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A Systematic Analysis Reveals Heterogeneous Changes in the Endocytic Activities of Cancer Cells

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Abstract

Metastasis is a multistep process requiring cancer cell signaling, invasion, migration, survival, and proliferation. These processes require dynamic modulation of cell surface proteins by endocytosis. Given this functional connection, it has been suggested that endocytosis is dysregulated in cancer. To test this, we developed In-Cell ELISA assays to measure three different endocytic pathways: clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin-independent endocytosis and compared these activities using two different syngeneic models for normal and oncogene-transformed human lung epithelial cells. We found that all endocytic activities were reduced in the transformed versus normal counterparts. However, when we screened 29 independently isolated non-small cell lung cancer (NSCLC) cell lines to determine whether these changes were systematic, we observed significant heterogeneity. Nonetheless, using hierarchical clustering based on their combined endocytic properties, we identified two phenotypically distinct clusters of NSCLCs. One co-clustered with mutations in KRAS, a mesenchymal phenotype, increased invasion through collagen and decreased growth in soft agar, whereas the second was

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enriched in cells with an epithelial phenotype. Interestingly, the two clusters also differed significantly in clathrin-independent internalization and surface expression of CD44 and CD59. Taken together, our results suggest that endocytotic alterations in cancer cells that affect cell surface expression of critical molecules have a significant influence on cancer-relevant phenotypes, with potential implications for interventions to control cancer by modulating endocytic dynamics.

Introduction

Tumor cell growth and metastasis involve changes in cell–cell and cell–matrix interactions, survival and proliferative signaling, and nutrient uptake, all of which depend on plasma membrane receptors and transporters (1, 2). Signaling from the cell surface and the interactions of cells with each other and their environment are dynamically regulated by the endocytosis of signaling, adhesion, and nutrient receptors. Consequently, it has been suggested that endocytosis is dysregulated in cancer cells (3–5). Indeed, there are numerous examples of cancer-specific mutations in components of the endocytic machinery and/or changes in their levels of expression (6–10). It has also been reported that endocytic trafficking can be perturbed downstream of oncogenes such as p53 and Ras (11, 12).

Clathrin-mediated endocytosis (CME) and caveolae-mediated endocytosis (CavME) remain the best-characterized endocytic pathways, although other more recently discovered and mechanistically distinct pathways have been shown to mediate the uptake of different subsets of signaling, adhesion, and nutrient receptors, as well as regulate the surface expression of membrane transporters (13–15). These alternate pathways, generally referred to as clathrin-independent endocytosis (CIE), include the recently discovered clathrin- and dynamin-2 (Dyn2)-independent uptake into so-called clathrin-independent carriers (CLIC), which involve the small GTPases Rac1, Cdc42, and Arf6 (14–18). To what extent these CIE pathways contribute to the endocytic capacity of the cell remains unclear, as some studies suggest they are the major pathway for bulk uptake (17), whereas a more recent study suggests that CME can account for virtually all bulk uptake (19).

Past studies of endocytosis in cancer cells have focused primarily on CME and CavME, and these have been studied, individually, in only a few cancer cell lines. Hence, it is unknown whether endocytic activities are selectively or randomly altered in cancers. Moreover, few studies have correlated the activities of specific endocytic pathways with changes in cellular behavior such as migration, adhesiveness, or proliferation. To address these issues, we have systematically and quantitatively analyzed multiple endocytic activities across a clinically diverse and molecularly characterized panel of non–small cell lung cancer (NSCLC) cell lines (20, 21). Our studies reveal significant heterogeneity across cell lines and endocytic pathways, which we utilize to test for correlations between specific endocytic activities and alterations in cellular processes related to cancer, including proliferation, adhesion, and migration.

Materials and Methods

Cell lines and culture

HBEC30KT and the NSCLC cancer cell lines were generated as previously described (20). HBEC3KT and their oncogene-transformed derivatives were developed by the Minna lab (22). All NSCLC lines used in this study were obtained from the Hamon Cancer Center Collection (UT Southwestern Medical Center) and maintained in RPMI-1640 (Life Technologies) supplemented with 5% FCS at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. All cell lines have been DNA fingerprinted using the PowerPlex 1.2 Kit (Promega) and are mycoplasma free using the e-Myco Kit (Boca Scientific). Culture media were purchased from Life Technologies. Human bronchial epithelial cell (HBEC), NSCLC, and Human retinal epithelia ARPE-19 cell lines were obtained from the ATCC and cultivated in complete KSF medium, RPMI-5% FBS (Sigma), or in DMEM/F12-10% FBS, respectively.

