBMCs were incubated for 2 h in plastic plates before the flask was washed intensively to remove any nonadherent cells. After 4 d of incubation in serum-free media (X-VIVO 10; BioWhittaker) supplemented with 1% autologous serum, adherent cells were washed with PBS and cultured in standard DMEM-based media for three to six additional days to generate monocyte-derived macrophages, phenotyped to be CD14+, CD11a+, CD3−, CD19−, and CD19+ using the following mAbs: allophycocyanin-conjugated mouse anti-human CD14 (61D3) and IgG1 (P3.6.2.8.1) (both from ebioscience); FITC-conjugated mouse anti-human CD3 (HT3a), CD11a (H111), IgG2a (G155-178), and IgG1 (MOPC-21); and PE-conjugated mouse anti-human CD19 (HIB19), CD56 (B159), and IgG1 (MOPC-21) (all from BD Pharmingen). Peripheral blood human T and B cells were isolated by negative selection from healthy donor PBMCs using magnetic beads (Pan T or B Cell Isolation Kit II; Miltenyi Biotec).

The human hepatocarcinoma HuH7 and HepG2, the human acute monocytic leukemia THP-1, and the B-lymphoblastoid 721.221 (221), and the mouse lymphoblastoid-like mastocytoma P815 cell lines were used untransfected or were stably transfected (Gene Juice; Merck Millipore or Microporator; Labtech International) to express GPI-anchored GFP (19).

Cells were cultured in standard DMEM-based media supplemented with 10% FBS, 2 mM l-glutamine, 10 mM HEPES, 50 U/ml penicillin, and 50 μg/ml streptomycin (all from Life Technologies). Freshly isolated T cells were stimulated for 24 h with 150 U/ml human rIL-2 (Roche). Where indicated, macrophages and HCCs were pretreated for 24 h with 75 μM 18-α-glycyrrhetinic acid (18-α-GA; in DMSO) or 5 μM manumycin A (in methanol; both from Sigma); for 2 h with 1 μM latrunculin A (in DMSO; Merck), 5 μM nocodazole (in DMSO; Sigma), 10 μg/ml breflidin A (in methanol; Merck), or 10 μg/ml polyclonal Ab anti-scavenger receptor class B member 1 (Novus Biologicals); or for 30 min with 1 mM 2-octanol.
bodies were added to 2.5 × 10^6 DNA marker Draq5 (1).

were confirmed to be apoptotic bodies by staining a small fraction with the centrifugation steps, as previously described (22). Collected microparticles calculated as: 100 * (median fluorescence intensity of recipient cells)/(median expression at 1 min of coculture (denoted "fold change").

To image the cellular distribution of exosomes, macromolecules were stained with 2 μM CellTrace Far Red DDAO-SE (Molecular Probes) and cocultured with HuH7 cells (ratio 1:1). Cells were fixed after 1 min or 5 h, and only viable single cells were sorted using a multiparametric gating strategy based on forward scatter/side scatter, live/dead cell discrimination using a dead cell stain (100 nM Sytox Blue; Invitrogen), macrophage and HuH7 fluorochrome markers. Cell-Trace- and GFP-sorted cells were checked for purity by flow cytometry, with the purity of macrophages and HuH7 cells always >98.5% (FACS Aria; Diva software; BD Bioscience).

Flow cytometric analysis of intercellular interactions and molecular exchange

Macroparticles were labeled with 1 μM 1',3'-diodoacetad-3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiD; Molecular Probes), 1 μg/ml EZ-Link Sulfo-NHS-Biotin (Pierce), or 1 μM the specific RNA-dye F22 (a styryl dye specific for RNA that remains nonfluorescent until it binds RNA) (20) or were transfected with 50 nM scramble siRNA targeted to HuH7 (1 nM of 1 μM scramble siRNA targeted to HuH7 (13)). Fluorescent macrophages were then washed and left to rest at 37°C for 30 min or overnight (when transfected). Cells were detached, washed, and cocultured with HuH7, P815, or 221 cells in a fresh plate. In Transwell experiments, HuH7 cells were cultured above or below a Transwell membrane (0.4-μm pore membrane) and, where appropriate, to the level of macrophages and HuH7 cells always >98.5% (FACS Aria; Diva software; BD Bioscience).

Imaging

For imaging, macrophages were stained with 2 μM CellTrace Far Red DDAO-SE (Molecular Probes). Cells were imaged in PBS in eight-well chambered coverglasses (Chambered Borosilicate Coverglass; Lab-Tek) at 37°C, 5% CO2 by confocal microscopy (TCS SP5 RS; Leica) using excitation dry objective (N.A. = 0.5). Brightness and contrast were changed in some images solely for clarity of figures; analysis was carried out on unprocessed images (Velocity; Improvision).

