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Microvesicle entry into marrow cells mediates tissue-specific changes in mRNA by direct delivery of mRNA and induction of transcription

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Abstract

Objective—Microvesicles have been shown to mediate intercellular communication. Previously, we have correlated entry of murine lung-derived microvesicles into murine bone marrow cells with expression of pulmonary epithelial cell-specific mRNA in these marrow cells. The present studies establish that entry of lung-derived microvesicles into marrow cells is a prerequisite for marrow expression of pulmonary epithelial cell-derived mRNA.

Methods/Results—Murine bone marrow cells co-cultured with rat lung, but separated from them using a cell-impermeable membrane (0.4 micron pore size), were analyzed using species-specific primers (for rat or mouse). These studies revealed that surfactant B and C mRNA produced by murine marrow cells were of both rat and mouse origin. Similar results were obtained using murine lung co-cultured with rat bone marrow cells or when bone marrow cells were analyzed for the presence of species-specific albumin mRNA after co-culture with rat or murine liver. These studies show that microvesicles both deliver mRNA to marrow cells and also mediate marrow cell transcription of tissue-specific mRNA. The latter likely underlies the longer term stable change in genetic phenotype which has been observed. We have also observed microRNA in lung-derived microvesicles and

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studies with RNase-treated microvesicles indicate that microRNA negatively modulates pulmonary epithelial cell-specific mRNA levels in co-cultured marrow cells. In addition, we have also observed tissue-specific expression of brain, heart and liver mRNA in co-cultured marrow cells suggesting that microvesicle-mediated cellular phenotype change is a universal phenomena.

Conclusion—These studies suggest that cellular systems are more phenotypically labile than previously considered.

Keywords

Adult stem cells; Stem cell-microenvironment interactions; Microvesicles

Introduction

Cell-derived membrane-enclosed vesicles have been shown to affect the phenotype of putative target cells under different conditions. Different terms have been used to describe these vesicles, including exosomes (1), microvesicles (2), ectosomes (3), membrane particles (4), exosome-like particles (5) and apoptotic vesicles (6). Vesicles have been characterized by size, density in sucrose gradients, electron microscopy, sedimentation, lipid composition, main protein markers and intracellular origin (7). However, all vesicle preparations are heterogeneous in nature as different protocols allow for enrichment of different vesicle types (7). We have studied vesicles sedimented at 100,000g, which would include exosomes and microvesicles as classically described, and have found that the mode of electron microscopic tissue preparation changed vesicle morphology dramatically, with cup-shaped vesicles or irregular-shaped, electron dense vesicles seen with different approaches. We will use the generic term “microvesicle” to encompass these populations of vesicles, realizing the heterogeneity of most reported vesicle populations.

Microvesicles may be secreted by activated normal cells (3–5) and have been found to transfer CD41, integrins and CXCR4 (6,8–10) as well as HIV and prions (11,12) between cells. Embryonic stem cell-derived microvesicles have been reported to reprogram hematopoietic stem/progenitor cells by horizontal transfer of mRNA and protein (13). Similarly, tumor-derived microvesicles can transfer surface determinants and mRNA to monocytes (8). Apoptotic bodies from irradiated Epstein-Barr Virus (EBV)-carrying cell lines have been shown to transfer DNA to a variety of co-cultured cells by integrating copies of EBV, resulting in high expression of EBV-encoded genes EBER and EBNA1 in recipient cells (14). Extracts from T lymphocytes containing transcription factor complexes can induce fibroblasts to express lymphoid genes (15). In addition, microvesicles derived from endothelial progenitor cells can induce a vascular phenotype both *in vitro* and *in vivo* through delivery of microvesicles (16). Recently, Prokopi et al. have published that the previously-described endothelial progenitor cell may represent mononuclear cells which have consumed platelet-derived microvesicles (17).

After marrow transplant into irradiated mice, lung cells with both lung and marrow-specific markers are present (18–20). We showed that when marrow cells are co-cultured with lung, but separated from lung by a cell-impermeable membrane (0.4 microns), marrow expresses pulmonary epithelial cell-specific mRNA (21). This effect was partially RNase sensitive. Ultracentrifugation of lung conditioned media yielded microvesicles, which contained pulmonary epithelial cell-specific mRNA. Incubation of marrow cells with these microvesicles induced elevations of pulmonary epithelial cell-specific mRNA and there occurred entry of the microvesicles into a minority of these cells. In addition, marrow cells co-cultured with lung showed an increased capacity to convert to pulmonary epithelial cells after transplantation. These studies suggested that the phenotypic alterations in marrow might be due to a direct

transfer of mRNA within lung-derived microvesicles (LDMV) into marrow cells. However, the persistence of lung-derived mRNA elevations in marrow three weeks after exposure to lung was a point against simple mRNA transfer, since it could be assumed that any transferred mRNA would have been degraded by this time.

