A chemical genomic study identifying diversity in cell migration signaling in cancer cells

Shigeyuki Magi, Etsu Tashiro & Masaya Imoto

Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan.

The aim of this study was to analyze the diversity and consistency of regulatory signaling in cancer cell migration, using a chemical genomic approach. The effects of 34 small molecular compounds were assessed quantitatively by wound healing assay in ten types of migrating cells. Hierarchical clustering was performed on the subsequent migration inhibition profile of the compounds and cancer cell types. The result was that hierarchical clustering accurately classified the compounds according to their targets. Furthermore, the cancer cells tested in this study were classified into three clusters, and the compounds were grouped into four clusters. An inhibitor of JNK suppressed all types of cell migration; however, inhibitors of ROCK, GSK-3 and p38MAPK only inhibited the migration of a subset of cell lines. Thus, our analytical system could easily distinguish between the common and cell type-specific signals responsible for cell migration.

Cell migration is central to many physiological processes, including development, tissue remodeling, and immune responses, and is also a required step in cancer metastasis. When a cell moves, multiple intracellular signaling networks control cell morphology. Signaling can be initiated through receptor tyrosine kinases, G protein-coupled receptors (GPCRs), integrin, and other receptors. These receptors are upregulated by extracellular stimuli that induce the activation of one or more intermediate signaling network branches. Finally, this signaling reaches the Rho family of small GTPase proteins. Many molecules and pathways have been implicated in intermediate signaling. For example, the Ras/Raf/MEK/ERK pathway has been reported to enhance cell motility. In addition to the Ras/Raf/MEK/ERK pathway, a phosphoinositide 3-OH kinase (PI3K)/Akt pathway is widely known to regulate cell migration. This pathway is considered to be necessary for both Cdc42- and Rac1-induced cell motility and invasiveness, and it regulates the expression of Snail, which can increase cell motility. Jun NH2-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38MAPK) have also been reported to play important roles in the signaling mechanisms involved in migration. The role of Rho family small GTPase proteins, which is considered to constitute the final stage of the migration-signaling network, is known to regulate actin nucleation and polymerization. In particular, RhoA, Rac1, and Cdc42 are the major regulators of cytoskeletal remodeling. Activation of RhoA increases cell contractility and leads to the formation of focal adhesions and stress fibers. Rac1 and Cdc42 activation induce the lamellipodia and filopodia, respectively. Thus, the core elements of the intracellular migration-signaling network have been demonstrated.

However, it is likely that signaling molecules regulating cell migration in one cancer cell may not regulate cell migration in other genetically distinct cancer cells. Indeed, the PI3K/Akt pathway, but not the MEK/ERK pathway, has been shown to be critical for prostate cancer cell migration. Other studies have reported that the constitutive activation of the MEK/ERK pathway by oncogenic mutations of BRafV600E significantly induced cell migration through activation of RhoA GTPase. In addition, the role of the Rho family of proteins in cell migration depends on specific cellular circumstances. The migration of several types of cancer cell is based on reorganization of the actin cytoskeleton, but their requirements for Rho and Rac signaling differ. With respect to a particular subset of cancer cells, cells migrated in a Rac-dependent manner, but Rho signaling was not essential. With respect to another subset of cancer cells, the inhibition of Rho/Rock signaling inhibited cell migration. Thus, although the same basic process of cell migration is induced, each type of cancer cell brings about migration in different contexts using distinct molecular repertoires. Therefore, understanding the diversity and commonality of signaling pathways that regulate cell migration in various cell types is important not only for basic research into cell migration, but also for the development of anti-metastatic anti-tumor drugs.
To address this issue, we utilized the chemical genomic approach in which chemical inhibitors were used as probes to mimic loss-of-function phenotypes by inhibiting target protein activity; that is, if a chemical inhibitor suppresses the cell migration of one type of cancer cell, the target protein of the inhibitor can be considered as being involved in the mechanism of cell migration of that type of cell. This chemical genetic approach is easily applicable to different cell models; therefore, it can determine which signaling molecule is universally involved in the migration mechanism in several types of cancer cells, and which one is specifically involved in each type of cell. In the present study, we first examined the effects of various chemical inhibitors on cell migration in several cancer cell models, and subsequently obtained chemosensitive migratory profiles and undertook cluster analysis to classify the signaling molecules and their inhibitors as being either common to all cancer cells or specific to certain cell types.

