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Prediction of Sphingosine 1-Phosphate-Stimulated Endothelial Cell Migration Rates Using Biochemical Measurements

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Abstract

The ability to predict endothelial cell migration rates may aid in the design of biomaterials that endothelialize following implantation. However, the complexity of the signaling response to migration-promoting stimuli such as sphingosine 1-phosphate (S1P) makes such predictions quite challenging. A number of signaling pathways impact S1P-mediated cell migration, including the Akt and Src pathways, which both affect activation of the small GTPase Rac. Rac activation promotes the formation of lamellipodia, and thus should be intimately linked to cell migration rates. In immortalized endothelial cells, expression of proteins that inhibit Akt, Src, and Rac (PTEN, CSK, and β 2-chimaerin, respectively) was decreased using RNA interference, resulting in increases in the basal level of activation of Akt, Src, and Rac. Cells were scrape-wounded and stimulated with 1 μ M S1P. The timecourse of Akt, Src, and Rac activation was followed over 2 h in the perturbed cells, while migration into the scrape wound was measured over 6 h. Rac activation at 120 min post-stimulation was highly correlated with the mean migration rate of cells, but only in cells stimulated with S1P. Using partial least squares regression, the migration rate of cells into the scrape wound was found to be highly correlated with the magnitude of the early Akt peak (e.g., 5–15 min post-stimulation). These results demonstrated that biochemical measurements might be useful in predicting rates of endothelial cell migration.

Keywords

Endothelial cell; Migration; Modeling; Partial least squares regression; Biochemistry

INTRODUCTION

The long-term blood compatibility of vascular grafts and endovascular stents may be improved by the presence of a surface layer of endothelial cells.^{6,9} A number of bioengineering strategies are available to promote the migration of endothelial cells onto the surfaces of blood-contacting devices after implantation, including the delivery of angiogenic factors such as VEGF, bFGF,

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ELECTRONIC SUPPLEMENTARY MATERIAL

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or sphingosine 1-phosphate (S1P) from biologically modified surfaces.^{18,22,43,48,49} However, no single strategy has proven to be entirely satisfactory, perhaps because no single growth factor or adhesion protein is solely responsible for the normal healing of breaches in the endothelium. Thus, the temporally controlled delivery of multiple factors may be necessary, but this may lead to an unwieldy optimization problem. For example, S1P-mediated migration of endothelial cells *in vitro* is enhanced by VEGF or physiologic levels of fluid shear stress, but S1P has almost no effect on cells stimulated with both VEGF and shear stress.¹⁵ Furthermore, the *in vitro* migration response to S1P is different in blood plasma than in cell culture medium, and is affected by the density and identity of adhesion ligands.^{2,47}

Engineering methods may help deconvolute the migration response of endothelial cells in the presence of a myriad of stimulatory factors. The application of systematic network analysis may aid in the design of devices by allowing useful predictions of the nonlinear migration response resulting from stimulation with combinations of factors. For example, knowledge of the activation states of all relevant growth factor receptors or integrins might allow construction of a predictive model.^{37,39} However, the number of potentially relevant receptors is quite large and thus difficult to characterize experimentally. An alternative approach is to look downstream of cell-surface activation at signaling events immediately distal to receptor activation, since receptor-mediated signals tend to converge on a few common pathways.

RhoGTPases (Rho, Rac, and Cdc42) greatly impact endothelial cell migration,¹⁰ with activation of Rac-GTP strongly correlated with endothelial hapto- and chemotaxis.^{41,42} However, different variants of constitutively active Rac (V12 vs. L61) have opposite effects on endothelial cell migration.^{14,42} In neutrophils, very low levels of Rac-GTP significantly decrease chemotaxis,¹¹ while a decrease in Rac activity of approximately 30% serves as a switch between random and persistent migration in both fibroblasts and endothelial cells.³⁵ At the other extreme, high levels of Rac-GTP inhibit cell migration, e.g., during cell spreading.³⁶ Optimal levels of Rac activity may be needed for rapid cell migration, but the presence of a complex nonlinear relationship may mean that Rac alone will be difficult to use as a predictor of cell migration rates.

We hypothesized that Rac, Akt, and Src activity together may yield better predictions of cell migration, as Akt, Src, and Rac are particularly important in S1P-mediated cell responses.^{12, 25–27} Akt and Src family kinases are activated in endothelial cells by fluid shear stress, receptor tyrosine kinases, G protein-coupled receptors, and focal adhesions.^{20,21,40,45} In essence, these factors together may better reflect the overall activation state of multiple growth factor receptors, mechano-receptors, G protein-coupled receptors, and integrins that as a whole drive the cell migration response.

To study endothelial cell migration as a multivariate function of Akt, Src, and Rac activities, we collected an extensive biochemical and biophysical dataset across a range of cellular perturbations. Since direct knockdown of Akt, Src, or Rac could lead to very low levels of activation that might not be relevant in highly stimulated migrating cells, we decreased expression of inhibitory accessory proteins, resulting in increased basal levels of active Akt, Src, and Rac. The accessory proteins targeted with shRNA were phosphatase and homolog deleted on chromosome ten (PTEN), c-terminal Src kinase (CSK), and β 2-chimaerin. These perturbations allowed us to examine a range of activation states and use the biochemical data from these states to construct a mathematical model linking cell migration to the biochemical measurements.

Including the activation states of all three proteins (Akt, Src, and Rac) in a partial least squares regression (PLSR) model allowed prediction of cell migration in both S1P-stimulated and unstimulated cells. The model suggested that the best predictors of cell migration rates were

early levels of active Akt (5–15 min after scrape wounding/S1P stimulation) or late levels of active Src (2 h after scrape wounding/S1P stimulation). The correlation between the level of active Akt 15 min post-stimulation and cell migration rates at 6 h was further demonstrated using low concentrations of the PI3K inhibitor LY294002. On the basis of this experimental approach in endothelial cells, we propose that Rac is a poor general predictor of migration due to its nonlinear behavior, while measurement of phosphorylated Akt alone is a useful predictor, perhaps because it serves to poll the state of signal cascade activation in the cell.