In the present studies, additional murine tissues were studied for marrow cell mRNA induction and underlying mechanisms for the observed tissue-specific mRNA expression in marrow were addressed. Our studies indicate that while there is direct mRNA transfer, there is also transcriptional induction of tissue-specific mRNA in marrow. In addition, mRNA expression may be modified by the inhibitory actions of microRNA transferred to cells in microvesicles.

Materials and methods

Experimental animals

All studies were approved by the Institutional Animal Care and Use Committee at Rhode Island Hospital. Six-to-eight week-old male C57BL/6 mice and Fischer-344 rats (Jackson Laboratories) were used. Euthanasia was performed using CO₂ inhalation followed by cervical dislocation.

Radiation injury

C57BL/6 mice were exposed to a single dose of 500 centigrey (cGy) total body irradiation (TBI) using a Gammacell 40 Exactor Irradiator at 110 cGy/minute (MDS Nordion).

Tissue collection

For solid organ harvest, blood was flushed using ice-cold 1x Dulbecco's Phosphate-Buffered Saline (1xPBS, Invitrogen). Lungs, hearts, livers and brains were collected and placed in 1xPBS supplemented with 5% heat-inactivated fetal calf serum (HICFS, Hyclone). For whole bone marrow (WBM) cell harvest, tibiae, femurs, iliac crests and spines were collected and crushed and bone fragments removed using a 40µm cell strainer.

Solid organ co-culture

20×10⁶ WBM cells were plated in six-well culture plates (Allegiance) filled with Fisher's Medium (Invitrogen) supplemented with 20% horse serum (Cambrex BioScience) and 0.1% hydrocortisone (Invitrogen) (Dexter media). Solid organs were minced and 100mg were placed on top of a cell-impermeable well insert (0.4 micron pore size, Millipore). Co-culture plates were incubated at 33°C/5% CO₂ for seven or 14 days then co-cultured WBM was harvested.

Cell free conditioned media (CM)

Minced organs were placed on top of cell-impermeable well inserts in Dexter media-filled culture plates, incubated at 33 °C/5% CO₂ for seven days and the CM (media below the insert) was used for co-culture with WBM cells. Alternatively, CM was ultracentrifuged (100,000g, one hour, 4°C) in a Thermo Scientific Sorval WX Ultra series ultracentrifuge and the pelleted material (UCF pellet) resuspended in 1xPBS.

Isolation of LDMV

The UCF pellet derived from lung conditioned media (LCM) was resuspended in 1xPBS (one lung-derived UCF pellet in 500 ul). An equal volume of the cell membrane dye PKH26 (Sigma), diluted 1:250, and the cell cytoplasm dye CFSE [5(6)-CFDA, SE; CFSE (5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester) (Molecular Probes), final concentration of 0.02 µM, were added and incubated for 15 minutes, 37°C. LDMV were isolated from the labeled pellet using a 5 laser Becton Dickinson/Cytopeia Influx High Speed Cell Sorter

modified with a small particle detector in the forward scatter channel. CFSE was excited at 488nm and detected through a 528/38 bandpass filter. PKH26 was excited at 561nm and detected through a 624/40 bandpass filter. Single stained controls indicated no compensation was necessary between the 2 dyes. CFSE+/PKH26+ events were defined as LDMV.

Transmission electron microscopy

UCF pellets or LDMV were fixed with 3% glutaraldehyde in 0.15M sodium cacodylate buffer (Electron Microscopy Sciences) then post-fixed in 1% osmium tetroxide (Electron Microscopy Sciences). Samples were diced into 1.5mm cubes and covered with a 3% agar solution. Excess agar was removed then samples were dehydrated through a graded series of acetone and embedded in Spurr's epoxy resin (Ladd Research Industries). Ultra-thin sections were then prepared, retrieved onto 300-mesh thin bar copper grids and contrasted with uranyl acetate and lead citrate. Sections were examined using a Morgagni 268-transmission electron microscope and images collected with an AMT Advantage 542 CCD camera system.