**Results**

**Determination of appropriate experimental conditions for the wound healing assay.** To select the cell models used in this study, sixteen cell lines, including colon carcinoma, esophageal carcinoma and lung cancer, were assessed with regard to their migration ability in response to migration factors using a wound healing assay13. The assay conditions of each cell line were optimized by examining migration factors such as growth factors, cell number required to maintain a confluent cell monolayer, and an assay duration that clearly revealed the extent of motility. We found out that the eight cell lines were suitable for use in a migration assay under the conditions indicated in Table 1 (see also the Methods section). We also confirmed that both number of alive and dead cells in each condition were not clearly increased in optimized assay condition. The other cell lines tested were not affected by migratory stimuli or could not be scratched. Among the eight cell lines selected, EC17 cells migrated without extracellular stimulation, indicating that EC17 cells secrete chemoattractants into the media, and acquire motility by autocrine signaling. Conversely, others required the addition of migration factors, such as epidermal growth factor (EGF), conditioned medium from EC17 cells (EC17-CM), or serum (Figure 1a). A431 cells and EC109 cells migrated in response to both EGF and EC17-CM. Figure 1b shows the morphology of migration in these cell lines. A431 cells and EC109 cells moved together in sheet-like structures (collective migration), whereas the other cell lines showed a fibroblast-like spindle-shaped morphology and migrated individually like mesenchymal cells (mesenchymal migration).

**Signaling pathway regulating for cell migration differs among three cancer cell lines.** Next, to examine whether our analytical system could distinguish between common signals responsible for cell migration in the cancer cells tested and cell type-specific signals, using signal transduction inhibitors, a test was done using A431 cells, EC109 cells or TT cells that were randomly selected to analyze their migration ability. This was conducted following treatment with three different inhibitors for 16 h, wound areas were observed and photographed under microscopy. (b) Images of cell lines treated with migratory stimuli. Cells were photographed 10 h after stimulation. The scale bar represents 50 μm.

| Table 1 | The experimental conditions of the wound healing assays for each cell line |
|---------|-----------------------------|
| **Cell line** | **Origin** | **Cell number (cells/well)** | **Migration factor** |
| A431 | Human epithelial carcinoma | $7.5 \times 10^4$ | EGF 3 ng/ml or EC17-CM |
| EC17 | Human esophageal carcinoma | $7.5 \times 10^4$ | None |
| EC109 | Human esophageal carcinoma | $7.5 \times 10^4$ | EGF 3 ng/ml or EC17-CM |
| HT1080 | Human fibrosarcoma | $7.5 \times 10^4$ | FBS [2%] |
| TE8 | Human esophageal carcinoma | $7.5 \times 10^4$ | EGF 3 ng/ml |
| TT | Human medullary thyroid carcinoma | $7.5 \times 10^4$ | EGF 3 ng/ml |
| 3Y1 | Rat fibroblast | $7.5 \times 10^4$ | FBS [5%] |
| B16 | Mouse melanoma | $2.2 \times 10^4$ | EC17-CM |

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kinase inhibitors; PI3K inhibitor, Rho-associated kinase (ROCK) inhibitor and EGF receptor kinase inhibitor. The reason why we focused on the inhibitors of PI3K and ROCK for this test was that PI3K and ROCK were expected to reveal cell type-specific effects on migration. This is because they have been reported to be involved in regulation mechanisms of cell migration that are initiated downstream to growth factor signaling in a subset of cancer cells\(^{5,14}\), although they were also reported to be dispensable for migration or membrane ruffling in certain conditions\(^{15,16}\).