Quantitative real-time PCR assays

miRNAs were isolated from total RNA (mirVana PARIS kit; Ambion) and reverse transcribed (TaqMan MicroRNA Reverse Transcription Kit; Applied Biosystems). Large RNAs were isolated with TRIzol reagent (Invitrogen) and reverse transcribed (TaqMan MicroRNA Reverse Transcription kit; Applied Biosystems). cDNAs were amplified by RT-PCR (TaqMan Universal PCR Master Mix; Applied Biosystems). Expression assays (TaqMan Assay; Applied Biosystems) were used to quantify the levels of different RNAs as follows: hsa-miR-122-5p, hsa-miR-142-3p, hsa-miR-223-3p, hsa-miR-22-3p, hsa-miR-22-5p, RNU48, hsa-miR-122, H03303072_pr, hsa-miR-142 H03303162_pr, hsa-miR-223 H03303017_pr, hsa-miR-425 H03303154_pr, stathmin-1 H03017551_g, insulin-like growth factor 1 receptor H00609566_m1, ras homolog gene family, member B H03676562_s1, ephrin-A1 H03058886_m1, and 18S RNA. Quantitative PCR was conducted in triplicate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s (7500 Fast Real-Time PCR System; Applied Biosystems). Cycle thresholds were normalized to an internal control: RNU48 for miRNA and 18S RNA for precursor of miRNA (pri-miRNA) and miRNA assays. The amount of RNA was calculated using the ΔΔCT method (21); the level of expression of an RNA was normalized to the adapted internal control (denoted "relative expression") and, where appropriate, to the level of expression at 1 min of coculture (denoted "fold change").

Apoptotic bodies

Macroparticles were isolated from supernatant of 2.5 × 10^6 macrophages, as previously described (9). Exosomes were then added to 2.5 × 10^6 HuH7 cells for 1 min or 5 h. Cells were washed and collected for qRT-PCR or imaged by confocal microscopy with 1 μM Draq5.

Exosomes

Exosomes were enriched from supernatant of 2.5 × 10^6 macrophages, as previously described (9). Exosomes were then added to 2.5 × 10^6 HuH7 cells for 1 min or 5 h. Cells were washed and collected for qRT-PCR. To image the cellular distribution of exosomes, macromolecules were stained with 2 μM CellTrace Far Red DDAO-SE (Molecular Probes) and cocultured with HuH7 cells (ratio 1:1). Cells were fixed in 4% PFA (Thermo Scientific), permeabilized with 0.05% saponin/PBS (Sigma), stained with a mouse anti-human CD63 mAb (H5C6, IgG1; BD Pharmingen), followed by an Alexa Fluor 568-conjugated goat anti-mouse IgG secondary Ab (IgG1; Invitrogen), and imaged by confocal microscopy.

High-density lipoprotein assays

A total of 2 × 10^6 HuH7 cells were cultured alone or with macrophages for 1 min or 5 h. At the end of the coculture, supernatants were collected, and cells were lysed. Supernatants and cell lysates were centrifuged for 5 min at 20,000 × g and assayed for the presence of high-density lipoproteins (HDLs) (HDL and LDL/VLDL Cholesterol Assay Kit; Abcam).

Sponge constructs

Sponges were designed following the method developed by Khvortsev et al. (23). The oligonucleotides used to generate the scramble sponges and anti-miR-223 sponges bulged multiple miRNA antisense binding sites constructs were synthesized as [Phos]GTCCCTATCTACTGCTACTGATGCTGGTCGTTATCTCTGCTCAGGG and [Phos]GTCCCTACTGGCTGGTATCTGACTCAGGGTCATCGGG and assayed for the presence of high-density lipoproteins (HDLs) (HDL and LDL/VLDL Cholesterol Assay Kit; Abcam).

Antagonomers

Antagonomers were synthesized with 2'-O-methyl oligonucleotides (Integrated DNA Technologies) as scramble antisense, 5'-mA*CmG*AmC*mAmC*AmG*CmC*mCmU*mG*mU*mG*mU*mG*AmG*CmC*mCmU*mCmU*mCmG*C*G-3' and miR-223 antisense, 5'-mA*mG*mG*mG*mU*mA*T*T*T*G*A*C*A*A*A*-3'. Macrophages were transfected with 50 nM modified oligonucleotides (GenMute siRNA Transfection reagent; SigmaGen Laboratories).

Dual-luciferase reporter assay

Plasmids encoding a portion of the 3'-untranslated region (3'UTR) of stathmin-1 (STMN1) linked to the firefly luciferase protein—full length, truncated, and mutated—were described previously (24). Firefly luciferase constructs were cotransfected with Renilla luciferase vector control (Promega) into HuH7 cells. Where indicated, HuH7 cells were stably expressing sponges, and macrophages were transfected with antagonomirs. Twenty-four hours after transfection, macrophages and transfected HuH7 cells were detached, washed, and co-cultivated (ratio 1:3) for 1 min, 5 h, or 24 h in fresh wells. Luciferase activities were measured consecutively (Dual-Luciferase Assay; Promega), and the relative luciferase activity was assessed (firefly activity/Renilla activity) or 24 h (firefly activity/Renilla activity)_{min}.