RT-PCR analysis

Total RNA was isolated using the RNeasy Mini or Micro Kit (Qiagen). RNA was measured for quantity and quality using a Nanodrop ND/1000 spectrophotometer (Thermo scientific). All RT-PCR equipment and reagents were purchased from Applied Biosystems. RNA was used to produce cDNA using the High Capacity cDNA Transcription Kit. Reactions were performed using a 9800 Fast Thermal Cycler; one 10 minute cycle, 25°C; one 120 minute cycle, 37°C; one 5 minute cycle, 85°C. cDNA pre-amplification reactions were performed using 12.5µl of a pooled mixture of all 20x primer assays (final concentration: 0.2x, each assay, see below), 25µl TaqMan Preamp Master Mix and 12.5 µl cDNA; one 10 minute cycle, 95°C; fourteen 15 second cycles, 95°C; one 4 minute cycle, 60°C. Gene expression was analyzed using the 7900HT Fast Real-Time PCR System. Murine 20x primer assays included β2 microglobulin (Mm00437762_m1), surfactants A (Mm00499170_m1), B (Mm00455681_m1), C (Mm00488144_m1), and D (Mm00486060_m1), glial fibrillary acidic protein (Mm00546086_m1), CCSP (Mm00442046_m1), aquaporin-5 (Mm00437578_m1), β3 tubulin (Mm00727586_s1), albumin (Mm00802090_m1), serum amyloid A1 (Mm00656927_g1), troponin I (Mm00437164_m1), troponin T2 (Mm00441922_m1), myosin light polypeptide 2 (Mm00440384_m1). Rat assays included β2 microglobulin (Rn00560865_m1), surfactant B (Rn00684785_m1), surfactant C (Rn01466216_g1), albumin (Rn00592480_m1). RT-PCR reactions included 20x assay mix (β2 microglobulin or a target gene), 2x TaqMan PCR Master Mix and cDNA; one 10 minute cycle, 95°C; forty 15 seconds cycles, 95°C; one 1 minute cycle, 60°C. Duplicate reactions of the target and housekeeping genes were performed simultaneously for each cDNA template analyzed. A cycle threshold (CT) value was obtained for each sample and duplicate values averaged. The $2^{-\Delta\Delta CT}$ method was used to calculate relative expression of each target gene (22). To calculate $2^{-\Delta\Delta CT}$ for target genes with no expression in the control group, a CT value of 40 was assigned so that a relative quantity of the target gene could be reported. The control group used for all comparisons was WBM cultured alone.

Proteomic analysis of LDMV

Proteomic analysis of LDMV were performed by nano-LC-ESI-MS/MS (nano-LC from LC Packings/Dionex, and Qstar XL from Applied Biosystems). Separated proteins (by 1 or 2-D electrophoresis) were excised from the gel and digested with trypsin (23). Tryptic digests were fractionated with a reversed-phase column and the column eluate introduced onto a Qstar XL mass spectrometer via ESI. Candidate ion selection and data collection were performed as previously described (24). Protein identifications were performed with Protein Pilot (Applied Biosystems) using the murine "RefSeq" databases from NCBI. For quantitative comparison

of LDMV isotope-coded affinity, labeling (iTRAQ) combined with LC-ESI-MS/MS were used. After iTRAQ labeling, LDMV were mixed together and fractionated with a strong cation exchange TopTip (PolyLC, Inc.). The dried eluates were used for subsequent LC-ESI-MS/MS. Standard information-dependent acquisition (IDA) of MS and MS/MS spectra during nano-LC fractionation of the peptide mixture were performed. Peptides and proteins were identified and quantified using Protein Pilot.

microRNA microarray

Total RNA was collected from LDMV, WBM exposed to LDMV in culture or WBM cultured alone, using the miRNeasy kit (Qiagen). Samples were sent to Exiqon (Reading, MA) for microRNA microarray analysis. Analysis was performed on 150ng of total RNA. After passing sample QC on a Bioanalyser2100, samples were labeled using the miRCURY™ Hy3™/Hy5™ power labeling kit, hybridized on the miRCURY™ LNA Array (v.11.0) and analyzed for the presence of the nucleotide signals in the expected range. Quantified signals were normalized using the global LOWESS (LOcally WEighted Scatterplot Smoothing) regression algorithm.

LDMV surface protein determination

LCMV was ultracentrifuged and the UCF pellet was resuspended in 1XPBS at a concentration of 1×10^7 LDMV / ml (assuming 1×10^6 microvesicles per lung). Equal aliquots were labeled with one of the following antibodies (final concentration: 0.5 ug antibody/ 1×10^6 microvesicles): anti-mouse CD49e ($\alpha 5$ integrin) AlexaFluor 647 (Biolegend), anti-human/mouse CD49f ($\alpha 6$ integrin) AlexaFluor 647 (Biolegend), rat IgG2a κ AlexaFluor 647 (isotype control for $\alpha 5$ and $\alpha 6$ integrin, Biolegend), allophycocyanin (APC) rat anti-mouse CD184 (CXCR4) (BD Pharmingen), APC rat IgG2b (isotype control for CXCR4, BD Pharmingen), anti-mouse CD107a (LAMP-1) AlexaFluor 647 (eBioscience), rat IgG2a AlexaFluor 647 (isotype control for LAMP-1, eBioscience). Samples were incubated on ice for 15 min then washed by ultracentrifugation. Antibody-positive LDMV were detected by fluorescence activated cell sorting (FACS) relative to a relevant isotype control. The quantity of antibody-positive LDMV, expressed as a percentage of all sorted LDMV, was determined.

