Investigating Chemoattractant-Specific Temporal Activation of mTORC2–PKC Signaling Pathways in Human Breast Epithelial Cells

Abstract

Directed cell migration is regulated by extracellular chemoattractants that activate intracellular signaling pathways controlling cytoskeletal dynamics. The mechanistic target of rapamycin complex 2 (mTORC2) is a key regulator of cell motility and is commonly assessed through AKT phosphorylation at S473. Although mTORC2 activation is typically evaluated by AKT phosphorylation, it remains unclear whether distinct chemoattractants generate unique temporal mTORC2 activation responses and differentially engage downstream PKC pathways.

Human breast epithelial MCF10A cells were stimulated with chemoattractants EGF, IGF-1, or LPA at various times over a 60 minute time interval. Western blotting was used to quantify phosphorylated and total AKT (S473), PKCδ (T505), and PKCζ (T410). EGF induced a sawtooth AKT phosphorylation profile, whereas IGF-1 and LPA produced more sustained activation patterns. Downstream PKC phosphorylation also varied by stimulus: IGF-1 stimulation was associated with early PKCδ phosphorylation, EGF stimulation produced a delayed PKCζ phosphorylation followed by decline below base cell levels.

These findings support a model in which chemoattractant identity is reflected in stimulus-specific temporal mTORC2 signaling dynamics and associated downstream PKC phosphorylation patterns. Understanding how signal timing may contribute to distinguishing extracellular cues may inform broader efforts to regulate cell migration in physiological processes such as wound healing and immune responses, as well as in pathological contexts including cancer metastasis.

Introduction

Directed cell migration is essential for normal physiological processes such as development, wound healing, and immune surveillance. However, dysregulated migration contributes to cancer metastasis, the primary cause of cancer-related mortality. Cells migrate in response to extracellular chemical gradients known as chemoattractants, which activate intracellular signaling pathways that regulate cytoskeletal remodeling and polarity (Liu et al., 2014).

The mechanistic target of rapamycin complex 2 (mTORC2) is a central regulator of cell motility. mTORC2 influences actin organization and cell polarity through phosphorylation of downstream effectors, including AKT and members of the protein kinase C (PKC) family. mTORC2 activity is commonly assessed by measuring phosphorylation of AKT at serine 473 (S473), which serves as a widely accepted readout of complex activation (Sarbassov et al., 2005).

Although mTORC2 is known to regulate migration-associated signaling, most studies focus on overall pathway activation rather than temporal signaling dynamics. It remains unclear whether distinct chemoattractants generate unique time-dependent patterns of mTORC2 activation and whether these dynamics differentially engage downstream PKC pathways. Because multiple chemoattractants converge on shared signaling pathways (Smith et al., 2020), differences in activation timing may serve as an information-encoding mechanism by which cells distinguish extracellular cues within shared signaling networks.

This study examined how three chemoattractants (epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), and lysophosphatidic acid (LPA)) influence the temporal activation of mTORC2 and downstream PKC pathways in human breast epithelial cells. It was hypothesized

that distinct chemoattractants would produce unique time-dependent mTORC2 activation profiles and selectively engage downstream PKC branches. Figure 1 summarizes the simplified signaling framework tested in this study.

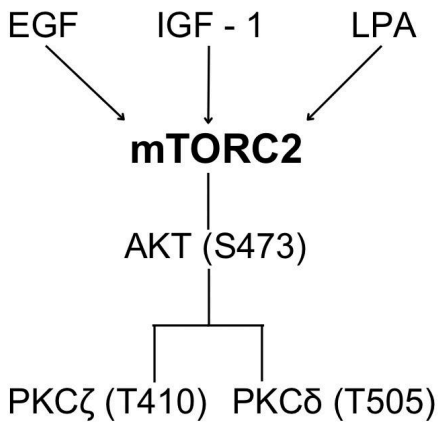

Figure 1. Chemoattractants (EGF, IGF-1, and LPA) were used to stimulate MCF10A cells, and mTORC2 pathway activation was assessed by AKT phosphorylation at S473, with downstream signaling evaluated using PKCδ (T505) and PKCζ (T410) phosphorylation. Figure created by the author using Canva (2026).

Research Question and Rationale

1) Do different chemoattractants activate mTORC2 with distinct timing patterns?

2) Does mTORC2 activate different downstream PKC proteins depending on the stimulus?

Most studies evaluate pathway activation as a static event rather than examining how activation timing varies across stimuli. Because multiple chemoattractants converge on shared signaling pathways, it remains unclear whether cells distinguish extracellular cues through differences in temporal signaling dynamics rather than pathway identity alone.

