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Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells

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Exosomes are vesicles of endocytic origin released by many cells. These vesicles can mediate communication between cells, facilitating processes such as antigen presentation. Here, we show that exosomes from a mouse and a human mast cell line (MC/9 and HMC-1, respectively), as well as primary bone marrow-derived mouse mast cells, contain RNA. Microarray assessments revealed the presence of mRNA from approximately 1300 genes, many of which are not present in the cytoplasm of the donor cell. *In vitro* translation proved that the exosome mRNAs were functional. Quality control RNA analysis of total RNA derived from exosomes also revealed presence of small RNAs, including microRNAs. The RNA from mast cell exosomes is transferable to other mouse and human mast cells. After transfer of mouse exosomal RNA to human mast cells, new mouse proteins were found in the recipient cells, indicating that transferred exosomal mRNA can be translated after entering another cell. In summary, we show that exosomes contain both mRNA and microRNA, which can be delivered to another cell, and can be functional in this new location. We propose that this RNA is called "exosomal shuttle RNA" (esRNA).

Exosomes are small (50–90 nm) membrane vesicles of endocytic origin that are released into the extracellular environment on fusion of multivesicular bodies (MVB) with the plasma membrane[1]. Many cells have the capacity to release exosomes, including reticulocytes[2], dendritic cells[3], B cells[4], T cells[5], mast cells[6], epithelial cells[7] and tumour cells[8]. The functions of exosomes are not completely understood, although it has been shown that exosomes can participate in the signalling events contributing to antigen presentation to T cells[4] and the development of tolerance[9]. Several mechanisms have been hypothesized describing the interactions of exosomes and recipient cells. Exosomes can bind to cells through receptor–ligand interactions, similar to cell–cell communication mediating, for example, antigen presentation[4]. Alternatively, exosomes could putatively attach or fuse with the target-cell membrane, delivering exosomal surface proteins and perhaps cytoplasm to the recipient cell[10,11]. Finally, exosomes may also be internalized by the recipient cells by mechanisms such as endocytosis[12].

Exosomes were isolated from a mast-cell line (MC/9), primary bone marrow-derived mast cells (BMMC) and a human mast-cell line (HMC-1) through a series of microfiltration and ultracentrifugation steps modified from what has been previously described[4]. To confirm that the structures studied indeed are exosomes, they were examined by electron microscopy (Fig. 1a), flow cytometric analysis (FACS; Fig. 1b), and proteomic analysis (see Supplementary Information, Table S1). The electron micrographs of the exosomes revealed rounded structures with a size of approximately 50–80 nm, similar to previously described exosomes[4,13–15]. The identity of the studied vesicles was further confirmed as exosomes by FACS analysis (Fig. 1b), which show the presence of the surface protein CD63 — a commonly used marker of exosomes. Finally, extensive protein analysis of the MC/9 exosomes was performed on multiple samples using LC-MS/MS technology. A total of 271 proteins were identified (see Supplementary Information, Table S1) from three preparations of the isolated vesicles, of which 47 proteins were present in all three samples. More importantly, a large number of the proteins found in the preparations were the same as proteins previously identified in exosomes produced by other cells (that is, exosomes derived from intestinal epithelial cells, urine, dendritic cells, microglia, melanoma, T-cells and B-cells). In particular, 60% of the 47 proteins found in all samples of mast-cell exosomes have been previously found in other types of exosomes. Moreover, 39% of the 271 total proteins found in the analysed exosome samples have also been previously found in other types of exosomes. Thus, the electron microscopy, the FACS, and the detailed protein analyses each provided significant evidence in favour of the identification of the isolated vesicles as exosomes.

The presence of nucleic acids was examined in exosomes derived from MC/9, BMMC and HMC-1 cells to define a potential mechanism by which exosomes may mediate cell–cell communication. These assessments showed that isolated exosomes contain no DNA (see Supplementary Information, Fig. S1). However, substantial amounts of RNA were detected by agarose gel electrophoresis, spectrophotometry

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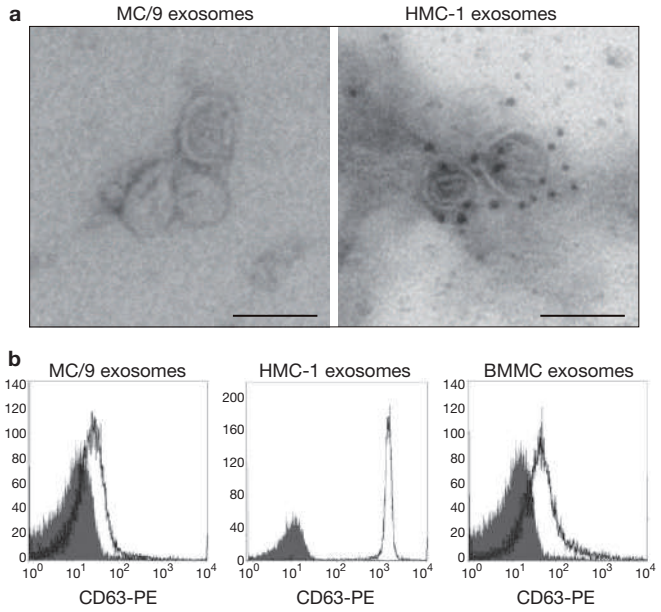

Figure 1 Identification and characterization of exosomes. Exosomes were isolated using repeated centrifugation, filtration and ultracentrifugation. (**a**) Electron micrographs of MC/9 and HMC-1 exosomes. The image shows small vesicles of approximately 50–80 nm in diameter. HMC-1 exosomes are immunogold labelled with anti-CD63. The scale bar indicates 100 nm. (**b**) For FACS analysis, exosomes (open trace) from MC/9, HMC-1 and BMMC or negative control (filled trace) were incubated with latex beads and stained with anti-CD63 PE.

and Bioanalyzer (Fig. 2). The MC/9 exosomal RNA was heterogeneous in size, but contained no or little ribosomal RNA (18S- and 28S- rRNA) compared to the donor cells. Importantly, small RNA was detected in the exosomes (Fig. 2a). The presence of poly-adenylated RNA in exosomes was also confirmed by synthesis of oligo d(T) primed cDNA (Fig. 2b). As a control, parallel samples were treated with RNase before cDNA synthesis. These results showed that exosomes from both BMMC and MC/9 cells contained presumptive mRNA in sufficient quantities to support cDNA synthesis. Importantly, RNA was also detected in the human mast-cell line, both by identification of radioactive ^{3}H-uracil (data not shown), and on an agarose gel (Fig. 2c). To confirm that the RNA is confined inside the exosomes, RNase treatment of the MC/9 exosomes was performed in solution. The results showed no difference in RNA degradation between RNase-treated and control exosomes (Fig. 2d), arguing against RNA being present on the exterior of exosomes. Importantly, exogenous RNA added to exosomes was degraded by the added RNase. Furthermore, exosomes treated with trypsin followed by separation of exosomes and the supernatant by ultracentrifugation, revealed that that exosomal RNA is resistant to trypsin treatment, again arguing that the RNA is confined inside of exosomes and not on external structures or macromolecules (Fig. 2e). In parallel, it was shown that anti-CD63 bound to exosomes was removed by trypsin treatment (Fig. 2f). To further examine the purity of exosomes, isolated exosomes were floated in a sucrose gradient. RNA detection on an agarose gel and detection of CD63 protein as an exosome marker by western blot analysis, and DNA detection, was performed on the different fractions. Interestingly, the results revealed that RNA and CD63 protein colocalized at the characteristic density of exosomes (1.11–1.21 g ml^{-1}; see Supplementary Information, Fig. S1). Moreover, no DNA was detected in any of these fractions.