Figure 2a presents the effect of these three inhibitors on the EGF-induced motility of A431 cells, EC109 cells, and TT cells. The extent of the cell motility was quantified by the measurement of the cell-free area in a photograph. The quantified value was calculated over a fixed period of time, and was termed the ‘migration inhibition score (MIS)’ (Figure 2b). These results indicated that the EGF receptor kinase inhibitor, AG1478, inhibited the EGF-induced migration of all three cell lines, as expected. The PI3K inhibitor and LY294002 suppressed the EGF-induced migration of A431 cells and EC109 cells, but not of TT cells, indicating that PI3K plays a critical role in EGF-induced cell migration in A431 cells and EC109 cells. In contrast, the ROCK inhibitor, Y27632, suppressed migration only in A431 cells and TT cells, indicating that ROCK is indispensable for EGF-induced cell migration in A431 cells and TT cells but not in EC109 cells. Thus, our analytical system using chemical inhibitors of signal transduction easily distinguished between common and cell type-specific signals responsible for cell migration.

**Two-way cluster analysis of migration inhibition score.** To reveal the diversity and generality of regulatory signaling in cancer cell migration, we tested the effect of 34 different signal transduction inhibitors on the migration of ten types of cells, as shown in Table 1. Table 2 lists the names of the chemical inhibitors of signal transduction used in this study, the experimental concentrations of each inhibitor, and their modes of action. Each inhibitor was used at three concentrations, the highest one being a concentration just below the level that would affect cell viability. Using these chemical inhibitors under the stated concentrations, we carried out two highly reproducible, independent experiments on each cell line (\(r = 0.94\), p-value < 2.2 × 10\(^{-16}\), Figure 3), and provided a final dataset by averaging the data points from the two experiments. The MIS dataset is shown in Supplementary Table 1. Then a hierarchical cluster analysis was performed. The results are displayed in the form of a heat map and a tree diagram (Figure 4). The heat map employs a gradient color scale from green, indicating MIS = 0, to magenta, indicating MIS = 1.0, interpolated over black indicating MIS = 0.5.

As a result of these experiments, the characteristic features of cell migration affected by chemical inhibitors in cancer cells were...
Cluster A consisted of three types of cells and cell migration properties: B16 cells, HT1080 cells, and 3Y1 cells; their cell migration displayed lower sensitivities to the inhibitors tested in this study than the others (Figure 4b). Cluster B consisted of cell migration of A431 cells and EC109 cells stimulated with either EGF or EC17-CM. The EGF-induced chemosensitive migratory profile of these cells was similar to that induced by EC17-CM. Cluster C consisted of three types of cells: EC17 cells, TE8 cells and TT cells.

It was expected that the chemical inhibitors that targeted the same molecule would be clustered into the same tree. Indeed, PD169316 and SB203580 as p38MAPK inhibitors, herbimycin A and radicicol (Hsp90 inhibitors), LY294002 and wortmannin (PI3K inhibitors), paclitaxel and vinblastine (tubulin binders), and alendronate and risedronate (farnesyl diphosphate (FPP) synthase inhibitors), were all clustered into the same position (indicated by gray boxes). These results indicate that our chemical genomic approach was able to classify the chemical inhibitors based on their respective modes of action, similar to previous studies on the chemosensitivities of cancer cells17–19.

Furthermore, the chemical inhibitors used in this study were classified into four general clusters (Figure 4b), and each inhibitor in Figure 4b can be linked to its target molecule. We also displayed the relationships of the targets of the inhibitors as a non-root phylogenetic tree (Figure 4c). The inhibitors grouped into cluster 1 contained the 5-lipoxygenase-activating protein (FLAP) inhibitor, MK886, the vacuolar-type proton-ATPase (V-ATPase) inhibitor, bafilomycin A, and the FPP synthase inhibitors, the bisphosphonates. These inhibitors showed little inhibitory effect on cell migration in almost all cell types, thus the target molecules of these compounds had little bearing.
on the regulating mechanisms of cell migration tested in this study. Cluster 2 contained Y27632, AG1478, the p38MAPK inhibitors, the chk1 inhibitor, SB218078 and so on. Most of these compounds showed a stronger inhibitory effect on cell migration, classified into migration type clusters B and C, in contrast to cluster A. Therefore, the target molecules of these compounds were not involved in the migration of HT1080 cells and 3Y1 cells but they did regulate cell migration in the subset of cell lines grouped into clusters B and C. Cluster 3 contained SP600125 and the cytoskeleton-affecting compounds. This group of inhibitors affected all types of cell migration, indicating that not only cytoskeletal molecules, but also JNK, are common regulators of cell migration, irrespective of cell type. Cluster 4 contains the Hsp90 inhibitors, the MEK inhibitor, U0126, and the PI3K inhibitors. These inhibitors also suppressed migration in all types of cell with different potencies depending on cell type. Thus the target molecules of these inhibitors also played a common role in all types of cell migration.