MATERIALS AND METHODS

Endothelial Cell Culture

All cell culture reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted. Human aortic endothelial cells (HAEC) were purchased from Clonetics, Inc (Walkersville, MD) and cultured in endothelial growth medium (EGM; MCDB131 medium supplemented with 10 ng/mL epidermal growth factor, 10 μ g/mL heparin, 1.0 μ g/mL hydrocortisone, 1% penicillin–streptomycin, 5% fetal bovine serum (FBS), and 12 μ g/mL bovine brain extract (Clonetics)). S1P was purchased from Biomol (Plymouth Meeting, PA). Serum-free medium was MCDB131 supplemented with 1% penicillin–streptomycin. For time-lapse microscopy, cells were cultured in Leibovitz's L-15 medium (Gibco) $\pm$ 1 μ M S1P. HAEC were immortalized with two rounds of amphotropic retroviral infection (pBABE-hygro-hTERT). HAEC were allowed to recover in fresh EGM for 48 h, after which 100 μ g/mL hygromycin was added for selection. The resulting cells were named HAEC-hT.

Wound Healing Migration Assay

Cells were rinsed once with warm PBS and serum starved for 2 h (0% FBS). Following starvation, cells in tissue culture dishes were mechanically wounded by swiping a high-density poly(ethylene) comb across the surface of the tissue culture plate seven times. The wounds were 230 ± 32 μ m wide, resulting in a removal of $49 \pm 7\%$ of the total cells from the plate. An average of five cell bodies was between each wound edge. After rinsing the cells once with warm serum-free medium, cells were stimulated with L-15 medium (phenol red-free) $\pm$ 1 μ M S1P and allowed to migrate for 6 h into the wounds. For migration assays used to populate the model, images of one wound area per plate were captured using time-lapse microscopy every 2 min on an Olympus X71 inverted microscope ($\times 10$ objective, $n = 4$ –9). For validation experiments, cells were plated in 24-well tissue culture plates for 24 h. After serum starvation for 4 h, cells were pre-treated with LY294002 (Calbiochem, 0–10 μ M as indicated) for 30 min and then wounded, rinsed, and stimulated $\pm$ 1 μ M S1P. Images were captured on a Zeiss Axio-Observer equipped with a Ludl automatic stage and environmental chamber. Metamorph software was used to capture four wound images per well every 10 min for 6 h ($n = 4$ biological replicates, 16 wounds total per condition). The distance traveled by the wound edge was quantified with ImageJ. For long-term wounding assays (24 h), cells were first serum starved (0.1% FBS) for 12 h and then stimulated with low serum medium (0.1% FBS), 100 nM S1P, or complete growth medium. A scrape wound was made using a P1000 plastic pipette tip. After rinsing, micrographs of the wounds were captured using a $\times 4$ objective and the cells were cultured in a CO₂-rich environment for 24 h. Micrographs of the same wound area were captured after 24 h and the number of cells that migrated into the area was counted and is reported as a cell density.

Rac Activity Assay (ELISA-based)

The concentration of Rac-GTP was measured using the Rac GLISA assay (Cytoskeleton, Inc) per manufacturer's protocol. The optimum lysate concentration for the endothelial cells used in this study was determined to be 0.75 mg/mL. All data were normalized to serum-starved shLuciferase control cells.

Construction of shRNA Vectors

As described previously, a joint PCR protocol was followed to construct the pFLRu-nYFP vectors containing gene-specific shRNA.²⁸ An Excel-based prediction program⁴⁶ was used to generate human gene-specific shRNA oligonucleotides against PTEN (forward primer: 5'-GTGGAAAGGACGAAACACC GCCAGCTAAAGGTGAAGATATATTCAAGAGAT ATATC-3', reverse primer: 5'-TCCAGCTCGAGA AAAAGCCAGCTAAAGG TGAAGATATATCTCTT GAATATATC-3'), β 2-chimaerin (forward primer: 5'-GTGGAAAGGACGAAACACCGCTGCCGAGT ACATTCAATTCAAGAGATTGAAA-3', reverse primer: 5'-), and CSK (forward primer: 5'-GTGGAA AGGACGAAAC ACCGTACCCAGCAAATGGGC ATTTCAAGAGAATGCC-3', reverse primer: 5'-). Joint U6-shRNA PCR product was cloned into pFLRu-nYFP at *Xba*I/*Xho*I sites using the Roche Rapid Ligation kit. Positive clones were confirmed by DNA sequencing.

Lentiviral Infection of Endothelial Cells

Lentivirus was produced by transfecting 293T cells (10 cm dish) with 5 μ g pFLRu vector and 5 μ g packaging vectors (8:1 ratio of pHR'8.2DR and pCMVSV-G) using 25 μ L of Transit LT1 (Mirus Bioscience). After 24 h, 12 mL EGM was added to the 293T cells. The following day, virus was collected and syringe filtered (0.45 μ m). HAEC-hT (75–80% confluent) were infected with virus by adding 6 mL of fresh viral supernatant to 10-cm² dishes along with 6 mL of EGM and 1 μ g/mL of protamine sulfate. Fresh EGM was added to HAEC-hT after 6–12 h. Infection was repeated the next day. Infected HAEC-hT were screened for positive RNAi-containing virus transduction by fluorescence microscopy. Cells that were greater than 90% YFP positive were used in subsequent experiments. In some cases, cells containing transduced RNAi viruses were selected by using 1.5 μ g/mL of puromycin for 1 day or 0.5 μ g/mL of puromycin for 3 days.

