We tracked the extracellular fate of proteins of pulmonary origin using the technique of Stable Isotope Labeling of Amino acids in Cell culture (SILAC) in cell-impermeable transwell culture systems. We find that irradiation to murine lung and lung-derived cells induces their release of proteins that are capable of entering neighboring cells including primary murine bone marrow cells as well as prostate cancer and hematopoietic cell lines. The functional classification of transferred proteins was broad and included transcription factors, mediators of basic cellular processes and components of the nucleosome remodeling and deacetylase (NuRD) complex including metastasis associated protein 3 (MTA3) and retinoblastoma binding protein 7 (RBBP7). In further analysis we find that RBBP7 is a transcriptional activator of E-cadherin and that its intercellular transfer leads to decreased gene expression of downstream targets such as N-cadherin and vimentin. SILAC generated data sets offer a valuable tool to identify and validate potential paracrine networks that may impact relevant biologic processes associated with phenotypic and genotypic signatures of health and disease.

Cellular communication forms the basis of eukaryotic complexity and better understanding of how cells influence and are influenced by their neighbors in health and disease is needed. Among the various signals involved in cellular cross talk are numerous proteins that bind to membrane receptors and initiate cascades, such as those involving Notch (1) and Wnt signaling (2). While several methods, such as protein affinity chromatography, affinity blotting and immunoprecipitation exist to identify protein based cellular interactions, those currently in widespread use rely upon proteomic analyses in artificial systems (e.g. yeast two-hybrid), employ manipulations that may conceivably affect protein function (e.g. insertion of protein tags) or rely upon potentially hazardous reagents (e.g. radioactive isotopes). Recently, the technique of Stable Isotope Labeling with Amino acids in Cell culture (SILAC) has been introduced as a mass spectrometry (MS) based strategy to quantitatively interrogate the proteome (3). SILAC employs either normal or heavy isotope-substituted amino acids that can be metabolically incorporated into proteomes, which can then be quantitatively identified with global, differential MS analysis. As with radioactive labeling of proteins using isotopes such as $^{35}$S-methionine, SILAC labeled proteins do not differ in structure or function compared to their unlabeled counterparts. An obvious advantage of stable isotopes is their safety profile and their ability to be easily used in cell culture (4) and more recently, in whole animals (5).
Standard SILAC experiments usually involve the selective labeling of two populations of cells with heavy and light amino acids, respectively. Cells are then mixed and proteomes are subsequently harvested, digested and analyzed by MS. Binary analysis of the MS spectra which appear as a series of peptide pairs allows discrimination of cellular origin (higher mass peaks representing proteins from heavy labeled cells) and protein abundance (relative intensity of peak) (6). In this manner, SILAC has been used to identify substrates for extracellular proteases (7, 8) and proteins with altered expression levels in malignant cells (9, 10) as well as to decipher protein-protein interactions (11, 12) and differences in post-translational modifications (13, 14). These studies indicate that the field of quantitative proteomics, while still in its infancy, has the potential to uncover regulatory cascades in health and disease.

Here, we extend the utility of SILAC to identify proteins secreted by one cell type and absorbed by another in a contact-free coculture system employing murine lung cells with murine hematopoietic or human cancer cell lines and primary murine stem cells. Our interest in developing this application derives from an obvious need to better understand intercellular protein communication. For example, take the broad clinical challenge of cellular therapy and tissue regeneration. While ample work has been done on generating appropriate cells, monitoring in vivo fate and measuring biological and clinical endpoints of therapy, very little is known regarding how exogenously introduced cells interact with their new tissue microenvironment. Similarly, consider the metastatic foci of certain cancers. A great deal of knowledge is emerging on the genetic re-programming necessary to convert a cell from its normal to malignant to metastatic state. Yet, upon leaving its tissue of origin, what cues, if any, do organs of eventual destination communicate to a metastatic cell (15)?

We summarize experiments using SILAC to catalogue a protein interactome relevant to stem cell and cancer biology. We find irradiation injury to lung cell lines or primary lung tissue ex vivo leads to the secretion of proteins that have not been previously regarded as secretagogues including the retinoblastoma binding protein 7 (RBBP7). In depth molecular investigation revealed that RBBP7 of lung origin is transferred into a wide range of neighboring cells including primary bone marrow, hematopoietic and cancer cell lines. Interestingly, the cellular transfer of RBBP7 leads to genotypic changes associated with the mesenchymal-epithelial transition (MET) including the relative reduction of levels of N-cadherin, fibronectin and vimentin (16, 17). In further analysis, we find that RBBP7 serves as transcriptional activator of the E-cadherin gene by binding to its promoter region thereby adding a further layer of complexity to its transcriptional control.