LDMV co-culture with marrow-derived myeloid and lymphoid cells

Low density marrow cells (<1.077 g/cm³) were isolated by discontinuous density centrifugation using Optiprep (Accurate Chemical), washed and resuspended at 10^8 cells/ml in 1XPBS/5% HIFCS. Cells were incubated with APC-conjugated anti-CD11b and anti-Gr-1 and Pacific Blue-conjugated anti-B220 and anti-CD4 antibodies (BD Pharmingen, $1 \mu\text{g}/10^6$ cells) for 15 minutes on ice. Propidium iodide (PI, Sigma) was added (1:1000 dilution) and all PI-/APC+ cells (myeloid cells), and PI-/Pacific Blue+ (lymphoid cells) were identified by FACS. An equal number of PI-, unlabeled WBM cells were also collected. 5×10^5 WBM, myeloid or lymphoid cells were cultured in Dexter media, with/without LDMV (one LDMV-to-two cells) and harvested 48 hours later. Cells cultured with LDMV were separated by FACS. All live (PI-) LDMV-containing cells (CFSE+/PKH26+) and cells not containing LDMV (CFSE-/PKH26-) were collected. All PI- cells not cultured with LDMV were also collected. Cells were analyzed by RT-PCR. Target gene levels were expressed as a fold difference compared with PI- WBM, myeloid or lymphoid cells not cultured with LDMV.

Transcription blocking experiments

Lung (from 500 cGy-irradiated mice), WBM co-cultures were established. WBM was also cultured without lung. After seven days, WBM was collected and placed in secondary culture with Dexter media containing Actinomycin-D (Sigma) resuspended in dimethyl sulphoxide (DMSO, Sigma), final concentration of $10 \mu\text{g}/\mu\text{l}$, or an equal volume of DMSO. Alternatively, α -Amanitin (Sigma) resuspended in DMSO, final concentration of $10 \mu\text{g}/\mu\text{l}$, was used. Cells

were incubated for an additional 24 (α -Amanitin) or 36 hours (Actinomycin-D) then collected for RT-PCR analysis. Target gene levels were expressed as a fold difference compared with WBM cultured without lung, Actinomycin-D or α -Amanitin. In parallel Actinomycin-D experiments, LDMV were used in place of lung tissue (one LDMV-to-two cells). Target gene levels were expressed as a fold difference compared with WBM cells cultured without LDMV or Actinomycin-D.

RNase experiments

LCM and LDMV from 500 cGy-irradiated mice were treated with RNase A/T1 (Ambion, final concentration: 2U/ml RNase A, 80U/ml RNase T1) for 1 hour, room temperature, or with 1xPBS. WBM was co-cultured with LCM or LDMV for seven days then treated with SUPERase-In RNase Inhibitor (Ambion, final concentration: 1U/ml) and analyzed by RT-PCR. RNA was also analyzed using a Bioanalyser2100 confirming the absence of degraded RNA. Target gene levels were expressed as a fold difference compared with WBM cultured without lung, LDMV or RNase.

Hybrid co-culture experiments

Lungs, liver and WBM cells were collected from mice and rats. Mouse or rat lungs or livers were co-cultured with mouse or rat WBM cells for seven days and co-cultured WBM analyzed by RT-PCR. Target gene levels were expressed as a fold difference compared with mouse or rat WBM cells cultured without mouse or rat lung or liver. Species-specific primers for surfactants B and C and albumin were used and confirmed to be species-specific.

Statistical analysis

Data were analyzed using the Student's *t*-test in cases where there were fewer than six measurements within the parent group. Wilcoxon rank sum test was also performed in cases where there were six or more measurements within the parent group. We considered results to be statistically significant when $p < 0.05$ (two-sided). Data were presented as mean $\pm$ one standard error.

Results

Co-culture of marrow with lung, brain, liver and heart

Cell-free conditioned media (CM) was prepared from lung, brain, liver and heart tissue of 500 cGy-irradiated mice. Murine WBM was then cultured with CM for 14 days and analyzed by RT-PCR, focusing on a variety of tissue-specific mRNA (Figure 1A). WBM cultured in lung CM (LCM) showed increased expression of mRNA for surfactants A, B, C, and D (Sp-A, B, C and D), clara cell specific protein (CCSP) and aquaporin-5 (Aq-5) compared to WBM cultured alone ($p < 0.05$, $n=6$) (figure 1B). Except for the brain-specific neurofilament heavy chain (NFH), WBM cultured in LCM did not express mRNA characteristic of other tissues. Similarly, WBM cultured in brain CM expressed $\beta 3$ tubulin, glial fibrillary acidic protein and NFH mRNA, but showed no expression of heart, liver or lung-specific mRNA. WBM cultured in liver CM expressed only albumin mRNA and no other tissue-specific mRNA. WBM cultured in heart CM expressed the heart-specific mRNA myosin light chain 2, tropinin I and tropinin T2, but no other tissue-specific mRNA, except for relatively low levels of Sp-B.

