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14. ABSTRACT

Successful wound healing requires the recruitment and migration of distinct cell types to the wound followed by re-epithelialization of the surface of injured tissue. Interventions that enhance the migration of effector cells to the site and temporally increase epithelialization can be clinically relevant. Cell attachment and adhesion molecules necessary for cell migration include all four members of the RIPP complex, consisting of the proteins Rsu1, Integrin Linked Kinase (ILK), PINCH, and Parvin. The correct association of these proteins in a functional complex depends on their phosphorylation by serine threonine kinases of the protein kinase C (PKC) family and the process can be enhanced or inhibited by modulating the levels of the RIPP complex proteins as well by regulating their serine and threonine phosphorylation. Our data indicate that the expression of the RIPP proteins is required for migration of human keratinocyte cell line *in vitro*. In addition, the phosphorylation of RIPP proteins contributes to their association and complex formation. For Rsu1- the sites include serine 264 and 268. Formation of the RIPP complex depends on appropriate signals that derive from cell adhesion including from PKC pathway and, hence, the inhibition of PKC blocks Rsu1-PINCH1 association, PINCH1-ILK association, and cell migration. Depletion of RIPP proteins also results in activation of PKA signaling and consequent disruption of regulatory networks impacting integrin function and actin polymerization.

15. SUBJECT TERMS

Wound healing, cell migration, protein kinase C, protein kinase A

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Introduction

The ultimate goal of this work is directed toward improved treatment protocols to accelerate wound healing. The rationale is that clinical interventions that enhance the migration of effector cells to wound sites may hasten epithelialization and are relevant for wound healing. Successful wound healing requires the recruitment and migration of distinct cell types to the wound followed by re-epithelialization of the surface of injured tissue. Interventions that either enhance the migration of effector cells to the site or temporally increase epithelialization are potentially clinically relevant. This study will determine if a complex of proteins required for epithelial cell migration can be manipulated in tissue culture and a mouse wound model to enhance re-epithelialization. The animal studies will employ a gel encapsulated release system in wounds to deliver biomodulators for regulation of specific protein expression in migrating epithelial cells [1].

The cell attachment and adhesion molecules necessary for this cell migration that are the focus of the study includes members of the RIPP complex, an intracellular adhesion complex consisting of the proteins Rsu1, Integrin Linked Kinase (ILK), PINCH, and Parvin [2, 3]. The RIPP complex serves a major intracellular effector complex for integrin-mediated adhesion and migration. ILK binds to the cytoplasmic domain of the beta integrins via its carboxyl terminal domain; the other members of the complex are found in a complex with ILK. The complex is linked to the epidermal growth factor receptor (EGF-R) signaling by members of the Nck family of signaling proteins. This is significant because EGF is a major chemo-regulator of epithelial cell migration in wound healing. In addition, the correct association of these proteins in a functional complex depends on their phosphorylation by serine threonine kinases including those of the protein kinase C (PKC) family, suggesting that they may serve as “druggable” targets affecting the re-epithelialization of wounds [4]. The PKC η isoform has been particularly singled out as a modulator of epithelial cell migration implicating it as the potentially relevant PKC isoform. The above cited study also demonstrated that the depletion of PKC η directly altered epithelial cell migration *in vitro* [5].

The above studies have led to the formulation of the following hypothesis: Epithelial cell migration into wounds can be temporally regulated by the association of Rsu1-ILK-PINCH1-Parvin into a functional complex. This process can be enhanced or inhibited by modulating the levels of the proteins that constitute the RIPP complex as well by regulating their serine and threonine phosphorylation. The topical administration of pharmacological regulators of serine and threonine protein kinase(s) may influence RIPP complex formation locally in wounds.

