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

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Abstract

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, lipids or in vesicles including apoptotic bodies and exosomes. However, there remains little understanding of the function of nucleic acid transfer between human cells. Here, we report that human macrophages transfer microRNAs (miRNAs) to hepato-carcinoma cells (HCCs) in a manner that required intercellular contact and involved gap junctions. Two specific miRNAs efficiently transferred between these cells - miR-142 and miR-223 - both endogenously expressed in macrophages and not HCCs. Transfer of these miRNAs influenced post-transcriptional 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 lead us to propose that intercellular transfer of miRNA from immune cells could serve as a new defense against unwanted cell proliferation or tumor growth.

Keywords

monocytes/macrophages; cell activation; tumor immunity

Introduction

The idea that RNA can transfer between cells was first hypothesised in the 1960s (1)but has gained greater traction more recently through several studies showing an exchange of small RNAs between plant cells and, to a lesser extent, between animal cells (2). A few studies have described the transfer of RNA between human cells, via an association with lipid complexes (3), trapped in apoptotic bodies (4), enclosed in microvesicles (5-10) or in a contact-dependent manner (9, 11). However, direct intercellular transfer of endogenous human miRNA - and how that may be important for specific functions of human cells - has been poorly documented.

Macrophages are the most abundant immune cell in the liver, where they are called Kupffer cells (12). They establish direct contact with hepatocytes (13), that is fundamental to maintain liver homeostasis (14). However, relatively little is known about how they communicate with or influence the parenchymal hepatocytes that they are in contact with.

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Macrophages have been shown to directly transfer cellular components, such as membrane lipids and proteins to other cells (15, 16), and very recently, Ismail et al. also showed that macrophages secrete microvesicles containing miRNAs which can influence neighbouring monocytes (17). Here, we report that endogenous miRNAs can transfer from macrophages to hepato-carcinoma 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.

Material 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 non-adherent cells. After 4 days of incubation in serum-free media (X-vivo 10; Bio-Whittaker) supplemented with 1% autologous serum, adherent cells were washed with PBS and cultured in standard DMEM-based media for 3 to 6 extra days to generate monocyte-derived macrophages, phenotyped to be $CD14^+$, $CD11a^+$, $CD3^-$, $CD56^-$, and $CD19^-$ using mAbs as follows: APC-conjugated mouse anti-human CD14 (61D3) and IgG1 (P3.6.2.8.1) (both from eBioscience); FITC-conjugated mouse anti-human CD3 (HIT3a), CD11a (HI111), IgG2a (G155-178) and IgG1 (MOPC-21); 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 hepato-carcinoma HuH7 and HepG2, the human acute monocytic leukemia THP-1, the B-lymphoblastoid 721.221 and the mouse lymphoblast-like mastocytoma P815 cell lines were used untransfected or stably transfected (Gene Juice; Merck Millipore or Microporator; Labtech international) to express glycosylphosphatidylinositol (GPI) -anchored GFP(19).

Cells were cultured in standard DMEM-based media supplemented with 10% FBS, 2 mM L-glutamine, 10 mM Hepes, 50 $U{\cdot}mL^{-1}$ penicillin and 50 $\mu g{\cdot}mL^{-1}$ streptomycin (all from Gibco). Freshly isolated T cells were stimulated for 24 h with 150 $U{\cdot}mL^{-1}$ human recombinant IL-2 (Roche). Where indicated, macrophages and HCCs were pre-treated for 24 h with 75 μM 18-alpha-GA (in DMSO; Sigma) or 5 μM manumycin A (in methanol; Sigma), or for 2 h with 1 μM latrunculin A (in DMSO; Merck) or 5 μM nocodazole (in DMSO; Sigma), 10 μg/ml brefeldin A (in methanol; Merck) or 10 μg/ml polyclonal antibody anti-SR-BI (Novus Biologicals), or for 30 min. with 1 mM 2-octanol (in EtOH; Sigma) or 100 μM oleamide (in DMSO; Sigma) or with the appropriate concentrations of methanol, DMSO or EtOH. These reagents were then also present throughout the co-culture of cells.

Cell sorting

Primary macrophages or THP-1 were labelled with a fluorescent tracer (2 μM CellTrace Far Red DDAO-SE; Molecular Probes) and co-cultured with HuH7 (ratio 1:1). Cells were collected after 1 min. or 5 h and only viable single cells were sorted using a multiparametric gating strategy based on FSC/SSC, live/dead cell discrimination using a dead cell stain (100

nM Sytox Blue; Invitrogen) and macrophages and HuH7 fluorescent markers, respectively. CellTrace and GFP sorted cells were checked for purity by flow cytometry, with the purity of macrophages and HuH7 always being > 98.5% (FACSAria; Diva software; BD Bioscience).

Flow cytometric analysis of intercellular interactions and molecular exchange

Macrophages were labelled as follows: 1 μM 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD; Molecular Probes) or 1mg/ml EZ-Link Sulfo-NHS-Biotin (Pierce) or 1μM of the specific RNA-dye F22 (a styryl dye specific for RNA which remains non-fluorescent until it binds RNA)(20) or transfected with 50 nM scramble interfering RNA labelled with Cy5 (Cy5-scramble-siRNA; Sequence 5′-[5Cy5]rArCrGrArUrArCrArUrArGrCrCr-3′; Integrated DNA Technologies - GenMute siRNA Transfection reagent; SignaGen Laboratories). Fluorescent macrophages were then washed and left to rest at 37°C for 30 min. or overnight (when transfected). Cells were then detached, washed and co-cultured with HuH7, P815 or 221 in a fresh plate. In transwell experiments, HuH7 were cultured above or below a transwell membrane (0.4-μm-pore membrane; Costar), with macrophages added only to the compartment above the transwell membrane.

After co-culture, cells were analysed by flow cytometry (Fortessa II; BD Biosciences); The percent of fluorescent molecules exchanged was calculated as: 100 * (MFI recipient cells)/(MFI donor cells), where MFI is the Median Fluorescence Intensity.

