High-Content Analysis of Cell Migration Dynamics within a Micropatterned Screening Platform

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Cell migration is a fundamental biological process that is dynamically regulated by complex interactions between the microenvironment and intrinsic gene expression programs. Here, a high-throughput cell migration assay is developed using micropatterned and dynamically adhesive polymer brush substrates, which support highly precise and consistent control over cell–matrix interactions within a 96-well cell culture plate format. This system is combined with automated imaging and quantitation of both cell motility and organization of the F-actin cytoskeleton for high-content analysis of cell migration phenotypes. Using this platform to screen a library of 147 epigenetic inhibitors identifies a set of EZH2-specific compounds that promote cytoskeletal remodeling and accelerates keratinocyte migration through derepression of an epithelial to mesenchymal transition-like gene expression program. Together, these studies establish the high-throughput, micropatterned assay as a powerful tool for discovery of novel therapeutic targets and for dissecting complex gene–environment interactions involved in wound repair.

1. Introduction

Cell migration is a fundamental biological process that is essential for tissue morphogenesis, wound repair, and immune responses, as well as pathologic processes such as cancer cell invasion. It involves a close interaction between cells and extracellular matrix (ECM) proteins, whereby integrin-mediated adhesion to the ECM and contractile forces within the cytoskeleton facilitate cell motility over or through the matrix.[1,2] The affinity of integrin receptors for specific ECM proteins,[3,4] as well as the mechanical[3,4] and structural properties of the cellular microenvironment,[5,6] interact with intrinsic gene expression programs to regulate and direct migration.[7] The ability to dissect and study these complex interactions is therefore essential to understanding normal and pathogenic migratory processes.

One of the most common and simplest in vitro methods for studying cell migration is the “scratch assay,” which uses a pipette tip or needle to create a linear wound within a monolayer of cells. More sophisticated versions of this method include the zone exclusion assay, where a silicone stopper or stencil defines a cell-free region and upon removal, cells migrate into the empty space.[8] While these assays are simple to perform, they are often limited to low-throughput analyses, and creating consistent and reproducible scratches is a significant challenge. A few studies have performed high-throughput cell migration screens,[7,9] but a major disadvantage of many existing models is still the poor control over cell–matrix interactions within the wound area, where serum or cell-secreted proteins adsorb nonspecifically to cell culture surfaces.

Microfabrication technologies provide a means to precisely control both geometry and composition of cell culture substrates. We previously employed micropatterned poly(oligo(ethylene glycol) methacrylate) (POEGMA) brush coatings to create highly stable and protein resistant surfaces and to study the role of cell–ECM interactions in keratinocyte differentiation.[10,11] More recently, we extended this technology to create a cell migration assay in which photocatalyzed thiol-yne “click” reactions were used to couple adhesive ligands to the POEGMA coatings and induce cell migration.[12] and similar approaches have been employed by other groups.[13–15] By covalently coupling small ECM-mimetic peptides (e.g., RGD) to a protein-resistant background, this system allows the composition of the underlying migration surface to be specifically controlled. In the present study, we now describe the scale-up of this dynamically adhesive micropattern model to establish a novel, high-throughput cell migration assay (HCMA) with tunable cell–ECM interactions. To demonstrate the biological applicability of this system, we used the HCMA to screen a library of epigenetic inhibitors and uncover new insights into the cross-talk between intrinsic regulators of gene expression and extracellular adhesive cues.

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2. Results

2.1. HCMA Design and Validation

The overall workflow of the HCMA involved 1) preparation of micropatterned substrates and plate assembly, 2) photoactivated coupling of ECM-mimetic peptides, and 3) high-content image processing and analysis (Figure 1A). Micropatterned POEGMA surfaces were first generated on large glass substrates with the dimensions of a standard well plate, using microcontact printing and surface initiated polymerization, and then modified with alkyne groups as previously described.[11,12] The micropatterned substrates were then bonded to a bottomless 96-well plate using a silicone-based adhesive, sterilized, and seeded with keratinocytes.[16] Here, we created arrays of 400 µm circular islands of POEGMA-alkyne brushes across the substrate. As the POEGMA brushes are highly resistant to protein adsorption and cell adhesion, keratinocytes only adhered to the inter-islands areas, resulting in ≈8–12 analyzable “microwounds” per well of the 96-well plate.