If different chemoattractants trigger distinct mTORC2–PKC activation profiles over time, cells may be able to tailor migration-related signaling depending on the external cue. Measuring PKC

pathways alongside AKT may reveal stimulus-specific pathway engagement not captured by AKT phosphorylation alone.

Importance and Societal Relevance

Understanding how signaling timing encodes extracellular information may improve insight into how cells regulate directed migration. Cell migration is essential for processes such as immune cell trafficking, wound healing, and embryonic development, and dysregulated migration contributes to pathological conditions including cancer metastasis. Identifying mechanisms by which cells generate stimulus-specific signaling responses may inform broader efforts to modulate migration-related behaviors in both physiological and disease contexts. By investigating how mTORC2 signaling dynamics encode chemoattractant identity, this study contributes to broader efforts to understand how signaling timing influences cellular decision-making in complex microenvironments.

Hypotheses / Predicted Outcomes

1. Different chemoattractants will produce unique time-dependent patterns of AKT (S473) phosphorylation
2. Differences in activation timing (not just signal strength) will distinguish between downstream activation of different PKC proteins
3. If activation timing matters, changes over time may help distinguish how cells respond to different chemoattractants

These predictions are based on prior literature suggesting stimulus-specific differences in receptor signaling kinetics and downstream effector utilization.

Materials and Procedure

1. Cell Culture

MCF10A human breast epithelial cells were used as a non-cancerous model system. Cells were maintained under standard culture conditions according to established laboratory protocols.

2. Chemoattractant Stimulation

Cells were stimulated with one of three chemoattractants: (1) Epidermal growth factor (EGF), 200 ng/mL; (2) Insulin-like growth factor 1 (IGF-1), 200 ng/mL; (3) Lysophosphatidic acid (LPA), 5 μM. Cells were harvested at 0, 2, 10, 15, 30, and 60 minutes following stimulation.

3. Protein Extraction and Western Blotting

Cell lysates were collected at each time point. Western blot analysis was performed to assess phosphorylation and total protein levels of: (1) AKT (phospho-S473 and total AKT); (2) PKC δ (phospho-T505 and total PKC δ); (3) PKC ζ (phospho-T410 and total PKC ζ).
Fluorescence-based antibody detection was performed using LiCor and Sapphire imaging systems.

4. Quantification and Normalization

Band intensities were quantified using ImageJ software. Phosphorylated protein levels were normalized to corresponding total protein levels. For graphical representation, time-based curves were normalized to the maximum signal observed within each chemoattractant condition.

5. Replication

AKT phosphorylation experiments were performed with two biological replicates (N = 2). PKC δ and PKC ζ phosphorylation experiments were performed with one biological replicate (N = 1). Due to limited biological replication, statistical analyses were not performed, and results are presented descriptively. The overall experimental workflow is summarized in Figure 2.

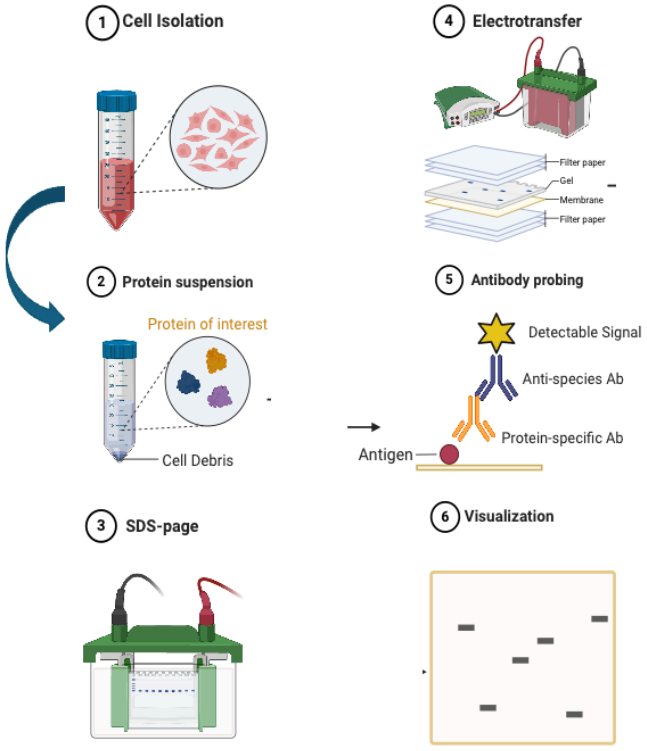

Figure 2. Experimental workflow used to assess mTORC2 pathway activation following chemoattractant stimulation. MCF10A human breast epithelial cells were stimulated with EGF (200 ng/mL), IGF-1 (200 ng/mL), or LPA (5 µM) across a 60 minute time interval. Cells were lysed at defined time points, and phosphorylation of AKT (S473), PKCδ (T505), and PKCζ (T410) was assessed by Western blot. Band intensities were normalized to total protein levels. Figure created by Isaiah Toth using BioRender (2025) based on the findings of the author.