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% CO_2, by confocal microscopy (TCS SP5 RS; Leica) using excitation wavelengths of 488, 546, and 633 nm with a 20× 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 (Volocity; Improvision Ltd).

Quantitative Real-Time PCR assays (qRT-PCR)

miRNAs were isolated from total RNA (mirVana PARIS kit; Ambion) and reverse transcribed (TaqMan miRNA reverse-transcription kit; Applied Biosystems). Large RNAs were isolated with trizol (Invitrogen) and reverse transcribed (High Capacity Reverse Transcription kit; Applied Biosystems). cDNAs were amplified by reverse transcription 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-425-5p, RNU48, hsa-miR-122 Hs03303072_pri, hsa-miR-142 Hs03303162_pri, hsa-miR-223 Hs03303017_pri, has-miR-425 Hs03303194_pri, stathmin-1 Hs01027515_gH, insulin-like growth factor 1 receptor Hs00609566_m1, ras homolog gene family, member B Hs03676562_s1, ephrin-A1 Hs00358886_m1 and 18S rRNA. Quantitative PCR was conducted in triplicate at 95°C for 10 min., followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec (7500 Fast Real-Time PCR System; Applied Biosystems). Cycle thresholds were normalized to an internal control, RNU48 for miRNA and 18S ribosomal RNA for pri-miRNA and mRNA assays. The amount of RNA was calculated using the ΔΔCT method (21), where the level of expression of an RNA was normalised to the adapted internal control (denoted ‘relative expression’), and where appropriate to the level of expression at 1 min. of co-culture (denoted ‘fold change’).

Apoptotic Bodies

Microparticles were isolated from supernatant of 2.5×10^6 macrophages treated for 16 h with 3.5 μg/ml cycloheximide (Sigma) by sequential centrifugation steps as previously described(22). Collected microparticles were confirmed to be apoptotic bodies by staining a small fraction with the DNA marker Draq5 (1 μM; New England BioLab). Unstained apoptotic bodies were added to 2.5×10^5 HuH7 cells for 1 min. or 5 h, before HuH7 cells were washed and collected for analysis by 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 2.5×10^5 HuH7 cells for 1 min. or 5 h. Cells were washed and collected for qRT-PCR. To assess the polarisation of exosomes, macrophages were stained with 2 μM CellTrace Far Red DDAO-SE (Molecular Probes) and co-cultured with HuH7 cells (ratio 1:1). Cells were fixed in 4% PFA (Thermo Scientific), permeabilised with 0.05% saponin/PBS (Sigma) and stained with a mouse anti-human CD63 mAb (H5C6, IgG1; BD Pharmingen) followed by an Alexa Fluor 568-conjugated goat anti-mouse IgG1 secondary antibody (IgG1; Invitrogen) and imaged by confocal microscopy.

HDL assays

2×10^5 HuH7 were cultured alone or with macrophages for 1 min. or 5 h. At the end of the co-culture, 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 HDLs (HDL and LDL/VLDL Cholesterol Assay Kit; Abcam).

Sponge constructs

Sponges were designed following the method developed by Kluiver et al.(23) The oligonucleotides used to generate the scramble sponges and anti-miR-223 sponges bulged multiple miRNA antisense binding sites (MBS) constructs were respectively synthesised as [Phos]*GTCCC*TATCTACCTGCACTCCGATGCTCTGTTATCTACCTGCACTCCGATGCTCTG*GG* and [Phos]GTCCCTGGGGTATTCATAAACTGACAAATTTGGGGTATTCATAAACTGACAGG. Plasmids were then screened for having the correct insert by PCR using the following primers (Eurofin MWG): Forward TTTATCCAGCCCTCACTCC and Reverse TTGTGTAGCGCCAAGTGCC. A sponge construct coding for 2 × 2 bulged MBS (8 non perfect miRNA antisense sites) were transfected in HuH7 cells (Gene Juice; Merck Millipore). Stable transfectants were selected with 1 μg/ml puromycin (Sigma).

AntagomiRs

AntagomiRs were synthesised with 2′-*O*-methyl oligonucleotides (Integrated DNA Technologies) as Scramble antisense - 5′-mA*CmG*AmUmA*CmAmU*AmG*CmCmU*CmU*CmU*GmA*CmG*C-3′ and miR-223 antisense - 5′-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; SignaGen Laboratories).

Dual-Luciferase Reporter Assay

Plasmids encoding a portion of the 3′UTR of STMN1 linked to the firefly luciferase protein, both full length, truncated and mutated, were previously described(24). Firefly luciferase constructs were co-transfected with *Renilla* luciferase vector control (Promega) into HuH7.

Where indicated, HuH7 were stably expressing sponges, and macrophages were transfected with antagomiRs. 24 h after transfection, macrophages and transfected HuH7 were detached, washed and co-incubated (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 as:

$$(\text{Firefly Activity}/\textit{Renilla}\ \text{Activity})_{5\text{ h or }24\text{ h}}/(\text{Firefly Activity}/\textit{Renilla}\ \text{Activity})_{1\text{ min.}}$$

Proliferation Assays

10^4 HuH7, untransfected or transfected with anti-miR-223 or control scramble sponges, were seeded in triplicate and co-cultured with macrophages, transfected or not with either scramble or anti-miR-223 antagomiRs (ratio 1:3) in presence of 1 μCi of [^{3}H]-thymidine (Perkin Elmer) per well. Cells were harvested (Harvester 96 Mach II M; Tomtec) after 4 days and cell proliferation, assessed by [^{3}H]-thymidine uptake, was measured in a beta scintillation counter (1450 MicroBeta TriLux; Wallac).