**EXPERIMENTAL PROCEDURES**

*Cell lines, media and SILAC labeling conditions*- Mouse lung originated MLG cell line (ATCC, CCL-206) was cultured in SILAC® DMEM labeling medium, designated as “Heavy” medium, containing 10% dialyzed FBS and supplemented with 100 mg/ml [U-13C6]-L-Lysine (K+6) and 100 mg/ml [U-13C6, 15N4]-L-Arginine (R+10) and 100X L-Glutamine (Invitrogen, Carlsbad, CA). Mouse bone marrow originated FDCP1 cell line (DSMZ, ACC 368), was cultured in DMEM medium (Invitrogen), designated as “Light” medium, which contains 10% dialyzed FBS and supplemented with 100 mg/ml L-Lysine, 100 mg/ml L-Arginine, 100X L-Glutamine and 10% WEHI conditioned media. MLG cell line was expanded in “Heavy” medium for six doublings (approximately six days) to achieve complete labeling of cellular proteins with heavy labeled amino acids, while FDCP1 cells were maintained in “Light” medium.

*Cell culture conditions*- SILAC labeled MLG cells were exposed to 500 cGy total irradiation using a Gammacell 40 Exactor Irradiator at 110 cGy/minute (MDS Nordion, Ottawa, ON, Canada), as described previously (18). After irradiation, cells were washed three times with 1X phosphate buffered solution (PBS) and then placed on the top of Millicell
culture plate insert (Millipore, Billerica, MA), while FDCP1 cells were plated beneath the membrane of well inserts. “Light” medium was added to both top and bottom. Culture plates were incubated at 37°C in 5% CO₂ for 48 hours after which FDCP1 and MLG cells were removed for independent downstream analysis. For experiments involving primary murine tissue, mice were exposed to 500 cGy total body irradiation (TBI) using a Gammacell 40 Exactor Irradiator at 110 cGy/minute (MDS Nordion). Control mice received no TBI. Mice were sacrificed and their lungs were harvested and processed after TBI. Bone marrow (BM) from non-irradiated mice was isolated and viable cells were quantified, as described previously (18). Transwell culture of minced lung tissue and BM was carried our for 48 hours. Prostate cancer cells (DU145WT) and breast cancer cells (MDA-MB-231) were co-cultured with irradiated MLG cells for 2 days by following the same procedures mentioned above for FDCP1. Trypan blue staining (Invitrogen) was used to measure cell viability and levels of cellular proliferation were quantified by MTT assay (Sigma, St. Louis, MO).

**Protein extraction and separation**- After harvesting, FDCP1, BM and DU145WT cells were then washed once with 1X PBS before treatment with CellLytic® M reagent (Sigma) for 15 minutes on a shaker. The lysed cells were centrifuged for 15 minutes at 16,000 g to pellet the cellular debris. Protein-containing supernatant was collected. Micro BCA Protein Assay Kit (Pierce, Rockford, IL) was used to determine protein concentrations. Protein samples were run on NuPAGE® 4-12% Bis-Tris precast Gel (Invitrogen) by using XCell SureLock® Mini-Cell (Invitrogen). The gel was stained by SimplyBlue® SafeStain (Invitrogen). The entire sample lane from the destained gel was cut into ten equal gel pieces for fractionation. Each piece was washed three times with 50% acetonitrile/50% HPLC grade water prior to mass spectrometric analysis.

**Mass spectrometry and quantitative analysis**- Gel pieces were washed, reduced with DTT and alkylated with iodoacetamide. Gels were then digested with modified trypsin (Promega, Madison, WI) at pH=8.3 overnight at 37°C with shaking. The resulting peptide mixtures from each gel piece after extraction were analyzed separately by data dependent microcapillary reversed phase liquid chromatography tandem mass spectrometry (LC/MS/MS) with a high resolution hybrid quadrupole-time-of-flight QSTAR i qTOF mass spectrometer (Applied Biosystems, Foster City, CA) operated in positive ion mode. Self-packed 10 cm length × 75 μm diameter C18 columns were used at a flow rate of 275 nL/min with a gradient of 5% B (acetonitrile) to 38% B over 1 hour. The buffer was 0.1% formic acid. MS/MS spectra were searched against the reversed Swiss-Prot protein database to identify proteins using the Paragon algorithm in ProteinPilot® (Applied Biosystems) software. Proteins with at least two unique peptides matching the forward database were initially accepted as valid identification followed by manual inspection of the MS/MS data. Posttranslational modifications were also considered during database searches. The ratio of heavy to light (H: L) peptides were analyzed from the MS scan and calculated by the ProteinPilot® software. In cases where the ratio of the light and heavy peptides was difficult to determine because of low signal in one of the states, a conservative value of 20 (for up-regulated proteins) or 0.05 for down-regulated proteins was used (9). Each protein SILAC ratio was expressed as the average of all the peptide SILAC ratios per protein integrated across all ten LC/MS/MS datasets.