Proliferation assays

A total of 10^5 HuH7 cells, untransfected or transfected with anti-miR-223 or control scramble sponges, was seeded in triplicate and cocultured with macrophages, transfected or not with either scramble or anti-miR-223 antagonomirs (ratio 1:3), in the presence of 1 μCi [3H]thymidine (PerkinElmer) per well. Cells were harvested (Harvester 96 Mach II M; Tomtec) after 4 d, and cell proliferation, as assessed by [3H]thymidine uptake, was measured in a beta scintillation counter (1450 MicroBeta Trilux; Wallac).

Statistical analysis

The Mann–Whitney U test was used to assess all data (GraphPad; Prism). Mean values are shown, and bars represent SEM.
Results

Intercellular transfer of RNA from macrophages to HCCs

To test which types of cell components transferred between macrophages and HCCs, primary human monocyte–derived macrophages were labeled as follows: surface membrane was marked with fluorescent lipid DiD, or surface proteins were biotinylated, or RNA was stained with the specific dye F22 (20), or cells were transfected to take up Cy5-scramble-siRNA. The differently labeled macrophages were then cocultured with other cells to study the transfer of cell components to hepatic tumor HuH7 cells, the EBV-transformed human B cell line 221, or the mouse lymphoblast-like mastocytoma cell line P815, each transfected to express GPI-anchored GFP so that they can easily be distinguished from macrophages (as in all experiments that follow, unless stated otherwise). The amount by which each label—marking lipids, proteins, or RNA—transferred to these different acceptor cells was assessed by flow cytometry (Fig. 1A, Supplemental Fig. 1A).

After 5 h of coculture, 7.8 ± 1.9% of fluorescent lipid initially loaded on macrophages, as well as 2.8 ± 1.6% of the biotinylated surface proteins, transferred to recipient HuH7 cells (Fig. 1A). More surprisingly, 15.1 ± 6.2% of F22, a dye that specifically binds RNA (20), and 3.4 ± 0.7% of the labeled small RNA also transferred from macrophages to HuH7 cells (Fig. 1A). The amount of transferred material was far lower when the acceptor cells were 221 or P815 cells. Thus, the extent to which different cellular components transferred was dependent on the nature of the recipient cells; especially striking was the unexpectedly high level of transfer of RNA from macrophages to HCCs.

A Transwell porous membrane was used to allow direct contact between macrophages and HuH7 cells in the upper chamber, whereas other HuH7 cells occupied the lower chamber so that they were exposed to the same solution but did not directly contact the macrophages. Only those cells in direct contact with macrophages acquired lipids, membrane proteins, or RNA (Fig. 1B). Similar levels of each cellular component were also seen to transfer from the macrophage cell line THP-1, again blocked by the presence of a Transwell membrane (Supplemental Fig. 1B). In addition, confocal microscopy of HCCs cocultured for 5 h with primary macrophages or THP-1 cells (Supplemental Fig. 1C) revealed that transferred fluorescent components accumulated, at least to some extent, in cytoplasmic vesicles (Fig. 1C). Together, these data indicate that macrophages transfer cell components to HuH7 cells, including RNA, in a cell contact–dependent manner. Nevertheless,
it is difficult to formally rule out the possibility that some free dye may transfer in these experiments; and importantly, the use of Cy5-scramble-siRNA only reports on the labeled RNA molecules that we added to the cells, not those that the cells produce endogenously. Thus, these initial data were taken to be hypothesis forming. Next, we directly tested whether specific RNAs transferred between macrophages and HuH7 cells.

Specific miRNAs transfer from primary macrophages to HCCs

To test whether macrophages transfer their own miRNAs to HuH7 cells, we first established which miRNAs were endogenously expressed in macrophages and not HCCs. We tested both cell types for the endogenous expression of different miRNAs, as in previous research (25). We found that miR-142 and miR-223, previously reported to work together to regulate gene expression (26), were both highly expressed in the human macrophage cell line THP-1 (Supplemental Fig. 1D) and primary macrophages, with very little expression detectable in HuH7 cells (Fig. 2A). In contrast, macrophages expressed a lower level of miR-122 compared with HuH7 cells, and the level of expression of miR-425 was similar in both cell types.