Western Blot Analysis

In all experiments, cells were rinsed with room temperature D-PBS and serum starved in MCDB131 (0% FBS) for 2 h prior to stimulation. Following starvation, cells were stimulated with 1 μ M S1P, mechanical wounding, or both. In the case of mechanical wounding, cells were immediately rinsed post-wounding with 6 mL of serum-free MCDB131 before stimulation. Cells were lysed after 0, 5, 15, 30, 60, or 120 min at 4 °C. After washing twice with ice-cold PBS, cells were solubilized in RIPA buffer (10 mM Tris-HCl, pH 7.4; 150 mM NaCl; 10 mM MgCl₂; 0.35% deoxycholate; 5% glycerol; 0.1% SDS; 1% NP-40; 10 μ g/mL aprotinin/leupeptin; 2 mM PMSF; and 50 μ M sodium orthovanadate) or Rac GLISA lysis buffer (supplemented with protease inhibitors, 2 mM PMSF, and 50 μ M sodium orthovanadate, Cytoskeleton, Inc). Total protein was measured in duplicate using the Precision Red protein assay (Cytoskeleton, Inc). Equal amounts of protein (20 μ g) were fractionated by SDS-PAGE (10%) and transferred to a PVDF membrane. Following a blocking step, blots were incubated overnight with primary antibody diluted 1:1000 for PTEN (138G6, Cell Signaling Technology), pan-Akt (9272, Cell Signaling Technology), pan-phosphoS473 Akt (9271, Cell Signaling Technology), CSK (sc-286, Santa Cruz), 1:500 for Src (sc-19, Santa Cruz), 1:2000 for phospho-Y419 Src (2101, Cell Signaling), or 1:10,000 for anti-actin (MAB1501, Chemicon) in 5% bovine serum albumin + 0.02% sodium azide in TBS-T at 4 °C. After washing, an HRP-conjugated secondary antibody (goat anti-rabbit IgG (1:5000) or rabbit anti-mouse IgG (1:20,000)) was added to the blocking buffer for 1 h at 25 °C. Proteins were visualized using ECL substrate (GE Bioscience). To determine equal loading, membranes probed for phospho-specific antigens were stripped using 10 mL of stripping buffer (62.5 mM Tris-HCl, pH 6.7; 2% SDS; and 100 mM β -mercaptoethanol) for 30 min at 55 °C. The stripped membranes were then washed twice with 50 mL of TBS-T for 10 min, blocked in 5% nonfat milk for 1 h, and incubated in primary antibody overnight at 4 °C. Densitometry was completed

using ImageJ. For validation experiments, cells were serum starved and then pretreated with LY294002 as indicated for 30 min prior to stimulation with 1 μ M S1P for 15 min. Cells were then lysed and processed as above.

Multivariate Analysis

Biochemical and migration measurements were divided into two data matrices. The biochemical data made up the dependent data matrix, **X**, and the average or median values of the migration data made up the response matrix, **Y**. A “full” model was constructed using the average values across all experiments, totaling eight vectors of biochemical data (one per cell type $\pm$ 1 μ M S1P). In the full model, each vector of biochemical data corresponded to mean measurements across all timepoints and biochemical variables. For the “resampled” model, one datapoint from each timepoint/biochemical variable was randomly selected to produce a new vector, with 75 new data vectors produced for each cell type $\pm$ 1 μ M S1P. Finally, the integral of each pseudo-timecourse (Akt, Rac, and Src) was calculated from the mean values and included in the dataset, expanding the **X** matrix to 600×21 [(75 data vectors $\times$ 8 cell types) $\times$ ((6 time-points + 1 AUC) $\times$ 3 biochemical variables)]. PLSR analysis was performed in SIMCA-P + 12.0 (Umetrics). Full models were cross-validated by 50 random permutations as allowed by the software to produce standard errors of prediction. Variance in split-set models was evaluated by Simca-P+ as described.⁸ The cumulative “goodness of fit” (R^2Y) and the fraction of variation in the migration responses that was captured by the model (Q^2Y) were used to evaluate differences between models. Weighted coefficients are reported with 95% confidence intervals.

Statistical analysis

Migration data are presented as either mean $\pm$ standard deviation. To evaluate differences in wound closure (both wound edge distance traveled and rate of wound closure), data were processed by ANOVA and compared by Tukey's HSD test.

The biochemical data approximately followed a normal distribution, on the basis of the distribution of the means calculated by bootstrap sampling (200 samples with replacement) of the original data (not shown). In short, the means of 200 distributions that were randomly sampled from the original data with the option of re-using individual datapoints were evaluated for a normal distribution. This was performed to gain confidence that statistical tests appropriate for a Gaussian distribution could be employed in the study. Data are presented as mean $\pm$ SEM. Statistical differences were evaluated using ANOVA and the Tukey's HSD test.

RESULTS

Telomerase Immortalization Does Not Affect Endothelial Cell Response to S1P

To facilitate large-scale biochemical analysis of human endothelial cell migration, human telomerase reverse transcriptase (hTERT) was used to immortalize early passage primary human aortic endothelial cells (HAEC-hT, Supplementary Figs. 1A, B). Migration of HAEC-hT in a scrape wound assay was not different from primary HAEC in low serum medium, stimulation with 100 nM S1P, or in complete growth medium (Supplementary Fig. 1C). Under conditions of fluid shear stress, hTERT-immortalized endothelial cells increased their migration speed in response to S1P similarly to primary HAEC (Supplementary Fig. 1D). In sum, these data demonstrated that telomerase immortalization of HAEC did not alter the ability of these cells to migrate in response to serum, S1P, and mechanical stimuli.

Rac Activity Increases in Serum-Starved PTEN-, CSK-, or β 2-Chimaerin-Depleted Cells, but Decreases upon Stimulation with 1 μ M S1P

To develop a predictive model of cell migration, RNAi was used to manipulate the activities of Akt, Src, and Rac. We reasoned that direct knockdown of Akt, Src, or Rac variants would lead to very low levels of activity that might not be relevant in highly stimulated migrating cells. Therefore, we decreased expression of inhibitory accessory proteins, resulting in increased basal levels of active Akt, Src, and Rac (Supplementary Fig. 2). The accessory proteins targeted with shRNA were β 2-chimaerin, PTEN, and CSK, which are known inhibitors of Rac, Akt, and Src activity, respectively.^{4,17,33} These perturbations allowed us to examine a range of activation states and use the biochemical data from these states to construct a mathematical model.