Risk and Safety

This study was conducted under supervised laboratory conditions at the University of Arizona. All cell culture and biochemical procedures were performed in accordance with established laboratory safety protocols.

Immortal human breast epithelial cells and recombinant DNA (rDNA) are classified as Biosafety Level 2 (BSL2). Standard BSL2 precautions were followed, including: use of personal protective equipment (lab coat, gloves, eye protection when needed), proper biological waste disposal (the material is either incubated for 15 minutes in 10% bleach and then discarded or placed in a designated biohazard container for decontamination by autoclaving), and use of biosafety cabinets for cell culture procedures in a designated cell culture room that is kept locked. Chemical reagents used in Western blot procedures, including electrophoresis buffers and antibody solutions, were handled in accordance with institutional chemical safety guidelines and Material Safety Data Sheet (MSDS) recommendations. No human or animal subjects were directly involved in this study. All experimental procedures were conducted under mentor supervision in a regulated research laboratory environment.

Data Analysis and Results

1. mTORC2 Activation Over Time Assessed by AKT (S473) Phosphorylation

Representative Western blots were used for data analysis and were imaged using a chemiluminescent detection system. Phosphorylation of AKT at serine 473 (S473) was used as a readout of mTORC2 activation. Phosphorylation of PKCδ (T505) and PKCζ (T410) was examined as downstream targets of mTORC2 signaling.

EGF stimulation produced a rapid, transient increase in AKT phosphorylation (Figure 3, top two rows, left). In contrast, IGF-1 and LPA produced more sustained activation patterns (Figure 3, top two rows, middle and right).

The blots provide an overall visual representation of protein activation. To enable precise comparison between conditions, band intensity was measured and normalized to total protein levels.

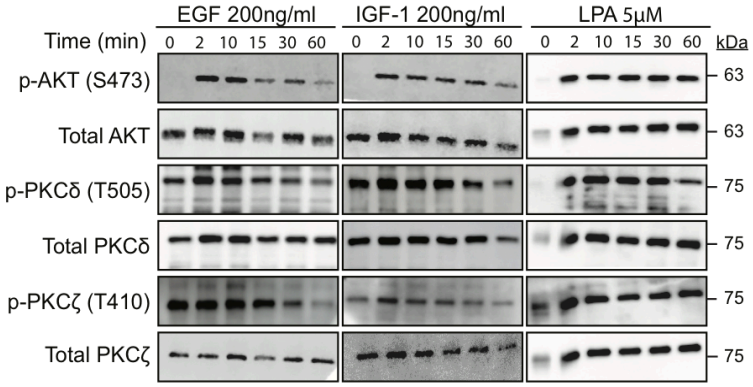

Figure 3. Representative Western blot analysis of AKT (S473) phosphorylation following chemoattractant stimulation. MCF10A cells were stimulated with EGF, IGF-1, or LPA across a 60 minute time interval. Phosphorylated AKT (S473) and total AKT levels were assessed by Western blot. Total AKT served as a loading and normalization control. AKT experiments were performed with N = 2 biological replicates. Figure created by Isaiah Toth using BioRender (2025) based on the findings of the author.

2. mTORC2 Activation Over Time Reveals Stimulus-Specific AKT Dynamics

Phosphorylation of AKT at serine 473 (S473) was used as a readout of mTORC2 activity. Quantitative analysis of p-AKT levels over time revealed distinct activation kinetics depending on the chemoattractant stimulus.

EGF stimulation triggered a rapid and transient increase in p-AKT, characterized by an early peak followed by a progressive decline. In contrast, IGF-1 and LPA produced more sustained p-AKT activation profiles.

Quantitative analysis confirms that mTORC2 activation kinetics are stimulus-specific, with EGF exhibiting transient dynamics and IGF-1 demonstrating sustained signaling.

These findings indicate that while all three chemoattractants activate mTORC2, the temporal dynamics of activation differ between stimuli (Figure 4).