Next, cells were cocultured and separated by FACS (purity > 98.5%; Supplemental Fig. 2A). Efficient separation of these cell types after sorting was confirmed by testing the level of expression of mRNA specific to macrophages and HuH7 cells, specifically Mac-1 and cytokeratin 19 (Supplemental Fig. 2B, 2C) (e.g., the macrophage-specific mRNA Mac-1 was not detected in HuH7 cells after cell sorting; Supplemental Fig. 2C). The amount of miR-142 and miR-223 in sorted THP-1 cells (Supplemental Fig. 1D, 1E) or macrophages remained relatively stable before and after coculture (Fig. 2B). However, the level of both miR-142 and miR-223 increased dramatically in HuH7 cells upon coculture with macrophages (Fig. 2C). In contrast, there was little, if any, change in the level of miR-122 and miR-425 detected in HuH7 cells (Fig. 2C). The specific changes in the levels of miR-142 and miR-223 were truly in the HuH7 cells, rather than due to the presence of contaminating macrophages, because HuH7 cells deliberately spiked with varying percentages of macrophages did...
not replicate the changes in miRNAs detected (Supplemental Fig. 2D).

The amount of miR-142 and miR-223 in HuH7 cells increased after coculture with macrophages across multiple donors tested, with a fold increase of 12–1984, depending on the donor (Fig. 2D). In contrast, the level of miR-122 and miR-425 in HuH7 cells did not change significantly after coculture, across donors (Fig. 2D). Primary human B and T cells also expressed high levels of miR-142 and miR-223, similar to macrophages (Fig. 2E). However, after coculture with B or T cells, HuH7 cells contained only a low amount of miR-142 and very little, if any, miR-223 (Fig. 2F). Together, these data establish that HuH7 cells express high levels of miR-142 and miR-223 following coculture with human macrophages.

We next set out to determine whether the increase in macrophage miRNA detected in HuH7 cells was due to transfer of miRNA or synthesis of nascent miRNA triggered by contact with macrophages. First, we tested whether miRNAs would be detected in recipient cells after coculture with macrophages that had been chemically fixed so that surface proteins still interact with other cells but dynamic processes, such as secretion, are inhibited. Fixed macrophages were still able to interact with HuH7 cells and form conjugates (Supplemental Fig. 3A). Small changes in the level of miR-425 and miR-122 in HuH7 cells after coculture were not inhibited by fixation of the donor macrophages, suggesting that these changes may be the result of interactions between surface molecules. However, the dramatic increases in levels of miR-142 and miR-223 in HuH7 cells after coculture were prevented by chemical fixation of macrophages (Fig. 3A). This indicates that an upregulation in these miRNAs requires more than mere contact with proteins at the macrophage cell surface.

Of course, chemical fixation influences many processes, including the recruitment of proteins to the contact interface; therefore, to more directly test whether miR-142 and miR-223 were directly acquired from macrophages or newly synthesized in HuH7 cells, we assayed for the processing of these miRNAs in HuH7 cells. pri-miRNAs are several hundreds of nucleotides long and are produced in the nucleus prior to the action of the Drosha protein. Knockdown of Drosha itself would be one way to inhibit processing of miRNAs, but we found that this was lethal for HuH7 cells. Instead, the expression level of each pri-miRNA was assessed directly in donor and recipient cells by RT-PCR.

After 5 h of coculture with macrophages, the precursors of miR-142 and miR-223 could not be detected in HuH7 cells (Fig. 3B). Their expression was readily detected in macrophages, confirming that our assay for these precursors worked. Also, pri-miR-425 and pri-miR-122 were detected in both HuH7 cells and macrophages (Fig. 3B). In fact, there was little, if any, change in the levels of expression of the pri-miRNAs tested in either cell type following coculture (Fig. 3C). Thus, the high levels of miR-142 and miR-223 in HuH7 cells after coculture with macrophages does not derive from new synthesis of these miRNAs, demonstrating that specific endogenous miRNAs transfer from macrophages to HuH7 cells.

**miRNAs are transferred to HCCs in a contact-dependent manner**

Several possible mechanisms have been suggested to facilitate intercellular transfer of RNA (27). One possible route for intercellular transfer of miRNAs is through apoptotic bodies following donor cell death (4). To test for this, HuH7 cells were cultured with macrophages or apoptotic bodies isolated from macrophages that had been treated with cycloheximide to trigger apoptosis (Supplemental Fig. 3B, 3C). HuH7 cells have the ability to endocytose environmental particles and, indeed, when incubated with apoptotic bodies derived from 10-fold more macrophages than usually used, 25.1 ± 2.8% of HuH7 cells showed a positive staining for one or several apoptotic bodies (Fig. 4A). However, the percentage of HuH7 cells containing apoptotic bodies did not increase when the cells were cocultured with macrophages compared with when HuH7 cells were cultured alone (Fig. 4A). More importantly, the level of miR-142 and miR-223 did not increase in HuH7 cells after incubation for 5 h with apoptotic bodies derived from 10-fold more macrophages than usually used in coculture experiments (Fig. 4B). These findings exclude the possibility that miR-142 and miR-223 transfer via apoptotic bodies in our experiments.