In other experiments, when liver tissue was co-cultured with WBM, there was relatively selective expression of albumin mRNA in co-cultured WBM after 6, 24 and 48 hours and 7 days of co-culture. In a similar fashion, when lung tissue was co-cultured with WBM for 6, 24 and 48 hours, there was relatively selective expression of Sp-C, Sp-D (data not shown).

CM made from lung, liver, heart and brain tissue of 500 cGy-irradiated mice was ultracentrifuged (UCF) and the pellet that formed (UCF pellet) was found to contain mRNA specific to its tissue of origin (Figure 1C). With the exception of a few mRNA not specific to brain or liver found in their UCF pellets, a similar degree of general tissue specificity was observed. In addition, the UCF pellet derived from lung (Figure 1D), brain (Figure 1E), heart (Figure 1F) and liver (Figure 1G) contained particles that were consistent with the appearance of microvesicles by transmission electron microscopy. These data indicate CM from specific tissues induces tissue-specific mRNA expression in marrow cells. CM made from each tissue contains mRNA species, which are generally tissue-specific, as well as microvesicles.

Characterization of LDMV by electron microscopy, proteomic analysis

We have used the cell membrane dye PKH26 and cytoplasm dye CFSE to label particles contained within the LCM UCF pellet (Figure 2A–C). PKH26+/CFSE+ particles can be identified and isolated by FACS. These particles have the morphologic appearance consistent with microvesicles by transmission electron microscopy (Figure 2D). Proteomic analysis on samples revealed that LDMV contain 75 distinct proteins. The majority of these proteins are involved in the biogenesis and trafficking of microvesicles (25–31). Included in these are annexins (II, V and VI), tubulin (α 1, α 2, β 2, β 5), actin (β , γ), clatherin, ezrin, radixin and ras-related protein Rap 1b. Other proteins identified in LDMV are heat shock protein 70, histones (H3, H2B, H2A), ferritin light chain and milk fat globule EGF-8. Among the lung-specific secreted proteins identified in LDMV was CCSP.

Characterization of surface proteins on LDMV

LCM UCF pellet was labeled with APC or AlexaFluor 647 (AF)-conjugated antibodies to different adhesion proteins. APC or AF-positive LDMV were then identified and collected by FACS and their frequency quantified relative to a relevant isotype control (Figure 2G,J). We found subpopulations of LDMV expressing a variety of adhesion proteins, including CXCR4 (0.61% of all sorted particles, Figure 2E), alpha 6 (0.28%, Figure 2H) and alpha 5 integrin (2.05% of all sorted particles, Figure 2I). These data suggest that specific receptor-ligand interactions might facilitate the uptake of LDMV into marrow cells. In addition, 20.83% of all sorted particles were positive for Lysosomal-Associated Membrane Protein-1 (LAMP-1, Figure 2F), a protein found in exosomes, indicating that among the lung cell-derived vesicles present in our preparations are exosomes.

Marrow cells consuming LDMV express pulmonary epithelial cell mRNA

WBM cells or marrow cells of a myeloid (My, Gr-1+/CD11b+) or lymphoid (Ly, CD4+/B220+) lineage were cultured with LDMV for 48 hours. Live (propidium iodide negative) cells containing LDMV (R2, PKH26+/CFSE+) or no LDMV (R1, PKH–/CFSE–) were isolated by FACS (Figure 3A). The presence or absence of fluorescent particles within target marrow cells was confirmed by fluorescence microscopy. WBM containing LDMV expressed Sp-B, Sp-C and CCSP mRNA. WBM cultured with LDMV, but containing no LDMV, had none of these mRNAs. My cells containing LDMV had high levels of Sp-B and CCSP mRNA while My cells cultured with LDMV, but containing no LDMV, had only low levels of Sp-B mRNA. Ly cells containing LDMV had high levels of Sp-B and CCSP mRNA while Ly cells cultured with LDMV, but containing no LDMV, had only low levels of Sp-B mRNA (Figure 3B). These data indicate that cells consuming LDMV in culture have elevated levels of pulmonary epithelial cell mRNA. A higher proportion of My cells consumed LDMV compared with Ly and WBM cells, indicating a myeloid preference of LDMV uptake. Interestingly, Sp-C was expressed in WBM, but not in the separated marrow cells. This suggests specific partitions between marrow cell populations and the possibility that Sp-C might be selectively expressed in non-differentiated cells or stem cells.