To trigger cell migration into the microwound area, we employed a photoactivated “click” reaction in which the free thiol group of a cysteine-containing peptide is covalently coupled to the alkyne group of the POEGMA brushes upon

Figure 1. Development of the high-throughput cell migration assay (HCMA). A) Workflow of the HCMA: 1). Micropatterned substrates containing POEGMA-alkyne microwounds are bonded to bottomless 96-well plates followed by sterilization and cell seeding. 2). Collagen-mimetic peptide (GFOGER) is covalently coupled to POEGMA-alkyne by photoactivated click chemistry. 3). Cell migration into the microwound area is imaged and quantified. Scale bar = 100 µm. B) Heat map of microwound closure by keratinocytes across 96-well plate (excluding column 1 and 12 due to nonuniform light exposure at edges) over 24 h time course. Data represent mean area and standard deviation of microwounds (n = 8–12 per well).
exposure to 365 nm light and in the presence of a photoinitiator (Irgacure 2959).[12] To induce keratinocyte migration we used a triple helical collagen mimic peptide, which contains the GFOGER motif and specifically targets the α2β1 integrin receptor.[17] A reaction concentration of 1 mg mL⁻¹ GFOGER was sufficient to support keratinocyte adhesion and spreading on nonpatterned surfaces (Figure S1, Supporting Information), and rapid migration and wound closure (<24 h) in the micropattern model (Figure 1A; Movie S1, Supporting Information).

To quantify cell motility within the HCMA, we developed a custom MATLAB script that measures the area of individual microwounds by applying a range filter and segmenting bright field images (Figure S2, Supporting Information). We then induced keratinocyte migration across an entire 96-well plate and collected bright field images using the IN Cell 2200 automated imaging system (GE). We observed consistent and reproducible wound closure throughout the plate (Figure 1B), demonstrating that this model system is a robust platform for high-throughput analysis of cell migration.

2.2. High-Content Screening of Epigenetic Regulators of Migration

We next used the HCMA to scan a small molecule library of 147 epigenetic inhibitors and gain new insight into the epigenetic regulation of keratinocyte migration, for which little is currently known. Following cell seeding, each compound was applied to a single well (10 × 10⁶ m final concentration per manufacturer's recommended dose) across duplicate plates and incubated overnight prior to photoactivation. Control treatments included no compound (untreated), 0.1% DMSO (negative control – carrier), no UV (positive control – blocker), 1 × 10⁻⁶ M Latrunculin B (positive control – blocker), and 10 ng mL⁻¹ epidermal growth factor plus 100 ng mL⁻¹ insulin-like growth factor (EGF + IGF1; positive control – activator). All wells were reacted with 1 mg mL⁻¹ GFOGER to induce migration, and wound closure was monitored by automated bright field imaging at 0, 2, 4, 7, and 24 h postactivation and quantitative image analysis. This approach allowed us to obtain both kinetic information and statistical measurements of wound closure (n = 8–12 microwounds per well, excluding well edges; Supporting Information).

Positive controls confirmed accelerated wound closure with the EGF + IGF treatment and a complete block of migration with no UV or Latrunculin treatment (Figure 2A). There also appeared to be a slight inhibitory effect of DMSO compared to the untreated condition. We then focused on the 7 h time point to assess the response to the epigenetic inhibitors, as it was the most sensitive to both positive and negative regulation of keratinocyte motility. The average change in microwound area compared to 0 h was calculated for each well, and inhibitor treatments were considered hits if there was a statistically significant difference in area compared to DMSO controls for both duplicate plates (Figure 2B,C). Based on these stringent criteria, we identified 11 blockers and 36 activators of keratinocyte migration, and these compounds corresponded to a range of different epigenetic targets (Figure 2C). Compound toxicity was identified for three compounds (Garcinol, Zebularine, and BIX01294) where the image analysis failed, and these conditions were excluded from subsequent analysis.