Another possible mechanism responsible for miRNA transfer is by exosomes or microvesicles (5–10, 17). To test for this, exosomes were isolated from a coculture of 10-fold more macrophages and HuH7 cells than usually used in other coculture experiments, and this large number of exosomes was subsequently added to fresh HuH7 cells. These exosomes were taken up by HuH7 cells, as evidenced by flow cytometry showing that HuH7 cells contained exosomes that had been prelabeled with an anti-CD63 mAb (Supplemental Fig. 3D). However, incubation with this large number of exosomes led to only a small increase in the amount of miR-142 and miR-223 detected in HuH7 cells (Fig. 4C).

Exosomes can also be delivered directly at the synapse after polarization (27). However, brefeldin A, which inhibits the secretion of exosomes (28), did not influence the level of miRNA detected in HuH7 cells after coculture with macrophages (Fig. 4C). In addition, cell conjugates were imaged at different times of coculture, when exosomes were stained with an anti-CD63 mAb. There was no polarization of exosomes toward the contact between macrophages and HuH7 cells (Supplemental Fig. 3E, 3F). Together, these results indicate that exosomes are not a major pathway for intercellular transfer of miRNA in these experiments.

Another possibility is that miRNA could be transferred through HDLs (3). HDLs were detected in cultures of HuH7 cells incubated alone or with macrophages (Supplemental Fig. 3G). However, only a very small amount of miR-142 and miR-223 transferred via the supernatant taken from a coculture of macrophages and HuH7 cells (Fig. 4D). Furthermore, blockade of scavenger receptors class B member 1, which functions as the receptor for HDLs on HuH7 cells, using a polyclonal Ab, did not significantly inhibit the transfer of miR-223 and miR-142 to HuH7 cells (Fig. 4D). In addition, treating the cells with manumycin A, which enhances miRNA export to HDLs and inhibits the release of exosomes (3, 29), did not influence the transfer of miR-223 and miR-142 to HuH7 cells (Fig. 4D). Together, these data indicate that HDLs did not play a major role in the transfer of miRNA in these experiments. Indeed, a Transwell membrane was able to fully block the transfer of miR-142 and miR-223 from macrophages to HuH7 cells (Fig. 4E), consistent with experiments using the RNA-specific dye F22 (Fig. 1B), indicating that the bulk transfer of miRNAs required direct contact between cells.

Treating the cells with latrunculin A, which disrupts the actin microfilaments of the cytoskeleton, decreased the efficiency of transfer of miRNAs, whereas treatment with nocodazole, which interferes with microtubule polymerization, did not influence the transfer (Fig. 4F). These data indicate that cytoskeletal processes can affect the transfer of miRNAs, consistent with the requirement for direct cell contact and perhaps directed secretion (9, 11). Unexpectedly, 18-α-GA, which blocks gap junction activity (30), reduced the efficiency of transfer of miR-142 and miR-223 by 78.6 ± 8.5% and 59.2 ± 14.6%, respectively (Fig. 4G). Two other inhibitors of the gap junctions, 2-octanol and oleamide (31), also
decreased the transfer of miR-142 and miR-223, to a similar extent for miR-142 and even more efficiently for miR-223, in comparison with 18-o-GA (Fig. 4G). Thus, taken together, these data establish that endogenous macrophage miRNAs transfer to HuH7 cells in a manner dependent on cell–cell contacts and gap junctions.

Transferred miRNAs are functional and inhibit HCC proliferation

miR-142 has been studied relatively little, but it was established previously that miR-223 is commonly repressed in HCCs (24). Moreover, it was shown that miR-223 targets the mRNAs of STMN1 (24) and insulin-like growth factor-1 receptor (IGF-1R) (32), which both influence cellular proliferation (33, 34). Therefore, we assayed for changes in expression of these two mRNAs in HuH7 cells following coculture with macrophages. Compared with HuH7 cells cultured alone, the level of expression of both STMN1 and IGF-1R mRNAs in HuH7 cells remained the same after 5 h but decreased significantly with 24 h of coculture (Fig. 5A). RhoB and Ephrin A1, also previously described as targets for miR-223 (3), were not affected by the coculture with macrophages (Supplemental Fig. 4A).

To test whether a decrease in the level of mRNA was caused by the intercellular transfer of miRNAs, HuH7 cells (not transfected to express GFP-GFP) were transfected to stably express sponges (along with GFP) that can inhibit the activity of specific miRNAs by design (23). Cells were transfected to express either a transcript containing multiple tandem binding sites for miR-223, so that they sequester the miRNA, or a control scrambled version of the miRNA. Strikingly, downregulation of STMN1 mRNA in HuH7 cells triggered by coculture with macrophages was abrogated in cells expressing anti–miR-223 sponges (Fig. 5B). Cells expressing control scramble sponges downregulated STMN1 mRNA to the same extent as did untransfected cells.