Body

Task 1. Determination of the RIPP protein phosphorylation profile in HaCAT cells following exposure to chemical inducers of PKC η . The sites of phosphorylation on PINCH1 and Rsu1 proteins with and without PKC activation will be identified by ^{32}P labeling of HaCAT cells and, if feasible, primary human keratinocyte cultures cells. Cells will be treated with specific activator of PKC (phorbol ester) and/or inhibitors of PKC (BIM, calphostin). In addition, cells will be labeled for recovery of PINCH1 and Rsu1 following siRNA-mediated depletion of individual PKC homologs; this approach will detect contributions of all PKC proteins to the phosphorylation events. PINCH1 and Rsu1 will be recovered using specific antibodies for the respective proteins; the detection of phosphorylation and quantitation of phosphate addition will be performed by SDS-PAGE, transfer to PVDF membrane, autoradiography and β -scanning of membrane. The amount of PINCH1 and Rsu1 in the immunoprecipitates will be determined by western blot of the filters. This will establish the specific PKC proteins and their activation pathways leading to phosphorylation of PINCH1 and Rsu1 as well as cell stimulation conditions for Task 2.

Rsu-1 is phosphorylated at consensus PKC sites. Analysis of the predicted amino acid sequence of Rsu-1 identified consensus sites for phosphorylation by PKC, PKA and casein kinase II. The phosphorylation of PINCH1 and Rsu1 proteins with and without PKC activation was performed by ^{32}P labeling of cells and stimulation with specific activator of PKC (phorbol ester) in presence or absence of

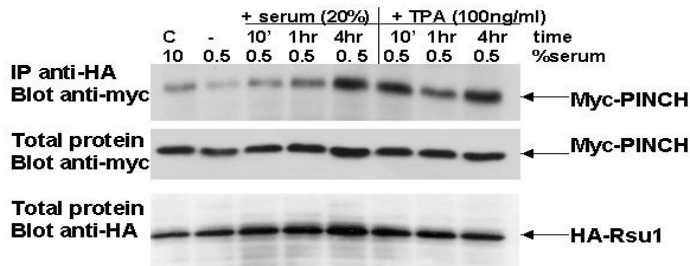


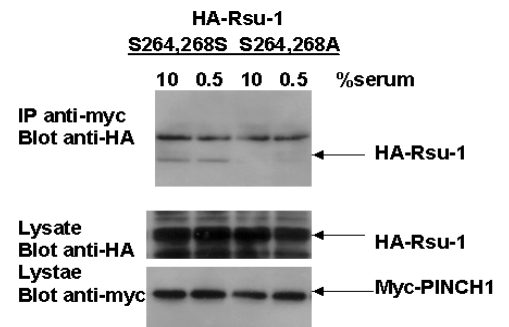
Figure 4. Cells transfected with a HA-Rsu1 and myc-PINCH1 plasmids. 36 hours post transfection the serum concentration was reduced to 0.5% except in the control well. Following 16 hours of serum starvation the cells were stimulated with either 20% serum or TPA. Cell lysates were harvested at 10min, 1 hr and 4 hours post stimulation. The HA-Rsu1 protein was immunoprecipitated with anti HA antibody. The immunoprecipitates (top panel) were analyzed by western blotting with anti myc Ab to detect associated myc-PINCH1 protein. The second and third panels show the amount of myc-PINCH1 or HA-Rsu-1 in 10% of each lysate

Additional studies have demonstrated that mechanical detachment of cells from substrate also blocks the association of Rsu1 and PINCH1. Detachment also inhibits numerous signal transduction pathways including Akt, suggesting that the detachment-induced block of Rsu1-PINCH1 binding may also result from decreased phosphorylation. Detachment results in elevated cAMP due to change in phosphodiesterases (8).