In an independent follow-up experiment, we confirmed selected hits targeting a range of different epigenetic regulators, including one strong blocker (α-hydroxyglutaric acid – histone methylation), one weaker blocker (Trichostatin A – histone acetylation), one strong activator (Mirin – DNA damage repair), and one weak activator (Suramin – DNA topoisomerase, only statistically significant in one replicate). Cells were treated with the original 10 × 10⁻⁶ m dose plus a tenfold lower dose of 1 × 10⁻⁵ m. Both activators increased migration compared to DMSO controls only at the higher dose used in the original screen, while both blockers inhibited cell migration at the high and low doses (Figure 2D; Movies S2–S4, Supporting Information). Interestingly, Suramin slightly reduced wound closure at the lower dose, which may reflect potential off target and false positive effects of this inhibitor with different dose dependencies.[18] Taken together, these studies confirmed the findings of the initial screen and demonstrate the applicability of the HCMA for quantitative analysis of cell migration in high throughput.

As the actin cytoskeleton is a main orchestrator of cell motility, we combined the migration data with high-content image analysis of the F-actin cytoskeleton and nuclei to look for potential phenotypic relationships associated with the responses to different inhibitors. At the final time point (24 h), all plates were fixed, stained with phalloidin and 4',6-diamidino-2-phenylindole (DAPI), and imaged. Image analysis was then used to define and quantify 24 different phenotypic parameters, including overall cell and nuclear morphology, as well as F-actin fiber intensity and structure within different cellular compartments (Figure 3A). We analyzed the effects of all epigenetic inhibitors and examined the relationships between these parameters using principal component analysis and hierarchical clustering (Figure 3B; Table S1, Supporting Information). Through this analysis we identified peripheral F-actin fluorescence as having the strongest negative correlation with cell migration (Table S1, Supporting Information), and there were two main clusters of phenotypic responses that were distinguished by parameters associated with F-actin intensity levels and F-actin spatial variation, as measured by the standard deviation in intensity per cell (Figure 3B).

In addition, there were significant differences in the phenotypic relationships between compounds that activated or blocked migration. For example, there was a stronger positive correlation between peripheral F-actin intensity at the edge of the cell and nuclear area in the blockers compared to the activators, while the activators displayed a stronger positive correlation between cell migration and spatial variation in peripheral F-actin intensity compared to the blockers (Figure S3, Supporting Information). The relationship between nuclear size and peripheral F-actin is intriguing given recent findings on the roles of nuclear stiffness and positioning in cell migration[19] and may reflect direct changes in nuclear mechanics induced by chromatin remodeling. Most notably, we observed significantly lower levels of F-actin at the periphery of cells treated with migration activators compared to either migration blockers or nonsignificant treatments (Figure 3C–E), consistent with the concept that disassembly of cell–cell junctions is required for rapid epithelial cell migration.[20] Together, these results demonstrate that the HCMA is compatible with high-content image analysis and can be used to develop quantitative relationships between complex cellular phenotypes.
A

Migration controls

Time after PA (hours)

Average Area (mm²)

0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
0.55
0.60
0.65
0.70
0.75
0.80
0.85
0.90
0.95
1.00

- Un-treated
- DMSO
- EGF + IGF
- Latrunculin B
- No UV

B

Time after PA (hours)

0
2
4
6
8
10
12
14
16
18
20
22
24

N=1
N=2

Average Area per treatment (mm²)