Microarray assessments of MC/9 exosomal RNA confirmed the presence of approximately 1,300 different mRNA transcripts in the MC/9 exosomes. The identified mRNA in exosomes was approximately 8% of the mRNA detected in the donor cells (16,000 mRNA; Fig. 3a–d; see Supplementary Information, Table S2, and see Accession numbers in Methods). The gene profile analysis displayed essential differences in the level of mRNA transcripts from exosomes versus donor cells (Fig. 3c, e). The most abundant transcripts in the exosomes were generally different from the abundant transcripts in the donor cells. Interestingly, 270 gene transcripts were identified that were only present in exosomes, but not detectable in their donor cells, according to Affymetrix DNA–chip analysis (Fig. 3d and see Supplementary Information, Table S3). Cluster analysis also showed differences in expression of mRNA in samples from MC/9 cells versus their respective exosomes (Fig. 3e). A network-based analysis (Ingenuity software; http://www.ingenuity.com) suggested that a key function of the exosomal mRNA transcripts is "cellular development, protein synthesis, RNA post-transcriptional modification". This network involves 47 gene products, all encoded by mRNAs present in exosomes (Fig. 3f).

The mRNA present in exosomes was demonstrated to be functional (i.e., capable of encoding polypeptides in support of protein synthesis) using a rabbit lysate *in vitro* translation assay (Fig. 3g). Briefly, total mRNA was isolated, translated to proteins and separated by two-dimensional PAGE. Seven novel protein spots were excised and analysed by LC-MS/MS, revealing the presence of the mouse proteins Cox5b, Hspa8, Shmt1, Ldh1, Zfp125, Gpi1 and Rad23b (Fig. 3g). These results show that the exosomes carry intact and functional mRNA that can produce protein in the presence of functional protein machinery. In contrast, incubation of mast-cell exosomes in medium supplemented with L-^{35}S-methionine demonstrated that the exosomes do not themselves have the components necessary for autonomous protein synthesis (data not shown).

Our identification of RNA being present in exosomes supports the hypothesis that exosomes may represent a vehicle by which one cell communicates with another, actually delivering RNA and, in turn, modulating recipient-cell protein production. This would, of course, be a novel and more intricate way by which mammalian cells communicate with each other. Moreover, if exosomes interact with recipient cell through specific receptor–ligand interactions, this venue of communication could be expected to be cell type specific. Our studies have demonstrated that exosome-derived RNA can be transferred to other mast cells, but not to CD4 cells (Fig. 3h). Specifically, exosomes containing ^{3}H-labelled RNA were generated by exposing MC/9 cells to ^{3}H-uracil. After coculture for 24 h with unlabelled recipient cells, the radioactive RNA was quantified by scintillation counting. These transfer studies showed that labelled RNA in MC/9 exosomes was transferable to MC/9 cells (11 ± 3 fold change, $P = 0.02$), to HMC-1 cells (12 ± 3 fold change, $P = 0.02$), but not to CD4 cells (2 ± 1 fold change, P = not significant; Fig. 3h). Recently, several groups have shown that circular membrane fragments called microvesicles also may be involved in cell–cell communication, and may contain mRNA[16]. Microvesicles are larger than exosomes (up to 1 μm) and are shed from the cell membrane, in contrast to the more intricate production of exosomes through inward budding of endosomes. Nonetheless, the suggestion that microvesicle mRNA can be delivered to other cells[17,18] provides the foundation for our proposed transfer mechanism by exosomes.

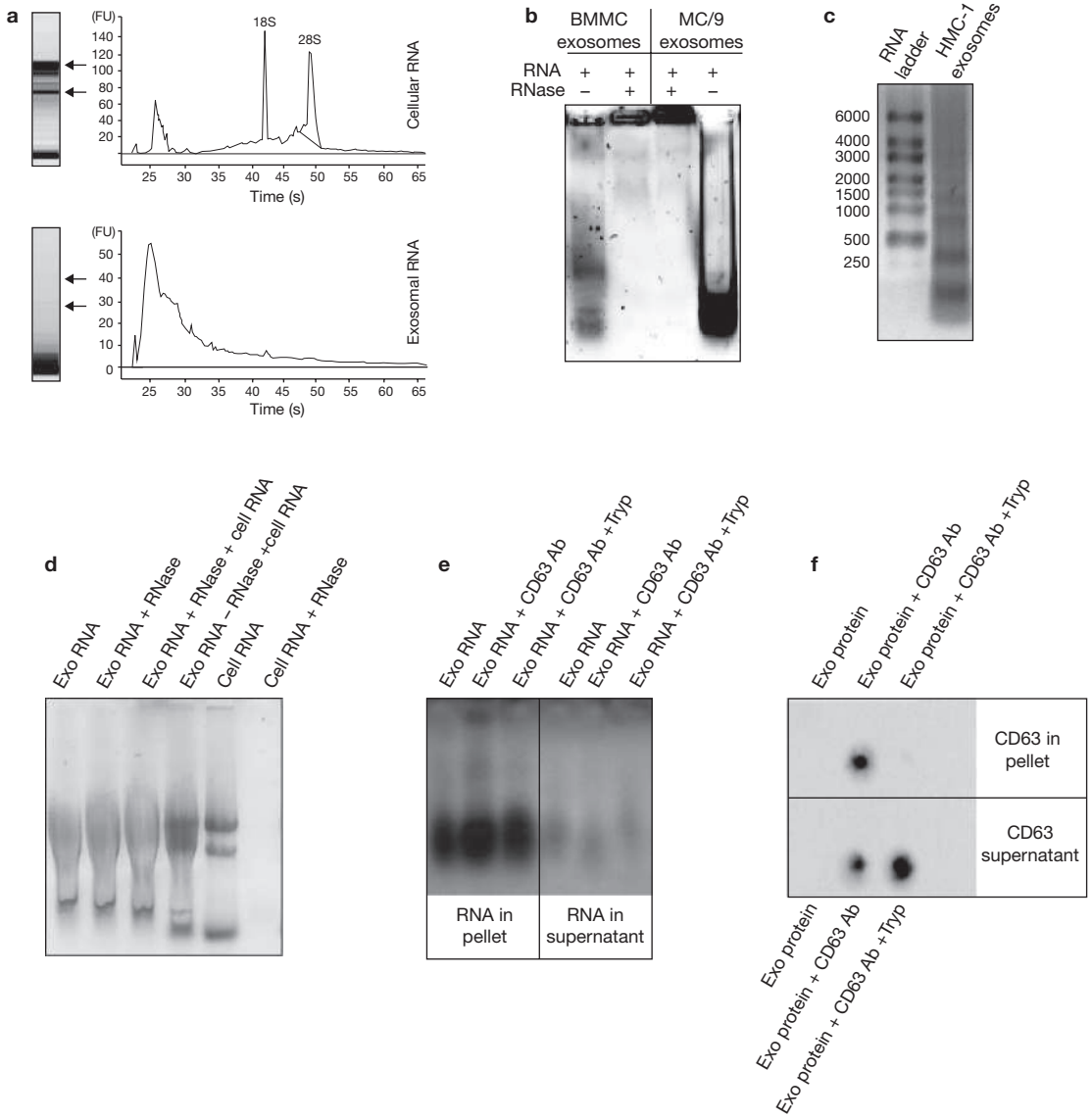

Figure 2 Exosomes contain multiple and heterogeneous RNA species. (**a**) RNA from MC/9 derived exosomes and their donor cells was detected using Bioanalyzer. The MC/9 exosomes contain a substantial amount of RNA, but no or very low amount of ribosomal RNA (18S- and 28S- rRNA) compared to their donor cells. Large amounts of small RNA (<30 nucleotides) were also detected in the exosomes and in their donor cells. (**b**) The presence of polyadenylated mRNA in exosomes from both MC/9 and BMMC was determined by conversion of mRNA to cDNA, using oligo d(T) primer and reverse transcriptase in the presence of radioactive CTP. As a negative control, samples were treated with RNase before cDNA synthesis. The product was separated on a 0.8% agarose gel and visualized using a phosphoimager. (**c**) HMC-1 exosomal RNA on a 1% agarose gel stained with ethidium bromide. (**d**) Intact MC/9 exosomes (exo) treated with RNase show no difference in RNA degradation compared to control exosomes. Cellular RNA added to the exosomes was degraded on RNase treatment. (**e**) MC/9 exosomes treated with trypsin (tryp) show no difference in RNA content compared to control, indicating that no macromolecules containing RNA are attached to the exosomes. Ab, antibody. (**f**) Anti-CD63 antibodies added to exosomes detach after trypsin treatment. An uncropped image of the scan is shown in the Supplementary Information, Fig. S3.