Statistical Analysis

Mann–Whitney U was used as statistical test for all data (GraphPad software; Prism). Mean values are shown and standard error bars are standard error of the mean (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 labelled as follows: (i) surface membrane was marked with fluorescent lipid DiD, or (ii) surface proteins were biotinylated, or (iii) RNA was stained with the specific dye F22 (20), or (iv) cells were transfected to take up a small RNA conjugated to the fluorescent dye Cy5 (Cy5-scramble-siRNA). These differently labelled macrophages were then co-cultured with other cells: the human HCC HuH7, to study the transfer of cell components to hepatic tumor cells, but also the EBV-transformed human B cell line 721.221 (221), or the mouse lymphoblast-like mastocytoma cell line P815, to test in parallel the transfer to other human tumor cells, respectively human or murine cells - each transfected to express GPI-anchored GFP so that they can be easily 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 then assessed by flow cytometry (Figure 1A and Supplementary Figure 1A).

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

The presence of a Transwell porous membrane was used to allow direct contact between macrophages and HuH7 in the upper chamber while 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 these cellular

components (Figure 1B). Similar levels of transfer 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 (Supplementary Figure 1B). In addition, confocal microscopy of HCCs co-cultured 5 h with primary macrophages or THP-1 (Supplementary Figure 1C) revealed that transferred fluorescent components accumulated, at least to some extent, in cytoplasmic vesicles (Figures 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-RNA 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 and we next tested directly whether or not specific RNAs transferred between macrophages and HuH7.

Specific miRNAs - not all - transfer from primary macrophages to HCCs

To test whether or not macrophages transfer their own miRNAs to HuH7, 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 -223, previously reported to work together to regulate gene expression (26), were both highly expressed in the human macrophage cell line THP-1 (Supplementary Figure 1D) and primary macrophages, with very little expression detectable in HuH7 (Figure 2A). In contrast, macrophages expressed a lower level of miR-122 compared to HuH7 and the level of expression of miR-425 was similar in both cell types.

Cells were co-cultured and separated by FACS (purity >98.5% - Supplementary Figure 2A). Efficient separation of these cell types after sorting was confirmed by testing the level of expression of mRNA specific to macrophages and HuH7, specifically Mac-1 and cytokeratin 19 (CK19) (Supplementary Figures 2B-C). That is, for example, the macrophage-specific mRNA Mac-1 was not detected in HuH7 after cell sorting (Supplementary Figure 2C). The amount of miR-142 and -223 in sorted THP-1 (Supplementary Figures 1D-E) or macrophages remained relatively stable before and after co-culture (Figures 2B). However, the level of both miR-142 and -223 increased dramatically in HuH7 upon co-culture with macrophages (Figure 2C). In contrast, there was little, if any, change in the level of miR-122 and -425 detected in HuH7 (Figure 2C). The specific changes in levels of miR-142 and -223 were truly in the HuH7, rather than the presence of contaminating macrophages, because HuH7 deliberately spiked with varying percentages of macrophages did not replicate the changes in miRNAs detected here (Supplementary Figure 2D).

The amount of miR-142 and -223 in HuH7 increased after co-incubation with macrophages across multiple donors tested; with a fold increase of 12 to 1,984 depending on the donor (Figure 2D). In contrast, the level of miR-122 and -425 in HuH7 did not significantly change after co-culture, across donors (Figure 2D). Primary human B and T cells also express high levels of miR-142 and -223, similar to macrophages (Figure 2E). However, after co-culture with B or T cells, HuH7 contained only a low amount of miR-142 and very little, if any, miR-223 (Figure 2F). Together, these data establish that HuH7 express high levels of miR-142 and -223 following co-culture with human macrophages.

We next set out to determine whether or not the increase in macrophage miRNA detected in HuH7 was due to transfer of miRNA or synthesis of nascent miRNA triggered by contact with macrophages. First we tested if miRNAs would be detected in recipient cells after co-culture 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

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macrophages were still able to interact with HuH7 and form conjugates (Supplementary Figure 3A). Small changes in the level of miR-425 and -122 in HuH7 after co-culture were not inhibited by the 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 -223 in HuH7 after co-culture, were prevented by chemical fixation of macrophages (Figure 3A). This indicates that an upregulation in these miRNAs requires more than that 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 and so to more directly test whether or not miR-142 and -223 were directly acquired from macrophages or newly synthesized in HuH7, processing of these miRNA in HuH7 was assayed for. Precursors of miRNA, pri-miRNAs, are several hundreds of nucleotides long and 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. Thus instead, the expression level of each pri-miRNA was assessed directly in donor and recipient cells by RT PCR.

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

miRNA are transferred to HCCs in a contact-dependant 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 here, HuH7 were cultured with macrophages or apoptotic bodies isolated from macrophages that had been treated with cycloheximide to trigger apoptosis (Supplementary Figures 3B-C). HuH7 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 (Figure 4A). However, the percent of HuH7 containing apoptotic bodies did not increase when the cells were co-cultured with macrophages compared to when HuH7 were cultured alone (Figure 4A). More importantly, the level of miR-142 and -223 did not increase in HuH7 after incubation for 5 h with apoptotic bodies derived from ten-fold more macrophages than usually used in co-culture experiments (Figure 4B). These findings exclude the possibility that miR-142 and -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 here, exosomes were isolated from a co-culture of ten-fold more macrophages and HuH7 than usually used in other co-culture experiments and this large number of exosomes were subsequently added to fresh HuH7. These exosomes were taken up by HuH7, evidenced by flow cytometry showing HuH7 contained exosomes that had been pre-labeled with an anti-CD63 mAb (Supplementary Figure 3D). However, incubation with this large number of exosomes led to only a small increase in the amount of miR-142 and -223 detected in HuH7 (Figure 4C).