**Western blotting**- Cellular protein was harvested, quantified by micro BCA assay and run on NuPAGE® 4-12% Bis-Tris precast Gel (Invitrogen) by using XCell SureLock® Mini-Cell (Invitrogen). After electrophoresis, the resolved proteins were transferred onto PVDF membrane by using XCell II® Blot Module. Upon the completion of transfer, standard protocols of WesternBreeze® Chemiluminescent Detection Kits (Invitrogen) were followed and developed on ECL Hyperfilm (GE Healthcare, Piscataway, NJ) according to manufacturer’s instructions. Image acquisition of western blot were performed through Epson scan (Version 2.68a) by using an Epson Perfection 4990 photo
scanner operating at film mode with the following settings: 256 gray shades and a resolution of 600 dpi. To quantify bands, QuantityOne® (Bio-Rad Laboratories, Hercules, CA) basic (Version 4.62) was used to measure densitometry. Levels of beta-actin were used to normalize protein input. Antibodies used in this study were: Anti-Beta-actin, anti-RBBP7 antibody and anti-MTA3 from Bethyl Laboratories (Montgomery, TX), anti-NSDHL from Atlas Antibodies AB (Stockholm, Sweden), anti-hnRNP G from Santa Cruz Biotechnology (Santa Cruz, CA), anti-Hsp86 from Abcam (Cambridge, MA), anti-PRP4 from ProteinTech Group (Chicago, IL), Anti-E-Cadherin from BD Biosciences (San Jose, CA).

Membrane protein digestion- One hundred million viable FDCP1 cells either cultured alone or co-cultured with irradiated MLG cells for 48 hours were treated in 1 ml of PBS (pH 7.5) for 10 min with trypsin (final concentration at 10µg/ml, Sigma) on ice (19). Cell surface proteins released by these mild protease treatments were separated from the cells by low-speed centrifugation (200 x g, for 7 min). The remaining cellular material was subjected to protein extraction as described above.

Real-time PCR- Total RNA was extracted using TRIZol® (Invitrogen) and then purified by RNasy™ Mini Kit (Qiagen, Valencia, CA). The concentrations of all RNA samples were determined by spectrophotometry. Equal amount of total RNA were used for reverse transcription and PCR, which was carried out on a thermocycler gradient (Eppendorf, Westbury, NY) by using RT² First Strand Kit (SuperArray, Frederick, MD) following standard protocols. Primers were designed by using Real-time PCR (TaqMan) Primer Design online tools available at https://www.genscript.com/ssl-bin/app/primer. Primer sequences are provided in Supplemental Table S2. Real-time PCR was performed on Mastercycler® ep realplex (Eppendorf). All reactions were performed in 96-well plates with the following reagents in a final volume of 25µl: 1µl of primers (5µM each for forward and reverse) and 2X Maxima® SYBR Green qPCR Master Mix from Fermentas (Glen Burnie, MD). 10ng of cDNA was added to this mixture. Triplicate reactions of the target and housekeeping genes were performed simultaneously for each cDNA template analyzed. The PCR reaction consisted of an initial enzyme activation step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A cycle threshold value (Ct) value was obtained for each sample and triplicate sample values were averaged. The 2-△△Ct method was used to calculate relative expression of each target gene. Data were analyzed using Student’s t-test. We considered results to be statistically significant when p<0.05 (two-sided). Real-time PCR products were also verified by running on 1.5% agarose gel, stained in 1xTAE with added GelStar® (Lonza, Basel, Switzerland) then visualized under UV transilluminator.

tRNA and cell transfection- siRNA pools containing three distinct siRNAs specific against mouse RBBP7 gene and mouse MTA3 gene and control siRNA were purchased from Santa Cruz Biotechnology. Nucleofector II device and Cell Line Nucleofector® Kit L (Amaxa, Gaithersburg, MD) were used for transfecting FDCP1 cells. Nucleofector program D-017 was used as described by the manufacturer. Each transfection used 1 µg siRNAs. After electroporation, cells were incubated in a humidified 37°C/5% CO₂ incubator for 48 hours before analyzing gene expression.

Chromatin Immunoprecipitation (ChIP)- ChIP assay was performed according to protocols supplied by Millipore’s EZ-ChIP® Kit. Anti-RBBP7 was used as the capture antibody. Normal rabbit IgG (Cell Signaling, Danvers, MA) was used as negative control. Anti-RNA Polymerase II included in this kit was used as positive control antibody.

Dual-glo luciferase assay- Three serially deleted promoter regions of mouse E-cadherin, spanning from -428, -377 and -293 nucleotides to translation starting site, were PCR amplified and cloned into pGL3-Basic vector (Promega). Dual-Glo® Luciferase Reagent (Promega) was used according to the manufacturer’s instructions. Luminescence was measured by TopCount NXT® Microplate Scintillation and
Luminescence Counter (PerkinElmer, Waltham, MA).

**Histology**- Morphological changes of cells were observed under microscopy with 20x power magnification. Immunofluorescence microscopy was performed with cells on coverslips that were fixed in -20°C cold methanol for 5 minutes. After fixation, cells were washed with PBS twice and blocked in 1.5% BSA/PBST for 30 minutes at room temperature. Then cells were incubated with primary antibodies E-cadherin and RBBP7 for 1 hour at room temperature. After washed in PBS for 3 times, cells were incubated with secondary antibody Alexa Fluor 594 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG for 1 hour and stained with DAPI for 5 minutes. The images were viewed and recorded by Olympus BX40 and SPOT Flex (Diagnostic Instruments, Version 4.5).