Lung cells transfer transcriptional elements to marrow cells in co-culture

WBM cells were co-cultured with lung or LDMV from 500 cGy TBI-irradiated mice then collected, washed and cultured for an additional 36 hours in media containing Actinomycin-D (Act-D, 10ug/ml), an agent which blocks transcription by preventing elongation by RNA polymerase, or an equal volume of diluent (DMSO). RT-PCR analysis was performed on co-cultured WBM using WBM cultured without lung or Act-D as a control population (control) (Figure 4A). Sp-B mRNA levels were elevated in WBM co-cultured with lung but these levels decreased after exposure to Act-D in culture ($P=0.012$, student's t-test, $n=12$) (Figure 4B). Conversely, Sp-C, Sp-D and CCSP mRNA levels were elevated in WBM co-cultured with lung, but these levels increased after exposure to Act-D in culture ($P<0.01$, Wilcoxon, $n=12$). A similar trend was seen in WBM co-cultured with LDMV (Figure 4C) where Sp-B mRNA levels decreased 91-fold after exposure to Act-D and Sp-C, Sp-D and CCSP mRNA levels increased after exposure to Act-D (150, 130, 3.6-fold, respectively, Figure 4D). Similar experiments were performed where WBM cells were co-cultured with lung then cultured for an additional 24 hours in media containing α -Amantin (10ug/ml), an agent which blocks transcription by directly inhibiting RNA polymerase II. Sp-B mRNA levels decreased 5.9-fold after exposure to α -Amantin (Figure 4E) and Sp-C, Sp-D and CCSP mRNA levels increased after exposure to α -Amantin (110, 1.8, 6.8-fold, respectively, Figure 4F). Both increases and decreases in different lung specific mRNA were observed in response to transcriptional blockade, suggesting that transfer of a lung or LDMV-derived factor results in a complex modulation of transcription.

Next, we established hybrid co-cultures using rat or mouse lung co-cultured with either mouse or rat WBM. Co-cultured WBM was analyzed by RT-PCR using rat or mouse species-specific primers for Sp-B and Sp-C, with rat or mouse WBM cultured alone as controls. Mouse Sp-B and Sp-C mRNA, but not rat, were found in mouse lung (data not shown) or mouse WBM co-cultured with mouse lung (Figure 5A). Likewise, rat Sp-B and Sp-C mRNA, but not mouse, were found in rat lung (data not shown) or rat WBM co-cultured with rat lung (Figure 5C). Mouse WBM co-cultured with rat lung had elevated levels of both mouse and rat Sp-B and Sp-C mRNA compared to control (WBM cultured without lung, $P<0.001$, Wilcoxon, $n=14$, Figure 5B), suggesting direct transfer of mRNA and also transfer of a transcriptional regulator. Rat WBM co-cultured with mouse lung had elevated levels of both rat and mouse Sp-B and Sp-C mRNA compared to control ($P<0.01$, Wilcoxon, $n=14$, Figure 5D), again suggesting direct transfer of mRNA and also transfer of a transcriptional regulator. Altogether, these data suggest that elevated levels of pulmonary epithelial cell mRNA in co-cultured WBM may be due to direct uptake of lung-derived mRNA and to the transfer of transcriptional regulators driving *de novo* mRNA synthesis. The same trend was noted in WBM cells co-cultured with liver cells. Mouse albumin (alb) mRNA, but not rat, was found in mouse liver (data not shown) or mouse WBM co-cultured with mouse liver (Figure 5E). Likewise, rat alb mRNA, but not mouse, was found in rat liver (data not shown) or rat WBM co-cultured with rat liver (Figure 5G). Mouse WBM co-cultured with rat liver had elevated levels of mouse and rat alb mRNA compared to control cells (WBM cultured without liver, $P<0.01$, Wilcoxon, $n=6$, Figure 5F). Rat WBM co-cultured with mouse liver had elevated levels of rat and mouse alb mRNA compared to control ($P<0.01$, Wilcoxon, $n=6$, Figure 5H). These findings speak to the universality of the information transfer that occurs in the context of our co-culture system.

RNase treatment of LCM, LDMV prior to co-culture influences marrow cell mRNA content

LCM or LDMV pretreated with RNase A/T1 was co-cultured with WBM (Figures 6A,C). Sp-B mRNA levels in WBM co-cultured with RNase-treated LCM or LDMV were lower than levels found in WBM co-cultured with untreated LCM ($P=0.035$ student's t-test, $n=6$) or LDMV ($P=0.019$ student's t-test, $n=6$). Conversely, Sp-A, Sp-C, Sp-D and CCSP mRNA levels in WBM co-cultured with RNase-treated LCM or LDMV were higher compared with levels found

in WBM co-cultured with untreated LCM or LDMV ($P < 0.05$ student's t-test, all comparisons, $n = 5$ or 6) (Figures 6B,D). These data suggest that RNase-sensitive factors, transferred from lung to marrow, possibly via LDMV, influence marrow cell mRNA levels. The increase in mRNA suggests that an inhibitor of mRNA might have been degraded by RNase, while the decreases in Sp-B indicate that this might have been due to direct transfer of mRNA which was degraded by RNase.