Antibodies and reagents

Anti-TfnR (HTR-D65) monoclonal antibody was produced in hybridoma as in ref. 23. Anti-CHC (sc-12734) and anti-Dyn-2 (sc-64000) antibodies were purchased from Santa Cruz Biotechnology. FITC-conjugated anti-CD44 (G44-26) and anti-CD59 (p282-H19) monoclonal antibodies were obtained from BD Pharmingen. Horseradish peroxidase (HRP)– and AlexaFluor-conjugated antibodies were purchased from Life Technologies. Biotinylated albumin (#A8549), OPD (#P1536), nystatin (#N6261), poly-l-Lysine (#P1536), fibronectin (#F1141), laminin (#L2020), and hyaluronic acid (#H5388) were obtained from Sigma-Aldrich. Rat-tail collagen (#354236) and streptavidin-POD were purchased from BD Biosciences and from Roche, respectively. Fluoromount G and paraformaldehyde (PFA) were purchased from Electron Microscopy Sciences.

Transferrin receptor, albumin, CD44, and CD59 internalization

TfnR, CD44, or CD59 internalizations were performed using receptor-specific mAbs. We used biotinylated albumin to measure Cav-ME. Cells were seeded at a density of 2.8×10^4 cells/well on collagen-coated 96-well plates and grown overnight. For assays, cells were washed (3× PBS) and incubated with 40 µL PBS⁴⁺ (PBS supplemented with 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, 5 mmol/L glucose, and 0.2% BSA) containing 4 µg/mL mAb or 30 µg/mL biotinylated albumin at 37°C for the indicated time points before being immediately cooled to 4°C to arrest internalization and washed to remove unbound ligand (3× PBS). The remaining surface-bound ligand was removed by acid washes (4× 1 minute 0.2 mol/L acetic acid, 0.2 mol/L NaCl, pH 2). Cells were washed with PBS and then fixed in 4% PFA (Electron Microscopy Sciences) in PBS for 10 minutes at 4°C and 10 minutes at room temperature. Cells were then permeabilized with 0.1% Triton-X100/PBS for 5 minutes, washed, and then blocked with 5% BSA/casein for 1 hour. Internalized albumin was assessed using streptavidin-POD (Roche). Internalized D65, CD44, and CD59 mAbs were assessed using a goat anti-mouse HRP-conjugated antibody and further developed with 200 µL OPD. The reaction was stopped by addition of 50 µL 5 mol/L H₂SO₄. The absorbance was read at 490 nm (Biotek Synergy H1 Hybrid Reader). Well-to-well variability in cell number was accounted for by normalizing the reading at 490 nm with a

BCA readout at 560 nm (Biotek Synergy H1 Hybrid Reader). The fraction of internalized ligand was calculated relative to the initial total surface-bound ligand at 4°C (without the acid wash step) measured in parallel for all the assays. Data represent mean $\pm$ SD from four independent experiments, each performed in triplicate.

To visualize internalization by fluorescence microscopy, HBEC30KT and HCC4017 cells were seeded in 8-well chambers. The same procedure as for the In Cell ELISA was used to generate "Surface-bound" and "Internalized" ligands except that after permeabilization the cells were incubated with suitable Alexa-Fluor-labeled secondary antibodies and then mounted with Fluoromount G. Images were captured using a 60 $\times$ objective mounted on a Ti-Eclipse inverted microscope equipped with a CoolSNAP HQ2 monochrome CCD camera (Photometrics).

For *siRNA-mediated* inhibition of endocytosis, RNAiMAX transfection reagent (Life Technologies) was used to deliver siRNA targeting Dyn2 (1:1 mixture of Dyn2_1: 5'-CCGAAUCAAUCG-CAUCUUCUU-3' and Dyn2_2: 5'-GACAUGAUCCUGCAGUU-CAUU-3') or clathrin heavy chain (CHC; ref. 24) to H1299 cells, following the manufacturer's instructions. We used All Star Negative siRNA as a control. Cells were used 72 hours after siRNA transfection. For inhibition by nystatin, H1299 cells were preincubated in the absence (control) or presence of 25 μ g/mL nystatin for 30 minutes at 37°C before internalization assays were performed as described above except the PBS⁴⁺ contained nystatin.

Proliferation, adhesion assays, and 3D migration assays

The following microscopy-based assays were performed in 96-well black plates with clear bottom (PerkinElmer #600525).

Proliferation—A total of 2,000 cells/well of each cell line were dispensed, in triplicate. Five hours after plating, cells from the first plate—"Day 0" plate—were fixed with 4% PFA and stored at 4°C. The same operation was repeated on days 1 through 5. Cell nuclei were stained for 30 minutes with 10 μ g/mL Hoechst/PBS solution. Cell proliferation was expressed as the ratio of cell counts on "Day 5"/"Day 0" plates.

Adhesion—96-well plates were incubated with 40 μ L/well for 1 hour containing 10 μ g/mL BSA, 0.01% poly-L-Lysine, 50 μ g/mL rat tail collagen, 10 μ g/mL fibronectin, 50 μ g/mL laminin or incubated overnight with 1 μ g/mL hyaluronic acid. After plates were washed with PBS, 15,000 cells/well were plated and allowed to adhere for 30 minutes at 37°C. Floating cells were then removed by three washes with PBS. Cells were fixed with 4% PFA and nuclei were stained for 30 minutes with 10 μ g/mL Hoechst/PBS solution. Cell adhesion represents the number of cells per condition.