Transduction of HAEC-hT with β 2-chimaerin-targeted shRNA significantly reduced β 2-chimaerin expression as measured by quantitative RT-PCR (Supplementary Figs. 2A, 2B), as there was no available specific antibody. This manipulation resulted in a significant increase in GTP-bound Rac in serum-starved cells vs. a control Luciferase shRNA-transduced cells (Fig. 1), indicative of loss of the Rac-GAP activity of β 2-chimaerin. Active Rac levels were measured by Rac-GTP ELISA,²⁴ which was validated by comparison with traditional immunoprecipitation of Rac-GTP using the protein-binding domain of PAK (Supplementary Fig. 3).

HAEC-hT were also transduced with shRNA targeting PTEN. This resulted in a $69 \pm 13\%$ decrease in protein expression (Supplementary Fig. 2C), and a 2.8 ± 0.7 -fold increase in phosphorylated Akt (S473) in serum-starved PTEN-deficient cells, vs. control cells, consistent with a decrease in PTEN lipid phosphatase activity (Supplementary Fig. 2D). PTEN depletion also resulted in a significant increase in Rac activity in serum-starved cells (Fig. 1).

Finally, CSK protein expression was decreased $72 \pm 11\%$ in HAEC-hT using CSK-targeted shRNA (Supplementary Fig. 2C). Depletion of CSK resulted in a 2.5 ± 0.5 -fold increase in basal phosphorylation of Src on Y416, its activation site (Supplementary Fig. 2D). Similar to the β 2-chimaerin- and PTEN-deficient cells, the depletion of CSK significantly increased the Rac-GTP levels in serum-starved HAEC-hT (Fig. 1).

To further determine the effect of PTEN, CSK, and β 2-chimaerin depletion on the kinetics of Rac activation in response to migration stimuli, shRNA-expressing HAEC-hT were scrape wounded in the presence or absence of 1 μ M S1P. Note that in these studies, multiple scrape wounds were made per well, such that almost 50% of the cells were removed from the plates, with an average of five cell bodies between wounds (see “Materials and Methods”). This provided enough cells for biochemical measurements of wounded cells and allowed us to distinguish between the effects of wounding and the effects of S1P alone. In control cells expressing a shRNA targeting Luciferase (i.e., nonspecific control), scrape wounding alone immediately stimulated Rac activity (5 min post-wounding), while the addition of 1 μ M S1P in combination with wounding led to a significant increase in active Rac after 2 h (Fig. 2). Unlike control cells, scrape wounding of PTEN-, CSK-, or β 2-chimaerin-deficient cells did not result in a further increase in Rac activity from the already increased basal level (Fig. 2). Moreover, in PTEN-, CSK-, or β 2-chimaerin-deficient cells, activation of Rac was not increased in the presence of 1 μ M S1P (Fig. 2). However, addition of 1 μ M S1P to wounded β 2-chimaerin-deficient cells resulted in a significant *decrease* in Rac activity vs. serum-starved cells at all timepoints (Fig. 2).

S1P Stimulation Increases Cell Migration in PTEN- and CSK-, but not in β 2-Chimaerin-Deficient Cells

Since Rac activity is required for S1P-mediated aortic endothelial cell migration,^{12,27} and the kinetics of Rac activation were altered in HAEC-hT depleted of PTEN, CSK, or β 2-chimaerin, we used time-lapse video microscopy to determine how re-endothelialization of scrape wounds was affected. Immediately after wounding, about 15% of the cells at the wound edge exhibited a rounded morphology, and rapid spreading of the cells into the wound area was frequently observed. Therefore, the first hour of each movie was disregarded so as to remove any potential artifacts from cell spreading. When control shLuciferase HAEC-hT were stimulated with 1 μ M S1P at the time of wounding, the average distance traveled by the leading row of cells was $53.8 \pm 8.5 \mu\text{m}$ vs. $20.9 \pm 5.3 \mu\text{m}$ in the absence of S1P after 6 h (Fig. 3a). The wound healing rate was then measured by fitting the wound edge distance to a cubic equation and taking the derivative with respect to time. The wound healing rate of S1P-stimulated cells was significantly higher than unstimulated cells from 2 h onward and was independent of time (Fig. 3b). Conversely, the wound healing rate of unstimulated cells decreased with time, reaching a steady state 2.5 h after wounding (Fig. 3b). In contrast to control cells, the wound closure rate of all S1P-stimulated PTEN-, CSK-, or β 2-chimaerin-deficient cells decreased with time (Figs. 3c–3e).

Migration rate was independent of PTEN, CSK, or β 2-chimaerin depletion in the absence of S1P (Fig. 4a), despite the high Rac activity in these cell lines measured at 120 min post-wounding (Fig. 2). The mean wound healing rate was calculated between 1 and 6 h, referred to herein as the wound closure rate. The addition of 1 μ M S1P significantly increased the wound closure rate in PTEN- or CSK-deficient cells, but not in β 2-chimaerin-deficient cells (Fig. 4a; $p = 0.143$ for β 2-chimaerin-deficient cells). The distance traveled by the wound edge increased upon addition of S1P in all cell types (Fig. 4b). The wound closure rate and the distance traveled by the wound edge were significantly decreased in all perturbed cells compared to control cells.

We asked if Rac activity at 120 min post-stimulation was indicative of long-term cell migration (6 h), and found that upon S1P stimulation, there was a high correlation ($r = 0.96$) between the concentration of active Rac and the mean rate of cell migration (Fig. 5). However, in the absence of S1P, the rate of endothelial cell migration was not related to Rac activity. Thus, Rac activity alone should not be a robust predictor of endothelial scrape wound migration.