LDMV contain microRNA

Total RNA from LDMV, WBM cells co-cultured with LDMV (LDMV+WBM) and WBM cells cultured without LDMV (WBM) was used for microRNA microarray analysis. 185 distinct microRNA species were present in LDMV and 35 had levels that were significantly different in LDMV and LDMV+WBM compared with levels present in WBM (Figures 6E,F). Using the Sanger microRNA registry (<http://microrna.sanger.ac.uk/sequences>) potential gene targets for these microRNA species were identified and 8 (Figures 6, yellow shading) were found to have homologies with Sp-A, Sp-B, Sp-C, Sp-D, CCSP or, Aq-5. These data would suggest that microRNA may be among the RNase-sensitive transcription elements transferred via LDMV, acting as modulators for pulmonary epithelial cell gene expression in marrow cells.

Discussion

We and others have shown that murine marrow transplanted into lethally-irradiated mice resulted in the presence of marrow-derived lung cells with epithelial cell markers (18–20). This was initially considered to represent “stem cell plasticity”, which became a controversial area of research (32). The mechanisms underlying these observations are unknown but may include transdifferentiation, dedifferentiation or derivation from rare predetermined stem cells. Cell-to-cell fusion provides one possible mechanism, but more subtle variants of fusion may result in phenotype changes observed in other settings. The presence of pulmonary epithelial cell-specific mRNAs in LDMV, the transfer of LDMV to cells associated with increased pulmonary epithelial cell-specific mRNA and protein expression and functional changes in phenotype, suggested that pulmonary epithelial cell-specific mRNA was directly transferred to marrow and might be the mechanism underlying the observed pulmonary cell phenotypes after marrow transplantation. The present studies show that LDMV transfer is directly related to the increased expression of pulmonary epithelial cell-specific mRNA in target marrow cells. Marrow cells separated by FACS on the basis of having consumed LDMV expressed pulmonary epithelial cell-specific mRNA, while marrow cells which had not taken up LDMV expressed little or none of these mRNAs. The uptake varied depending on marrow cell type, with predominant uptake by myeloid cells, indicating that uptake shows selectivity based on individual cell phenotype, possibly due to expression of surface epitopes which bind proteins expressed on LDMV. The observed Sp-C mRNA expression in WBM but not in myeloid or lymphoid cells further suggests that there may be LDMV targeting specific cell classes and that LDMV inducing Sp-C expression in target cells might be directed to non-myeloid and non-lymphoid cells, possibly erythroid cells or stem/progenitor cells.

Messenger RNA induction in co-cultured marrow was also shown with liver, heart and brain tissue and this mRNA expression was remarkably tissue-specific. When lung was co-cultured with marrow, pulmonary epithelial cell-specific mRNA was expressed. Except for one tissue-specific marker, brain, heart and liver mRNA was not expressed in these co-cultures. Marrow co-cultured with liver, heart or brain, with one exception, expressed only the tissue specific mRNA of the co-cultured tissue. Thus, the genetic change seen in these co-cultures appears to be relatively universal and highly specific for each organ under study.

The initial observations that LDMV contained pulmonary epithelial cell-specific mRNA and entered marrow cells and that marrow consuming LDMV expressed high levels of pulmonary

epithelial cell-specific mRNA, suggested that there was direct transfer of LDMV mRNA to marrow cells. However, the persistence of mRNA expression three weeks after exposure to LDMV was difficult to explain on this basis, since one might assume that transferred mRNA would be degraded by this time. Accordingly, we investigated the mechanism of this increased mRNA expression. Actinomycin-D and α -Amantin were used to block transcription and evaluate whether an LDMV-based transcriptional component was responsible for increases in mRNA expression. These agents did influence pulmonary epithelial cell-specific mRNA expression, but in most cases resulted in an increase rather than a decrease in mRNA expression, indicating the presence of complex transcriptional mechanisms. To directly assess whether transcription was involved in tissue-specific mRNA changes, we employed a hybrid mouse/rat co-culture system. Here, species-specific primers for Sp-C, Sp-B and albumin were used for RT-PCR analysis of co-cultures using rat lung or liver opposite mouse marrow or mouse lung or liver opposite rat marrow. In each instance, we observed marrow cell expression of rat and mouse pulmonary epithelial cell or liver-specific mRNA. These data indicated that there was direct transfer of liver or pulmonary epithelial cell-specific mRNA to marrow and that there was induced transcription of liver or pulmonary epithelial cell-specific mRNA in marrow cells.

We have shown the presence of protein species in LDMV, as have others (16,25–31). Using a SILAC approach (33), we have shown that proteins are transferred to marrow in co-culture. We and others have also shown the presence of microRNA in microvesicles/ectosomes (34–38). We found 185 species of microRNA within LDMV, eight of which had targets that might modify pulmonary epithelial cell gene expression, suggesting that the microRNA may modify mRNA levels in co-cultured marrow. MicroRNA classically exerts its effects by inhibiting mRNA but transcriptional effects have been observed, either inhibitory or stimulatory in nature (39–44). Thus, LDMV protein or microRNA might mediate transcriptional effects in co-cultured marrow.