To determine whether mouse proteins could be produced in human mast cells after transfer of mouse MC/9 exosomes, we examined the presence of mouse proteins in the recipient cell, by two-dimensional PAGE followed by MALDI-TOF. After incubation of the human cells with mouse MC/9 exosomes for 24 h, 96 new or enhanced protein spots were identified. Interestingly, three distinct mouse proteins were identified in human cells that were not present in MC/9 exosomes. These proteins were mouse CDC6 (O89033), mouse zinc finger protein 271 (P15620) and mouse CX7A2 (P48771; see Supplementary Information, Fig. S2 and Table S4). The mRNA of the first two proteins was present in two of the microarray experiments, and the last one was present in all four microarrays performed, suggesting that mRNA delivered by exosomes to a recipient cell can be translated into protein.

The presence of large amounts of small RNAs in exosomes (Fig. 2a) suggested that exosomes may contain microRNA (miRNA, 19–22 nucleotides). Therefore, miRCURY LNA Array was applied to identify the miRNA in exosomes. The results showed that exosomes carry approximately 121 miRNAs that have been found in all four experiments (Fig. 4 and see Supplementary Information, Table S5). Unlike cellular MC/9 miRNAs, the exosomal miRNAs originate from a selective number of genes,

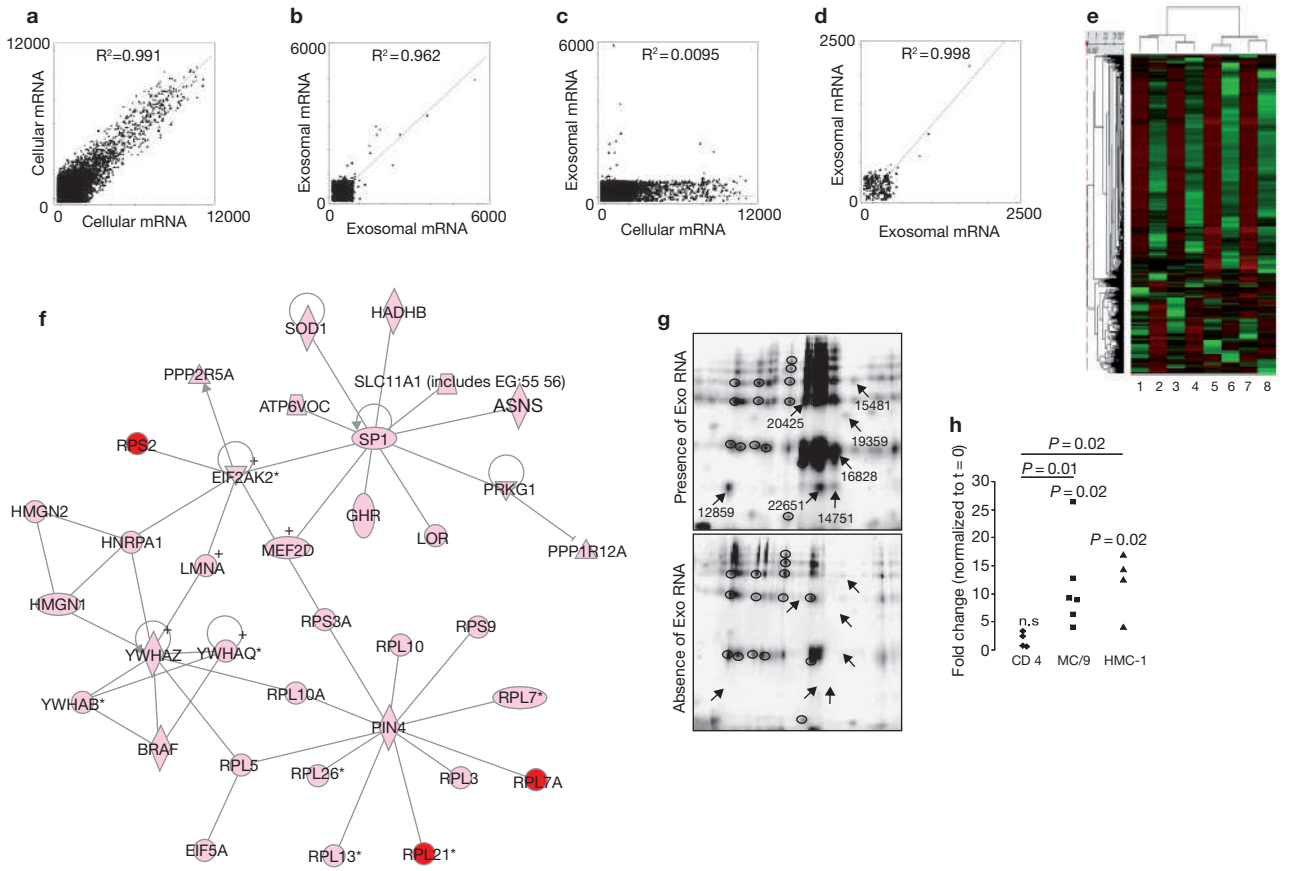

Figure 3 Characterization and transfer of exosomal RNA. Affymetrix mouse DNA microarray was applied to identify mRNA from MC/9 derived exosomes and cells. Each sample originated from double RNA preparations and quadruple DNA arrays were performed. (**a**, **b**) Correlation of gene expression signals within cells and within exosomes samples are shown. In total, 16,027 mRNA transcripts could be detected in the cells and 1272 mRNAs in the exosomes. (**c**) No relationship could be found between exosomal and cellular RNA. (**d**) Relationship of expression signal of the 248 genes that were only present in the exosomes, but not detectable in their donor cells. (**e**) Cluster analysis of exosomal mRNA (lanes 1, 3, 5 and 7) versus their respective donor cell mRNA (lanes 2, 4, 6 and 8) show clear differences in the expression of genes between cells and exosomes in the four samples analysed. (**f**) Network analysis using the Ingenuity software showed involvement of exosomal RNA in "cellular development, protein synthesis, RNA post-transcriptional modification" pathway, which includes 47 gene products. (**g**) The total RNA of MC/9 exosomes was isolated and *in vitro* translated to proteins, using a rabbit lysate protein machinery assay, and was subsequently separated by two-dimensional PAGE. A sample without exosomal RNA was treated equally and used as negative control. Seven newly produced proteins (indicated by arrows) were identified by LC-MS/MS to be of mouse origin. Circles were used for orientation. (**h**) The MC/9 exosomal RNA were labelled by ^{3}H-uracil and transferred to CD4, MC/9 and HMC-1 cells. The cells were harvested at 0 and 24 h, RNA was isolated and the radioactivity was quantified by scintillation. Results are presented as fold changes in radioactive RNA in CD4, MC/9 and HMC-1 cells at 24 h, normalized to the 0 h measurement. A sample without donor cells was treated equally and used as negative control (mean fold change for CD4 is 0.3, $n = 2$; for MC/9 is 2.5 ± 0.7, $n = 6$; and for HMC-1 is 1.6 ± 0.5, $n = 4$). The data show that the recipient MC/9 and HMC-1 cells take up the RNA from the added exosomes.

including *AP3M2*, *DGKA*, *PRKA1*, *SKP1P* and *FGFR1OP* (see Supplementary Information, Table S5). Some of the miRNAs were expressed at higher levels in exosomes than in the cells, again implying that some miRNA may be uniquely packed into exosomes.

In this study, we have identified different miRNAs, including *let-7, miR-1, miR-15, miR-16, miR-181* and *miR-375* in exosomes. Two of the best studied miRNAs are *lin-4* and *let-7*, which are involved in early (*lin-4*) and late (*let-7*) developmental timing[19,20]. In addition, the exosomal miRNAs *miR-1, miR-17, miR-18, miR-181* and *miR-375* have been suggested to have roles in angiogenesis, haematopoiesis, exocytosis and tumorogenesis[20,21], which may imply multiple functions of miRNAs shuttled by exosomes. In view of the extensive regulatory capacity of miRNAs, the finding that exosomal RNA can be shuttled between cells (Fig. 3h) suggests a novel mechanism by which mast cells may regulate the activity or differentiation of other mast cells. Collectively, the data suggest that the transfer of exosome-derived unique miRNAs or novel mRNAs to recipient cells is a previously undescribed mechanism allowing gene-based communication between mammalian cells.