A Linear Multivariate Model Predicts Endothelial Wound Closure Rate

Our results indicated that Rac activity alone cannot robustly predict endothelial cell migration rates. To determine if the combination of Rac, Akt, and Src activities predicted S1P-mediated endothelial wound healing, we measured the kinetics of Akt and Src phosphorylation in response to wounding with and without 1 μ M S1P. We also measured the kinetics of Rac, Akt, and Src activation in subconfluent mono-layers stimulated with S1P but not scrape wounded (S1P only). The complete dataset is shown in Fig. 6. Because evaluating relationships within highly dimensionalized data is difficult using simple graphical techniques, we employed a multivariate analysis technique to gain insight into the relationship between the biochemical dynamics and the migration responses. PLSR reduces the dimensionality of a dataset by identifying linear combinations of the biochemical measurements that capture the most information about the migration response.¹⁹ A PLSR model was constructed that included the mean value at each timepoint and the total activation (i.e., area under the curve) of all biochemical variables. Unlike Rac-GTP alone, the PLSR model was able to accurately describe the measured migration rates in both the presence and absence of S1P ($R^2 = 0.93$; Fig. 7a). The robustness of the modeling approach was cross-validated iteratively with a leave-one-out strategy using each of the RNAi-depleted cell lines, allowing calculation of model error.

The modeling framework was tested more vigorously by removing each of the RNAi-depleted cell lines individually, and building an entirely new “split-set” model with the remaining cell types. In this model, six datasets (e.g., shLuciferase, shPTEN, shCSK, all with and without 1 μ M S1P) were used to train the model, and the biochemical data from the remaining RNAi-depleted cell type was input to obtain a predicted wound closure rate (e.g., sh β 2-chimaerin with and without 1 μ M S1P). The split-set models (four in total) were able to adequately predict the measured migration rate, although some predictability was lost due to the inability of the model to accurately capture the maximal and minimal migration rates ($R^2 = 0.65$). A summary of the output from the four split-set models is shown in Fig. 7b.

The magnitude of the model regression coefficients associated with each biochemical signal quantifies their contribution to endothelial cell wound closure. In a model constructed using mean valued data, coefficient values indicated that early phosphorylation of Akt was positively correlated with cell migration rate (Fig. 7c). Early Rac and Src activation were negatively correlated, while late (120 min post-stimulation) Rac and Src activation were positively correlated with cell migration rate. However, the 95% confidence intervals of the coefficients were large and many intervals included zero. To better account for measurement errors, an alternative PLSR model was constructed using re-sampled, nonaveraged, biochemical, and migration metrics. The datasets were generated by randomly sampling with replacement from the measured biochemical and migration rate data. The expanded model consisted of 75 resampled data vectors for each RNAi-depleted cell type $\pm$ 1 μ M S1P across the six timepoints and three biochemical variables (i.e., activated Akt, Src, and Rac). In addition to the six timepoints, the area-under-the-curve was also calculated for each biochemical variable. A model containing three latent variables was able to adequately correlate the 12,600 resampled biochemical datapoints (75 data vectors $\times$ 8 cell types $\times$ 7 timepoints/area under the curve $\times$ 3 biochemical variables) to the migration rate data (“goodness of fit” (R^2Y) = 0.654, “predictability” (Q^2Y) = 0.629). The regression coefficients correlated well with the averaged data model (Fig. 7c), and the reduced confidence intervals confirmed that early Akt and late Src activities were positively correlated with wound healing rate but that late Rac activity (120 min) was not correlated with migration rate. Early Src and early Rac activities were still found to be negatively correlated with cell migration in the alternative model.

The purpose of the PLSR framework is to reduce the dimensionality of the data; therefore, we asked if a predictive model of endothelial wound healing could be constructed only from the most important signals. The variable importance to projection (VIP) provides a measure of how much of the response (wound healing rate) is captured by each signaling variable. If a VIP value is >1.0 , the biochemical signal contributes significantly to the model-predicted wound healing rate. Using only the eight VIP variables with values ≥ 1.0 in the original full model from Fig. 7a, listed in Table 1, good correlation was maintained, as expected due to the nature of dimensionality reduction by PLSR (Fig. 8a). Then, a new split-set PLSR model was constructed using only the eight signaling variables with VIP values ≥ 1.0 . The four split-set models were constructed as in Fig. 7b by leaving out one cell type at a time and constructing four models. The four split-set models were able to accurately predict wound healing rates ($R^2Y = 0.92$, $Q^2Y = 0.69$) to a similar extent as the model containing 21 signaling variables (Fig. 8b; compare with Fig. 7b).

Akt accounted for five of the eight variables with $VIP \geq 1.0$. A model was thus constructed using only Akt activity measurements. A full model was constructed and good correlation was found using the model constructed with the Akt data alone (Fig. 8c). The two variables with the highest VIP were phospho-Akt at 5 and 15 min post-stimulation. The value of the phospho-Akt peak at 15 min by itself was found to explain a large amount of the variance in endothelial cell migration rates ($R^2 = 0.86$; Fig. 8d). In comparison, little of the variance could be explained

by the starting or 120 min levels of phospho-Akt ($R^2 = 0.05$ and 0.003 , respectively, data not shown).

To experimentally validate the PLSR model predictions, we examined a range of Akt activation states using a chemical inhibitor. This was accomplished by treatment of HAEC-hT shLuciferase cells with 0.125 – $1.0 \mu\text{M}$ LY294002 prior to and during stimulation with $1 \mu\text{M}$ S1P (Figs. 9a, 9b). To test for off-target effects, LY303511, an inactive analog of LY294002, was employed at similar levels, which had no effect on Akt phosphorylation (Supplementary Fig. 4). Concentrations of LY294002 above 500 nM led to statistically significant decreases in cell migration in the presence of S1P, while $5 \mu\text{M}$ LY294002 was required to observe a statistically significant decrease with wounding alone (Fig. 9c). Higher doses of LY294002 (5 and $10 \mu\text{M}$) decreased the migratory response to S1P even further, but not to the level measured in the absence of S1P. In fact, cell migration was decreased by less than 30% when the phospho-Akt peak was nearly eliminated. However, the correlation between the height of the phospho-Akt peak at 15 min and cell migration in the presence of S1P was high ($r = 0.93$), consistent with the observations obtained using the perturbed cells.