0.020
0.046
0.072
0.098
0.124
0.150
0.176
0.202
0.228
0.254
0.280
0.306

C

| Compound name | Functional Inhibitory Target | Fold change |
|---------------|------------------------------|-------------|
| Latrunculin B | Negative control             | 0.39        |
| Dark (No UV)  | Negative control             | 0.50        |
| Etoposide     | Topoisomerase inhibitor      | 0.53        |
| 6-Thioguanine | Purine synthesis inhibitor   | 0.55        |
| α-Hydroxyglutaric Acid (sodium salt) | | 0.57 |
| 5-Methyl-2'-deoxycytidine | DNA methylation inducer | 0.59 |
| LAQ824        | HDAC inhibitor               | 0.61        |
| 2,4-Pyridinedicarboxylic Acid | Lysine demethylase inhibitor | 0.61 |
| Valproic Acid (sodium salt) | HDAC inhibitor | 0.63 |
| RVX-208       | BET inhibitor                | 0.68        |
| RGFP986       | HDAC inhibitor               | 0.68        |
| Trichostatin A| HDAC inhibitor               | 0.68        |
| Loniquinib    | DNA methyltransferase inhibitor | 0.74 |
| SB309         | HDAC inhibitor               | 1.36        |
| Tubastatin A  | HDAC6 inhibitor - microtubule acetylation | 1.37 |
| CAY10433      | HDAC inhibitor               | 1.44        |
| WDR6-0103     | Histone methyltransferase inhibitor | 1.47 |
| trans-Resveratrol | SIRT1 and HDAC activator | 1.47 |
| JIB-04        | Jumonji histone demethylase inhibitor | 1.48 |
| Panobinostat  | HDAC inhibitor               | 1.50        |
| DMOG          | Prolyl-4-hydroxylase inhibitor | 1.54 |
| GSK126        | Histone methyltransferase inhibitor | 1.57 |
| SGC-CBP30     | Bromodomain inhibitor        | 1.57        |
| Chaetocin      | Histone methyltransferase inhibitor | 1.62 |
| 2',3',5'-triacyethyl-5-Azacytidine | DNA methyltransferase inhibitor | 1.62 |
| SGC9845       | Histone methyltransferase inhibitor | 1.63 |
| AM-1 (sodium salt) | Arigine methyltransferase inhibitor | 1.64 |
| OTX1015       | Bromodomain inhibitor        | 1.64        |
| Suberoylhydroxamic Acid | HDAC inhibitor | 1.65 |
| 5-(5'-Adenosyl)-L-methionine chloride | DNA methylation inducer | 1.68 |
| UNC1999       | Histone methyltransferase inhibitor | 1.75 |
| UNC1531 (trifluoroacetate salt) | Histone methyltransferase inhibitor | 1.83 |
| (R)-PFI-2 (hydrochloride) | Histone methyltransferase inhibitor | 1.84 |
| 3-Deazaadenosine A | Histone methyltransferase inhibitor | 1.96 |
| GSK343        | Histone methyltransferase inhibitor | 1.96 |
| N-Oxalylglycine | Prolyl-4-hydroxylase inhibitor and | 1.96 |
| MS-275        | HDAC inhibitor               | 2.00        |
| UNC1215       | Histone methyltransferase inhibitor | 2.04 |
| Bromosporine  | Bromodomain inhibitor        | 2.13        |
| GSK2801       | Bromodomain inhibitor        | 2.17        |
| Pyroxamide    | HDAC inhibitor               | 2.20        |
| LINC942       | Histone methyltransferase inhibitor | 2.23 |
| Sinefungin    | DNA methyltransferase inhibitor | 2.26 |
| HPOB          | HDAC6 inhibitor - microtubule acetylation | 2.26 |
| RG-108        | DNA methyltransferase inhibitor | 2.31 |
| SAHA          | HDAC inhibitor               | 2.33        |
| EPZ05667      | Histone methyltransferase inhibitor | 2.40 |
| Mirin         | DNA repair inhibitor         | 2.75        |
| AK-7          | SIRT2 inhibitor              | 2.78        |
| EGF + IGF     | Positive control             | 3.50        |

D

Screen Validation

Wound Closure

- Suramin
- Mirin
- α-Hydroxyglutaric Acid
- Trichostatin A

1 μM

10 μM
2.3. EZH2-Dependent Regulation of Migration and Epithelial-Mesenchymal Transition

Out of 36 compounds that activated keratinocyte migration, 12 were histone methyltransferase inhibitors, including four EZH2-specific inhibitors. These results are consistent with previous observations of demethylation and polycomb down-regulation during wound healing and suggest that histone demethylation may help promote keratinocyte migration. We therefore selected two of the EZH2 inhibitors, EPZ005687 and GSK343, for further validation of the screening strategy and downstream analysis. We confirmed that treatment with both compounds accelerated keratinocyte migration using the standard scratch wound assay (Figure 4A), and that these inhibitors reduced global levels of the EZH2 histone modification, H3K27me3 (Figure 4B,C). In addition, both compounds induced a mesenchymal-like morphology, characterized by a loss of circumferential F-actin stress fibers (Figure 4D), higher aspect ratio cell shape (Figure 4E), and reduced circularity (Figure 4F), which were consistent with the results of the high-content screen (Figure 3E).