**RESULTS**

**Identification and validation of the cellular transfer of proteins**- We used an in vitro transwell cell culture system in which two cell types were jointly cultured but separated by a permeable membrane (0.4 µm pore size) to allow transport of secreted proteins but prevent inter-compartmental cellular migration or contact (Figure 1A). To optimize experimental techniques, we first used a “donor” murine lung fibroblast cell line (MLG) that had been cultured in SILAC medium for 6 days, corresponding to ~6 doubling times sufficient to label all proteins with [U-13C6]-L-Lysine and [U-13C6, 15N4]-L-Arginine. Cells were then extensively washed, irradiated and layered upon “acceptor” FDCP1 hematopoietic cell line that had been grown in identical cell culture media but with normal Lysine and Arginine. The effect of irradiation (500 cGy) on cellular health was assessed by serial trypan blue exclusion and MTT assays over 2 days. As seen in Fig 1B, radiation injury led to cell death and blunted proliferative responses in a statistically significant manner compared to non-irradiated cells maintained in similar conditions over the same time frame. After 48 hours of co-culture, FDCP1 cells were harvested and total cellular proteins were extracted, digested by trypsin and analyzed by LC-MS/MS using a hybrid quadrupole/time-of-flight (qTOF) mass spectrometer. A total of 2528 unique peptides were identified that initially mapped to 461 proteins (Supplemental Table S1-a). To increase the stringency of identification, we required that each protein associate with at least two unique peptides to be considered as valid identification from the reversed SwissProt database (20). Using these criteria, the total number of proteins was reduced to 383 (Supplemental Table S1-b). In order to unambiguously assign peptides as “heavy”, the threshold for peptide ratios (0.201±0.04) was calculated based on the average of ratios of all unique peptides detected and quantified and 0.205 was used as a cut-off value (Supplemental Table S1-c). Finally, the peptide ratios for each protein were averaged and we required that a ratio of >0.5 was needed in order to classify a protein as “heavy” (Supplemental Table S1-d). After applying the above mentioned criteria, a set of seven unique proteins were identified that could be confidently classified as being transferred from MLG cells to FDCP1 cells on the basis of LC-MS/MS data analysis using SILAC (Table A and Supplemental Table S3).

To independently validate this data set, we quantified the levels of six identified proteins for which commercial antibodies were available. Of these six proteins, western blot examination revealed that five proteins (NSDHL, RBBP7, HNRPG, MTA3 and PRP4) were indeed expressed at higher levels in co-cultured FDCP1 cells compared to control cells (Figure 2A). Interestingly, none of the proteins had been previously identified as secretagogues and their functional characterization ranged from metabolic enzymes, transcriptional factors to mediators of RNA splicing. We next sought to exclude the possibility that increased protein levels derived from increased transcription of their respective genes in FDCP1 cells mediated by components of the MLG secretome. Real-time PCR revealed no significant change of mRNA level of any of the six candidate proteins in co-cultured FDCP1 cells compared to FDCP1 cells cultured alone (Figure 2B). We next...
asked whether increased protein levels were simply due to their adsorption onto FDCP1 cell membranes or were indeed due to bona fide cellular entry. Trypsin digestion of co-cultured FDCP1 cells sufficient to destroy membrane adsorbed proteins was carried out and western blotting revealed no change at protein level (Figure 2C). Thus far, our coculture experiments had used irradiated MLG cells with FDCP1 cells. When irradiation was removed from the experimental flow, levels of the candidate proteins as well as their mRNA level were not increased in FDCP1 cells compared to relevant controls (Figure 2D). Furthermore, detailed analysis of supernatants from irradiated vs. non-irradiated cells revealed that only the former harbored detectable levels of the candidate proteins suggesting that the process of irradiation was critical for enabling protein release from MLG cells (Supplemental Figure S1). Taken together, the higher level of these five proteins in FDCP1 co-cultured cells appeared to result from the direct transfer from MLG cells to FDCP1 cells after the former received irradiative stress.

The interactome harbors modulators of E-cadherin- Two transferred proteins were retinoblastoma binding protein 7 (RBBP7), also referred to as histone-binding protein 7 or retinoblastoma (Rb) protein associated protein 46 (RbAp46) and metastasis associated protein 3 (MTA3). Both belong to the nucleosome remodeling and deacetylase (NuRD) complex (21, 22). Constituents of this complex are responsible for the deacetylation of lysine residues of core histones and also serve as transcriptional repressor complexes by associating with many different proteins, thus playing an important role in transcriptional regulation, cell cycle progression and developmental events (23, 24). RBBP7 belongs to a highly conserved WD-repeat protein (25) and plays an important role in chromatin remodeling, and regulation of cell proliferation and differentiation by binding directly to retinoblastoma (Rb) (26) and BRCA1 (27), which are known tumor suppress proteins.

To further probe the significance of increased expression of RBBP7 and MTA3 in co-cultured FDCP1 cells, we surveyed the literature on potential downstream effects of these two proteins. We noted that MTA3 increases the expression of E-cadherin by its potent repressive effect on snail transcription by interacting with histone deacetylases (22, 28). Thus, we profiled levels of E-cadherin in co-cultured FDCP1 cells, and observed an increase of both protein and mRNA levels (P<0.01) (Figure 3A). To validate these results, we introduced highly effective siRNAs against MTA3 and RBBP7 together into MLG cells and assessed E-cadherin expression in co-cultured FDCP1 cells. As expected, E-cadherin protein levels were decreased (Figure 3B). Next, we examined the effect of silencing MTA3 or RBBP7 individually. MTA3 knock down was associated with a 90% decrease in E-cadherin mRNA levels decreased (P<0.01) (Figure 3C). RBBP7 silencing was also associated with a significant reduction (60%, P<0.01) of E-cadherin mRNA levels (Figure 3D, left panel). These findings were validated by corresponding western blots (Figure 3D, right panel). Thus, our gene silencing studies suggested that RBBP7 may be acting as a regulator of E-Cadherin and that its effects were distinct from those of MTA3.