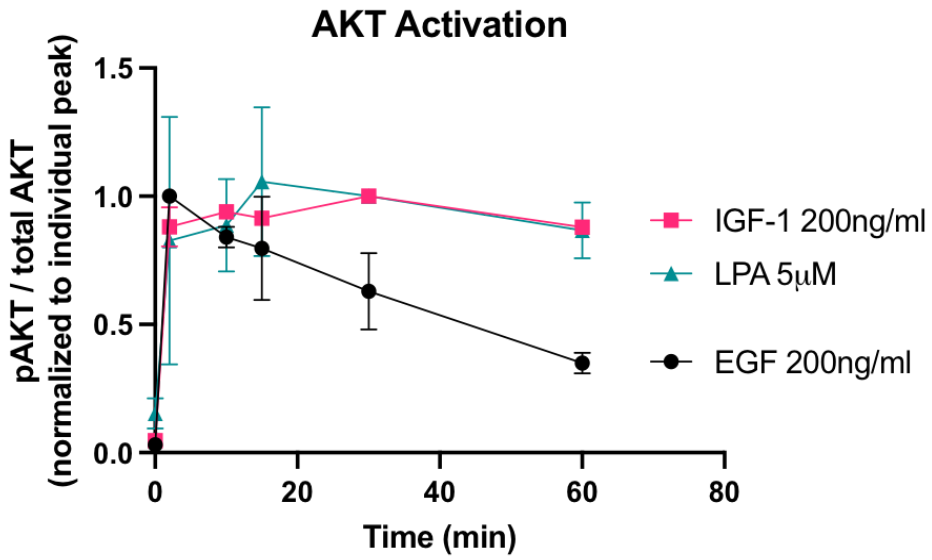

Figure 4. Quantification of AKT (S473) phosphorylation over time following stimulation with EGF, IGF-1, or LPA. Phosphorylation levels were normalized to total AKT and plotted relative to the maximum signal observed within each chemoattractant condition. EGF induced a rapid, transient response, whereas IGF-1 and LPA produced more sustained activation profiles. AKT experiments were performed with N = 2 biological replicates. Figure created by Isaiah Toth using BioRender (2025) based on the findings of the author.

3. Phosphorylation Is Chemoattractant-Specific

Downstream mTORC2 signaling was examined by measuring PKCδ phosphorylation at threonine 505 (T505). Quantitative analysis revealed stimulus-dependent differences in PKCδ activation dynamics. IGF-1 stimulation produced a rapid, early peak in p-PKCδ at approximately 2 minutes that decayed by 15 minutes. In contrast, EGF and LPA also induced early PKCδ phosphorylation by 2 minutes but lacked a distinct activation peak, as the decay was slower and phosphorylation levels remained elevated with respect to baseline throughout the 60-minute interval.

Although all stimuli activated mTORC2, only IGF-1 produced a rapid PKCδ phosphorylation transient (peak) event, indicating stimulus-specific downstream routing rather than uniform pathway activation. These results suggest that PKCδ phosphorylation patterns vary across chemoattractants under the conditions tested (Figure 5).

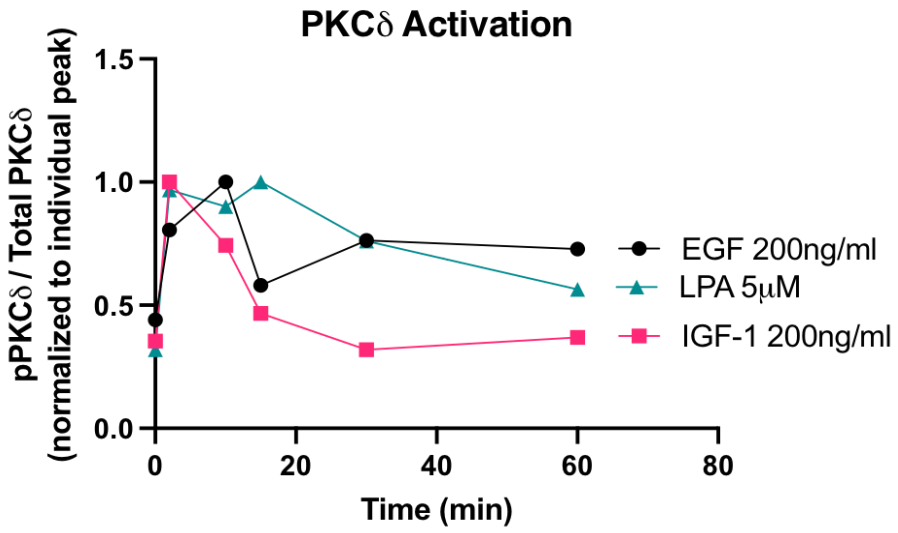

Figure 5. Quantification of PKCδ (T505) phosphorylation over time following stimulation with EGF, IGF-1, or LPA. Phosphorylation levels were normalized to total PKCδ and plotted relative to the maximum signal observed within each condition. IGF-1 stimulation was associated with an early phosphorylation peak. PKCδ experiments were performed with N = 1 biological replication. Figure by Isaiah Toth using BioRender (2025) based on the findings of the author.