Discussion

In the present study, we investigated some general and specific regulatory mechanisms of cell migration. To accomplish our objective, we assessed the effects of 34 different kinds of chemical inhibitors on the migration of ten types of cells using a wound healing assay, and subsequently performed a cluster analysis on the dataset. One significant aspect of this work is that each compound showed a characteristic cell type-specific inhibitory pattern on migration, and hierarchical clustering precisely classified the compounds according to their respective targets, such as p38, Hsp90, PI3K, tubulin and FPP synthase (Figure 4b, c). Therefore, our research could be applied to predict the mode of action of each compound. For example, moverastin and its derivative UTKO1 were classified into same cluster, in spite of the different functions of these two compounds, which have similar structures. We previously reported that moverastin was an inhibitor of farnesyltransferase20, and it inhibited cell migration by inhibiting farnesylation in H-Ras. On the other hand, UTKO1 was reported not to inhibit farnesyltransferase21, but it directly bound to 14-3-3\(\text{f}\), and inhibited the interaction between the 14-3-3 proteins and Tiam1, a protein that has been reported to be a Rac-specific GEF. This resulted in the inhibition of Rac122. Therefore, it has been demonstrated that the migration of epithelial cells requires Taim1-mediated Rac1 activation. However, because our profiling data demonstrated that moverastin and UTKO1 were classified in the same cluster, we examined the possibility that moverastin could bind to 14-3-3\(\text{c}\), and obtained similar results (data not shown). Therefore it is likely that moverastin might inhibit cell migration not only by inhibiting protein farnesylation, but also by inhibiting Taim1-mediated Rac1 activation. In addition, because the CysLT1 antagonist MK571 was classified into the same cluster as moverastin and UTKO1, this raised the possibility that CysLT1 signaling might be closely related to Taim1-mediated Rac1 activation. We therefore examined this potential mode of action, and found that EGF-induced Rac1 activation was regulated by CysLT1 signaling (unpublished...
data). Thus, our profiling data appeared to be helpful in the mechanistic study of cell migration. At the same time, our clustering data indicated that xanthohumol was grouped in the same category as the Ca\(^{2+}\) ionophore, A23187. Recently, xanthohumol was reported to bind to and inhibit valosin-containing protein (VCP)\(^{12}\), resulting in the induction of ER stress. Although A23187 is also a well-known inducer of ER stress\(^{4,25}\), other ER stress-inducing compounds such as tunicamycin and thapsigargin were classified into different clusters from xanthohumol and A23187. Therefore, an interpretation of the clustering data must be made with great caution.

The characteristics of cell migration based on chemical inhibitor-sensitivity profiles were grouped into three clusters (clusters A, B, and C), and chemical inhibitors were classified into four general groups (clusters 1 to 4, Figure 4). Although the motilities of several cell lines we tested are upregulated by extracellular stimuli such as EGF, they migrated a little without stimulation (Figure 1a). Additionally, EC17 cells did not require extracellular stimulation to migrate. Thus we just have to evaluate and discuss chemical inhibitor-sensitivity profiles as total effects on “basal” and “stimulated” motility. JNK inhibitor, tubulin and actin polymerization inhibitors in cluster 3 showed a potent inhibitory effect on migration of all cell types, indicating that JNK is a common and crucial signaling molecule regulating cell migration. Indeed, JNK was reported to modulate migration in a broad range of cell types\(^{7,8}\), such as keratinocytes, neuronal cells\(^{19}\), and many cancer cell lines\(^{26,27}\). Because dynamic reorganization of the actin cytoskeleton is considered to be key to the cell’s capacity to migrate\(^{28}\), JNK may be indispensable for the phosphorylation of paxillin and F-actin polymerization\(^{7,8}\).