3D migration assay—Assays were performed using 1 μ g/mL bovine collagen and 2.5% FBS as chemoattractant, following the experimental procedure described in ref. 25. Microscopy images of nuclear staining were taken at 50 μ m steps from 0 to 150 μ m into the collagen plug. Invasion index was calculated as the sum of cell counts at 50, 100, and 150 μ m over cell counts at 0 μ m.

Cell nuclei were visualized using a 20× (migration assays) or 10× (proliferation and adhesion assays) air objective mounted on a Ti-Eclipse inverted microscope (Nikon) driven by NIS Elements V4.13 software and quantified using the "Object Count" feature of NIS Elements software.

Growth in soft agar—Anchorage-independent growth in soft agar was performed by seeding cells in 0.4% noble agar on top of a 1% layer. After 3 weeks, cell foci were stained with a Crystal Violet solution and extensively washed with water to remove excess stain. Foci were then counted manually using the Cell Counter tool on ImageJ software.

Statistical analysis and hierarchical clustering

A hierarchical clustering algorithm (26) was used to group the 29 NSCLC cell lines based on their collective endocytic activities. For this purpose, we took the averaged endocytic activity across replicates for each cell line and each pathway (Supplementary Table S1), and then used these values to calculate Pearson correlation distance metrics, average linkages (26), and then generated the hierarchical clustering results using GENE-E software from GenePattern (27) with default settings. The hierarchical tree identified two clusters. We tested the significance of the clustering result using the R package sigclust by using the 2-means clustering index as a statistic to test the null hypothesis that a group of samples is from a single Gaussian distribution (28). Migration and growth in soft-agar enrichment analysis for two clusters were measured by the Fisher exact test to examine whether the two kinds of classification from categorical data are associated, which is best suited for small sample sizes and a 2×2 contingency table. We tested association of the two clusters with endocytic activities and the total surface protein expression using a Wilcoxon rank sum test, which compares the sums of ranks and is more robust to the presence of outliers. The statistical correlations between each endocytic activity and cell proliferation were calculated by the Spearman rank correlation test, which is less sensitive to outliers and queries the extent of dependence of one ranked variable on another. Correlations between KRAS mutations and endocytic activities were also measured by the Wilcoxon rank sum test.

Results

Choice of pathway-specific and lung cancer-related endocytic markers

CME and CavME have been extensively studied, and specific markers allowing the quantification of these endocytic routes are well described. We chose transferrin receptor (TfnR) for CME (29) and albumin, which binds to gp60 and is internalized by CavME (30). Although markers for clathrin- and caveolae-independent endocytotic pathways are less well characterized, glycoposphatidylinositol-anchored proteins (GPI-AP) are reportedly internalized by these routes (15, 17, 18). For our studies, we chose complement lysis restricting factor/CD59 (31), a GPI-AP that is involved in cellular processes critical to lung cancer cell survival and internalized via CIE (32, 33). Recent studies have identified the transmembrane glycoprotein, CD44, which is a hyaluronic acid receptor, as a cargo molecule specifically internalized by CLICs and recycled via GPI-enriched endocytic compartments (GEEC; refs. 15, 34). CD44 becomes more concentrated in flotillin-low or non-raft fractions, presumably corresponding to CLICs, in migrating breast cancer cells

(35). Interestingly, CD44 is expressed in NSCLC, but not in small cell lung cancer (36). Elevated expression levels of CD44 combined with low expression of CD24 (CD44^{high}/CD24^{low}) is an emergent prognosis tool in the clinic as it is associated with "stemness" features of cancer cells *in vivo* and *in vitro* (36–38). Together, these markers allowed us to directly measure the CME, CavME, and CIE activities in NSCLC cells.

Development of pathway-specific 96-well endocytosis assays

To efficiently and systematically measure multiple endocytic pathways using several NSCLC cell lines in parallel, we developed a new sensitive and quantitative method using 96-well plates, referred to as an In-Cell ELISA (see Materials and Methods). As ARPE-19 cells have been routinely used to study CME, we used this cell line to set up and validate the highthroughput assay. Upon incubation of ARPE-19 cells at 37°C with the anti-transferrin receptor antibody HTR.D65 (D65; ref. 39), we could detect the time and temperature-dependent accumulation (expressed as the fraction internalized at 37°C over the total surface bound at 4°C) of intracellular D65 (Supplementary Fig. S1A, left, quantification in B). The use of the bivalent anti-TfnR mAb, rather than transferrin, as ligand reduces rapid recycling (40) and enables us to focus our measurements on initial rates of internalization. As a negative control for internalization of surface-bound versus soluble mAb, we incubated these cells with a specific antibody against the T-cell marker CD8, which is not expressed on epithelial cells. As expected, we did not detect surface binding or any internalization of anti-CD8 in ARPE-19 cells (Supplementary Fig. S1A, right, quantification in B).