This study show that exosomes carry both mRNA and miRNA when released from mast cells, and that many of these RNA species seem to be specifically packaged exclusively into exosomes. We have also confirmed that the mRNA present in the MC/9 exosomes is translatable, encoding proteins that were often exosome specific. More importantly, our data suggest that exosomes are capable of shuttling RNA between mast cells, thus greatly increasing the complexity by which these cells communicate. Specifically, transfer of mRNA from one cell to another resulting in specific protein production, may provide the necessary signal(s) to modulate the function of the recipient cells. We were especially excited to find miRNA in the exosomes as the regulatory capacity of this group of RNAs is extensive[21]. Hypothetically, the 121 miRNAs that we have found in the mast cell

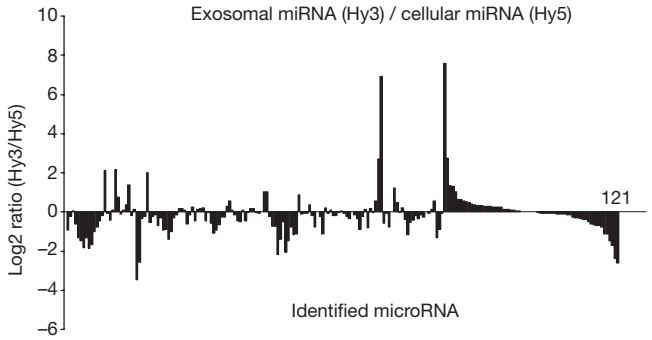

Figure 4 Identification of exosomal microRNA. The median hybridization signals of miRNA in exosomes and cells analysed by miRCURY LNA array ($n = 3$) are shown. Exosomes carry approximately 121 miRNAs, found in all four experiments (see Supplementary Information, Table S6). Some miRNA were found to be expressed to a greater extent in the exosomes compared to their donor cells.

exosomes may interfere with 24,000 mRNAs as it has been suggested that each species may interact with up to 200 mRNAs[22]. We also studied the ability of mast-cell exosomes to deliver RNA, but in theory other cells that also produce exosomes may send RNA between each other.

Cell–cell communication can occur by several means, including chemical receptor-mediated events, direct cell–cell contact and cell–cell synapses. Here, we suggest that a new mechanism of cell–cell communication can be delivery of RNA by transfer through exosomes, increasing the complexity by which cells may communicate. This genetic communication between cells may be occurring in the microenvironment, but could potentially also occur at a distance, by traffic of exosomes through the systemic circulation in a similar way to hormones. In fact, exosomes may be more effective in affecting a recipient cell if they deliver a specific mRNA or miRNA that can modify recipient cell protein production and gene expression. The ability of exosomes to deliver nucleic acids to cells at a distance also makes them ideal candidates as vectors for gene therapy — it may be possible to engineer exosomes to contain specific RNA or DNA to be delivered to cells. More importantly, the non-immunogenic character of the exosomes produced by the recipient's cells after *in vitro* processing could offer considerable advantages for gene therapy compared to current strategies, such as viral vectors. □

METHODS

Cells. MC/9 cells (ATCC, Manassas, VA), the human mast cell line HMC-1 (J, Butterfield, Mayo Clinic, /rochester, MN) and bone marrow mast cells (BMMC) were prepared and cultured as described by ATCC[23, 24] with some modifications. $CD4^+$ T cells were purified from mouse spleens by negative selection using the Spincep mouse $CD4^+$ T cell enrichment cocktail (Stemcell Technologies, Vancouver, Canada). Exosomes from MC/9 cells were released continuously, whereas HMC-1 cells were stimulated with calcium ionophore and BMMC cells were cultured in high concentration for exosome release.

Exosome purification. Exosomes were prepared from the supernatant of MC/9, BMMC and HMC-1 cells by differential centrifugations as previously described[4], with some modifications. Briefly, cells were harvested, centrifuged at 500*g* for 10 min to eliminate cells and at 16,500*g* for 20 min, followed by filtration through a 0.22 μm filter. Exosomes were pelleted by ultracentrifugation at 120,000*g* for 70 min. For mass spectrometry, the exosome pellet was washed once in PBS. Exosomes were measured for their protein content using BCA protein assay kit (Pierce, Rockford, IL). For the density gradient experiment, exosomes were floated in a sucrose gradient as previously described[4], with some modifications. For exosome yields, see Supplementary Information, Table S6.

Flow cytometry. For FACS analysis, exosomes from MC/9 and BMMC cells were adsorbed onto 4 μm aldehyde–sulphate latex beads (Interfacial Dynamics, Tualatin, OR) and incubated with CD63 antibody (Santa Cruz, Santa Cruz, CA) followed by secondary antibody, washed and analysed on a FACSscan (Becton Dickinson, San Diego, CA). For immunoisolation, HMC-1 exosomes were incubated with latex beads coated with purified anti-CD63 antibody (BD, Erembodgem, Belgium) or mouse IgG1 (Sigma-Aldrich, St Louis, MO), washed, incubated with PE-conjugated CD63 antibody (BD), washed and analysed.

Electron microscopy. Exosomes from MC/9 and HMC-1 cells were loaded onto formwar carbon-coated grids, fixed in 2% paraformaldehyde, washed and the HMC-1 exosomes were immunolabelled with anti-CD63 (BD) antibody followed by 10 nm gold-labelled secondary antibody (Sigma Aldrich). The exosomes were post-fixed in 2.5% glutaraldehyde, washed, contrasted in 2% uranyl acetate, embedded in a mixture of uranyl acetate (0.4%) and methyl cellulose (0.13%), and examined in a LEO 912AB Omega electron microscope (Carl Zeiss NTS, Oberkochen, Germany).

Protein analysis by LC-MS/MS. Exosome proteins were extracted and collected in the stacking part of a 10% SDS gel. Total proteins were cut from the gel, trypsinated and analysed using the LC-MS/MS by core facilities (http://www.proteomics.cf.gu.se). The analysis was repeated three times.

Autonomous translation assay. MC/9 exosomes (740 μg protein weight), cells (3×10^6) or extracted proteins (310 μg) were kept for 4 h in complete medium containing 10% of the standard concentration of methionine, and incubated in the presence of L-^{35}S-methionine (Amersham Biosciences, Piscataway, NJ), 10 mCi ml^{-1}, for 18 h to label newly produced proteins. Proteins were extracted, washed and the radioactivity was measured by scintillation counting.

Isolation of RNA, DNA and proteins. RNA, DNA and proteins were isolated using Trizol (Invitrogen, Paisley, UK) or RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufactures protocol. For copurification of microRNA and total RNA, the RNA was extracted using Trizol, followed by the RNeasy mini kit. Cells and exosomes were disrupted and homogenized in buffer RLT (Qiagen) and 3.5 volumes of 100% ethanol were added to the samples before use of the RNeasy mini spin column. The rest of the procedure was performed according to the manufactures protocol.

RNA detection. Detection of RNA was performed using agarose gel followed by ethidium bromide staining, by QC-RNA (http://www.exiqon.com), and by using Agilent 2100 Bioanalyzer (http://www.chem.agilent.com). For detection of RNA in BMMC exosomes, the cells were cultured in the presence of ^{3}H-uracil for 48 h, exosomes were isolated, washed, and the signal was detected by scintillation. For detection of mRNA in both BMMC and MC/9 exosomes, cDNA was synthesised using reverse transcriptase (Fermentas, St. Leon-Rot, Germany) in the presence of radioactive α-^{32}P-CTP, 10 mCi ml^{-1} (Amersham) according to manufactures recommendations. Total cDNA was separated on a 0.8% agarose gel, dried over night and visualized using a phosphoimager. To confirm that the RNA is confined inside the exosomes, MC/9 exosomes were treated with 0.4 μg μl^{-1} RNase (Fermentas) for 10 min at 37 °C. As a control, 5 μg cellular RNA was added to the exosomes before the RNase treatment. To elute any macromolecules bound to the exosomes, MC/9 exosomes were treated with 0.25% trypsin for 10 min at 37 °C. Anti-CD63 (Santa Cruz) was added to the exosomes before the trypsin treatment to determine whether external proteins were removed by the treatment. Exosomes were pelleted and RNA and proteins from the exosome pellet and supernatant were isolated and separated on gels. The anti-CD63 antibody was detected by horseradish peroxidase-coupled secondary antibody (Harlan Sera-lab, Bicester, UK).