Upregulation of E-Cadherin by RBBP7 does not involve known regulatory pathways- The factors that mediate E-cadherin expression are of intense interest given the central role E-cadherin plays in cancer biology with reduced expression leading to increased tumor aggressiveness and metastatic potential (29, 30). While the molecular pathways mediating E-cadherin loss are relatively well characterized whether it is by hypermethylation of its promoter region or transcriptional repression by a wide array of factors, relatively little is known regarding mechanisms that induce E-cadherin in mammalian cells (17). The complexity of the transcriptional control of E-cadherin derives from the sheer number of regulatory loops that control its activity at the genetic, epigenetic and transcriptional levels (30). Various regulators, mainly negative, have been reported, including E47 (31), Snail (SNAI1) (32, 33), Slug (SNAI2) (34, 35), TWIST1 (36), ZEB1 (37, 38) and ZEB2 (39, 40). To
determine whether any of the above transcription factors were involved in the regulation of E-cadherin under conditions of RBBP7 transfer in FDCP1 cells, we examined their mRNA levels by real-time PCR. Compared to FDCP1 cells cultured alone, we could not detect any significant change in the level of any of the above mentioned E-cadherin specific factors in co-cultured FDCP1 cells, including no detectable expression for slug (Supplemental Figure S2A). Furthermore, RBBP7 silencing was not associated with any increased expression of known repressors of E-cadherin (Supplemental Figure S2B). Additionally, there was no change in the protein level of histone deacetylase 1 (HDAC1), a subunit of the core histone deacetylase (HDAC) complex composed of HDAC1, HDAC2, RBBP4 and RBBP7 (21, 41) (Supplemental Figure S3). Based on these findings, it appeared unlikely that RBBP7 was regulating E-cadherin through previously identified suppressive transcriptional networks or through its association with the HDAC repressor complex.

**RBBP7 accumulates in the promoter region of E-cadherin** As previously described, many E-cadherin regulators, such as E47 and SNAIL1 exert transcriptional control by direct binding to the E-cadherin promoter region. The apparent increase in E-cadherin mRNA level in co-cultured FDCP1 cells led us to ask whether RBBP7 might also physically interact with the E-cadherin promoter. We surveyed the ~1200 base pair region upstream of the transcription start site (TSS) and 127 base pair downstream of TSS for putative RBBP7 binding sites by chromatin immunoprecipitation (ChIP). We detected robust (~8-fold enrichment) accumulation of RBBP7 around -428 to -297 bp upstream of the TSS, compared to relevant controls (Figure 4A). To further narrow down the promoter sequence motif involved in RBBP7 binding, three different lengths of the promoter region spanning from -428 (Ecad\(_{-428}\) -pro-luc), -377 (Ecad\(_{-377}\) -pro-luc) and -293 (Ecad\(_{-293}\) -pro-luc) to the translation start site were adjoined to sequences of firefly luciferase. After simultaneous transfection of siRNAs targeting RBBP7 along with ECad-pro-luc constructs into FDCP1 cells, we observed dramatic decreased (~80%) luciferase activity in the experiments involving Ecad\(_{-428}\) -pro-luc compared to cells transfected with control empty vector. We did not observe a significant change in luciferase expression in experiments involving the other reporter constructs (Figure 4B). Thus, reporter assay results suggest that the region between -428 to -377 of the E-cadherin promoter harbors a binding site for RBBP7.

**RBBP7 cellular transfer is not limited to FDCP1/MLG cells** Thus far, our work involved murine lung and hematopoietic cell lines. To determine whether intercellular transfer of RBBP7 is a generalized process, we next conducted similar co-culture experiments involving primary murine lung with bone marrow cells as well as MLG cells with human breast (MDA231) and prostate (DU145WT) cancer cell lines. Using a similar protocol, we found that co-culture led to the transfer of RBBP7 to bone marrow cells from irradiated primary lung tissue with attendant increase of E-cadherin protein expression (Figure 5, left panel). A similar relationship existed when irradiated murine MLG cells were co-cultured with the human prostate cancer cell line (Figure 5, right panel). Trypsin digestion did not alter RBBP7 levels in co-cultured BM or DU145WT cells and radiation was required for protein transfer. When similar experiments were repeated with a breast cancer cell line, we could not detect any transfer of RBBP7 (data not shown). At this juncture, it is unclear what cell specific factors mediate RBBP7 uptake and is a focus of further investigation.