This assay was adapted to measure CavME using biotinylated-albumin as ligand, and CIE using anti-CD59 or anti-CD44 mAbs as markers, and validated in the NSCLC line, H1299. We first confirmed that these different surface receptors utilized different pathways by siRNA knockdown of CHC, which is specifically required for CME and dynamin 2 (Dyn2), which is required for both CME and CavME (Fig. 1A). We also treated cells with the antifungal drug nystatin that disrupts the plasma membrane cholesterol and selectively inhibits CavME, but not CME. As expected, CME of TfnR was inhibited upon RNAi-mediated knockdown of either Dyn2 or CHC (Fig. 1B), whereas nystatin showed no effect. Also, as expected, uptake of albumin by CavME was inhibited upon RNAi knockdown of Dyn2 and treatment with nystatin (Fig. 1C), but was unaffected by CHC knockdown. Finally, uptake of CD44 and CD59 was not affected by either of these perturbations (Fig. 1D and E), confirming that these markers are internalized by a clathrin-, Dyn2-, and caveolae-independent mechanism. These data establish our ability to independently and selectively measure at least three distinct endocytic pathways.

Altered endocytic activity across pathways in isogenic normal and cancer cell lines

We next compared the endocytic activities in an NSCLC cell line (HCC4017) with those in a normal, nontumorigenic HBEC line, HBEC30KT, derived from the same patient. The HBEC30KT cells were immortalized by serial introduction of retroviral expression vectors encoding cyclin-dependent kinase 4 (Cdk4), which prevents premature growth arrest, and human telomerase reverse transcriptase (hTERT), which bypasses telomere-dependent senescence (41). The internalization rates of all four receptors, expressed as the fraction internalized after 5 minutes at 37°C over the total surface bound at 4°C, were reduced in the

NSCLC cells as compared with their normal HBEC30KT counterpart (Fig. 2A). This was confirmed by fluorescence microscopy (Fig. 2B–E), which also illustrates the uniformity in uptake among the cells within each line. These results suggest that endocytosis may indeed be altered in cancer cells.

Malignant transformation of HBEC alters endocytosis

To determine when changes in endocytosis might occur during the transformation process, we obtained normal HBEC30KT cell lines along with their derivatives with the following oncogenetic modifications common to lung cancer: (i) p53 deficiency triggered by stable shRNA-mediated knockdown of p53 (sh-p53), (ii) overexpression of activated *KRAS* (*KRAS*^{V12}), (iii) both sh-p53 and *KRAS*^{V12}, or (iv) sh-p53, *KRAS*^{V12}, and c-myc overexpression. It had been previously shown that all three oncogenic changes were necessary and sufficient for full oncogenic transformation of HBEC30KT cells as assessed by *in vitro* anchorage-independent growth and *in vivo* tumor formation after subcutaneous injection into NOD/SCID mice (22). Consistent with this, the transfected cells were morphologically indistinguishable from the parent HBEC30KT cells until all three oncogenic mutations were present (Fig. 3A), at which point they adopted a more elongated and irregularly shaped mesenchymal morphology similar to some NSCLC cell lines.

We measured the various endocytic activities in these cells, and consistent with the need for all three changes to induce tumor formation in mice, only the HBEC30KT^{sh-p53/KRASV12/c-myc} cell line exhibited decreased rates of uptake of all four markers (Fig. 3B). Interestingly, the different endocytic pathways were differentially affected during the transformation process. For example, whereas the rate of CavME did not significantly decrease until all three oncogenic changes were introduced, CME decreased linearly with each oncogenic modification. CD44 and CD59 uptake, both thought to report CIE, also respond differently to the oncogenic changes. Introduction of the *KRAS*^{V12} mutant was sufficient to decrease CD59-CIE activity to its lowest levels, whereas CD44-CIE activity was unaffected by expression of *KRAS*^{V12} alone, but was progressively reduced with the addition of sh-p53 and myc. Together, these data provide additional support that our assays measure distinct endocytic processes and establish that the different endocytic pathways can be differentially sensitive to oncogenic changes. Importantly, as we had observed in comparing patient-matched normal HBEC30KT cells with HCC4017 NSCLC tumor-derived cells (Fig. 2), we again found that full oncogenic transformation of otherwise syngeneic HBEC30KT cells resulted in decreased rates of all endocytic activities measured.