Task 2: The sites of phosphorylation on RIPP proteins PINCH1 and Rsu1 with and without PKC activation were analyzed by mass spectrometry following recovery using specific antibodies. Initial attempts were not successful and the analysis had to be repeated. The identification of specific sites by this method was not reproducible. This suggested to us that additional signal transduction pathways contribute to Rsu1 and PINCH1 phosphorylation. Recent data from informatic analyses indicate that Rsu1 threonine100 is a potential target of PKB and, hence, phosphorylation at this site could enhance binding of PINCH1 to Rsu1. In addition, proteins with highest predicted association to the COOH domain of Rsu1 include SH3 domains of p85 PI-3-kinase and Nck1. These predictions are relevant in light of the ability of ILK or ILK-complexed proteins to regulate the level of Ser473 phosphorylated Akt *in vitro*. While originally mammalian ILK was reported to function as a kinase, recent data demonstrates that ILK is a pseudokinase and adapter protein rather than exhibiting kinase activity per se [6]. A report in 2010 demonstrating that the phosphatase PP1a binds to PINCH1, via a site adjacent to the Rsu1 binding site on PINCH1, proposed that sequestration of PP1a by PINCH1 in the IPP complex inhibits dephosphorylation of Akt resulting in elevated Ser473 phosphorylated Akt [7]. We have constructed mutants in Rsu1 at threonine 100 for analysis of PINCH1 binding activity. Because this is based on newly available information these studies are still underway.

Task 3: Existing PINCH1 and Rsu1 phosphorylation site mutants will be tested, and some additional mutants constructed, for *in vitro* phosphorylation following activation of PKC pathway. This will confirm identities of the specific sites of phosphorylation.

Figure 5. Cells were co-transfected with a myc-PINCH1 plasmid + HA-Rsu1 plasmid, or HA-Rsu1 (S264,268A) plasmid. 36 hours post transfection the cells were incubated in 10% or 0.5% serum concentration. Following 16 hours of serum starvation cells were harvested. The myc-PINCH1 protein was immunoprecipitated with anti myc antibody and subjected to SDS-PAGE and western blotting with anti HA Ab to detect associated HA-Rsu1 protein. The bottom panel shows the amount of myc-PINCH1 and HA-Rsu1 or mutant HA-Rsu1 in each lysate prior to immunoprecipitation.



Plasmids encoding the mutant HA-Rsu1 proteins S4A and S264A/S268A were tested for the ability to co-immunoprecipitate with myc-PINCH in Cos1 cells. The results shown in Figure 5 indicated that

mutation of S264 and S268 blocked the co-precipitation with myc-PINCH1 suggesting that phosphorylation at these sites is required for the association in mammalian cells. Mutation of S4 did not affect co-immunoprecipitation with PINCH1. A recent report based on use of PKC inhibitors indicated that PKC activity is also required for PINCH1 and ILK association.

In light of the difficulties in regulating site specific phosphorylation of Rsu1 and PINCH1 in cultured cells a structural mutant of Rsu1 without ability to bind PINCH1 was constructed. The Rsu1 N91D single amino acid mutation altered the structure of the 2nd leucine rich repeat (LRR) in Rsu1, a region required

for binding to PINCH1. Rsu1 N91D was readily expressed in cells and did not bind to exogenously expressed PINCH1 or the endogenous PINCH1 protein. This mutant has been used to examine dependence of Rsu1 on PINCH1 interaction in migration.