Western blot. Total proteins were extracted and separated on polyacrylamide gels, before transfer to a nitrocellulose membrane (BioRad, Hercules, CA). The membrane was blocked, incubated with the CD63 antibody followed by the horseradish peroxidase-coupled secondary antibody and subjected to enhanced chemiluminescence.

Microarray. The microarray experiments were performed by SweGene (http://www.swegene.org/) according to Affymetrix microarray DNA-chip analysis. The program Spotfire DecisionSite 8.2 was used for gene-profiling analysis and Ingenuity was used for the network analysis.

MicroRNA array and profiling. Identification of microRNA was performed by the Exiqon company.

Transfer experiments. To label MC/9 exosome RNA, cells were cultured in complete medium supplemented with 1 μl ml^{-1} ^{3}H-uracil 72 h before exosome isolation. Exosomes were isolated and washed to remove free nucleotides. The exosomes were added to MC/9, CD4^{+} and HMC-1 cells in the ratio of 8:1 between donor cells and recipients. At 0 h and 24 h, cells were harvested and washed twice. RNA was isolated by RNeasy mini kit and the signal of radioactive RNA was measured using scintillation. Medium supplemented with 1 μl ml^{-1} ^{3}H-uracil absent from donor cells was treated equally and used as negative control.

***In vitro* translation.** The *in vitro* rabbit lysate translation kit (Promega Corporation, Madison, WI) was used according to manufacturers' recommendation to translate exosomal mRNA (0.5 μg) to proteins. A sample without exosomal RNA was used as negative control. After the translation procedure was accomplished, total proteins were separated using two-dimensional PAGE, BioRad instruments (Mini-protean 3cell). Newly produced proteins were identified using LC-MS/MS. The newly produced proteins of mouse origin were compared to the genes identified from the DNA microarray analysis.

***In vivo* translation.** MC/9 exosomes (1000 μg) were added to HMC-1 cells (8×10^{6}) at three different time points (0, 3, 6 h) and the cells were incubated for approximately 24 h. The HMC-1 cells were harvested, washed and the total proteins of the cells were separated by two-dimensional PAGE. A sample without exosomes was treated similarly and used as negative control. The newly produced proteins (96 spots) were cut and identified using MALDI-TOF.

Statistics. The Kruskal Wallis test followed by the Mann-Whitney test was used for the statistical analysis, where required, and a P value <0.05 was considered significant.

Accession numbers. Details about the microarray deposition can be found at http://www.ncbi.nlm.nih.gov/projects/geo/ (the GEO accession number is: GSE7275).

Note: Supplementary Information is available on the Nature Cell Biology website.

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AUTHOR CONTRIBUTIONS

H.V. and K.E., with contributions from A.B., performed all experiments. J.L. conceived the study and participated in the overall planning of the study, with support from all authors. All authors participated in the design of specific experiments and in writing the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details acompany the full-text HTML version of the paper online.