Exosomes can also be directly delivered at the synapse after polarisation (27). Here however, brefeldin A, which inhibits the secretion of exosomes (28), did not influence the

level of miR detected in HuH7 after co-culture with macrophages (Figure 4C). In addition, cell conjugates were imaged at different times of co-culture, where exosomes were stained with an anti-CD63 mAb. There was no polarisation of exosomes towards the contact between macrophages and HuH7 (Supplementary Figures 3E-F). 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 high density lipoproteins (HDLs) (3). Here, HDLs were detected in cultures of HuH7 incubated alone or with macrophages (Supplementary Figure 3G). However, only a very small amount of miR-142 and -223 transferred via the supernatant taken from a co-culture of macrophages and HuH7 (Figure 4d). Furthermore, blockade of Scavenger Receptors class B member 1 (SR-BI) that functions as the receptor for HDLs on HuH7, using a polyclonal antibody, did not significantly inhibit the transfer of miR-223 and -142 to HuH7 (Figure 4D). In addition, treating the cells with manumycin A, which enhances miRNA export to HDLs but also inhibits the release of exosomes (3, 29) didn't influence the transfer of miR-223 and -142 to HuH7 (Figure 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 -223 from macrophages to HuH7 (Figure 4E), consistent with experiments using the RNA specific dye F22 (Figure 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 while treatment with nocodazole, which interferes with microtubule polymerization, did not influence the transfer (Figure 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-alpha-glycyrrhetinic acid (18-alpha-GA), that 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 (Figure 4G). Two other inhibitors of the gap junctions, 2-octanol and oleamide (31), also decreased the transfer of miR-142 and -223, to a similar extent for miR-142 and even more efficiently for miR-223, in comparison with 18-alpha-GA (Figure 4G). Thus, taken together, these data establish that endogenous macrophage miRNAs transfer to HuH7 in a manner dependent on cell-cell contacts and gap junctions.

Transferred miRNAs are functional and inhibit HCC proliferation

miR-142 has been relatively little studied but it has been established previously that miR-223 is commonly repressed in HCCs (24). Moreover, it has been shown that miR-223 targets the mRNAs of stathmin-1 (STMN1) (24) and insulin-like growth factor-1 receptor (IGF-1R) (32), which both influence cellular proliferation (33, 34). We therefore assayed for changes in expression of these two mRNAs in HuH7 following co-culture with macrophages. Compared to HuH7 cultured alone, the level of expression of both STMN1 and IGF-1R mRNAs in HuH7 remained the same after 5 h, but decreased significantly with 24 h of co-culture (Figure 5A). RhoB and Ephrin A1 (EFNA1), also previously described as targets for miR-223 (3), were not affected by the co-culture with macrophages (Supplementary Figure 4A).

To test whether or not a decrease in the level of mRNA was caused by the intercellular transfer of miRNAs, HuH7 (here, not transfected to express GPI-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, down-regulation of STMN1-mRNA in HuH7 triggered by co-

culture with macrophages was abrogated in cells expressing anti-miR-223 sponges (Figure 5B). Cells expressing control scramble sponges down-regulated STMN1 mRNA to the same extent as untransfected cells.

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

To further test the consequences of miRNA transfer, macrophages were transfected with methyl-oligonucleotides expressing the complementary sequence of a miRNA of interest, here termed as antagomiRs (35-37) - directed against miR-223 or a control scramble miRNA. HuH7 co-cultured with cells expressing scramble control antagomiRs down-regulated luciferase activity similarly to untransfected cells, but expression of anti-miR-223 antagomiRs restored luciferase activity to a mild but statistically significant extent (Figure 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, and IGF-1R is implicated in several cancers and can aid the growth of tumors (32, 35). Here, we found that co-culture with macrophages reduced the proliferation of HuH7 by over 50%, as assessed by a standard thymidine incorporation assay (Figure 5F). Proliferation of HuH7 was not effected when co-cultured with fixed macrophages (Figure 5F). More importantly, proliferation of HuH7 was restored to some extent when co-cultured in presence of gap junction inhibitors 18-alpha-GA, 2-octanol, or oleamide (Figure 5G). To test directly whether or not 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 or anti-miR-223 antagomiRs in the macrophages (Supplementary Figures 4B-C). Indeed the reduced proliferation of HuH7 caused by co-culture with macrophages was partially restored by expression of anti-miR-223 sponges in the HuH7 (Figure 5H) or of anti-miR-223 antagomiRs in the macrophages (Figure 5I). Proliferation of another HCC line, HepG2, to which macrophages also transferred miRNA-142 and -223 (Supplementary Figures 4D-E), was equally decreased in presence of macrophages and importantly, proliferation of HepG2 was also restored in presence of sponges or antagomiRs to inhibit the activity of miR-223 (Figures 5F, H-I). 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 mRNAs and miRNAs can also transfer between cells. First demonstrated in plants and nematodes(2), the intercellular transfer of specific RNAs has been relatively recently observed between mammalian cells.

miRNAs can travel through body fluids and reach distant recipient cells via vesicles or association with HDL (42), or traffic across an immune synapse (9). However the functional importance for an intercellular transfer of endogenously expressed miRNAs between human cells has not been established. Here, we found that primary human macrophages efficiently transferred RNA to HCC as well as, in a less extend, to other tumor cells such as 221 cells. Likely, the transfer of miRNA is a common phenomenon, and the importance of the transformation level of the cells has now to 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 multi-directional 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 HCC. There is some specificity to which miRNAs transfer since miR-142 and -223 were acquired by HCCs but not miR-425. It remains unclear whether the specificity of transfer that we observe is the result of particularly high expression of miR-142 and -223 in macrophages compared to Huh7, or if this process is limited to certain miRNAs. Other RNAs, including other miRNAs must also transfer beyond those directly assayed for here and more broadly, transfer of miRNAs between cells could be commonplace. A screen to assess which miRNAs transfer between macrophages and HCC 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 - though the transfer of miR-142 and miR-223 was particularly efficient from macrophages to HCCs.

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

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, with a stronger influence, gap junctions, all effect 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-alpha-GA, 2-octanol or oleamide decreased the intercellular transfer of miR-142 and -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. reported previously that small synthetic RNAs can move through gap junctions composed of connexin 43 (Cx43) but not those comprised of other connexins, Cx32 and Cx26 (48). In the context of the cell types studied here, 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 already known to be involved in different immunological functions such as the secretion of cytokines or the antigen 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 HCC is not clear. microRNAs could occur traffic directly through connected gap junctions, perhaps in a manner that can allow peptides to transfer (44), or 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). Here we found that transfer of these miRNAs influences protein expression in HuH7. Specifically, miR-223 decreases expression of STMN-1, a protein that is usually only present at low levels in healthy hepatocytes, but gets expressed more highly in hepato-carcinomas (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 showing a decrease of 40% in the number of HuH7 cells upon transfection with miR-223 (24).