Systematic analysis of CME, CavME, and CIE in 29 NSCLC cell lines

To determine whether there are indeed systematic changes in endocytic activities associated with NSCLC, we measured the rates of uptake for all four endocytic pathways in NSCLC cell lines derived from 29 patients. These cell lines were chosen for their diverse molecular and clinical status (e.g., isolated from the primary tumor vs. metastases). All assays were performed in quadruplicate over multiple days with H1299 used as an internal control for each experiment (see Materials and Methods). The data, which are summarized in Supplementary Table S1 (avg. $\pm$ SD, $n = 4$ for each pathway in each cell line) and presented in Fig. 4A, show that the four endocytic activities varied significantly across all NSCLC

lines. This variability cannot be attributed to differences in surface expression of the four molecular markers, as the rates of uptake did not correlate with changes in surface expression of receptors (Supplementary Fig. S2). For comparison, the rates of uptake determined in normal HBEC30KT and HBEC3KT cells were plotted as solid squares and triangles, respectively (Fig. 4). As shown with the isogenic pairs (Figs. 2 and 3), the majority of NSCLC cell lines have lower rates of uptake across all four pathways compared with the nontransformed HBEC lines; however, several NSCLC cell lines had equal or higher rates of endocytosis especially in the CD44-CIE pathway, which exhibited the greatest cell-to-cell variability. Thus, although our analyses of isogenic normal and NSCLC lines showed a consistent and significant decrease in activity across all endocytic pathways, we find considerable heterogeneity in endocytic activities across a diverse panel of NSCLC cell lines.

Given this heterogeneity, we asked whether changes in internalization rates occurred randomly across the different endocytic routes, or if they positively or negatively correlated with one or more pathways. For this comparison, we ordered the 29 NSCLC cell lines from low to high, based on their rates of TfnR uptake and compared these rates to those measured for other pathways (Fig. 4B). We were unable to detect any correlation between CME or CavME and the other endocytic pathways. As expected, a similar pattern emerged when comparing the rate of uptake of the two CIE markers, CD44 and CD59. However, consistent with their differential response to the introduction of oncogenic changes (Fig. 3B), clear, quantitative differences were also detected. Thus, these four pathways appear to be differentially regulated in cancer cells.

Correlation of endocytic activities with cancer-related properties of NSCLC cell lines

Given the differential effects of loss of p53 in combination with overexpression of *KRAS*^{V12} and/or c-myc on endocytosis in HBEC3KT cells and their derivatives (Fig. 3B), we next extracted information regarding the status of these three oncogenes for the 29 NSCLC cell lines from existing databases (Fig. 5A; refs. 42–44). We wondered whether these oncogenic changes might correlate with their measured endocytic activities. The majority of the cell lines had functional mutations in p53, whereas only about half had functional mutations in *KRAS*, and only 5 had increased expression of c-myc. In contrast with the results obtained with syngeneic cell lines, the three NSCLC lines exhibiting all three oncogenic changes (H1792, Hcc44, and H2122) did not exhibit the lowest levels of endocytic activity for any of the pathways (see Supplementary Table S1). Indeed, there was no correlation between changes in activity of any of the endocytic pathways with p53 mutations or c-myc overexpression (Supplementary Fig. S3A and S3B). We could detect a small, but significant ($P < 0.1$, Wilcoxon rank sum test) correlation between *KRAS* mutations and a decrease in endocytic activity of both CIE pathways (Fig. 5B); however, there was no significant correlation between *KRAS* mutations and CME or CavME. Thus, we were unable to detect systematic changes in endocytic activity corresponding to specific oncogenic changes in our panel of NSCLC cell lines.

Several studies on individual cell lines have suggested roles for CME, CavME, and CIE in cancer progression (4, 6–9, 45, 46). Therefore, we next asked whether the heterogeneity

observed in pathway-specific internalization rates might relate to differential activities of the 29 cell lines assessed in a panel of *in vitro* cancer-relevant assays. For this purpose, we established 96-well assays to measure the following cellular processes (see Materials and Methods): (i) 3D migration through a collagen matrix; (ii) adhesion to different substrates (collagen, fibronectin, laminin, and hyaluronic acid); (iii) proliferation; and (iv) growth in soft agar. Once again we observed significant heterogeneity in these activities across the different NSCLC lines (Supplementary Table S2). We applied the Spearman rank correlation test to determine whether differences in individual endocytic activities correlated with changes in any of the above properties. We detected a significant negative correlation between proliferation and CIE pathway activity that was not seen for CME or CavME (Supplementary Fig. S4). However, we saw no significant correlations between 3D migration, adhesion or growth in soft agar, and changes in either of the four individual endocytic activities (Supplementary Figs. S5 and S6A). However, adhesion on one substrate was correlated with adhesion on another by Pairwise Pearson correlation comparison (Supplementary Fig. S6B), providing evidence for the validity of our measurements and demonstrating that the adhesion properties of these cells are not substrate specific.