Given that EZH2 inhibition induced a mesenchymal-like morphology in keratinocytes and a recent report demonstrated that loss of EZH2 in human embryonic stem cells promotes aberrant mesodermal differentiation, we hypothesized that EPZ005687 and GSK343 could be enhancing keratinocyte migration through derepression of mesenchymal genes. Analysis of existing ChIP-seq data sets for human keratinocytes from the ENCODE database revealed high levels of the H3K27me3 mark and EZH2 binding near the transcriptional start site of the classic EMT gene SNAI1 and throughout PITX2, which is a key regulator of mesendoderm development (Figure 5A). Moreover, treatment with EZH2 inhibitors resulted in a significant upregulation of both EMT markers SNAI1 and SNAI2, as well as PITX2 (Figure 5B). In contrast, the epithelial gene CDH1 had low levels of EZH2 binding and H3K27me3 and did not respond significantly to inhibitor treatment (Figure 5A,B). Taken together, these findings suggest that the EZH2 inhibitors promote keratinocyte migration and activation of a mesenchymal-like phenotype through epigenetic derepression of EMT genes.

3. Discussion

In summary, we have developed and validated a unique, micropatterned cell migration assay that supports high-throughput and high-content analysis of cell migration dynamics. We demonstrated the power of this platform by discovering novel regulators of keratinocyte migration in a compound-based screen and developing quantitative relationships between F-actin organization and cell migration for greater mechanistic insight. Through this approach, we identified EZH2 inhibitors as potent activators of keratinocyte migration and EMT-like phenotypes. While EZH2 has previously been shown to control epidermal differentiation, these findings suggest an additional function in the regulation of wound healing and EMT, which will be important areas for future investigation.

As an experimental platform, the major advantages of the HCMA include its compatibility with automated imaging systems, consistent and reproducible microwound geometries, multiple wounds per well for greater statistical power, the ability to independently tune micropattern geometry and specific cell–ECM interactions. Here, we demonstrated these properties using the collagen-mimetic sequence GFOGER; however, the thiol-yne click reaction is amenable to any other peptide combination, such as fibronectin or laminin-mimetic motifs. For example, our previous studies employed RGD peptides with a range of densities to tune the migration speed of fibroblasts. This tunability represents a unique feature in comparison to commercially available wound assays, and it will be crucial to the advancement of ECM biology, as this tool allows the use of several different ECM proteins and molecular motifs. In addition, because specific ligands are covalently coupled to the nonfouling POEGMA brushes, the direct effects of these cues on cell migration can be determined without confounding effects of nonspecific protein adsorption.

Like all model systems, however, the HCMA has a few notable limitations, such as the lack of a cellular injury, which may play a role in regulating certain aspects of cell migration, and the requirement for light-based activation of migration. Potential areas for future development include adaptation of the assay for genetic screens (e.g., siRNA), the combination with automated liquid handling systems for increased throughput, and the integration with single-cell tracking image analysis algorithms.

The HCMA has the potential to be a powerful tool for dissecting complex interactions between intrinsic and extrinsic regulators of cell migration and for the discovery and testing of new therapeutic compounds. In this context, targeting epigenetic gene regulation represents a novel and potentially effective strategy for enhancing wound repair. By affecting whole programs of gene expression through the inhibition of major epigenetic regulators (e.g., histone methyltransferases) it may be possible to more efficiently activate repair and regenerative processes. This approach could be highly advantageous for the treatment of chronic wounds as small molecule inhibitors could be delivered directly to the wound site through topical creams or dressings. However, the up-regulation of EMT genes...
A

Phalloidin → Whole cell - Inner cell = Edge

DAPI → Nuclei → Fiber and morphology analysis

B

Cluster 1

Cluster 2

C

Migration vs Peripheral F-actin

Migration Area Change 7hr

Nucleus area

Cell form factor

Cell aspect ratio

1.2

0.12

0.1

0.02

0.01

0.00

0

0

500

1000

1500

2000

2500

Peripheral F-actin Intensity (a.u.)