4. **PKCζ Displays Distince Temporal Regulation**

PKCζ phosphorylation at threonine 410 (T410) was measured as an additional downstream readout of mTORC2 signaling. Quantitative analysis revealed stimulus-specific temporal regulation of PKCζ phosphorylation.

EGF stimulation caused a delayed response in p-PKCζ during the first 10 minutes, followed by a rapid rise to peak activation at 15 minutes and a decline thereafter. p-PKCζ levels following EGF stimulation fell below the initial (0-minute) level at later time points.

IGF-1 produced a small peak in p-PKCζ at 2 minutes that declined almost as rapidly before returning to the initial (0-minute) level by 30 minutes.

LPA generated maximum phosphorylation of PKCζ at the 0-minute time point. Phosphorylation levels dropped rapidly to below half of maximum by 2 minutes, reached a minimum at 30 minutes, and then showed a slow rise to approximately half of maximum by 60 minutes. PKCζ dephosphorilates in response to LPA activation of mTORC2.

Together with the PKCδ results, these data demonstrate that chemoattractants do not merely alter signaling magnitude, but selectively route mTORC2 output through distinct PKC pathways in a timing-dependent manner (Figure 6).

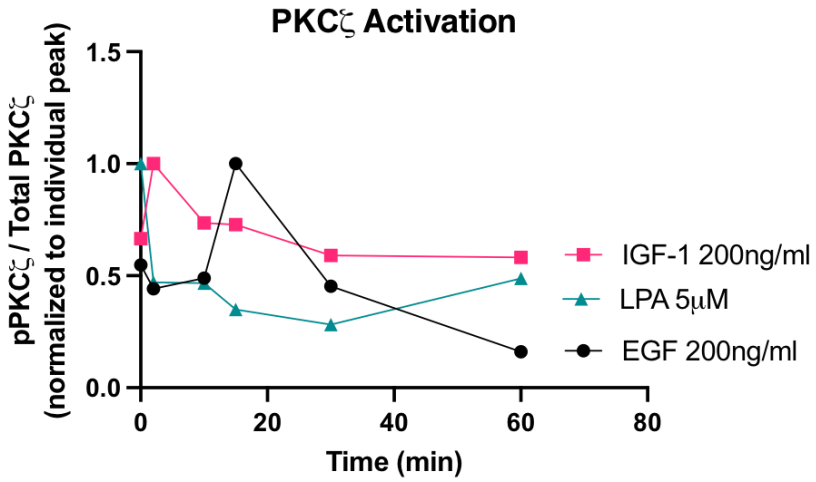

Figure 6. Quantification of PKCζ (T410) phosphorylation over time following stimulation with EGF, IGF-1, or LPA. Phosphorylation levels were normalized to total PKCζ and plotted relative to the maximum signal observed within each condition. EGF stimulation produced a delayed transient increase followed by decline below baseline, whereas IGF-1 and LPA exhibited minimal detectable phosphorylation under these conditions. PKCζ experiments were performed with N = 1 biological replication. Figure by Isaiah Toth using BioRender (2025) based on the findings of the author.

Discussion

This study examined whether distinct chemoattractants generate stimulus-specific temporal patterns of mTORC2 activation and whether these differences are associated with differential downstream PKC pathways engagement in human breast epithelial cells. Although all three chemoattractants tested (EGF, IGF-1, and LPA) activated mTORC2, as measured by AKT S473 phosphorylation, the timing, duration, and nature of activation varied across stimuli.

EGF stimulation produced a rapid and transient activation profile, while IGF-1 and LPA generated more sustained signaling responses. These results address the first research question and indicate that mTORC2 activation is not uniform across extracellular cues, but instead differs in its temporal structure. Because signaling kinetics can influence downstream pathway selection

and cellular responses (Manning & Cantley, 2007), these temporal differences may contribute to stimulus-specific signaling outputs.