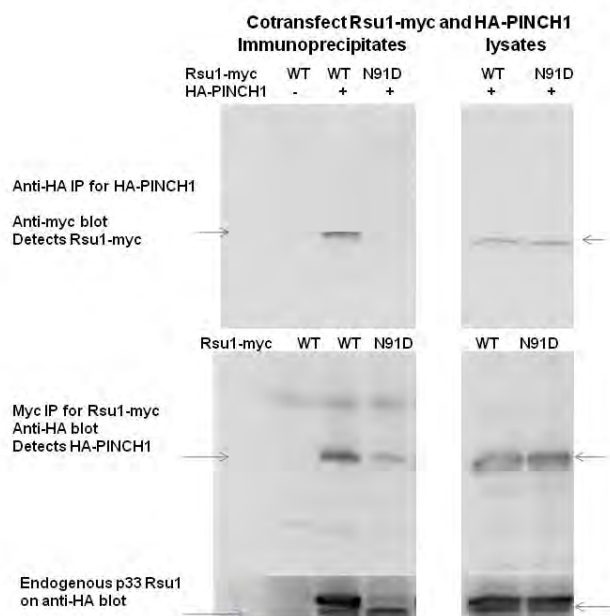


Figure 6. Cells were co-transfected with a HA-PINCH1 plasmid or empty vector + Rsu1-myc WT or N91D plasmid. 72 hours post transfection the cells were harvested. **(top panel)** The HA-PINCH1 protein was immunoprecipitated with anti-HA antibody, subjected to SDS-PAGE and western blotting with anti-myc Ab to detect associated Rsu1-myc protein. **(middle panel)** The Rsu1-myc protein was immunoprecipitated with anti-myc Ab and associated HA-PINCH1 was detected by western blotting. The **bottom panel** shows the amount of Rsu1-myc and endogenous Rsu1 in the HA-PINCH1 IPs. The right hand panels indicate the amount of specific tagged proteins in the cell lysates prior to immunoprecipitation.

Task 4. The effect of PKC activation and RIPP phosphorylation on HaCAT cell and primary human keratinocyte migration. The effect of PKC activation and RIPP phosphorylation on HaCAT cell and primary human keratinocyte migration will be analyzed. The parameters tested will include treatment of cells for PKC η activation and inhibition as well as siRNA-mediated depletion of specific PKC proteins. Depletion of Rsu1 protein inhibits cell migration *in vitro*. Cells were transfected using siRNA for Rsu1, PINCH1, ILK or negative control siRNA. The cells were plated in Oris^R migration plates containing an insert that was removed at 36 hours past migration to allow documentation and quantification of cell migration. The results (Figure 7) indicated that the depletion of PINCH1 or ILK inhibited cell migration in response to EGF. Rsu1 siRNA (Rsu1-3) that depletes all the Rsu1 variants (i.e.p33,p29,p27,p15) blocked migration but Rsu1 siRNA that depletes only full length p33 Rsu-1 does not block migration compared to the negative control siRNA. This identifies a role for Rsu1 alternative splice products in regulating migration *in vitro*. Note that depletion of Rsu1 results in reduction in Rsu1 and PINCH1, and that PINCH1 depletion reduces Rsu1 and ILK as well as PINCH1.

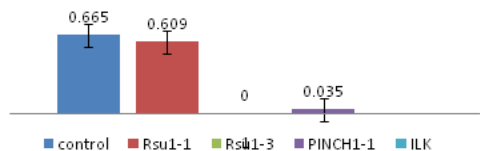
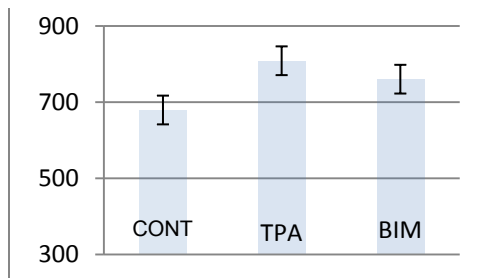


Figure 7. Cells were plated in Oris^R migration plates containing an insert that was removed at 36 hours past migration to allow documentation and quantification of cell migration. Cells were photographed at the beginning of the migration assay and at the conclusion 20 hours later. Quantitation was performed by staining cells and reading absorbance spectrophotometrically using a template to define the field of migration. The results are reported as the mean of four wells for each condition. Error bars represent standard error. T test result: Control:Rsu1-3, PINCH1-1 or ILK siRNAs $p < 0.005$

The effect of enhancement or inhibition of PKC signaling on human keratinocyte migration has been tested *in vitro*. HaCAT cells treated with TPA or inhibitors of PKC (BIM and calphostin C) were plated on Oris^R cell migration plates and the degree to which the cells migrated into the empty surface was recorded after 24 hours.