For an alternative approach to test whether downregulation of mRNAs in HuH7 cells can be attributed to the intercellular transfer of miRNAs from macrophages, HuH7 cells were transfected to express a luciferase reporter gene linked to the 3′UTR of the STMN1 mRNA or a truncated or mutated version of this 3′UTR, which lacks the target sequence for miR-223 (24). After coculture with macrophages for 5 h, a decrease in the activity of luciferase was evident in HuH7 cells transfected to express the wild-type 3′UTR-reporter gene, and this was decreased further after 24 h (Fig. 5C). There was no repression of luciferase activity in HuH7 cells transfected to express the truncated or mutated version of STMN1-3′UTR. In addition, coculture with macrophages that had been chemically fixed did not influence luciferase activity in HuH7 transfecants expressing the wild-type 3′UTR-reporter gene. Importantly, luciferase activity reduced by coculture with macrophages could be partially restored by the expression of anti–miR-223 sponges in HuH7 cells (Fig. 5D). This confirms that decreased activity of luciferase linked to the 3′UTR of STMN1 was caused by the intercellular transfer of miRNAs.

To further test the consequences of miRNA transfer, macrophages were transfected with methyl-oligonucleotides expressing the complementary sequence of an miRNA of interest, termed “antagomiRs” in this article (35–37), directed against miR-223 or a control scramble miRNA. HuH7 cells cocultured with cells expressing scramble control antagomiRs downregulated luciferase activity similarly to untransfected cells, but expression of anti–miR-223 antagomiRs restored luciferase activity to a mild, but statistically significant, extent (Fig. 5E). Together, these data establish that the intercellular transfer of macrophage miRNAs can influence protein expression in recipient HuH7 cells.

STMN1 is involved in cell cycle regulation, and its inhibition leads to a decrease in proliferation; IGF-1R is implicated in several cancers and can aid the growth of tumors (32, 35). In this study, we found that coculture with macrophages reduced the proliferation of HuH7 cells by >50%, as assessed by a standard thymidine-incorporation assay (Fig. 5F). Proliferation of HuH7 cells was not affected when cocultured with fixed macrophages (Fig. 5F). More importantly, proliferation of HuH7 cells was restored to some extent when cocultured in the presence of gap junction inhibitors 18-o-GA, 2-octanol, or oleamide (Fig. 5G). To test directly whether this change in cancer cell proliferation could be a direct consequence of the intercellular transfer of miRNAs, we again used two ways to interfere with this process: expression of anti–miR-223 sponges in HuH7 cells or anti–miR-223 antagomiRs in the macrophages (Supplemental Fig. 4B, 4C). Indeed, the reduced proliferation of HuH7 cells caused by coculture with macrophages was partially restored by expression of anti–miR-223 sponges in the HuH7 cells (Fig. 5H) or of anti–miR-223 antagomiRs in the macrophages (Fig. 5I). Proliferation of another HCC line, HepG2, to which macrophages also transferred miRNA-142 and miR-223
Supplemental Fig. 4D, 4E), was equally decreased in the presence of macrophages. Importantly, proliferation of HepG2 was also restored in the presence of sponges or antagomiRs to inhibit the activity of miR-223 (Fig. 5F, 5H, 5I). Thus, miRNAs from macrophages can directly inhibit the proliferation of HCCs.

Discussion

The transfer of membrane and proteins from immune cells to other cells has been well established (38–41). More recently, it has emerged that miRNAs and miRNAs can also transfer between cells. First demonstrated in plants and nematodes (2), the intercellular transfer of specific RNAs was observed relatively recently between mammalian cells. miRNAs can travel through body fluids and reach distant recipient cells via vesicles or association with HDL (42), or they can traffic across an immune synapse (9). However, the functional importance of an intercellular transfer of endogenously expressed miRNAs between human cells has not been established. We found that primary human macrophages efficiently transferred RNA to HCCs, as well as to other tumor cells, such as 221 cells, although to a lesser extent. Likely, the transfer of miRNA is a common phenomenon, and the importance of the transformation level of the cells must now be addressed.

Macrophages primarily delivered miRNAs to HCCs across an intercellular contact, although a small fraction of miRNA could transfer via isolated exosomes. Transfer of endogenous miRNAs through multidirectional soluble secretion would mean that all neighboring cells could be affected, whereas a cell contact–dependent transfer of miRNAs implies that macrophages can directly influence specific cells. Transfer of a specific RNA dye indicated that 10–15% of endogenous RNA could transfer from macrophages to HCCs. There is some selection to which miRNAs transfer, because miR-142 and miR-223 were acquired by HCCs, but miR-425 was not.