DISCUSSION

Given the central role of Rac in the regulation of lamellipodia formation and function,³² the concentration or localization of Rac-GTP must be quantitatively linked to cell migration rates. Previous results suggest a complicated, nonlinear relationship,³⁵ making it possible that the relationship between Rac-GTP and cell migration rates may shift following stimulation with different factors. For example, it is known that the typical biphasic migration response of cells plated on variably adhesive substrates changes following stimulation with EGF or S1P.^{29,47} This suggested to us that measurement of Rac-GTP combined with other measures of the general state of cell activation would be more predictive of cell migration rates. Indeed, adding measurements of Akt and Src phosphorylation resulted in a model that was valid for all of the four cell perturbations, with and without S1P stimulation. Furthermore, split-set submodels containing three of the four shRNA-containing cell lines could adequately predict the migration rate of the fourth cell line, validating the applicability of the modeling method to this dataset (Fig. 7b). Further validation of the PLSR model predictions was undertaken employing the PI3K inhibitor LY294002. These experiments confirmed that early Akt activation is well correlated with S1P-mediated endothelial wound healing. While others have shown that high concentrations of LY294002 moderately decrease S1P-mediated migratory responses,^{34,38} we demonstrated that a high correlation exists between the magnitude of the decrease in early Akt activation and subsequent cell migration.

Rac activity alone was a good predictor of cell migration in S1P-stimulated cells. In the absence of S1P, high levels of Rac activity in the RNAi perturbed cells did not result in a change in the rate of cell migration. Following stimulation with S1P, Rac activities actually *decreased* to levels that were correlated with the migration rate (Fig. 5). These results suggest the presence of multiple modes of cell migration with varying degrees of dependence on Rac-GTP. A basal migration mode may be present that does not depend on the level of Rac-GTP, such as in the case of cells that are wounded without the addition of exogenous S1P. Meanwhile, a stimulated migration mode may be present in which cell migration rates depend on Rac-GTP levels to maintain a high migration speed, or one in which Rac-GTP levels are regulated to levels appropriate to the rate of cell migration. Rac1 is required for chemotaxis of vascular endothelial cells in response to S1P or VEGF, but migration in serum-free conditions is independent of Rac1 expression, suggesting that a Rac1-independent migration mode is plausible in wounded cells in the absence of S1P stimulation.⁴⁴ The mechanism leading to the decrease in Rac-GTP that we measured following stimulation with S1P is currently unknown. However, one type of PI3K (PI3K γ) that is activated by G-protein $\beta\gamma$ subunits enhances the localization of a Rac-

GAP to the plasma membrane.⁵ S1P stimulation of endothelial cells leads to activation of PI3K β ,¹⁶ but subsequent Rac-GAP activation has not been characterized. The activation of an alternate Rac-GAP is plausible, as over 70 Rho family GAPs have been described, most of which are relatively uncharacterized.³ The most dramatic decrease in Rac activity was observed in S1P-stimulated sh β 2-chimaerin cells (Fig. 7). This suggests that β 2-chimaerin may shield Rac-GTP from the action of other Rac-GAPs, perhaps those linked to PI3K activity.

The main hypothesis of this study was that Rac, Akt, and Src activation could account for the general state of cell activation and aid in the prediction of cell migration rates. Akt and Src respond to a wide variety of stimuli, including shear stress, growth/chemotactic factors that signal through either receptor tyrosine kinases or G protein-coupled receptors, and adhesion through integrin receptors.^{20,21,40,45} Our previous results illustrated that signals through one pathway, e.g., S1P receptors, may become irrelevant in the presence of multiple stimuli (e.g., VEGF and shear stress).¹⁵ We suspected that this may not be reflected at the level of receptor activation, as phosphorylation of the VEGF or S1P receptors may be normal, but may be reflected by the levels of Akt and Src activation. Indeed, the five most important variables in predicting cell migration were all measures of the early peak in Akt activity. In the perturbed cells, the transient peak in Akt activity was present upon stimulation with S1P, but with lower amplitudes than the control cells. Thus, although the perturbed cells began with higher basal levels of Akt, the muted early Akt response to wounding and S1P was well correlated with lower wound closure rates. The LY294002 inhibitor experiment (Fig. 9) further supports a relationship between the migration response and PI3K/Akt activation.

Akt activation is generally associated with enhanced endothelial cell migration, but this is the first report that the relationship is linear. Pankov *et al.* found that mutation of beta1 integrins led to a decrease in Rac and Akt activation, resulting in an enhancement in the directionality of migration (D/T) and a decrease in migration velocity. D/T, migration velocity, and Rac activity could be recovered in the cells by transfection of constitutively active Akt. Interestingly, the level of constitutively active Akt in cells was highly correlated with migration velocity.³⁵ However, others have demonstrated that deletion of Akt1 in endothelial cells leads to less than a 50% decrease in S1P-mediated migration, although the activities of the Akt2 and Akt3 in the presence of S1P were not measured.¹ Localized activation of PI3K leads to the formation of the leading edge of a cell, which is predictive of the direction of migration, and persistence of migration is thus directly related to the lifetime of the leading edge.⁵⁰ Migration speed is more complex but is correlated with the plasticity of focal adhesions.¹³ Persistence and migration speed are governed by complex, dynamic processes and the connection to the early, transient Akt peak is not obvious. A possible explanation is that the perturbed cells were less capable of responding to the stimuli (scrape wounding or S1P) and this was reflected in the magnitude of the early Akt peak as well as the lower migration rates. Akt is known to phosphorylate the main S1P receptor in endothelial cells, S1P₁, but it is not known how this affects S1P signaling.²⁶ Receptor modifications of this type often influence receptor endocytosis and thus long-term elevated Akt activity in the perturbed cells could have led to a downregulation of the S1P receptor and a muted response to S1P.