Analysis of downstream PKC phosphorylation further revealed stimulus-associated differences. IGF-1 stimulation was associated with early PKCδ phosphorylation. In contrast, EGF stimulation produced detectable PKCζ phosphorylation that subsequently declined below baseline levels. LPA produced sustained AKT activation with minimal detectable PKCδ phosphorylation and dephosphorylation in PKCζ under the conditions tested. Together, these patterns are consistent with differential downstream pathway engagement depending on the chemoattractant. Because these findings are derived from quantitative, time-based phosphorylation time-based analysis, they describe stimulus-associated signaling dynamics rather than direct causal pathway relationships. Overall, the data support a model in which mTORC2 does not function solely as a binary signaling node. Instead, it appears capable of encoding extracellular cue identity through differences in activation timing and downstream routing. Such temporal encoding may provide one mechanism by which shared signaling pathways generate stimulus-specific cellular outputs.

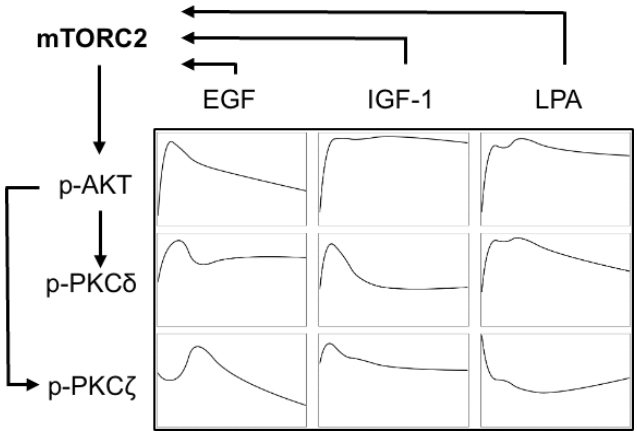

Figure 7. Different stimuli activate the same mTORC2 pathway, but with distinct timing patterns. These temporal differences influence which downstream PKC proteins are engaged, allowing cells to generate stimulus-specific responses. Figure by the author using Mathematica (2026).

Implications

The findings of this study indicate that activation of the same signaling pathway can produce different response patterns depending on the extracellular stimulus. Although mTORC2 was activated by all chemoattractants tested, the temporal characteristics of activation differed across conditions. These results suggest that cells may use signaling timing, rather than signal strength alone, to interpret environmental cues and regulate downstream responses.

Differences in activation timing may influence how cells organize their cytoskeleton and regulate movement. Because cell migration is essential for multiple biological processes, including immune cell trafficking to infection sites, wound healing, embryonic development, and cancer metastasis, variations in signaling dynamics could have meaningful functional consequences.

If signal timing alters how cells interpret external cues, then these differences may affect how persistently or aggressively cells migrate under different conditions.

Conclusion

All tested chemoattractants (EGF, IGF-1, and LPA) activated mTORC2, but with stimulus-specific temporal activation patterns. Although the same signaling pathway was engaged, the timing and duration of mTORC2 activation differed across extracellular cues. These differences were associated with distinct downstream PKC phosphorylation profiles, indicating that activation dynamics bias pathway engagement rather than producing uniform signaling outputs.

Together, these findings support a model in which mTORC2 functions as a temporal signal generator rather than a simple binary ON/OFF switch. Differences in activation timing appear to

influence downstream PKC pathway selection. More broadly, these results suggest that identical signaling pathways can produce distinct cellular outcomes through differences in response timing that direct pathway routing.

Limitations and Future Directions

PKC phosphorylation analyses were performed with limited biological replication (N = 1), and statistical testing was not conducted. Accordingly, PKC findings are presented as descriptive signaling trends within this experimental framework. Conclusions are based on phosphorylation dynamics rather than direct functional migration assays. mTORC2 activity was assessed using AKT (S473) phosphorylation as a proxy readout. Downstream analysis focused specifically on PKCδ and PKCζ, and additional signaling pathways were not examined. Experiments were performed in the MCF10A model under defined experimental conditions.

This study was intentionally designed to characterize temporal signaling dynamics rather than to establish direct functional or mechanistic causality. Future studies incorporating increased biological replication, statistical analysis, mechanistic perturbations (such as PKCδ or PKCζ inhibition or overexpression), and live-cell migration assays will be necessary to determine whether stimulus-specific temporal signals correspond to distinct functional migration behaviors. It will also be important to test whether temporal encoding extends to additional chemoattractants or other cell types.

Acknowledgements

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