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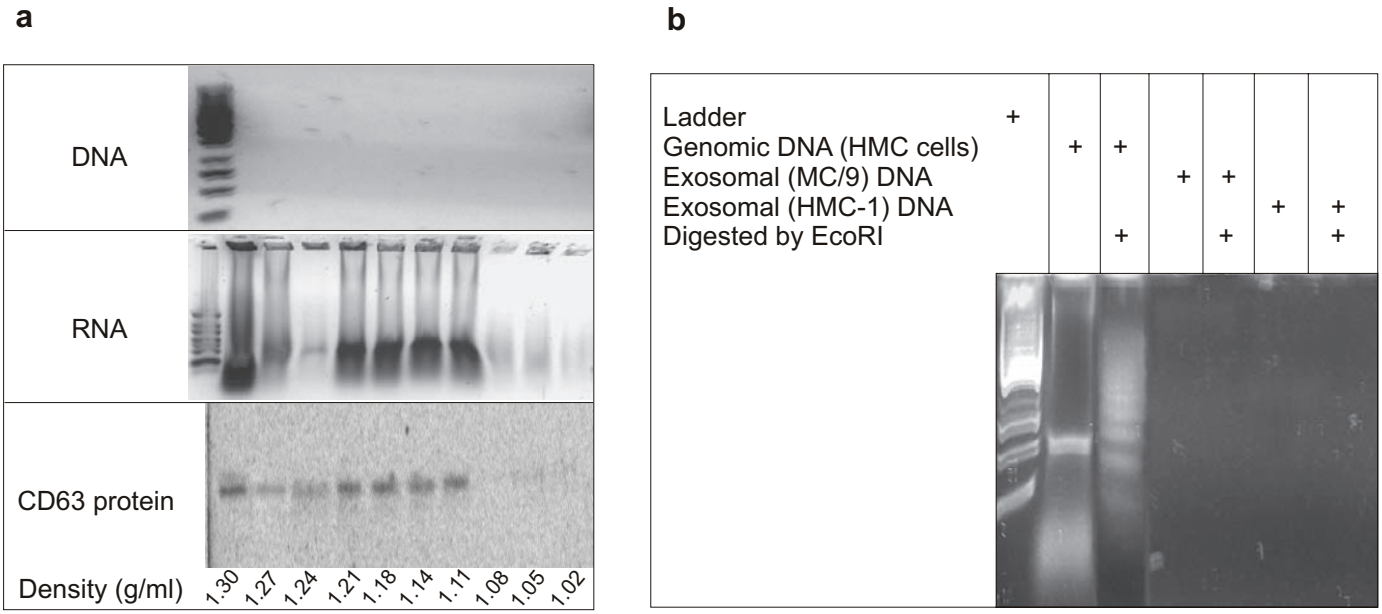
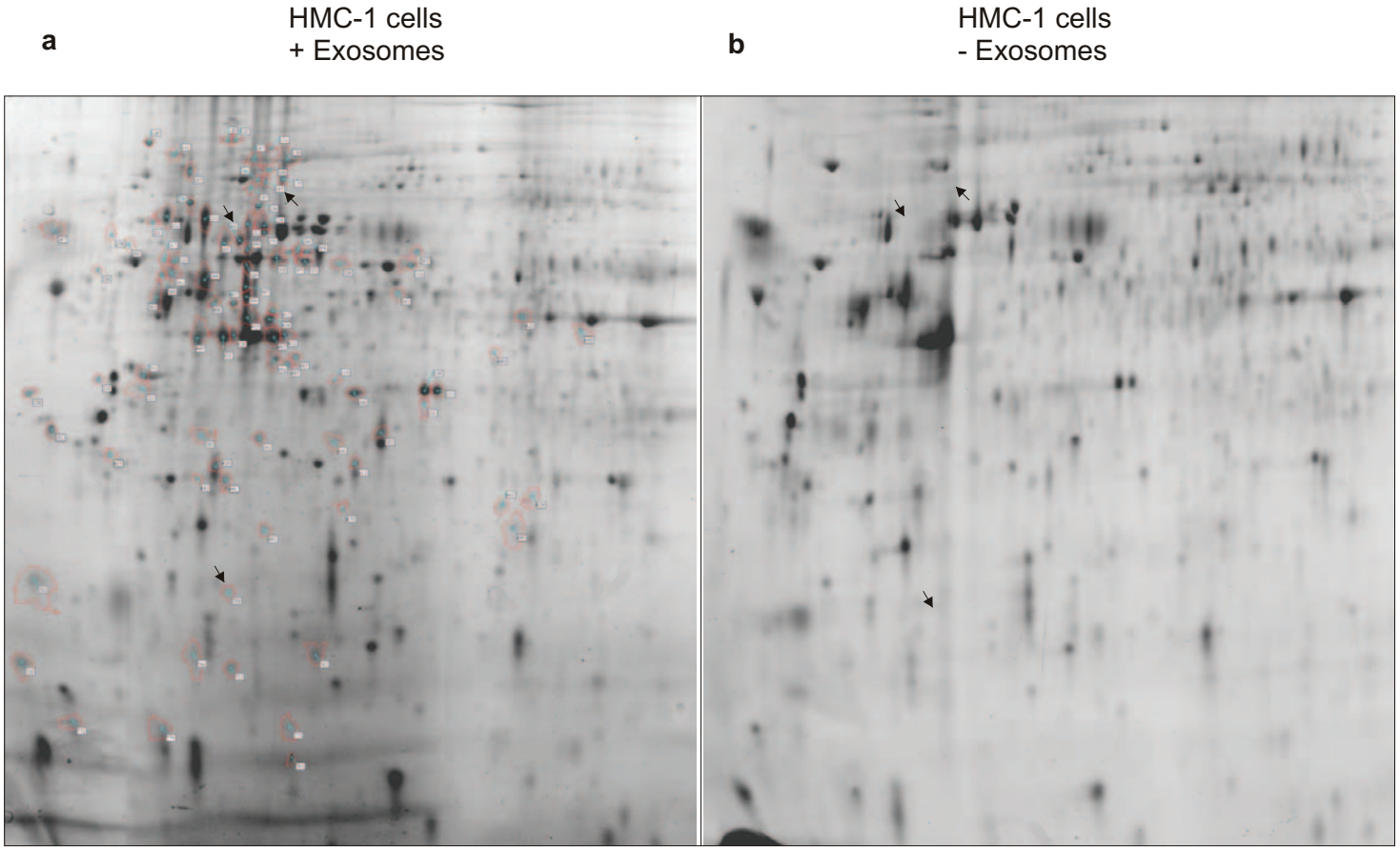
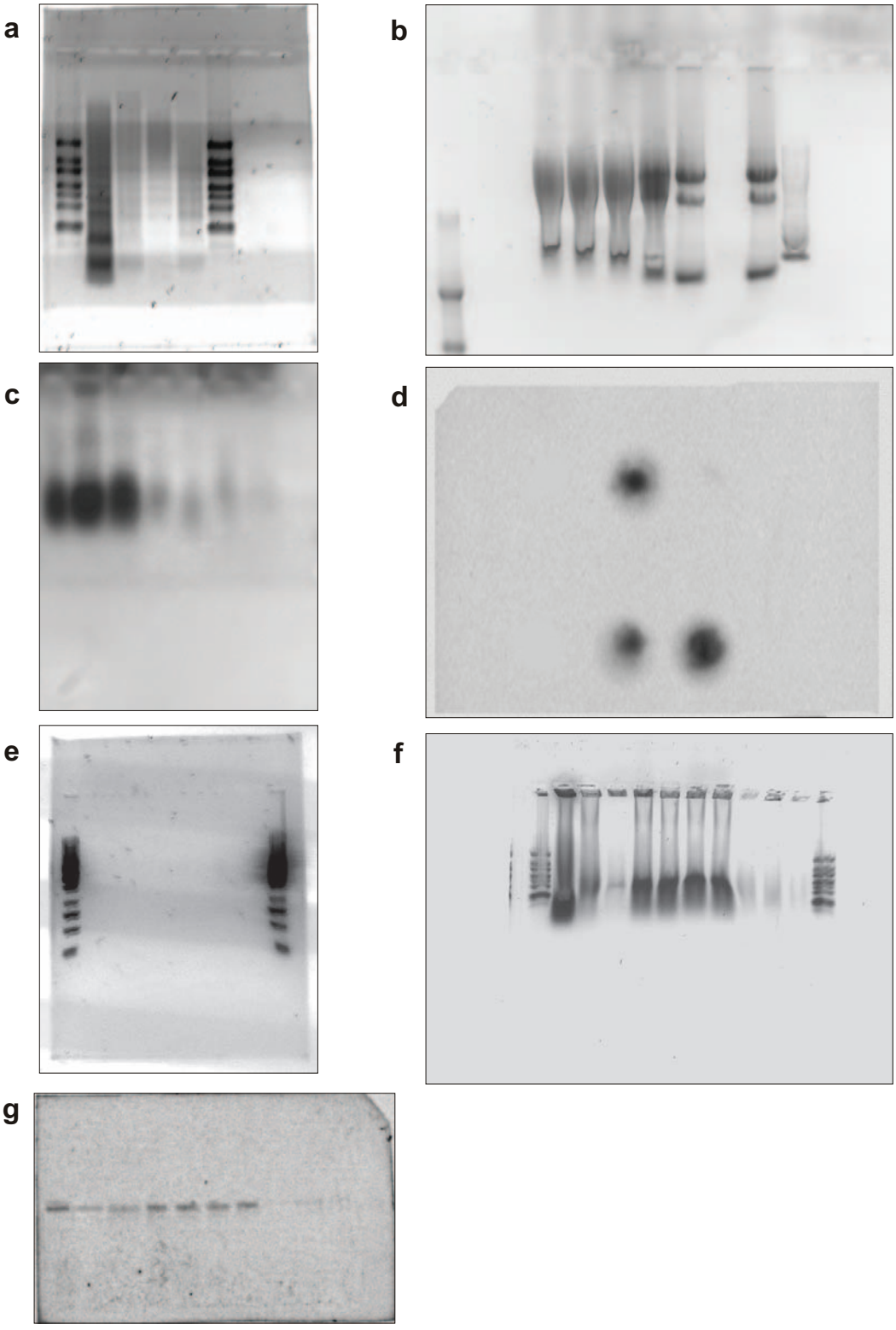


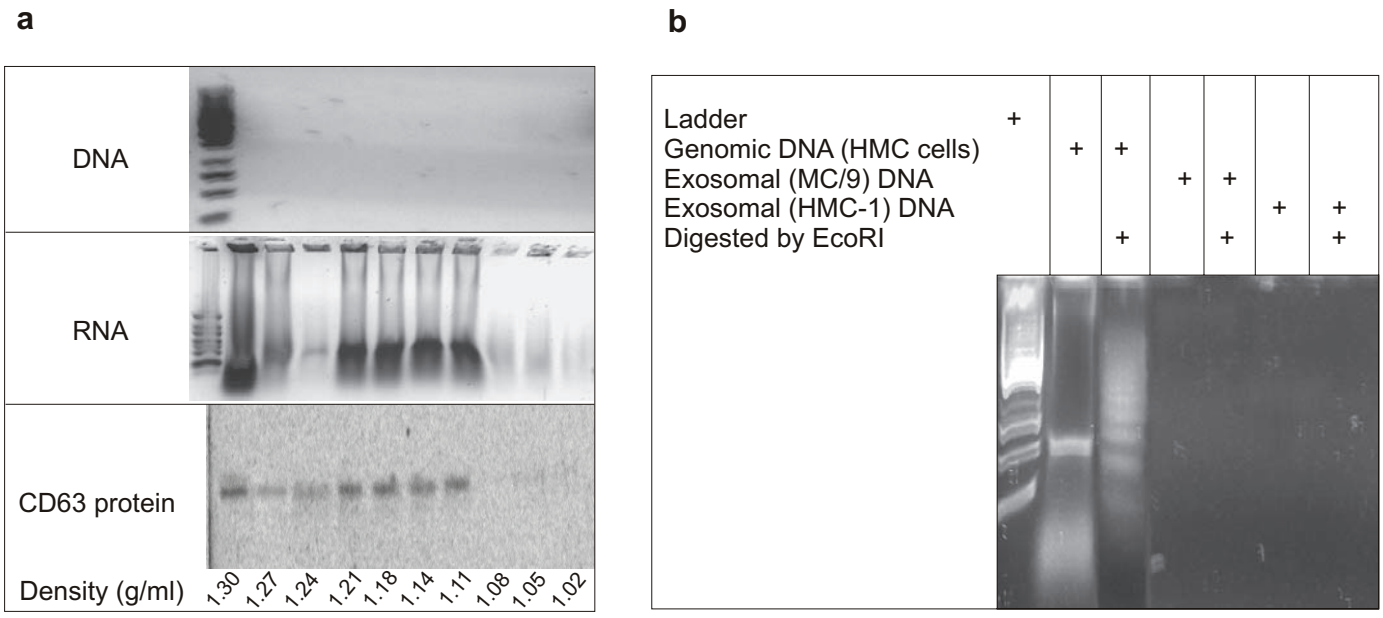
Supplementary Information, Figure 1.
Detection of DNA, RNA, and protein of MC/9 exosomes.
Exosomes were isolated as previously described in methods. (**a**) To further examine the purity of the exosomes, the exosome pellet harvested after the 120 000 g centrifugation was floated in a sucrose gradient (0.25-2M sucrose, 20 mM Hepes/NaOH, pH 7.2). The exosomes were dissolved in 2.5 M sucrose and the gradient was layered on top of the exosomes. The gradient was centrifuged at 100 000 g for 15 h according to previously published methodology6. Gradient fractions (10 x 3.8 ml) were collected from the bottom of the tube, and was diluted with 10 ml PBS and ultracentrifuged for 2h at 150 000 g (Beckman Ti70.1 rotor), and the pellets were dissolved in Trizol. The DNA, RNA and proteins from each fraction was extracted. No DNA could be detected in any fraction of the sucrose gradient. The RNA and CD63 protein was found at the characteristic density of exosomes (1.11-1.21 g/ml). RNA and CD63 protein could also be detected in fractions 1 and 2 (density 1.27-1.30 g/ml), probably because of aggregated exosomes. (**b**) To confirm absence of DNA in exosomes, DNA was extracted (Qiagen DNA easy tissue kit Cat No 69506) from HMC-1 cells and from exosomes from both MC/9 and HMC-1. Five microgram of the DNA from the cells and 50% of the sample volume from the exosomes were digested by the restriction enzyme EcoRI. The samples were separated in a 1% agarose gel. The results show that HMC-1 cells contain DNA, but the exosomes from both HMC-1 and MC/9 cells do not contain any DNA. Same results have been obtained using Trizol extraction method. For full scan see supplementary information figure 3.