Expression of other mRNAs, RhoB and Ephrin A1 (EFNA1), also previously described as targets for miR-223 (3), were not affected by the co-culture with macrophages. This likely reflects complexity in the miRNA system that we do not yet fully understand; mRNA targets are likely to vary in different cell types, as well as according to a cell's state of activation, and 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 micro-array. However, one difficulty with this is that many miRNAs are expressed both in macrophages and HCC, and it is not trivial to design a screening approach that is able to distinguish new-synthesised miRNAs from miRNAs acquired directly from other cells in contact.

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 contacts with hepatocytes (13, 52). Kupffer cells are bone marrow-derived monocytes which migrate from the blood to the hepatic tissue, transform into tissue macrophages and, amongst other immune functions, have a recognised anti-tumor 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 of the size of the hepatic metastasis (60). Thus, several lines of evidence indicate a role for macrophages in preventing the development of hepato-carcinomas. However, the interaction between macrophages and tumor cells are variable and not clearly understood. Macrophages form a very heterogeneous population with both pro- and anti-tumor properties. Macrophages can be anti-tumor through presentation of antigens to T cells while some tumor-associated macrophages are pro-tumorigenic (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 defence against tumour cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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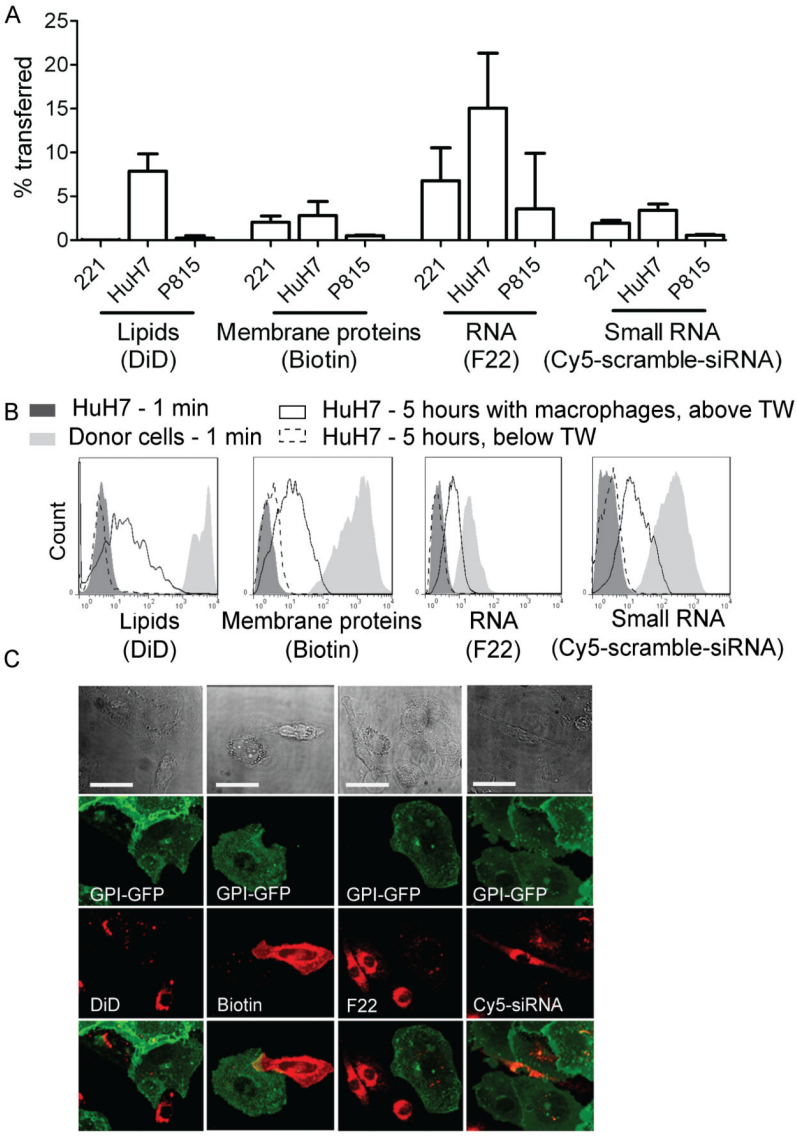

Figure 1. Macrophages transfer cell components, including RNA molecules, to hepato-carcinoma cells

(A) Macrophages were stained with the fluorescent lipid DiD or biotin or RNA dye F22, or were transfected with Cy5-scramble-siRNA, and co-cultured with 221, HuH7 or P815 transfected to express GPI-GFP for 1 min. or 5 h. Graph shows the percentage of fluorescence initially present in macrophages that had transferred to recipient cells as determined by flow cytometry. Error bars are SEM. n = 3. (B) HuH7 were cultured above or below a transwell membrane (TW), with macrophages stained as described in (A), added only to the compartment above the TW. After 1 min. or 5 h of co-culture, cells were analysed by flow cytometry. Light grey histogram shows the level of fluorescence in the donor cells at the beginning of the co-culture. Data are representative of 4 donors. (C) Donor macrophages were stained as described in (A) (red) and co-cultured with HuH7 (marked by GFP, green). After 5 h of co-culture, cells were analysed by confocal microscopy. Scale bars: 10 μm. n > 44 cells for each condition, from 6 independent experiments.