Distance not filled during 24 hr HaCAT migration



Migration of HaCAT Cells (% closure)

Untreated control	24.4%
TPA (10 ng/ml)	10.1%
BIM (20nM)	15.5%

Figure 8. TPA inhibits HaCAT cell migration. HaCAT cells harvested, exposed to media alone or inhibitor + media and plated in Oris^R migration plates containing a gel insert that dissolved within approximately 2 hours after cells were added to the wells. The insert prevented attachment of cells to a circular area in the center of each well; migration is required for the cells to fill the center. At 24 hours post plating the results of the migration were examined. The cells were fixed, stained and photographed to allow documentation and quantification of cell migration. Quantitation was performed by photographing microscopic images and applying measurement software to calculate distance remaining in the center of the well. The results are reported as the mean of four wells for each condition. Error bars represent standard error. T test: CONT:TPA, $p < 0.005$; CONT:BIM, $p < 0.05$. TPA (phorbol 12-myristate 13-acetate), BIM (bisindole maleimide 20 nM).

The modest changes detected in the experiments above prompted a more complete analysis of signal transduction pathway influence on migration using this assay. The inhibition of PI3-kinase or Mek-Erk signaling blocked migration as did inhibition of Rac or Rho kinase (ROCK), but the inhibition of PKA was most effective in enhancing migration. Moreover, inhibition of PKA or activation of Epac restored the Rsu1-specific defect in migration. Epac 1 and 2 are GEFs for the GTPases Rap1 and Rap2 which

contribute to integrin activation. Epac can be activated by cAMP, which also activates PKA activity, but 8-CPT-2Me-cAMP activates Epac without stimulating PKA.

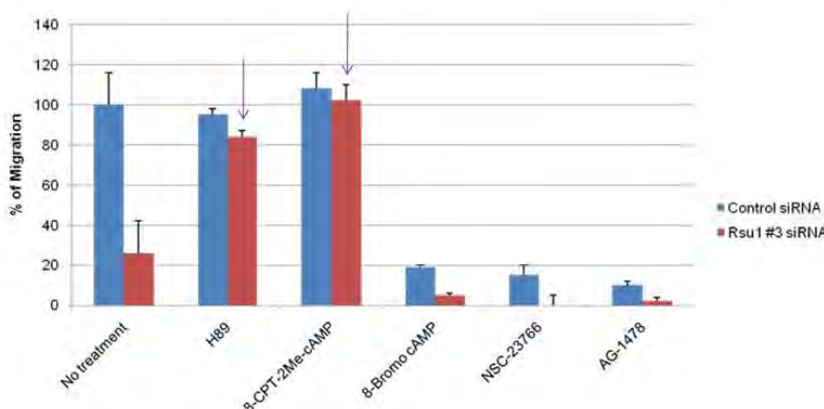
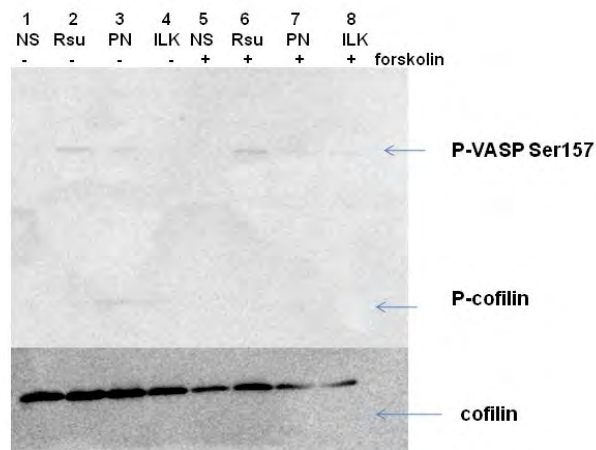