**RBBP7 transfer impacts the expression of genes involved in mesenchymal-epithelial transition** Regulatory insight on E-cadherin is welcome given the protein’s prominent role in cancer biology. Current models suggest that one of the first steps a malignant cell takes is down regulation of E-cadherin with the loss of adherens junctions enabling cancer cells to separate from one another and metastasize (17). However, once a malignant cell arrives at its destination, it must lodge and perhaps recover adherens activity (15). Thus, E-cadherin has emerged as a central player in the mesenchymal-epithelial transition (MET) and
the reverse epithelial-mesenchymal transition (EMT). We next assessed what role the physiologic transfer of RBBP7, resulting in higher levels of E-cadherin, played in MET/EMT. We quantified the levels of N-cadherin (CDH2), fibronectin (FN1) and vimentin (VIM), three genes whose expression levels are low in MET but high in EMT (17, 30). The three indicator genes were quantified in bone marrow cells that had been cultured with irradiated murine lung for 2 days. As assessed by real-time PCR, mRNA levels of N-cadherin (P < 0.05), and vimentin (P < 0.01) were significantly reduced, while levels of N-cadherin were increased (Figure 6A, left panel). As for co-cultured prostate cancer cells (DU145WT), mRNA levels of N-cadherin (P < 0.01), and fibronectin (P < 0.05) were significantly reduced, while levels of vimentin were reduced but did not attain statistical significance (Figure 6A, right panel). Next, we compared the morphology of both bone marrow and prostate cancer cells cultured alone and in the presence of irradiated primary lung. Microscopic analysis of the former did not reveal the classic changes associated with EMT (Figure 6B, left panel). In contrast, prostate cancer cells presented a slight cobblestone appearance lacking cellular extensions. This morphology suggests a low level of E-cadherin-based cell-cell adhesions indicative of a partial reversion back to an epithelial phenotype (Figure 6B, right panel). In support of this, immunofluorescence staining revealed higher levels of E-cadherin protein in prostate cancer cells (DU145WT) cultured with irradiated MLG cells compared to cells cultured alone (Figure 6C).

**DISCUSSION**

The goal of our experiments was to assess the utility of SILAC in deciphering the protein interactions of adjacent cells and to specifically identify the constituents of this interactome and the biologic relevance of intercellular protein transfer. The methodology we used and its associated findings raise several issues. Previously, the intercellular transfer of proteins has largely been studied by tracking the fate of selected proteins expressed in one cell type but not another in direct co-culture systems (42, 43). SILAC offers an advantage in that uniform labeling of the entire proteome of a given cell allows the design of co-culture conditions employing cells of diverse tissue origin thereby allowing the interrogation of the entire proteome. Standard SILAC procedures have traditionally used a 1:1 mix of “light” and “heavy” labeled samples. Our approach, however, utilized samples that were predominantly “light” but allowed to harbor “heavy” proteins that had been transferred. Data analysis and validation with western blot revealed that SILAC labeled proteins could be correctly identified using our approach.

Previous studies of intercellular protein transfer have identified several membrane associated proteins as likely participants in transfer such as glycosyl phosphatidylinositol (GPI)-anchored proteins (44, 45), the CCR5 chemokine receptor (43) and more recently, p-glycoprotein (43). Furthermore, direct cellular contact has been thought to be necessary for protein transfer (46). We had expected to identify previously known components of signal transduction cascades (e.g., proteins involved in G protein signal cascade or Wnt signaling pathway) that act through membrane receptor binding. Instead, our initial work using murine cell lines identified a panel of proteins of lung origin that underwent transfer to adjacent cells, a process that was reproducible in most but not all human and murine cell types examined. Interestingly, the transfer of one of the constituent proteins, RBBP7, was associated with the induction of a MET program mediated by increasing levels of E-cadherin. Our confidence of actual RBBP7 entry to recipient bone marrow and prostate cells derives from noting the downstream effects of RBBP7 which we found mediated E-cadherin activation by physically interacting with the latter’s promoter region. Our findings are noteworthy along several fronts. The considerable literature on mechanisms of E-cadherin genetic control has largely identified negative regulators and quantified their respective levels in malignant cells. A number of regulators, including E47, SNAL1, SNAL2,
act by binding specifically to E-box motifs, especially the so called E-pal regions (35, 47, 48), a unique array of tandem E-boxes, located between -86 to -75 bp in the promoter region of murine E-cadherin. We localized the site of RBBP7 binding to -428 to -377 bp by ChIP and luciferase reporter assays, a region that we found to contain an additional palindromic E-box (CACGTG) located between -401 to -396 bp. While we observed a direct relationship between levels of RBBP7 and E-cadherin, we cannot discount the fact that RBBP7 may be enhancing E-cadherin transcription by physically preventing the localization of E-box specific suppressive factors. Nonetheless, our results clearly add RBBP7 to the growing list of proteins that impact E-cadherin expression. Presently, few studies have examined the relative expression of RBBP7 in healthy and diseased tissues. A recent examination of breast carcinoma revealed relatively high levels of RBBP7 mRNA and protein (49). It would be interesting to determine whether a direct relationship exists between RBBP7 and E-cadherin in a broad sampling of malignant and normal tissue.