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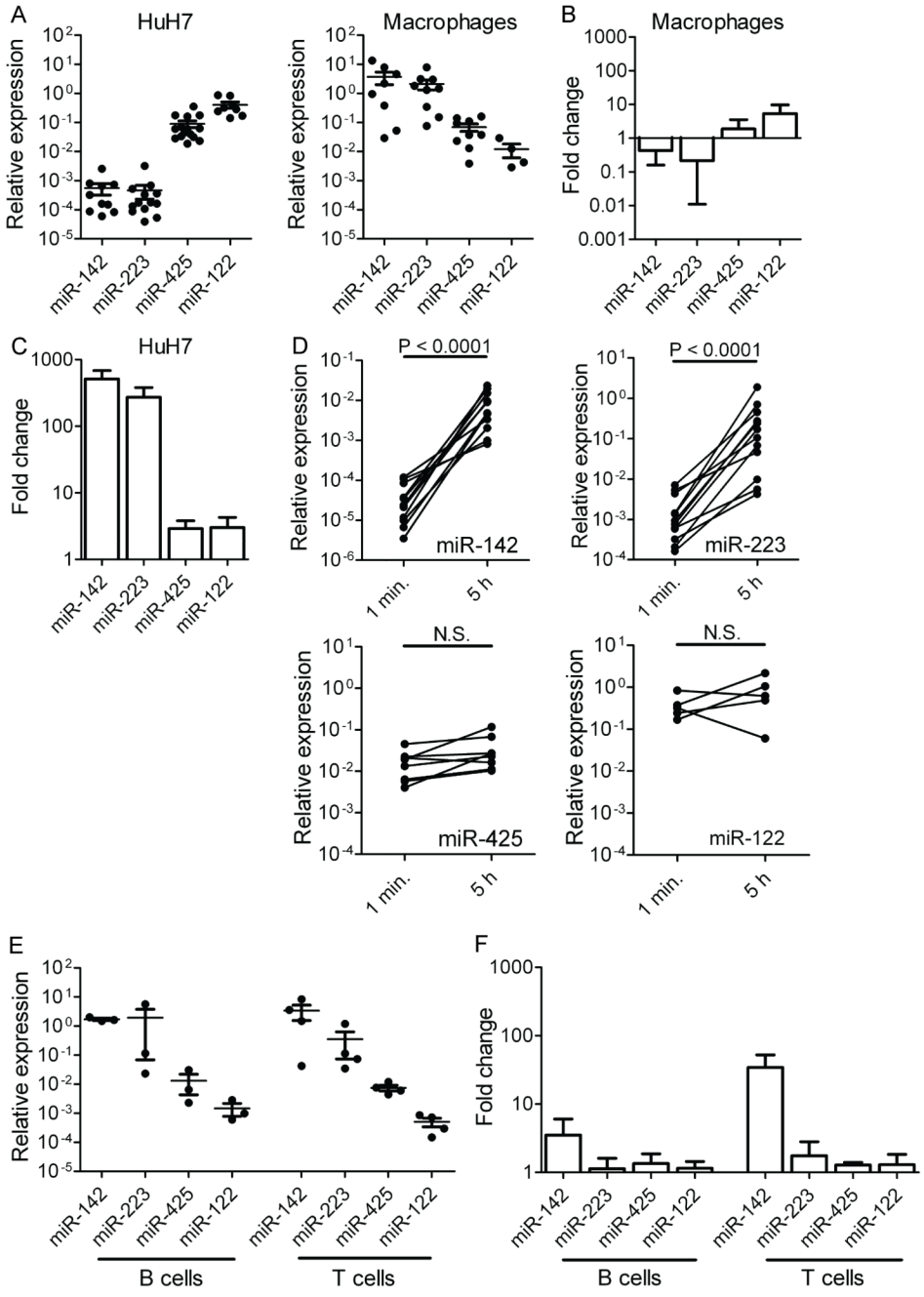

Figure 2. Specific miRNAs are transferred from macrophages to HCCs

Relative expression of miR-142, -223, -425 and -122 was assessed by quantitative Real-Time PCR (qRT-PCR) in macrophages and HuH7 alone (A). Fold change in the level of expression of miR-142, -223, -425 and -122 was assessed by quantitative Real-Time PCR (qRT-PCR) in isolated macrophages and HuH7 that had been co-cultured together for 1 min. or 5 h - macrophages are represented in (B) and HuH7 in (C). Data points represent individual experiments in (A); (B) n = 3; (c) n > 5. (D) Relative expression of each miRNA in HuH7, after 1 min. or 5 h of co-culture with macrophages was individually plotted. Connected data points represent individual experiments, each using cells from a different donor. Expression of miR-142, -223, -425 and -122 in primary human B and T cells alone

(E) or in HuH7 co-cultured with T or B cells for 1 min. or 5 h (F). Data points represent individual donors in (E); (F) B cells: n = 3, T cells: n = 4. Error bars are SEM. Significance was determined by a non-parametric Mann-Whitney U test.

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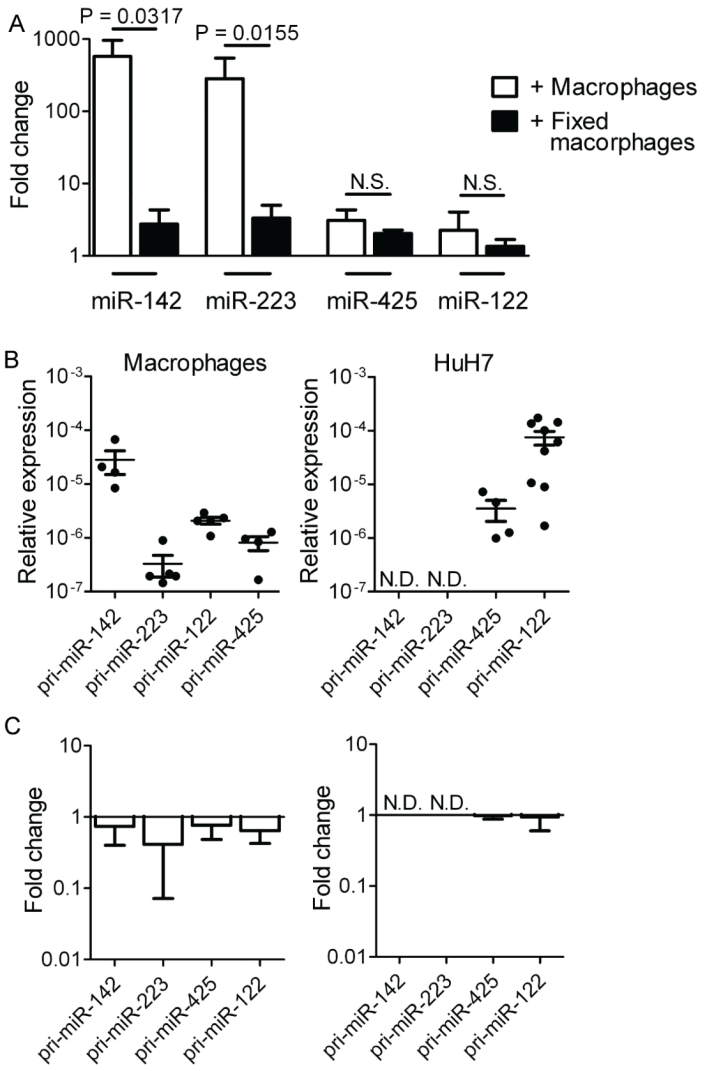