Supplementary Information, Figure 2
Proteomic results from transfer of MC/9 exosomes to HMC-1 cells.
Human mast cells HMC-1 were incubated with the mouse MC/9 exosomes (**a**) and without (**b**) for 24 hours. Proteins between the two gels were matched and 96 newly produced proteins were identified by MALDI-tof. Spots labelled with arrows are good candidates as mouse protein produced from exosomal mRNA. For further information see supplementary information, Table S5.



Supplementary Information, Figure 3.
Full scans of all key gel and western data.
Gels **a-d** are original scans from figure 2 in the manuscript, gels **e-g** are the original scans for supplementary figure 2.



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Supplementary Methods

Cells

MC/9 cells (ATCC) were cultured according to manufacturer's recommendations. To eliminate exosomes present in serum, Rat T-Stim and FBS were ultracentrifuged at 120 000 g for 90 min using a Ti70 rotor (Beckman optima LE-80k Ultracentrifuge). The human mast cell line HMC-1 (Dr Joseph Butterfield, Mayo Clinic, USA), was cultured in IMDM containing 10% FBS, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 2 mM L-glutamine and 1.2 mM alph-thioglycerol. For release of exosome, the HMC-1 cells were cultured in the presence of 1 µM calcium ionophore for 30 min. Bone marrow mast cells (BMMC) were prepared by culturing bone marrow cells from femurs of 7-10 wk old male BALB/c in the presence of IL-3 (R&D systems) as described previously[24]. After 4 weeks of culture, the cells were harvested and consisted of 96% pure MCs as analysed by morphology. During the last 48 h, BMMC were cultured at $3x10^6$ cells ml^{-1} in complete medium with ultracentrifuged FBS supplemented with 10 ng ml^{-1} IL-4 (R&D-systems), and in some experiments in the presence of 1 µl ml^{-1} ^{3}H-Uracil (Amersham Biosciences). For culture of $CD4^+$ T cells, mouse spleens were collected and passed through a 70µm followed by 30 µm filter. $CD4^+$ T cells were purified by negative selection using the Spincep® mouse $CD4^+$ T cells enrichment cocktail (Stemcell Technologies) according to the manufactures instructions. The purity of the $CD4^+$ T cells ranged from 89 to 91%, as analysed by flow cytometry. The cells were cultured in RPMI 1640 containing 10% FBS, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin at $1x10^6$ cells per ml in flat bottom 48 well plates.

Exosome purification

Exosomes were prepared from the supernatant of MC/9, BMMC and HMC-1 cells by differential centrifugations as previously described[4] with some modifications. Briefly, cells were harvested, centrifuged at 500 g for 10 min to eliminate cells and at 16 500 g for 20 min, followed by filtration through 0.22 µm filter to remove cell debris. Exosomes were pelleted by ultracentrifugation (Beckman Ti70 rotor) at 120 000 g for 70 min. For mass spectrometry, the exosome pellet was washed once in PBS. Exosomes were

measured for their protein content using BCA™ Protein Assay Kit (Pierce). For the density gradient experiment the 120 000 g exosome pellet was floated in a sucrose gradient (0.25-2 M sucrose, 20 mM Hepes/NaOH, pH 7.2). The exosomes were dissolved in 2.5 M sucrose and the gradient was layered on top of the exosome suspension. The gradient was centrifuged at 100 000 g for 15 h according to[4] with some modifications. Gradient fractions (10 x 3.8 ml) were collected from the bottom of the tube, diluted with 10 ml PBS and ultracentrifuged for 2 h at 150 000 g (Beckman Ti70.1 rotor), and the pellets were extracted by Trizol. For exosome yields, see Supplementary Information Table S1.

Flow cytometry

For FACS analysis, exosomes from MC/9 and BMMC cells were adsorbed onto 4-μm aldehyde/sulphate latex beads (Interfacial Dynamics) for 15 min in 30 μl PBS followed by 3 h with agitation in 200 μl PBS at RT (for MC/9; 25 μg exosomes per $1.5x10^5$ beads and for BMMC; exosomes from 10^7 cells per $1.5x10^5$ beads). The reaction was stopped by incubation in 100 mM glycine for 30 min. Exosome-coated beads were washed three times, incubated with CD63 antibody (Santa Cruz), washed twice, incubated with PE-conjugated secondary antibody (Santa Cruz), washed twice and analysed on a FACSScan (Becton Dickinson, San Diego CA). For immunoisolation, 4-μm-diameter aldehyde/sulphate latex beads were incubated with purified anti-CD63 mAb (BD) or mouse IgG1 (Sigma-Aldrich) under gentle agitation at RT overnight according to manufactures recommendation (Interfacial Dynamics). For FACS analysis, 30 μg of HMC-1 exosomes were incubated with 1.5×10^5 anti-CD63 or mouse IgG1 beads in 30 μl PBS at RT for 15 min, the volume was made up to 400 μl and the beads were incubated at 4 °C overnight under gentle agitation. The reaction was stopped by incubation in 100 mM glycine for 30 min. Exosome-coated beads were washed twice, incubated in 1% human serum at 4 °C for 15 min, washed twice and incubated with PE-conjugated CD63 antibody (BD), washed and analysed on a FACSScan (Becton Dickinson, San Diego CA).

Electron microscopy

The exosome pellets from MC/9 cells and HMC-1 cells resuspended in PBS were loaded onto formwar carbon coated grids (Ted Pella Inc, Redding, CA, USA cat no 01800N-F). Exosomes were fixed in 2% PF, washed and the HMC-1 exosomes were immunolabelled with anti-CD63 (BD) antibody followed by 10 nm gold labelled secondary antibody (Sigma Aldrich). The exosomes were post-fixed in 2.5% glutaraldehyde, washed, contrasted in 2% uranyl acetate, embedded in a mixture of uranyl acetate (0.8%) and methyl cellulose (0.13%), and examined in a LEO 912AB Omega electron microscope (Carl Zeiss NTS, Germany).