Finally, therapies proposing to use stem cells or those aiming to eradicate malignant foci require a better appreciation of the signals a diseased tissue emits to and receives from its healthy counterparts. A rationale understanding of this “interactome” has the potential to forward relevant therapeutics and to fill in current gaps in our understanding of the tissue milieu of regeneration or metastatic destruction. SILAC based methodologies as used here could easily be extended to characterize the interactome of biologically relevant cellular pairs such as malignant/normal cells isolated from a primary tumorous tissue. Such knowledge may shed light on the complexity of cellular communication in health and disease.

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FOOTNOTES
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The abbreviations used are: MS, mass spectrometry; SILAC, stable isotope labeling of amino acids in cell; RBBP7, retinoblastoma binding protein 7; BM, bone marrow; MET, mesenchymal to epithelial transition

FIGURE LEGENDS
Figure 1. A.) Overall experimental schema: MLG cells grown in SILAC medium were co-cultured with FDCP1 cells using a transwell system. MS analysis was used to identify proteins that had been transferred from MLG to FDCP1 cells. Similar analysis of protein transfer was performed for other cell types co-cultured in transwell systems including primary murine lung/bone marrow and MLG/prostate cancer or breast cancer cell lines. B.) Trypan blue exclusion staining and MTT based cell proliferation assays revealed that irradiation at 500 cGy led to decreased cellular survival and blunted proliferative responses in a significant manner. Experiments were performed in triplicate and are shown as mean values +/- S.D (* P<0.05, ** p<0.01).

Figure 2. Validation of intercellular protein transfer by western blot and real-time PCR. All western blots utilized beta-actin (ACTB) as a loading control. All western blot and PCR reactions were performed in triplicate and are shown as relative expression levels +/- S.D.
A.) MS analysis identified seven unique proteins as being transferred from MLG cells to FDCP1 cells. Commercial antibodies were available for six proteins. Conventional western blot was used to validate this data set and revealed an increase by densitometric analysis in the respective levels of all proteins except HSP86 in FDCP1 cells that had been co-cultured with irradiated MLG cells compared to FDCP1 cells cultured alone. Fold change in protein levels are noted below their respective identifiers. B.) Real-time PCR was used to quantify the mRNA levels of all transferred proteins and revealed no statistically significant difference in FDCP1 cells cultured alone compared to FDCP1 cells that had been cultured with irradiated MLG cells. C.) To exclude the possibility that increased levels of protein were due to adsorption onto plasma membranes rather than bona fide cellular entry, protein levels were quantified with or without trypsin digestion of
FDCP1 cells that had been cultured with irradiated MLG cells. Western blot revealed that trypsin treatment had no effect upon protein levels. D.) Requirement of irradiation of MLG cells for the intercellular transfer of RBBP7 and MTA3. When FDCP1/MLG co-culture was repeated using non-irradiated MLG cells, no appreciable increase in protein levels was noted by either Western blot (left panel) or real-time PCR (right panel) in co-cultured cells compared to FDCP1 cells cultured alone.

Figure 3. Effect of FDCP1/MLG co-culture on levels of E-cadherin (CDH1).
A.) FDCP1/MLG co-culture was associated with an increase in CDH1 as quantified by western blot (left panel) and real-time PCR (right panel). CDH1 mRNA levels increased by ~5-fold in co-cultured FDCP1 cells (** p<0.01).
B.) FDCP1 cells were co-cultured with irradiated MLG cells that had been treated with a combination of siRNA targeting MTA3 and RBBP7. Western blot of both proteins (left panel) confirmed the activity of their respective siRNA. Co-culture of siRNA treated MLG cells with FDCP1 cells led to a decrease in CDH1 protein levels in FDCP1 cells (right panel).
C.) Small interfering RNAs (siRNAs) were used against MTA3 and as expected, levels of CDH1 decreased by >90% (** P<0.01) in MTA3 silenced FDCP1 cells. siRNA transfection reduced MTA3 mRNA levels by 95% (* P<0.05) but had no effect upon an irrelevant mRNA such as RBBP7. All siRNA transfection used a pool of three gene specific RNA duplexes.
D.) RBBP7 levels were decreased ~80% (** P<0.01) by specific pooled siRNAs in FDCP1 cells but had no effect upon an irrelevant mRNA such as MTA3. RBBP7 silencing was surprisingly associated with reductions in CDH1 mRNA (60% reduction, ** P<0.01) (left panel) and protein (right panel).

Figure 4. Localization of RBBP7 to the promoter region of CDH1 by chromatin immunoprecipitation (A) and luciferase based promoter assays (B).
A.) Sheared chromatin from FDCP1 cells that had been crosslinked with formaldehyde was immunoprecipitated with anti-RBBP7 antibodies. Crosslinks were removed and the DNA was purified. The promoter region of RBBP7 (-1214 to +127) was arbitrarily deconstructed into 15 segments of ~100 nt each. Specific PCR primers were designed to amplify each component and reveal relative enrichment in sector I (* p<0.05), corresponding to nucleotides -428 to -297 with respect to the CDH1 transcription start site. All values are relative to nonimmune IgG and normalized to an intergenic control region. B.) The CDH1 promoter region corresponding to nucleotides -428 to -293 was further investigated by use of luciferase reporter constructs. Promoter fragments spanning from -428, -377 and -293 to translation start site (+127) were placed adjacent to firefly luciferase and transfected into FDCP1 cells along with siRNAs targeting RBBP7 or an irrelevant siRNA. Luciferase activity was reduced in the -428 fragment (* p<0.05). These findings in association with ChIP data suggest RBBP7 binding at regions -428 to -377 of the CDH1 promoter. All transfections were performed in triplicate and luciferase activity is expressed relative to that obtained in experiments involving control siRNA +/- S.D.