Hierarchical clustering based on endocytic activities reflects cancer-related properties

Previous studies have used hierarchical clustering analyses to classify NSCLCs based on histologic (47), transcriptional (48), and genetic differences (49). To gain more insight into cancer cell properties that might be linked to their endocytic activities, we took a similar approach and conducted unsupervised hierarchical clustering analysis (26) of all 29 cell lines based on Pearson correlation distance metrics and average linkage of their measured endocytic activities using the raw data presented in Supplementary Table S1 (see Materials and Methods). Hierarchical clustering based on the functional criteria of their collective endocytic behaviors identified two clusters (Fig. 6A). The significance of the clustering result ($P = 0.0037$) was tested using the R package *sigclust* (50). Whereas individual endocytic activities did not appear to correlate with other cancer cell properties, we were able to detect significant differences in cancer cell properties between the two clusters derived from analysis of their collective endocytic activities. Cluster 1 was enriched in NSCLC cell lines that expressed mutant *KRAS* (Fig. 6B) and mesenchymal cell lines based on a published 76 gene signature (51), whereas cluster 2 was enriched in NSCLC cell lines classified, based on their gene signature, as epithelial ($P = 0.02$, Fisher exact test).

NSCLC cells in the two clusters also differed in their ability to migrate through collagen and their growth in soft agar. Cluster 1 exhibited a greater ability to migrate through collagen as compared with cluster 2 (Fig. 6C, $P = 0.0641$, Fisher exact test as assessed by categorical correlation for migratory or nonmigratory behavior of the two clusters). However, the majority of NSCLC cell lines (54% of cluster 1 and 81% of cluster 2) failed to migrate under these conditions; thus, it is possible that other assays for cell migration might reveal more significant differences. Cluster 1 also appeared to less effectively grow in soft agar as compared with cluster 2 (Fig. 6D, $P = 0.117$). That this apparent difference did not reach statistical significance may be due to the qualitative nature of the assay and its saturation at 300 colonies/well. Consistent with this, only 31% of cluster 1 cell lines compared with 62% of cluster 2 cell lines exhibited high growth (i.e., at >300 colonies/well) in soft agar (P

= .079, two-sided Student *t* test). Together, these studies suggest that clustering of NSCLC cells based on functional criteria may be informative as to cancer cell properties. Clearly higher numbers of cell lines will need to be examined to confirm these associations.

A recent study has suggested a link between CD44 expression and *KRAS*-driven lung adenocarcinomas (52). Given our finding of the small but significant correlation between CD44 endocytosis and *KRAS* mutations (Fig. 5), the negative correlation between CD44 endocytosis and proliferation in 2D (Supplementary Fig. S4), and the apparent differences in proliferation on soft agar between clusters 1 and 2, we more closely examined uptake and surface expression of CD44 in the two clusters. We found that cluster 1 had significantly lower clathrin-independent endocytic activity and correspondingly higher surface expression of both CD44 (Fig. 6E and F) and CD59 (Supplementary Fig. S7A). In contrast, we did not detect systematic or significant differences in the other endocytic activities or changes in surface expression of their markers (Supplementary Fig. S7B and S7C) between the two clusters. Together, these data suggest that selective changes in endocytic activities, in particular clathrin-independent endocytosis, can dynamically alter surface expression of cancer-related molecules and affect cellular processes that contribute to cancer aggressiveness. However, such changes do not appear to occur systematically across endocytic pathways or in all NSCLC cells. Further studies will be needed to determine whether these changes affect clinical outcomes.

Discussion

Endocytosis of cell surface receptors can potentially control many activities related to cancer cell proliferation and migration, including nutrient acquisition, cell–cell and cell–matrix adhesion, receptor tyrosine kinase, and G-protein–coupled receptor signaling. Moreover, many components of the endocytic machinery are mutated or have altered expression in a number of cancers (4, 5, 53). Thus, it is generally assumed that endocytosis is somehow altered in cancer cells to enhance their proliferative and metastatic potential. Evidence in support of this concept derives primarily from analysis of the effects of perturbing specific endocytic pathways on signaling, proliferation, and/or migration in cancer cells (6, 7, 9, 45). Here, we sought additional support for this hypothesis through a more systematic and quantitative analysis of the endocytic activities of a large panel of NSCLC cell lines to determine whether there were consistent alterations in one or more endocytic pathway that might be linked to changes in cancer cell proliferation, including anchorage-independency, adhesion, or migration through a collagen matrix. We measured the uptake of four ligands via mechanistically distinct endocytic pathways in 29 independently isolated NSCLC cell lines and discovered a large degree of heterogeneity in these activities. Our results emphasize the inherent complexity and heterogeneity in cancer cell biology that can preclude drawing general conclusions from acute perturbation studies in single cell lines. Nonetheless, based on their overall endocytic properties, we could identify two phenotypically distinct clusters of NSCLC cell lines, which differed in their preponderance of *KRAS* mutations, epithelial or mesenchymal gene signatures, 3D migration, and anchorage-independent growth. These data suggest that changes in overall endocytic capacity might indeed influence cancer cell behavior and/or vice versa.