We also evaluated the effect of RNase on the expression of pulmonary epithelial cell-specific mRNA by marrow cells. RNase exposure, with one exception (Sp-B), increased pulmonary epithelial cell-specific mRNA expression in marrow cells. This can be explained by the removal of an inhibitory RNA species, although it is unclear if RNase is capable of penetrating LDMV. Regardless, the demonstration of microRNA in LDMV and the delivery of some of these microRNA to marrow suggests that the observed RNase effects may be on inhibiting the inhibitory effect of LDMV microRNA.

Initial profiling of the surface epitopes on the LDMV has shown the presence of CXCR4 and $\alpha 6$ and $\alpha 5$ integrins, suggesting possible mechanisms for binding of LDMV to different marrow cell populations.

These studies indicate the existence of a complex microvesicle-based cell regulatory network. Our previous work suggests that radiation-induced lung injury is important for the induction of LDMV production (21). Other studies have indicated that hypoxia (45), cytokines and coagulation-related thrombin expression (46), cytotoxic chemotherapy (47), complement activation (48), oxidative injury and shear forces (49) can all result in the increased release of microvesicles. Thus, cellular systems may determine cell phenotype at baseline and after various perturbations by differentially releasing microvesicles, which in turn exert their effects by delivering mRNA, inducing transcription and inhibiting mRNA (Figure 7). Further control of such a system can be envisioned by differential delivery of microvesicles based on ligand-receptor interactions between microvesicles and putative target cells. Altogether, these data indicate that cell regulatory systems and phenotypes may be quite fluid and less ordered than generally perceived.

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Figure 1. CM induces mRNA expression changes, contains microvesicles
(A) mRNA expression scale vs. control cells (WBM cultured alone). (B) mRNA in WBM co-cultured with CM vs. control. (C) mRNA in CM ultracentrifuged pellet vs. control. Electron microscopy of (D) lung, (E) brain, (F) heart, (G) liver CM ultracentrifuged pellets. Bar=100 nm, * P<0.05.

Figure 2. LDMV isolation, characterization

(A–C) LCM is made from lung cultured on cell-impermeable membranes, which is then ultracentrifuged. The pellet is labeled with PKH26, CFSE and separated by FACS to isolate LDMV. (D) Electron microscopy of LDMV. Populations of LDMV express (E) CXCR4, (F) LAMP-1, (H) α6 and (I) α5 integrin. (G,J) isotype controls.

Figure 3. LDMV affect marrow cell mRNA content

(A) LDMV are incubated with WBM, myeloid or lymphoid cells. Cells containing LDMV (R2, PKH26+/CFSE+) or no LDMV (R1, PKH-/CFSE-) are isolated by FACS. (B) Sp-B, C and CCSP mRNA levels of cells containing LDMV or no LDMV vs. control cells (cells not exposed to LDMV in culture).

Figure 4. Transcriptional blocking studies

WBM co-cultured with (A) lung or (C) LDMV is then cultured alone in media containing Actinomycin-D (Act-D, 10 ug/ml) or diluent (DMSO), (E) α-Amantin (10 ug/ml) or diluent. (B,D,F) mRNA levels of co-cultured WBM vs. WBM cultured without lung, LDMV and transcriptional blocking agents. * $P < 0.01$. No error bars displayed in graphs with only experiment.

Figure 5. Hybrid rat/mouse co-cultures

Mouse, rat-specific Sp-B, C mRNA in mouse WBM co-cultured with (A) mouse, (B) rat lung or in rat WBM co-cultured with (C) rat, (D) mouse lung. Mouse, rat-specific albumin mRNA in mouse WBM co-cultured with (E) mouse, (F) rat liver or in rat WBM co-cultured with (G) rat, (H) mouse liver. *P<0.01.

Figure 6. RNase studies, microRNA content of LDMV
WBM co-cultured with RNase-treated, untreated (A) LCM, (C) LDMV. (B,D) mRNA expression in co-cultured WBM vs. WBM co-cultured with untreated LCM, LDMV. *P < 0.05. microRNA expression in (E) LDMV, (F) WBM co-cultured with LDMV vs. WBM, microRNA species with homologies to pulmonary epithelial cell genes (yellow box).

Figure 7. LDMV-mediated transfer of genetic phenotype from lung to marrow: A proposed mechanism

LDMV enter marrow cells in co-culture. Pulmonary epithelial cell-specific mRNAs found in co-cultured marrow cells are a reflection of LDMV-derived mRNA species and mRNA induced from target cells by LDMV-based transcription factors. LDMV-derived microRNA degrades mRNA to further modulate the system.