Figure 5. The RBBP7/CDH1 axis. Co-culture of primary murine lung with bone marrow (BM) or MLG cells with DU145WT (a prostate cancer cell line) leads to RBBP7 protein transfer to recipient BM (left panel) and DU145WT cells (right panel) as determined by western blot. Co-culture had no effect on RBBP7 mRNA levels in BM or DU145WT cells (bottom panel).

Figure 6. Effect of RBBP7 transfer on the mesenchymal-epithelial transition. CDH1 is known to enforce MET by reducing levels of marker genes such as N-cadherin (CDH2), Fibronectin (FN1) and Vimentin (VIM). A.) Real-time PCR was used to quantify their levels. In bone marrow cells (left panel), co-culture was associated with decreased levels of CDH2 and VIM but not FN1 (**) P<0.01 or * P<0.05. In DU145WT cells (right panel), co-culture was associated with a decrease of all three EMT markers in DU145WT cells although the decrease of VIM was not statistically
significant. **B.** Acquisition of a more epithelial phenotype was further probed by characterizing *in vitro* phenotypic changes. Co-cultured BM cells (Left panel) showed fibroblastic morphology but no cobblestone appearance. Co-cultured DU145WT (Right panel), however, showed more typical mesenchymal-to-epithelial phenotypic changes with cobblestoning beginning to appear. **C.** Immunofluorescence staining of CDH1 (red) and RBBP7 (green) in co-cultured DU145WT cells revealed higher levels of both proteins compared to DU145WT cells that had been cultured alone.
| RefSeq Accession Number | Protein Names                      | Gene Names | Mass (Da) | Total Identified Peptides | Number of “Heavy” Peptides | Protein Ratios |
|-------------------------|-----------------------------------|------------|-----------|--------------------------|---------------------------|----------------|
| NP_035071               | NAD(P) dependent steroid dehydrogenase-like | NSDHL      | 40,686    | 4                        | 2                         | 5.14±0.02      |
| NP_033057               | Retinoblastoma binding protein 7   | RBBP7      | 47,790    | 6                        | 2                         | 3.45±0.01      |
| NP_033059               | RNA binding motif protein, X chromosome retrogene | HNRPG      | 42,162    | 8                        | 2                         | 2.58±0.0      |
| NP_034610               | Heat shock protein 1, alpha        | HSP86      | 84,788    | 15                       | 3                         | 1.42±0         |
| NP_663590               | Electron transferring flavoprotein, alpha polypeptide | ETFA       | 35,009    | 10                       | 2                         | 0.65±0.02      |
| NP_473423               | Metastasis associated 3            | MTA3       | 67,077    | 3                        | 3                         | 0.57±0.04      |
| NP_081573               | PRP4 pre-mRNA processing factor 4 homolog | PRP4       | 58,370    | 3                        | 2                         | 0.51±0.04      |

**Table A.** Candidate proteins thought to undergo transfer from MLG to FDCP1 cells based upon MS data.

Shown are the seven proteins, their molecular weight, number of “heavy” peptides as well as number of peptides associated with each protein. We required that each protein be associated with at least two heavy peptides and protein ratios >0.5 in order to be considered for downstream evaluation. In depth analysis of MS data for candidate proteins including MTA3 and RBBP7 are shown in Supplemental Table S3.
Figure 1

A

Grow in “Heavy” media

MLG cells

Expand cells for six doublings

Irradiation of cells

Wash with PBS, transfer to top of insert

Maintain in “Light” media

FDCP-1 cells

Co-culture in “Light” media for 48 h

Transfer to bottom of insert

Collect cells from bottom of transwell system, Extract proteins and Run SDS-PAGE

Excise bands and in-gel digestion

MS analysis and data verification

B

![Graph A](image1)

![Graph B](image2)
Figure 3

A

|       | FDCP1 | FDCP1/MLG |
|-------|-------|-----------|
| CDH1  |       |           |
| ACTB  |       |           |

B

|       | MLG/FDCP1 | MLG/FDCP1 |
|-------|-----------|-----------|
| MTA3  |           |           |
| RBBP7 |           |           |
| ACTB  |           |           |

C

![Graph showing relative expression of MTA3, CDH1, and RBBP7](image)

D

![Graph showing relative expression of CDH1, RBBP7, and MTA3](image)
Figure 4

A

B

E-box

Relative Luciferase Reading

-293

-377

-428

IgG

RBBP7

FDPC1-168-C

FDPC1-168-RBBP7
Figure 5
Figure 6

A

B

BM
BM/LUNG

DU145WT
DU145WT/MLG

C

DU145WT
DU145WT/MLG

DAPI
RBBP7
CDH1
merge
Intercellular transfer of proteins as identified by stable isotope labeling of amino acids in cell culture (SILAC)

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