(Fig. 4A) Percentage of cells containing apoptotic bodies, as stained by Draq5 and detected by confocal microscopy, for HuH7 cells alone, HuH7 cells cocultured with macrophages, or HuH7 cells cocultured with apoptotic bodies isolated from macrophages treated with cycloheximide (n > 707 cells for each condition, from six independent experiments). (B) Fold change in level of miR-142 and miR-223, assessed by qRT-PCR, in HuH7 cells cocultured for 5 h with isolated apoptotic bodies derived from macrophages treated with cycloheximide (n = 4). (C) Fold change in the level of miR-142 and miR-223 in HuH7 cells, as determined by qRT-PCR, following coculture for 5 h with macrophages, macrophages in the presence of 10 μg/ml breydelin A (Bref A), exosomes isolated from cultures of macrophages, or exosomes isolated from macrophages cocultured with HuH7 cells (n = 4). (D) Fold change of miR-142 and miR-223 in HuH7 cells cocultured for 5 h with macrophages, supernatant from macrophages, or supernatant from macrophages previously cocultured with HuH7 cells in the presence or absence of an Ab blocking HDL receptors or 5 μM manumycin A (n > 4). (E) Fold change of miR-142 and miR-223 in HuH7 cells cocultured for 5 h above or below a Transwell membrane (TW), with macrophages added only to the compartment above the TW (n > 4). (F) Fold change of miR-142 and miR-223 in HuH7 cells cocultured for 5 h with macrophages after being left alone or treated with 1 μM latrunculin A or 5 μM nocodazole (n = 4). (G) As in (F), except that cells were either left alone or pretreated and then cocultured with 75 μM 18-alpha-GA, 1 mM 2-octanol, or 100 μM oleamide (n = 4). Error bars are SEM. Significance was determined by a nonparametric Mann–Whitney U test. N.S., Not significant.
FIGURE 5. Intercellular transfer of miRNAs is functionally important. (A) Fold change of STMN1 and IGF-1R mRNA was assessed by qRT-PCR in HuH7 cells alone or cocultured with macrophages for 5 or 24 h \((n = 6)\). (B) As in (A), except that HuH7 cells were stably transfected to express anti–miR-223 sponges or control scramble sponges and analyzed for the expression of STMN1 mRNA \((n = 5)\). (C) HuH7 cells, transfected to express a plasmid encoding the wild-type, a control truncated or mutated 3'UTR of STMN1 fused to the firefly luciferase, along with another plasmid encoding the Renilla luciferase, were cocultured alone or with macrophages that were either alive or fixed in PFA. Luciferase activity was assessed after 5 or 24 h \((n = 6)\). (D) As in (C), but HuH7 cells were stably transfected to express anti–miR-223 sponges or control scramble sponges \((n = 6)\). (E) As in (C), but macrophages were transfected to express anti–miR-223 antagomiRs or control scramble antagomiRs \((n = 4)\). (F) Proliferation of HuH7 or HepG2 cells, cocultured with live or fixed macrophages, was measured by incorporation of [3H]thymidine over 4 d. Graph shows proliferation of HCCs in cocultures relative to HCCs cultured alone \((n \geq 7)\). (G) As in (F), except that where indicated, macrophages and HuH7 cells were pretreated for 24 h with 75 \(\mu M\) 18-aza-GA. (H) As in (F), except that HCCs were stably transfected to express anti–miR-223 sponges or control scramble sponges \((n = 3)\). (I) As in (F), but macrophages were transfected with anti–miR-223 antagomiRs or control scramble antagomiRs \((n = 4)\). Error bars are SEM. Significance was determined by a nonparametric Mann–Whitney \(U\) test. N.S., Not significant.
was not. It remains unclear whether the specificity of transfer that we observe is the result of particularly high expression of miR-142 and miR-223 in macrophages compared with HuH7 cells or whether this process is limited to certain miRNAs. Other RNAs, including other miRNAs, must also transfer beyond those directly assayed for in this study, and more broadly, transfer of miRNAs between cells could be commonplace. A screen to assess which miRNAs transfer between macrophages and HCCs is an important next goal, as is to compare and contrast the transfer of miRNAs between different cell types, including macrophages in other tissues. Indeed, we found that T cells and B cells also transfer miRNAs to HuH7 cells, although the transfer of miR-142 and miR-223 from macrophages to HCCs was particularly efficient.

The fold increase in the expression of miR-142 and miR-223 was dramatic in HuH7 cells; they naturally expressed a very low level of these miRNAs, which increased significantly upon coculture with macrophages. We detected a small decrease in these miRNAs in macrophages following coculture (Fig. 2B). However, it is important to note that a large fold increase in these miRNAs in HuH7 cells does not necessarily require a large fold decrease in the macrophages, because we assayed for relative fold changes in miRNA levels. Importantly, however, there was little, if any, change in the levels of expression of pri-miRs in either cell type following coculture, demonstrating that the higher levels of miR-142 and miR-223 in HuH7 cells after the coculture does not derive from new synthesis. This strongly implicates an intercellular transfer of endogenous miRNA from macrophages to HuH7 cells.