Heterogeneity in NSCLC cell lines confirms diversity of endocytic pathways

The functions and activities of different endocytic pathways are frequently measured after selective perturbation of one or other pathway. However, the effects of these perturbations can partially overlap making it difficult to distinguish endocytic pathways, especially in the case of the less well-defined clathrin-independent mechanisms. Indeed, the relationship between CLIC-mediated uptake of CD44 and GPI-anchored proteins and the arf6-dependent uptake of similar markers remains somewhat controversial (6, 15, 32, 54, 55). Adding to this complexity, it has been suggested that there might be cross-talk between endocytic pathways, such that inhibition of one might lead to upregulation of another. Such a reciprocal relationship has been recently suggested for CavME and CD44 uptake (56). Finally, a recent study suggested that CME accounts for >95% of all bulk endocytosis, including that of the GPI-AP, CD59 (19), and brought into question the physiologic relevance of alternate endocytic pathways. Our finding that at least three distinct endocytic pathways can be differentially up- or downregulated in an otherwise non-perturbed panel of NSCLC lines confirms that these pathways, including two poorly defined clathrin-independent pathways mediating the uptake of CD59 and CD44, respectively, can be differentially regulated and hence must be, at least in part, mechanistically independent.

Endocytosis and cancer

Unexpectedly, we observed a decrease in all endocytic pathways measured when we compared syngeneic normal HBEC cell lines with their tumorigenic counterparts. Having lower rates of endocytosis might benefit the NSCLC cell lines by maintaining higher surface levels of important plasma membrane molecules, including those for signaling and adhesion. However, this pattern did not hold across all NSCLC lines, highlighting the genetic and mechanistic diversity of cancer. Moreover, it is likely that endocytic activities of cancer cells will be influenced by different signaling environments *in vitro* and *in vivo*.

Previous work using siRNA knockdown of components of the endocytic machinery had suggested a link between CME and CavME and the ability of cancer cells to proliferate, adhere, and migrate. CME has been implicated in integrin (45) and receptor tyrosine kinases (57) endocytosis. In order for a cell to sustain directed forward migration, integrins and receptor tyrosine kinases, such as the EGFR, must be constantly internalized by CME and recycled to the leading edge of the cell. CavME has also been found to be important for the internalization of membrane-type 1 matrix metalloproteinase, which helps to degrade the ECM at the leading edge of a cell, (58), as well as integrins (59) contributing to the disassembly of focal adhesions. Correspondingly, knockdown of Dyn2 in prostate cancer cells prevented cell invasion in 3D and *in vivo* (9), and siRNA knockdown of Cav1 in NCI-H460 lung cancer cells causes an increased ability of these cells to migrate and invade *in vitro* (7). Thus, we were surprised to see no correlation between CME or CavME endocytic activity and these cancer cell processes. This difference likely reflects the effects of strong perturbation of these pathways as compared with the more subtle regulation and variations we observe in our panel of NSCLC cell lines. It is also possible that rather than global changes in CME and CavME, changes in the endocytosis of specific cargo molecules not used here to measure these pathways could be selectively altered to affect migration,

proliferation, and/or adhesion. For example, we detected an inverse relationship between CIE of CD44 and proliferation in 2D that was less evident when measuring CIE of CD59.

Better classification of NSCLCs would facilitate the diagnosis and treatment plans for individual patients. Numerous studies have applied unsupervised clustering methods to classify NSCLC cell lines based on their diverse patterns of mRNA or protein expression, epigenetic modifications, etc. (47–49). Here, for the first time, we apply this analysis to their measured diversity in a cell biological activity, endocytosis. Interestingly, despite our inability to correlate differences in individual endocytic activities with other cancer-related cellular properties (e.g., migration, adhesion, proliferation), hierarchical clustering based on the diversity of their collective endocytic activities identified two distinct NSCLC cell line clusters that co-cluster with other cancer-related properties. Cluster 1 appeared enriched in cell lines bearing KRAS mutations, a mesenchymal phenotype, an enhanced ability to migrate in collagen, reduced ability to grow in soft agar, and reduced CIE, in particular of CD44, leading its increased expression on the cell surface. Consistent with these findings, recent studies reported a role for CD44 in mediating NSCLC proliferation downstream of KRAS (52). It will be important to extend these analyses to other NSCLC cells to test the validity of these linkages and also to determine their functional and/or clinical relevance. Moreover, applying this approach to other NSCLC lines will enhance our statistical power and allow us to assess the functional significance of the "outliers" we detect among this initial sampling of 29 cell lines.