D

Peripheral F-actin Intensity

E

DMSO

TSA (blocker)

EPZ005687 (activator)

F-actin Intensity (a.u.)

NS  Blocker  Activator

*
in the studies presented here highlights the significant risk of simultaneously activating metastatic processes, and future work will require in-depth analysis of potential side effects. Key questions include the specificity, sensitivity and stability of the responses to varying doses and timings of treatments, as well as the effects on other cell types within the wound environment.

4. Conclusion

In this study, we describe the development of a high-throughput and high-content screening platform for cell migration using dynamically adhesive, micropatterned substrates. As proof-of-principle, we completed a compound-based screen of epigenetic inhibitors using this system. We identified novel...
Figure 5. EZH2 represses an EMT gene expression programme in keratinocytes. A) Tracks of ENCODE ChIP-seq data for H3K27me3 (blue) and EZH2 (red) enrichment at SNAI1, SNAI2, PITX2, and CDH1 in normal human keratinocytes (NHEK). BIGWIG files were downloaded from the Gene Expression Omnibus (Accession numbers GSM1003489 and GSM733701) and displayed using the WashU Epigenome Browser (http://epigenomewidget.Wustl.edu/browser/). B) Expression of SNAI1, SNAI2, PITX2, and CDH1 mRNA after 24 h treatment with inhibitors. Data represent the mean ± SEM of gene expression normalized to GAPDH, T-test \( p < 0.05 \) compared to DMSO (\( n = 3 \) experiments).
regulators of keratinocyte migration including EZH2 inhibitors, which enhanced wound closure through epigenetic derepression of EMT genes.

5. Experimental Section

Cell Culture: The nTERT human epidermal keratinocyte line\(^{(16)}\) was maintained in keratinocyte serum-free medium (KSFM) supplemented with 0.1 ng mL\(^{-1}\) EGF, bovine pituitary extract, and 1% penicillin/streptomycin. Cells were passaged weekly with 0.05% trypsin/EDTA, followed by inactivation with KSFM with 10% FBS. Keratinocytes were pelleted by centrifugation and replated in fresh KSFM. All cell culture reagents were from Thermo Fisher.

Fabrication of Polydimethylsiloxane (PDMS) Stamps: All chemicals were from Sigma Aldrich unless otherwise indicated. A large PDMS stamp that covered a 96-well sized glass plate, was generated using a modified photolithography procedure. A mechanical-grade silicon wafer (PI-KEM, Tamworth UK) of 6-inch diameter was spin coated (1000 rpm, 1 min) with SU-8 2010 and cured for 5 min at 100 °C. A photomask containing the 400 µm patterns was placed onto the SU-8 and exposed to UV light for 10 sec. The wafer was baked for 5 min at 100 °C (postexposure bake), then submerged in propylene glycol monomethyl ether acetate for 30 sec. The wafer was baked for 5 min at 100 °C within a 37 °C and 5% CO\(_2\) environmental chamber, and the same acquisition parameters mentioned above apply, with a sampling rate of 1 frame every 10 min. Composite images were first stitched together using the Developer Toolbox (GE) version 1.9.3, and image analysis of wound area and data visualization were performed on stitched images using a custom MATLAB script using specific commands from the Image Processing Toolbox (Figure 52, Supporting Information). Briefly, the image analysis script applied preprocessing histogram-based stretching (command stretchlim), followed by 2D adaptive noise-removal filtering (command weiner2), and a range filter to detect the microwounds (command rangefill). Thresholding was then applied based on the detection of last histogram peak corresponding to the microwound intensity (low response). Morphological binary operations were then applied (commands: imdilate > imfill > nclearbor). Initial time point binary images were saved as masks for subsequent time points. Next, segmented microwound properties were detected using the command regionprops and handled for subsequent analysis.