The only way in which we could completely inhibit the transfer of miRNAs was to separate the cells with a Transwell membrane. However, we identified that the actin cytoskeleton, exosomes, scavenger receptors, and gap junctions (to a greater degree), all affect the transfer of miRNA to some extent. Indeed, an inhibitor of actin polymerization, latrunculin A, significantly decreased the transfer of miRNAs from macrophages to HCCs. This may reflect the fact that actin rearrangements are to be likely to be involved in the process of miRNA transfer, as has been established for other types of secretion across immune cell contacts (43). Alternatively, the effect of latrunculin A on miRNA transfer may be a consequence of interfering with other processes, such as cell–cell conjugation. Surprisingly, blocking gap junctions with 18-α-GA, 2-octanol, or oleamide decreased the intercellular transfer of miR-142 and miR-223. Gap junctions are transmembrane channels that connect cytoplasm of adjacent cells to allow intercellular exchange of ions, second messengers, peptides, and nucleotides, such as cAMP, ADP, and ATP, with a limit of permeation of ~1.0–1.5 kDa (44–47). Valiunas et al. (48) reported that small synthetic RNAs can move through gap junctions composed of connexin (Cx)43 but not those composed of other Cxs: Cx32 and Cx26. In the context of the cell types used in this study, healthy hepatocytes are known to express Cx32 and Cx26 but not Cx43. However, Cx43 is upregulated in HCCs (49). Thus, it is possible that regulation of Cx43 expression is a key determinant for cells taking up miRNAs from macrophages. Cx43 is known to be involved in different immunological functions, such as the secretion of cytokines or Ag cross-presentation (44, 50), and the transfer of miRNAs as an immune cell effector function may be a new function for Cx43. The precise role of the gap junctions in the transfer of miRNAs between macrophages and HCCs is not clear. miRNAs could traffic directly through connected gap junctions, perhaps in a manner that allows peptides to transfer (44); alternatively, there could be an indirect effect due to some aspect of cell activation being dependent on gap junctions.

The miRNAs that transfer between macrophages and HCCs include miR-142, which has been little studied, and miR-223, which is known to directly impact on a large range of cell functions, including cell cycle regulation (24, 26, 32, 51). We found that transfer of these miRNAs influences protein expression in HuH7 cells. Specifically, miR-223 decreases expression of STMN1, a protein that is usually only present at low levels in healthy hepatocytes but is expressed more highly in hepatocarcinomas (24). Functionally, we established that transfer of macrophage miRNA dampens HCC proliferation. The ability of miR-223 to prevent proliferation of HCCs is also consistent with a previous study (24) showing a 40% decrease in the number of HuH7 cells upon transfection with miR-223.

Expression of other miRNAs, RhoB and Ephrin A1, also previously described as targets for miR-223 (3), were not affected by the coculture with macrophages. This likely reflects complexity in the miRNA system that we do not yet fully understand; miRNA targets are likely to vary in different cell types, as well as according to a cell’s state of activation; in addition, multiple miRNAs can act synergistically. It would be of great interest to study the intercellular transfer of miRNAs on a genome-wide larger scale (e.g., using a microarray). However, one difficulty with this is that many miRNAs are expressed both in macrophages and HCCs, and it is not trivial to design a screening approach that is able to distinguish newly synthesized miRNAs from miRNAs acquired directly by contact with other cells.

Another important next goal seeded by this study is to test for the functional transfer of miRNAs in vivo. The human liver contains 50–80% of the body’s macrophages (12), Kupffer cells, which are distributed in the hepatic sinusoids where they establish direct contact with hepatocytes (13, 52). Kupffer cells are bone marrow–derived monocytes that migrate from the blood to the hepatic tissue, transform into tissue macrophages, and, among other immune functions, have a recognized antitumor role (53–57). In vivo, Kupffer cells are attracted to tumor cells and interact with them (58). The degree of activation of Kupffer cells influences the number and the size of hepatic metastases following the injection of HCCs (59), and depletion of Kupffer cells results in an increase in the size of the hepatic metastasis (60). Thus, several lines of evidence indicate a role for macrophages in preventing the development of hepatocarcinomas. However, the interaction between macrophages and tumor cells is variable and not clearly understood. Macrophages form a very heterogeneous population with both pro- and antitumor properties. Macrophages can be antitumor through presentation of Ags to T cells, whereas some tumor-associated macrophages are protumorigenic (61). Our data indicate a new level of regulation by which macrophages can influence tumor progression: through transfer of miRNA.

In summary, we present evidence that macrophages transfer specific endogenous miRNAs to HCCs in a cell contact–dependent manner to directly impact protein expression, in turn functionally dampening cellular proliferation. These results lead us to suggest that transfer of endogenous miRNAs could be a novel mechanism of immune defense against tumor cells.

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