Protein analysis by LC-MS/MS

Exosome proteins were extracted and collected in the stacking part of a 10% SDS gel. Total proteins were cut from the gel, trypsinated and analysed using the LC-MS/MS by Core facilities (http://www.proteomics.cf.gu.se/). Briefly for the liquid chromatography an Agilent 1100 binary pump was used, together with a reversed phase column, 200 x 0.075 mm, packed in-house with 3 μm particles Reprosilpur C_{18}-AQ. To separate the peptides, the flow through the column was reduced by a split followed by a 50 min gradient of 0-50% CH_3CN. The nano-flow LC-MS/MS were done on a 7-Tesla LTQ-FT mass spectrometer (Thermo Electron) equipped with a nanospray source modified in-house. The spectrometer was operated in a data-dependent mode, automatically switching to MS/MS mode. MS-spectra were acquired in the FTICR, while MS/MS-spectra were acquired in the LTQ-trap. For each scan of FTICR, the three most intense, doubly or triply charged, ions were sequentially fragmented in the linear trap by collision induced dissociation. All the tandem mass spectra were searched by MASCOT (Matrix Science, London, UK) program to identify proteins. The analysis was repeated three times.

Autonomous translation assay

MC/9 exosomes (740 μg protein weight), cells ($3x10^6$) or extracted proteins (310 μg) were kept for 4 h in complete medium containing 10% of the standard concentration of methionine, and incubated in the presence of L-[^{35}S]-methionine (Amersham Biosciences), 10 mCi ml^{-1}, for 18 h to label newly produced proteins. Proteins were

extracted, washed three times using a 10 kDa ultra filter (Millipore) and radioactivity was measured by scintillation.

Isolation of RNA, DNA and proteins

RNA, DNA and proteins were isolated using Trizol® (Invitrogen) or RNeasy® mini kit (Qiagen) according to the manufactures protocol. For co-purification of microRNA and total RNA, the RNA was extracted using Trizol, followed by the RNeasy® mini kit. Cells and exosomes were disrupted and homogenized in Buffer RLT (Qiagen) and 3.5 volumes of 100% ethanol were added to the samples prior use of the RNeasy mini spin column. The rest of the procedure was performed according to the manufactures protocol.

RNA detection

Detection of RNA was performed using agarose gel followed by EtBr staining, by QC-RNA (www.exiqon.com), and by using Agilent 2100 Bioanalyzer® (www.chem.agilent.com). For detection of RNA in BMMC exosomes, the cells were cultured in the presence of ^{3}H-Uracil for 48h, exosomes were isolated, and washed with a 10 kDa cut-off column to eliminate unbound nucleotides. The exosomal RNA was isolated and the signal was detected by scintillation. For detection of mRNA in both BMMC and MC/9 exosomes, cDNA was synthesised using reverse transcriptase (Fermentas) in the presence of radioactive [α-^{32}P]-CTP, 10 mCi ml^{-1} (Amersham) according to manufactures recommendations. Total cDNA was run on a 0.8% agarose gel, dried over night, and visualized using phosphoimager. As control, samples were RNase treated before cDNA synthesis. To confirm that the RNA is confined inside the exosomes, MC/9 exosomes were treated with 0.4 µg µl^{-1} RNase (Fermentas) for 10 min at 37°C. As a control, 5µg cellular RNA was added to the exosomes before the RNase treatment. To elute any macromolecules bound to the exosomes, MC/9 exosomes were treated with 0.25% trypsin for 10 min at 37 ºC. Anti-CD63 (Santa Cruz) was added to the exosomes, before the trypsin treatment to determine whether external proteins were removed by the treatment. Exosomes were isolated by ultracentrifugation. RNA and proteins were isolated using Trizol. RNA was run on an agarose gel and the anti-CD63 antibody was detected by horseradish peroxidase-coupled secondary antibody (Harlan

Sera-lab) followed by enhanced chemiluminescence (Amersham Biosciences, Inc.) on a nitrocellulose membrane.

Western blot

Total proteins from the 10 fractions were extracted and run on polyacrylamide gels, before transfer to nitrocellulose membranes (BioRad). Membranes were blocked in TBS containing 0.5% skim milk, incubated with the CD63 antibody (Santa Cruz) followed by the horseradish peroxidase-coupled secondary antibody (Harlan Sera-lab), and subjected to enhanced chemiluminescence (Amersham Biosciences, Inc.).

Microarray

The microarray experiments were performed by SweGene (www.swegene.org/) according to Affymetrix microarray DNA chip analysis. The expression level Signals were scaled in GCOS 1.2 to give a median array intensity of 100. This was done to enable different arrays to be compared. The program Spotfire DecisionSite 8.2 (www.spotfire.com) was used for gene-profiling analysis. For the network analysis to identify biological mechanisms, the program Ingenuity was used (www.ingenuity.com).

MicroRNA array and profiling

Methods for purification of small RNA were described in the section of RNA isolation. Identification of microRNA was performed by the Exiqon company (www.exiqon.com). Briefly, the quality of the total RNA was verified by an Agilent 2100 Bioanalyzer profile. Two μg total RNA from the exosome and the donor mast cell (MC/9) samples were labelled with Hy3™ and Hy5™ fluorescent stain, respectively, using the miRCURY™ LNA Array labelling kit, following the procedure described by the manufacturer. The Hy3™-labeled exosome samples and a Hy5™-labeled mast cells were mixed pair-wise and hybridised to the miRCURY™ LNA array version 8.0, which contains capture probes targeting all human, mouse and rat miRNA listed in the miRBASE version 8.0. The hybridisation was performed according to the miRCURY™ LNA array manual using a Tecan HS4800 hybridisation station (Tecan Systems, Inc. San Jose, CA). The miRCURY™ LNA array microarray slides were scanned by a ScanArray 4000 XL

scanner (Packard Biochip Technologies, Billerica, MA ,USA) and the image analysis was carried out using the ImaGene 6.1.0 software (BioDiscovery, Inc,El Segundo, CA USA). The quantified signals were normalised using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm.

Transfer experiments

To label MC/9 exosome RNA, cells were cultured in complete medium supplemented with 1 µl ml^{-1} ^{3}H-Uracil 72 h before exosome isolation. Exosomes were isolated according to the isolation protocol and washed by ultra-filtration (10 kDa, Millipore) to remove free nucleotides. The exosomes were added to MC/9, $CD4^{+}$, and HMC-1 cells in the ratio of 8:1 between donor cells and recipients at the starting point of labelling. At 0 h and 24 h, cells were harvested and washed twice. RNA was isolated by RNeasy® mini kit and the signal of radioactive RNA was measured using scintillation. Medium supplemented with 1 µl ml^{-1} ^{3}H-Uracil absent from donor cells was treated equally and used as negative control.

In vitro translation

Total exosomal RNA was purified using RNeasy® mini kit and 0.5 µg was used for the translation. The *in vitro* rabbit lysate translation kit (Promega Corporation) was used according to manufactures recommendation to translate exosomal mRNA to proteins. A sample without exosomal RNA was treated equally and used as negative control. After the translation procedure was accomplished, total proteins were precipitated using acetone and determined using RC DC protein assay (BioRad). The protein content of the samples (presence and absence of the exosomal RNA) was compared using 2D-PAGE, BioRad instruments (Mini-protean®3cell) and recommendation. The 2D-gels were visualised using SyproRuby (BioRad) and digitalised using phosphoimager. Protein spots of the samples were compared and a selection of the newly produced proteins was cut, trypsinated, and identified using LC-MS/MS followed by MASCOT program search. The newly produced proteins of mouse origin were compared to the genes identified from the DNA microarray analysis.

In vivo translation

MC/9 exosomes (1000 μg) were added to HMC-1 cells (8 x 10^6) in three different time points (0, 3, 6 h) and the cells were incubated for approximately 24 h. The HMC-1 cells were harvested, washed, and the total proteins of the cells were separated by 2D-PAGE according to Core facility (www.proteomics.cf.gu.se). A sample without exosomes was treated equally and used as negative control. The newly produced proteins were detected using PDQUEST and 96 spots were cut and identified using MALDI-tof followed by MASCOT program search, according to Core facility (www.proteomics.cf.gu.se).

Statistics

Kruskal Wallis test followed by Mann-Whitney was used for the statistical analysis when required and a p-value <0.05 was considered significant.

Accession numbers

Details about the microarray deposition can be found at http://www.ncbi.nlm.nih.gov/projects/geo/ (the GEO accession number is: GSE7275).