Following the final bright field time point (24 h), all plates were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.2% Triton for 5 min, labeled with phallolidin-Alexafluor488 (1:1000) and DAPI (1:1000) for 1 h, and rinsed three times with PBS. Fluorescence images were acquired by automated imaging with the IN Cell 2200 (GE) using a 20x objective and analyzed with the Developer Toolbox (GE). Nuclei were identified from the 16-bit DAPI images by applying a nuclear segmentation setting followed by filling holes, clump breaking, selecting objects 50–500 µm\(^2\) and excluding objects with a mean grey level below 1500. Cells were identified from the 16-bit phallodin images by applying a minimum grey value threshold (>400 grey value), filling holes, clump breaking, and selecting objects 100–6000 µm\(^2\). Subcellular regions of interest were defined by eroding the whole cell mask by an average of 5 pixels and subtracting the eroded region (inner cell) from the whole cell to define the edge (peripheral) area. In addition, the DAPI mask was used to define regions of the cell in or around the nucleus. Individual F-actin stress fibers were identified using a phallodin threshold of greater than 500 grey values and size criteria of greater than 4 µm\(^2\) and longer than 6 µm. F-actin intensity, fiber size, fiber number, and fiber length were quantified within the whole cell and different compartments for all nucleated cells. Finally, overall size and aspect ratio of the cells and nuclei were calculated using the cell and DAPI masks.

Scratch Assay: Confluent layers of nTERT keratinocytes were cultured in a 24-well plate and pretreated with 4 µg mL\(^{-1}\) mitomycin C plus 10 × 10\(^{-6}\) M of EPZ005687, 10 × 10\(^{-6}\) M GSK343, or 0.1% DMSO (carrier control) for 5 h prior to scratching with a 20 µl pipet tip. Cells were immediately washed three times with phenol-free DMEM and KSFM. For the epigenetic screen, cells were treated with fresh inhibitors (10 × 10\(^{-6}\) M) immediately after photoactivation.

Cell Seeding and Activation of Migration: Keratinocytes were seeded into the micropatterned 96-well plates at 100 000 cells per well and let to adhere for 30 min, then gently rinsed with fresh media and let to spread overnight. For the epigenetic inhibitor screen, cells were treated with 10 × 10\(^{-6}\) M of each compound and let to incubate for 16 h the day after seeding (day 2).

To activate cell migration into the “microwounds,” substrates were functionalized with a collagen mimetic peptide GFOGER\(^{(17)}\) (Apptec, Louisville KY). KSFM was removed from each well and replaced with phenol-red-free Dulbecco’s modified Eagle media (DMEM) containing 1 mg mL\(^{-1}\) GFOGER and 0.5% IgG-urea 2959. Sterile AbsorbMax tape was used to cover the plates, and the coupling reaction was initiated by exposing the plate to 365 nm light from an light-emitting diode (LED) array (Cetoni, Korbussen Germany) for 2 min. After exposure, substrates were immediately washed three times with phenol-free DMEM and KSFM. For the epigenetic screen, cells were treated with fresh inhibitors (10 × 10\(^{-6}\) M) immediately after photoactivation.

High-Content Imaging and Quantification: Imaging was performed using the IN Cell 2200 microscope (GE, Boston MA). Bright field imaging with a 10x objective was used for the wound closure screen, and a 20x objective was used for fluorescence imaging. Six frames per well were acquired. For the epigenetic screen, imaging was performed at room temperature at 0, 2, 7, and 24 h (less than 30 min imaging time at each time point). For live-cell imaging, cells were maintained within a 37 °C and 5% CO\(_2\) environmental chamber, and the same acquisition parameters mentioned above apply, with a sampling rate of 1 frame every 10 min. Composite images were first stitched together using the Developer Toolbox (GE) version 1.9.3, and image analysis of wound area and data visualization were performed on stitched images using a custom MATLAB script using specific commands from the Image Processing Toolbox (Figure 52, Supporting Information). Briefly, the image analysis script applied preprocessing histogram-based stretching (command stretchlim), followed by 2D adaptive noise-removal filtering (command weiner2), and a range filter to detect the microwounds (command rangefill). Thresholding was then applied based on the detection of last histogram peak corresponding to the microwound intensity (low response). Morphological binary operations were then applied (commands: imdilate > imfill > nclearbor). Initial time point binary images were saved as masks for subsequent time points. Next, segmented microwound properties were detected using the command regionprops and handled for subsequent analysis.