The modeling approach has limitations. Although the perturbations to the cells were small and tended to increase the activities of the proteins of interest, the three factors PTEN, β 2-chimaerin, and CSK are not strictly specific for Akt, Rac, and Src, respectively. PTEN has protein phosphatase activity against FAK^{30,31} and the Src family kinase Fyn.⁷ β 2-chimaerin is directly phosphorylated by Src family kinases, inhibiting its GAP activity.²³ Nonetheless, specificity of the perturbations is not a requirement of the PLSR approach and it could be argued that the use of chemical inhibitors or direct knockdown of Akt, Rac, or Src would cause more severe perturbations. Future approaches that may complement the current study include

the use of combinations of growth factors in different concentrations or the use of inducible promoters to enhance the expression of Akt1, Rac1, or cSrc.

The PLSR approach itself also has limitations. The model linearizes the cue–response relationship, while the underlying biochemical processes are inherently nonlinear. PLSR models are also correlative and nonmechanistic, thus having the potential to fail as conditions deviate from those used to generate the model. The whole-cell measurements used here also reflect the challenge of studying the intracellular signaling cascades underlying cell migration. The concentration of active proteins within the migration machinery, e.g., the lamellipodium, may be significantly higher than in the cell body, and thus masked by the heterogeneity within the cell itself when using population level measurements. In addition, the variances associated with measuring Rac and Src activities were high compared to the change in their means following stimulation. The early Akt activation peak may have been more prominent in the model simply due to the strength and reliability of the signal. This illustrates an important limitation in the large-scale study of protein signaling networks. The need for robust measurement techniques and methods to ascertain spatially restricted activation of signaling intermediates certainly represent bottlenecks to deciphering network structure. In particular, measurement of GTP bound to small GTPases remains a challenge.

The results with the Akt inhibitor LY294002 demonstrate that early Akt activation is correlated with the rate of wound closure. However, experiments in our lab have also demonstrated that removal of SIP leads to a rapid decrease in the rate of cell migration (data not shown). Thus, it seems unlikely that the migration of cells at 6 h post-stimulation is solely due to the early Akt peak. Rather, the strength of the stimuli leading to cell migration may be reflected in the height of the early Akt peak. Instantaneous rates of cell migration will likely be dictated by the nearly concurrent levels of activation of some set of factors. The importance of the late Src peak in the PLSR model suggests this may be a good candidate, although a more precise method for the spatial measurement of Src activation would likely aid in exploring this link. However, to accurately predict cell migration under a wide variety of conditions, a mechanistic model of cell migration will likely be required. The development of such a kinetics-based model that takes into account localization of active factors within the migration machinery will be of great importance in advancing our understanding of cell migration. Until that time, as this study has shown, PLSR models may yield not only predictions but also insights on the basis of the relative importance of each factor in the model and the overall correlation of the model.

While the early Akt peak may prove to be useful to predict cell migration only under conditions similar to those explored here, the results do have implications for biomaterials design. If early phospho-Akt peak height proves to be a useful predictor of endothelial cell migration, Akt activation could be measured in cells migrating on materials, sequentially testing different combinations of factors. In this way, a large number of conditions could be screened, saving the tedious measurement of cell migration for the most promising combinations. It is likely that more complex measures will be needed to predict cell migration after stimuli with multiple growth factors or as the density of adhesion molecules on the material changes. It is possible that similar correlative relationships may be found by mathematical techniques such as PLSR. However, the systematic study of the kinetics of signaling cascades leading to migration, while more challenging, may in fact be more efficient than the methods used here.

CONCLUSION

Rac-GTP levels cannot be used as a simple marker of cell migration. This is likely due to the underlying biphasic migration response and the presence of multiple modes of cell migration. Perturbations to inhibitory accessory enzymes that affect Rac, Akt, and Src generally increased the activity of these proteins but did not enhance cell migration and generally hindered the

ability of the cells to respond to S1P stimulation. Without S1P stimulation, cell migration rates were low and unaffected by Rac-GTP levels. However, after S1P stimulation, Rac-GTP levels in the perturbed cells decreased to levels that were appropriate for the observed rate of cell migration. Finally, a model of the rate of wound closure was developed that relied primarily on early levels of active Akt. The model was predictive when developed using only part of the data. Using low levels of a chemical inhibitor, the decrease in cell migration correlated well with the decrease in phospho-Akt at 15 min post-stimulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CSK	C-terminal Src kinase
HAEC-hT	Human aortic endothelial cells immortalized with hTERT
hTERT	Human telomerase reverse transcriptase
PLSR	Partial least squares regression
PTEN	Phosphatase and homolog deleted on chromosome ten
S1P	Sphingosine 1-phosphate
VIP	Variable importance to projection

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FIGURE 1.

Perturbations increase basal levels of Rac-GTP that do not further increase with S1P stimulation. HAEC-hT transduced with RNAi targeting Luciferase (black), PTEN (white), CSK (light gray), or β 2-chimaerin (dark gray) were serum starved for 2 h and lysed or stimulated by wounding or wounding + 1 μ M S1P. Normalized Rac-GTP concentration at 120 min post-wounding. * $p < 0.05$ vs. control (shLuciferase) cells. Data are presented as mean $\pm$ SEM.

FIGURE 2.

Rac-GTP levels in perturbed cells stay level or decrease after wounding $\pm 1 \mu\text{M}$ S1P. The kinetics of Rac activation are shown for shLuciferase, shPTEN, shCSK, and sh $\beta 2$ -chimaerin cells after wounding (squares, solid line) or wounding + $1 \mu\text{M}$ S1P (circles, dotted line). The fold increase in Rac-GTP concentration was measured by Rac GLISA in serum-starved cells and at five timepoints post-stimulation (5, 15, 30, 60, and 120 min). * $p < 0.05$ vs. serum-starved cells. † $p < 0.05$ vs. wound only at that timepoint.

FIGURE 3.