Figure 3. miR-142 and -223 are not newly synthesised in HuH7 following co-culture with macrophages

(A) Fold change in the level of miR-142, -223, -425 and -122 present in HuH7 cells, assessed by qRT-PCR, following co-culture with macrophages that were either alive or fixed in paraformaldehyde (PFA). n = 4. (B) Relative expression of precursors of miR-142, -223, -425 and -122 in macrophages, and HuH7 analysed after 5 h of co-culture. Data points represent individual experiments. (C) Fold change of pri-miRNAs in macrophages and HuH7 cells following their co-culture. n > 3. N.D. - Not Detected. Error bars are SEM. Significance was determined by a non-parametric Mann-Whitney U test.

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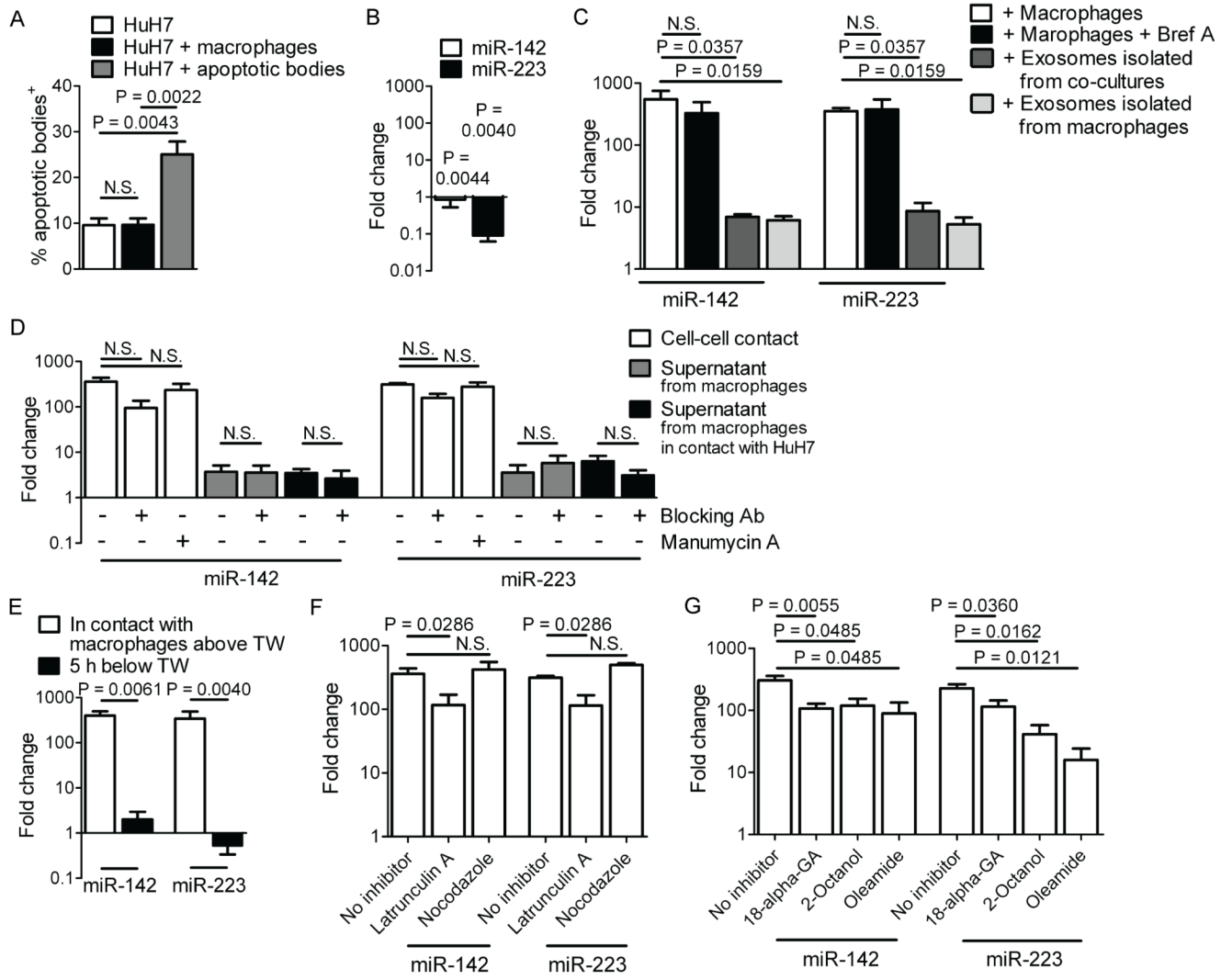

Figure 4. miRNAs are transferred via a direct contact between macrophages and HCCs