Following the final bright field time point (24 h), all plates were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.2% Triton for 5 min, labeled with phalloldin-Alexafluor488 (1:1000) and DAPI (1:1000) for 1 h, and rinsed three times with PBS. Fluorescence images were acquired by automated imaging with the IN Cell 2200 (GE) using a 20x objective and analyzed with the Developer Toolbox (GE). Nuclei were identified from the 16-bit DAPI images by applying a nuclear segmentation setting followed by filling holes, clump breaking, selecting objects 50–500 µm\(^2\) and excluding objects with a mean grey level below 1500. Cells were identified from the 16-bit phallodin images by applying a minimum grey value threshold (>400 grey value), filling holes, clump breaking, and selecting objects 100–6000 µm\(^2\). Subcellular regions of interest were defined by eroding the whole cell mask by an average of 5 pixels and subtracting the eroded region (inner cell) from the whole cell to define the edge (peripheral) area. In addition, the DAPI mask was used to define regions of the cell in or around the nucleus. Individual F-actin stress fibers were identified using a phallodin threshold of greater than 500 grey values and size criteria of greater than 4 µm\(^2\) and longer than 6 µm. F-actin intensity, fiber size, fiber number, and fiber length were quantified within the whole cell and different compartments for all nucleated cells. Finally, overall size and aspect ratio of the cells and nuclei were calculated using the cell and DAPI masks.

Scratch Assay: Confluent layers of nTERT keratinocytes were cultured in a 24-well plate and pretreated with 4 µg mL\(^{-1}\) mitomycin C plus 10 × 10\(^{-6}\) M of EPZ005687, 10 × 10\(^{-6}\) M GSK343, or 0.1% DMSO (carrier control) for 5 h prior to scratching with a 20 µl pipet tip. Cells were immediately washed three times with phenol-free DMEM and KSFM. For the epigenetic screen, cells were treated with fresh inhibitors (10 × 10\(^{-6}\) M) immediately after photoactivation.
were rinsed with fresh media and cultured for 24 h in inhibitor-containing media without mitomycin. Wounds were imaged at 0 and 24 h using a Nikon Eclipse TE2000-S microscope (Nikon, Kingdom Upon Thames, UK), and wound closure was measured as the percentage closure at 24 h compared to 0 h using Image J.

**Immunofluorescence:** Cells were fixed in 4% PFA for 10 min and permeabilized with 0.2% Triton-X100. Samples were blocked for one hour in 2% bovine serum albumin and incubated with anti-H3K27me3 (ab6002, 1:100, Abcam, Cambridge, UK) overnight at 4°C. Coverslips were washed and incubated for 1 h with goat anti-mouse secondary antibody Alexa Fluor 568 (1:1000, Thermo Fisher Scientific), Alexa Fluor 488-phalloidin (1:1000, Thermo Fisher Scientific) and DAPI. Coverslips were mounted onto glass slides with Mowiol and imaged with a 20x or 63x objective on a Zeiss 710 confocal microscope (Zeiss, Oberkochen Germany).

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR):** Total RNA was isolated from cells using the RNeasy Plus Mini Kit (Qiagen, Manchester, UK). cDNA was synthesized from 1 μg of total RNA using Quantitect Reverse Transcription kit (Qiagen). qRT-PCR was carried out using KAPA SYBR green PCR kit (KAPA Biosystem, Wilmington MA) and Applied Biosystems 7500 Real-time PCR system (Applied Biosystems, Foster City CA). Primers used were against SNAI1, SNAI2, PITX2, CDH1 and the housekeeping gene GAPDH (Table S1, Supporting Information). The mRNA expression level of each gene relative to GAPDH was calculated using the ΔΔCt method.

**Statistical Analysis:** The average and standard deviation of individual microwound areas were calculated for each well and compared to the DMSO control well using a two-tail T-test (unequal variance). Significance was determined by computing normalized P-values within each plate for both blockers and activators. The strongest blocker had a normalized P-value of 0 and DMSO equal to 1. The compounds with normalized P-values below 0.2 in both replicate plates were selected as hits. Principal components analysis and hierarchical clustering of normalized P-values were performed using the Clustvis on-line software (24 parameters total), and then confirmed data for all treatments from a single replicate was performed using the DMSO control well using a two-tail test (unequal variance). Significance was determined by computing normalized P-test (unequal variance).

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords**

animal replacement, cell migration, epigenetics, EZH2, high-throughput