Further work is necessary to directly explore the role and regulation of endocytosis in cancer progression. That many components of the endocytic machinery are dysregulated or mutated in cancer suggests a functional link. Our results suggest that dynamic regulation of the surface expression of important cancer molecules through endocytosis may, in some cases, contribute to the malignant properties of cancer cells. However, they also highlight cancer cell heterogeneity and reveal that the functional relationship between endocytic activities and cell migration, adhesion, and proliferation may be more complex than suggested by perturbation analyses of single cell lines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Measuring mechanistically distinct endocytic pathways in NSCLC H1299 cells. H1299 NSCLC cells grown in 96-well plates were incubated with the indicated antibodies or ligands, and the mechanistically distinct endocytic activities were measured using an In-Cell Elisa assay (see Materials and Methods). A, representative Western blot showing efficiency of siRNA-mediated knockdown of CHC or dynamin 2 (Dyn2) measured 72 hours after transfection of H1299 cells. GAPDH was used as a loading control. CME of D65 anti-TfnR mAb (B), CavME of biotinylated albumin (C), CIE of anti-CD44 mAb (D), or anti-CD59 mAb (E) were measured 72 hours after transfection with control (open circle), CHC (closed circle), or Dyn2 (closed square) siRNAs, or 30 minutes after incubation with nystatin (closed triangle). Data are expressed as the fraction of total surface-bound ligand internalized after incubation for the indicated time at 37°C ($n = 3$ independent experiments, each performed in triplicate $\pm$ SD). Two-tailed Student *t* tests were used to assess statistical significance. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

Figure 2.

Differential internalization of endocytic markers in matched patient-derived normal HBEC30KT and NSCLC (HCC4017) cells. A, comparison of the extent of internalization after 5 minutes at 37° C via the indicated endocytic pathways in nontumorigenic-HBEC30KT (black bars) and the NSCLC line (HCC4017, gray bars) derived from the same patient. Two-tailed Student *t* tests were used to assess statistical significance. ***, *P* < 0.0005. B–E, representative fluorescence images of total surface-bound and internalized (after 5 minutes at 37° C) ligands in HBEC30KT and HCC4017 cell lines assayed for CME of D65 anti-TfnR mAb (B), CavME of biotinylated-albumin (C), CIE of anti-CD44 mAb (D), or CIE of anti-CD59 mAb (E).

Figure 3.

Changes in endocytic activity accompany progressive oncogenic transformation of HBEC3KT cells. A, phase contrast images of nontumorigenic HBEC3KT cells and the same cells transformed by sh-p53 knockdown and/or mutant *KRAS*^{V12} and c-myc overexpression, as indicated. Morphologic changes relative to the parent HBEC3KT cells are only apparent when all three oncogenic transformations have been introduced. B, comparison of internalization via CME, CavME, and CIE in nontumorigenic HBEC3KT, HBEC3KT^{sh-p53}, HBEC3KT^{KRASV12}, HBEC3KT^{sh-p53/KRASV12}, and HBEC3KT^{sh-p53/KRASV12/c-myc} cells. Rates of internalization in HCC4017 NSCLC cells, from Fig. 2, are also shown for comparison. Data shown are average values for the fraction of surface-bound ligand internalized after 5 minutes at 37° C for three independent experiments, each performed in triplicate. Two-tailed Student t tests were used to assess statistical significance compared with the parent HBEC3KT (ns, not significant; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$).

Figure 4.

Summary of data for the activities of four distinct endocytic pathways in 29 NSCLC cell lines. A, mechanistically distinct endocytic activities were measured in 29 NSCLC cell lines, as described in Fig. 1. Each data point represents the average endocytic activity ($n = 4$ independent experiments, each performed in triplicate) for a different cell line. The closed square and triangle represent the internalization rate of normal, HBEC30KT, and HBEK3KT cells, respectively. B, the 29 cell lines were ordered from low to high CME of Tfn (top), and the other endocytic activities for each cell line are shown in the three bottom plots. Although the extent of CIE of CD44 and CD59 was related, neither CIE nor CavME covaried with CME activity.

Figure 5.

Relationship between endocytic activity and genetic alteration associated with cancer cell oncogenic transformation. A, the mutation status of p53 and *KRAS* and overexpression status of *MYC* in the 29 NSCLC cell lines, as derived from ref. 42. B, correlation between *KRAS* mutation status and endocytic activity in all four pathways. Wilcoxon rank sum tests were used to assess statistical significance, as indicated.

Figure 6.

Endocytic activities identify two phenotypically distinct clusters of NSCLC cell lines. A, the 29 NSCLC cell lines clustered into two distinct groups after unsupervised learning based on endocytic activity in all four pathways. B, NSCLC cell lines in cluster 1 were enriched in those bearing *KRAS* mutations (+) and exhibiting a mesenchymal phenotype (M), whereas cells in cluster 2 were enriched in those exhibiting an epithelial phenotype (E). C, invasion ratio for each cell line was measured as the fraction of cells migrating up through a collagen matrix toward serum (see Materials and Methods). D, rate of growth in soft agar reported as colonies/well (see Materials and Methods). Each bar represents the average activity ($n = 3$ independent experiments, each performed in triplicate) for each cell line indicated and clustered in A. E and F, comparison of the extent of internalization of CD44 through CIE after 5 minutes at 37° C (E) and total surface binding of CD44 measured at 4° C in cluster 1 and cluster 2 (F). Wilcoxon rank sum tests were used to assess statistical significance, as indicated.