Scrape wound healing migration rate diminishes over time in perturbed cells. (a) The migration of control (shLuciferase) HAEC-hT into a scratch wound area in the absence (squares, solid line) or presence (circles, dashed line) of 1 μ M S1P was tracked every 2 min with time-lapse microscopy (6 h). The distance traveled by the wound edge was quantified every 30 min using ImageJ. $*p < 0.05$ vs. cells not treated with S1P at that timepoint. (b) The rate of wound closure is significantly increased in control HAEC-hT treated with 1 μ M S1P. The instantaneous rate of wound closure was calculated independently for each experiment by fitting a cubic polynomial to displacement curves and solving for the derivative at each timepoint. $*p < 0.05$ vs. cells not treated with S1P over the course of that hour. (c–e) The rates of wound closure for: (c) PTEN-, (d) CSK-, and (e) β 2-chimaerin-deficient cells are shown in the absence (squares, solid lines) and presence (circles, dashed lines) of 1 μ M S1P. The effects of S1P stimulation appear to diminish with time in the perturbed cells, unlike in the shLuciferase cells shown in (b). In all panels, data are shown as mean $\pm$ standard deviation.

FIGURE 4.

Scrape wound healing migration rates are lower in perturbed cells in the presence of S1P. Serum-starved PTEN-, CSK-, or β 2-chimaerin-deficient endothelial cells were scrape-wounded in the absence or presence of 1 μ M S1P and the extent of re-endothelialization was recorded using time-lapse microscopy. (a) The average rate of wound closure was determined by taking the mean of the instantaneous derivatives calculated in Figs. 3b–3e. The first hour of each experiment was disregarded to allow for cell spreading. (b) The addition of 1 μ M S1P (black bars) increases the total distance traveled by each wound front in all cell types. * p < 0.05 for S1P-stimulated (black bars) vs. unstimulated cells (white bars). ** p < 0.05 vs. control (shLuciferase) cells + 1 μ M S1P.

FIGURE 5.

Rac activity at 120 min post-stimulation is correlated with migration rate in the presence of S1P. In the absence of S1P, the level of active Rac is not predictive of migration. However, upon the addition of 1 μ M S1P (triangles), the Rac-GTP concentration at 120 min post-stimulation highly correlated with migration rate ($r = 0.96$).

FIGURE 6.

Effect of mechanical wounding and S1P stimulation on Rac, Akt, and Src activity. Normalized, time-dependent active protein concentration in control (shLuciferase), PTEN-deficient (shPTEN), CSK-deficient (shCSK), and β 2-chimaerin-deficient (sh β 2-chimaerin) endothelial cells in response to mechanical wounding (diamonds, solid line), wounding + 1 μ M S1P (squares, dotted line), or 1 μ M S1P (circles, solid line). Rac-GTP was measured using ELISA, and phospho-specific antibodies were used to detect active levels of Akt and Src by Western blot. Data are presented as mean $\pm$ SEM for at least three independent replicates of responses at 0, 5, 15, 30, 60, and 120 min post-stimulation.

FIGURE 7.

Partial least squares regression analysis predicts that early Akt activity is an important contributor to migration rate. (a) Experimentally measured rates of migration were successfully predicted ($R^2 = 0.93$) using a PLSR model (“full model”) constructed with mean valued biochemical data (Fig. 7, wound and wound + S1P only) and migration rate (“wound closure rate”, Fig. 4). (b) The full model was validated by constructing “split set” models using mean valued data collected with only three of the four cell types, and then predicting the migration rate of the fourth cell type based upon its biochemical dataset. The predicted migration rate for the “left out” cell type from each model (four in total) is summarized. The dashed lines in (a) and (b) represent perfect correlation. (c) Weight coefficient values of the first latent variable in PLSR models consisting of either eight mean-valued datasets (solid bars) or 600 re-sampled

datasets (white bars) (see “Materials and Methods”). Coefficients for protein activation measurements are shown for all timepoints (0, 5, 15, 30, 60, and 120 min post-stimulation) and total activation (AUC), as measured by the integral of the activation curve. Coefficients are shown with 95% confidence intervals. For both (a) and (b), the horizontal error bars represent standard deviation. For (a), the vertical error bars represent cross-validated standard error of prediction. For (b), the vertical error bars represent jack-knifed 95% confidence intervals.

FIGURE 8.

Alternative PLSR models confirm that early Akt measurements are most highly correlated with migration rates. (a) PLSR model predictions from a reduced-factor model containing only signaling variables with VIP > 1.0 from the full model (see Table 1). (b) The “VIP > 1” model was validated by constructing split set models, as was done in Fig. 8b. (c) PLSR model predictions from a reduced-factor model containing only Akt variables. (d) Correlation between cell migration rates and the magnitude of the phospho-Akt peak at 15 min. For (a–c), the horizontal error bars represent standard deviation. For (a) and (c), the vertical error bars represent cross-validated standard error of prediction. For (b), the vertical error bars represent jack-knifed 95% confidence intervals.

FIGURE 9.

Chemical inhibition of PI3K with low levels of LY294002 demonstrate the correlation between early Akt activation and subsequent cell migration. (a) Increasing concentrations of LY294002 lead to a gradual decrease in phospho-Akt 15 min post-stimulation with 1 μ M SIP. (b) Quantification of results from (a). (c) Scrape wound closure was measured in shLuciferase HAEC-ht. Statistically significant decreases in cell migration were observed with 5 and 10 μ M LY294002 without SIP. In the presence of SIP, statistically significant decreases were found with ≥ 0.5 μ M LY294002. Cell migration in the presence of SIP was well correlated with phosphoAkt at 15 min ($r = 0.93$).

TABLE 1

Individual signaling variables with VIP > 1.0 that contribute the most information to the prediction of endothelial cell wound closure rate.

Biochemical variable	Variable importance to projection (VIP)
p-Akt-S473 (15 min)	2.04
p-Akt-S473 (5 min)	1.81
p-Akt-S473 (AUC)	1.58
p-Akt-S473 (30 min)	1.31
p-Akt-S473 (60 min)	1.14
Rac-GTP (0 min)	1.10
Rac-GTP (15 min)	1.01
p-Src-Y416 (120 min)	1.00