In contrast, some of the chemical inhibitors classified into cluster 2 (AG1478, tunicamycin, the CysLT1 antagonist MK571, Moverastatin and UTK01) affected the migration of cell lines of epithelial origin (EC109 cells, A431 cells, EC17 cells, TT cells, TE8 cells) in clusters B and C, but did not affect the migration of cell lines of mesenchymal origin (HT1080 cells and 3Y1 cells) placed in cluster A. This suggests that there is an essentially different regulatory mechanism of cell migration between cells of these two origins. The regulatory mechanism of cell migration of B16 cells appeared somewhat similar to that of cell lines of mesenchymal origin when compared to those with an epithelial origin.

Moreover, although EGF did induce migration of cells of epithelial origin (TT cells, TE8 cells, A431 and EC109 cells), differential sensitivities to several inhibitors was observed in A431 and EC109 cells in cluster B and TT cells and TE8 cells in cluster C. These results suggest that the EGF signaling pathway leading to migration of A431 and EC109 cells in cluster B was not identical to that of TT cells and TE8 cells in cluster C. Furthermore, EC17-CM also induced migration of A431 and EC109 cells with a similar chemosensitive profile to that of cell lines of mesenchymal origin when compared to those with an epithelial origin.

In summary, we have shown that JNK is a signaling molecule common to all types of cell migration, and many molecules have diverse functions in the migration of particular types of cancer cells. We determined this using a chemical genomic approach. Our approach can be used as a tool for understanding the diversity and similarities in cancer cell migration signaling, opening up the potential for revealing novel molecular targets in cancer therapy.

Methods

Cell culture. Y1 and HT1080 cells were maintained in Dulbecco’s Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.1 g/l kanamycin, 100 units/ml penicillin G, 0.6 g/l L-glutamine, and 2.5 g/l NaHCO\(_3\). A431 cells were maintained in DMEM supplemented with 5% calf serum (CS), 0.1 g/l kanamycin, 100 units/ml penicillin G, 0.6 g/l L-glutamine, and 2.5 g/l NaHCO\(_3\). B16 cells were maintained in DMEM supplemented with 8% FBS, 0.1 g/l kanamycin, 100 units/ml penicillin G, 0.6 g/l L-glutamine, and 2.5 g/l NaHCO\(_3\). For routine culture, cells were incubated in a standard humidified incubator at 37°C in 5% CO\(_2\).

Reagents. A23187, AG1478, ALLN, cytochalasin D, okadaic acid, rapamycin, and Y27632 were purchased from Calbiochem. Thapsigargin was purchased from Santa Cruz Biotechnology. AA861, bafilomycin A, LY294002, mevastatin, MG132, MK886, PD190354, SB203580, SB258826, SB600125, tunicamycin, U0126, wortmannin, and epidermal growth factor were purchased from Sigma. Paclitaxel, radicicol, and vinblastine were purchased from Wako Pure Chemical Industries, Ltd. Herbimycin A, meroveratin, and xanthohumol was purified from cultures of Streptomyces sp. in our own laboratory. Leptomycin B
Preparation of conditioned medium (CM) from EC17 cells. EC17 cells (1.0 × 10⁶ cells) were seeded in 100 mm dish. The following day, the medium was replaced with 10 ml of RPMI 1640 containing 1% FBS. After 24 h, the medium was recovered and sterilized by filtration.

Wound healing assay. A confluent monolayer of cells in a 48-well plate was scratched with a micropipette tip to create a cell-free zone in each well, about 1 mm in width. The medium was replaced with RPMI1640 with 1% FBS with or without test compound, and cells were either treated with the migratory stimulus or not treated with it to serve as controls. After a fixed period of time, cells were observed and photographed under a microscope. The experimental conditions for each cell line are described as Table 1. Wound areas were quantified using ImageJ software. After photographing, cells were trypsinized and collected, and cell viability was determined by trypan blue dye exclusion assay. Average migration inhibition scores were calculated from two independent experiments.

Cluster analysis. The value of migration inhibition was ordered according to the experimental conditions of cell migration or the compounds used. These profiles were analyzed by hierarchical clustering (using the method of Ward’s linkage based on Euclidean distance) and visualized using a heat map using the R Project package (http://www.R-project.org).

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**Author contributions**

M.I. is responsible for project planning and experimental design; S.M. performed all of the experiments and statistical analyses; S.M., E.T. and M.I. co-wrote the paper.

**Additional information**

Competing financial interests: The authors declare no competing financial interests.

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