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 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 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 endocytic 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.

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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). 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 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-H119) 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 PBS 4°C (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). For visualization by fluorescence microscopy, HBEC30KT and HCC4017 cells were seeded in 8-well chambers. The procedure was the same as that for the Live/Dead Kit, with the addition of 5 μg/mL Hoechst 33342 (Millipore) and 10 μg/mL propidium iodide (Thermo Fisher Scientific) to stain cell nuclei every 24 hours. Images were captured using a ×40 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'-GCAAAUCAACUCCACU-3' and Dyn2-2: 5'-GACUUGAUCUGGUCAGUGGU-3') or clathrin heavy chain (CHC; ref. 24) to the H1299 cells, following the manufacturer’s instructions. We used All Star Negative control siRNA as a control. Cells were used 72 hours after siRNA transfection. After inhibition by nystatin, H1299 cells were pre-incubated in the absence (control) or presence of 25 μg/mL nystatin for 30 minutes at 37°C before internalization assays were performed as described above except the PBS™ containing 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 seeded, and triplicate. Five hours after plating, cells from the first plate were irradiated with 1 Gy to kill any residual cells that had not adhered to the plate and the plates were transferred to a humidified CO2 incubator. Cells were then allowed to grow for 5 days and 100 μL of CellTiter-Glo (Promega) was added to the plate and incubated at room temperature for 1 hour before being measured. The absorbance was read at 490 nm (Biotek Synergy H1 Hybrid Reader).

Adhesion. 96-well plates were coated with 40 μL/well for 1 hour containing 10 μg/mL BSA, 0.01% poly-L-Lysine, 50 μg/mL rat tail collagen, 10 μg/mL fibronectin, 50 μg/mL laminin or incubated with 1 mg/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 with 30 μg/mL Hoechst/PBS solution. Cell proliferation was expressed as the ratio of cell counts on “Day 5”/“Day 0” plates.

3D migration assay. Assays were performed using 1 mg/mL bovine collagen and 2.5% PBS as chemoattractant, following the experimental procedure described in ref. 25. Microscopy images of nuclear staining were taken at 50 μm steps from 0 to 150 μm into the collagen plug. Invasion index was calculated as the sum of cell counts at 50, 100, and 150 μm over cell counts at 0 μm. Cell nuclei were visualized using a ×20 magnification (magnification) or ×40 (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.
**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 endocytic pathways are less well characterized, glycophosphatidylinositol–anchored proteins (GPI-AP) are reportedly internalized by these routes (15, 17, 18). For our studies, we chose complement lysis restricting factor/C59 (31), 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 knockout 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, nonmalignant 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.

**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 high-throughput 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-CDS59 or anti-CDS4 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 in Cancer Cells

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 HBEC3K7p53-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 CME 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 KRASV12 mutant was sufficient to decrease CD59-CIE activity to its lowest levels, whereas CD44-CIE activity was unaffected by expression of KRASV12 alone, but was progressively reduced with the addition of sh-p53 and c-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 HBEK30KT cells with HCC4017 NSCLC tumor-derived cells (Fig. 2), we again found that full oncogenic transformation of otherwise syngeneic HBEK3KT 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. ± 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 HBEK30KT and HBEK3KT 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 HBEK 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 TnR 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<sup>V12</sup> 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 sigclus (50). Whereas individual endocytic activities did not appear to correlate with other cancer cell properties, we were able to detect significant

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<sup>V12</sup> 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<sup>sh-p53</sup>, HBEC3KT<sup>KRASV12</sup>, HBEC3KT<sup>sh-p53/KRASV12</sup>, and HBEC3KT<sup>sh-p53/KRASV12/c-myc</sup> 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).
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 $\geq300$ 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 ($P = 0.079$, 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

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.
When measuring CIE of CD59.

between CIE of CD44 and proliferation in 2D that was less evident

adhesion. For example, we detected an inverse relationship

Better classification of NSCLCs would facilitate the diagnosis

Numerous studies have applied unsupervised clustering methods to classify NSCLC

cell lines based on their diverse patterns of mRNA or protein

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 can-
cer-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

tolecules 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: S.R. Elkin, N. Bendris, J.D. Minna, S.L. Schmid

Development of methodology: S.R. Elkin, N. Bendris, C.R. Reis

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