(A) Percent of cells containing apoptotic bodies, as stained by Draq5 and detected by confocal microscopy, for HuH7 alone or co-cultured with macrophages or co-cultured with apoptotic bodies isolated from macrophages treated with cycloheximide (CHX). n > 707 cells for each condition, from 6 independent experiments. (B) Fold change in level of miR-142 and -223, assessed by qRT-PCR, in HuH7 co-cultured for 5 h with isolated apoptotic bodies derived from macrophages treated with CHX. n = 4. (C) Fold change in the level of miR-142 and -223 in HuH7, as determined by qRT-PCR, following co-culture for 5 h with macrophages, macrophages in presence of 10 μg/ml brefeldin A (Bref A), or with exosomes that were isolated from cultures of macrophages or isolated from macrophages co-cultured with HuH7. n = 4. (D) Fold change of miR-142 and -223 in HuH7 co-cultured for 5 h with macrophages, supernatant form macrophages or supernatant from macrophages previously co-cultured with HuH7, in the presence or absence of an antibody blocking HDL receptors or 5 μM manumycin A. n > 4. (E) Fold change of miR-142 and -223 in HuH7 co-cultured for 5 h above or below a TW, with macrophages added only to compartment above the TW. n > 4. (F) Fold change of miR-142 and -223 in HuH7 co-cultured 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 pre-treated and then co-

cultured with 75 μM 18-alpha-GA or 1 mM 2-octanol or 100 μM oleamide. n = 4. Error bars are SEM. Significance was determined by a non-parametric Mann-Whitney U test.

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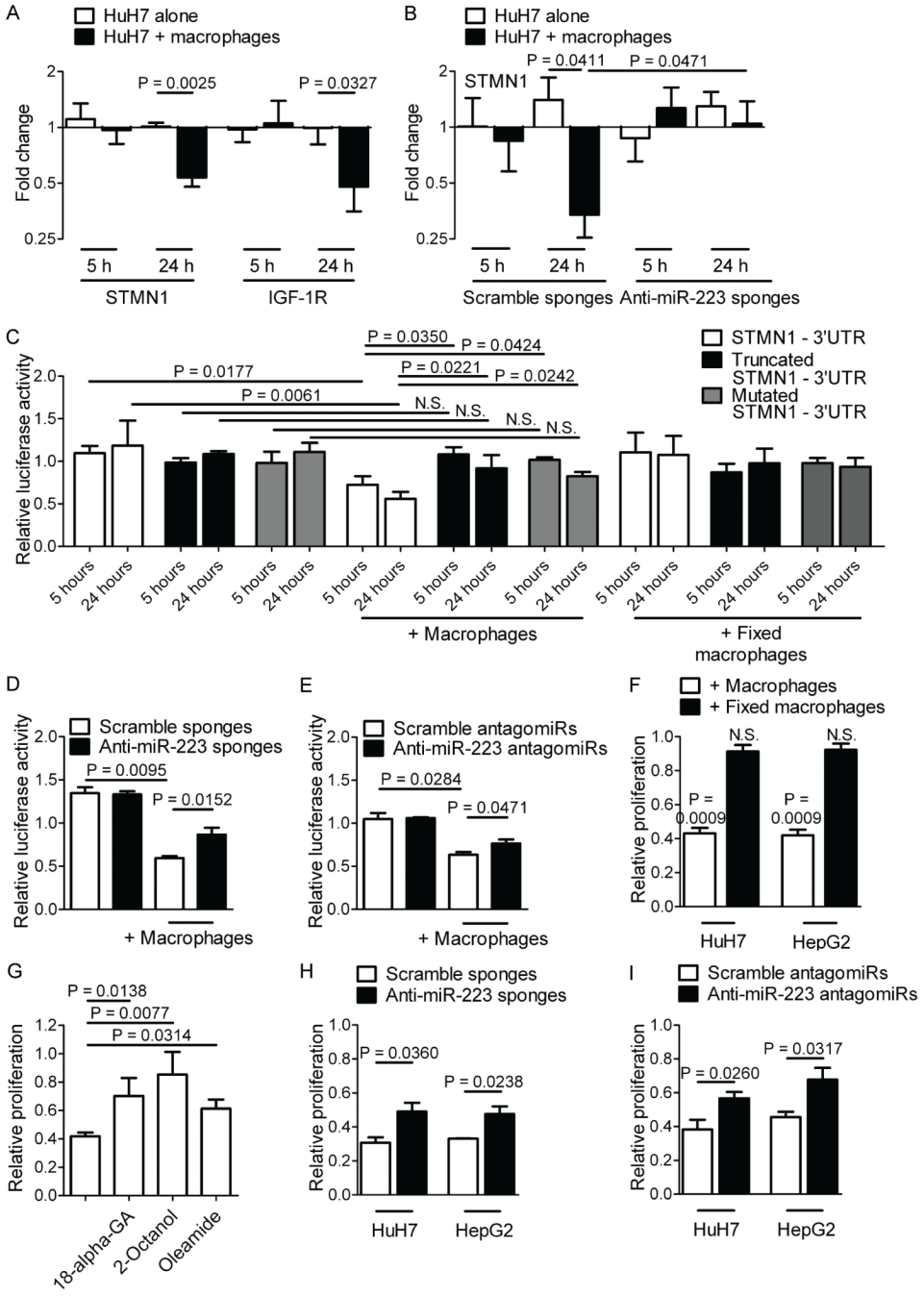

Figure 5. Intercellular transfer of miRNAs is functionally important

(A) Fold change of STMN1 and IGF1-R mRNA was assessed by qRT-PCR in HuH7 cells alone or co-cultured 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 analysed 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 co-cultured 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 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 co-cultured with live or fixed macrophages was measured by incorporation of [3H]-thymidine over 4 days. Graph shows proliferation of HCCs in co-cultures relative to HCCs cultured alone. n > 7. (G) As in (F), except that where indicated, macrophages and HuH7 were pre-treated for 24 h with 75 μM 18-alpha-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 non-parametric Mann-Whitney U test.