Figure 9. Inhibition of PKA or activation of EPAC enhanced migration in Rsu1 knockdown cells. Cells were plated in Oris^R migration plates containing an insert that was removed at 36 hours post plating to initiate migration and allow documentation and quantification of cell migration. Quantitation was performed by staining cells and reading absorbance spectro-

photometrically using a template to define the field of migration. The results are reported as the mean of four wells for each condition. Inhibitor concentration: H89-100 μ M, 8-Br-cAMP-100 μ M, 8-CPT-2Me-cAMP-100 μ M, NSC-23766-50 μ M, AG-1478-10 μ M. Error bars represent standard error. T test result: Rsu1 KD in No treatment control:H89 or 8-CPT-2Me-cAMP $p < 0.005$.

These findings were supported by analysis of cAMP levels in Rsu1 and PINCH1 knockdown cells as well as the analysis of phosphorylation of PKA targets (figure 10). cAMP levels were elevated in Rsu1- or PINCH1-depleted cells and this corresponded to an increase in phospho-VASP.

Cells were transfected using siRNA for Rsu1, PINCH1, ILK or negative control siRNA. Lysates were analyzed by western blotting for targets of PKA phosphorylation: phospho-VASP(ser157) or phosphor-cofilin in the presence and absence of forskolin to stimulate PKA. As seen in figure 10 cells depleted of Rsu1 and PINCH1 exhibit elevated phospho-VASP(ser157) at a site of PKA and PKC phosphorylation even in the absence of cAMP increase and PKA activation by forskolin treatment. This indicates that



RIPP protein-depleted cells have elevated VASP phosphorylation likely leading to inhibition of actin polymerization. The cause of elevated cAMP level and resulting PKA activation in the knockdown cells is not known at this time. However, this is an ongoing line of inquiry and we are focusing on role of phosphodiesterases as well adenylyl cyclases and GPCRs[8].

Figure 10. Cells were transfected with the indicated siRNA and 96 hrs post transfection were harvested with or without a 15 minutes exposure to forskolin (20 μ M). Blots were reacted with anti-phosphoVASP specific for serine 157, phospho-cofilin or total cofilin. NS-non specific, RSU-Rsu1, PN-PINCH1, ILK-integrin linked kinase.

Task 5. HaCAT cell will be infected with viral vectors encoding wild type and phosphorylation site and phospho-mimetic mutants of PINCH1 and Rsu1 following siRNA-mediated knockdown of the respective endogenous protein to test the effect of the mutants on *in vitro* migration of cells. The results will determine the role of PINCH1 and Rsu1 phosphorylation on migration and will identify the mutants of PINCH1 and Rsu1 to be used in *in vivo* wound healing studies.

Migration experiments: Cells were seeded in migration plates. Following removal of the central plug the migration of cells into central area of the wells migration was monitored by quantitative microscopic (light and fluorescent) imaging. Both PKC η activation or inhibition as well as siRNA-mediated depletion of specific PKC proteins resulted in only modest changes in migration.

The lentiviral vectors expressing Rsu1 and mutants were constructed and the replication defective virus was purified. Cells were infected with viral vectors encoding wild type and phosphorylation site and phospho-mimetic mutants of Rsu1 following siRNA-mediated knockdown of the respective endogenous protein. Rsu1 phosphorylation had only a moderate effect on migration *in vitro*. However, expression of Rsu1-N91D in a Rsu1-depleted cell background restored migration to a similar degree as the WT Rsu1 suggesting that an Rsu1 function independent of PINCH1 binding can contribute to this process.

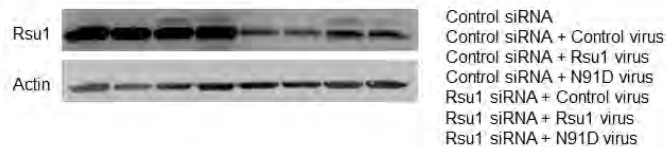
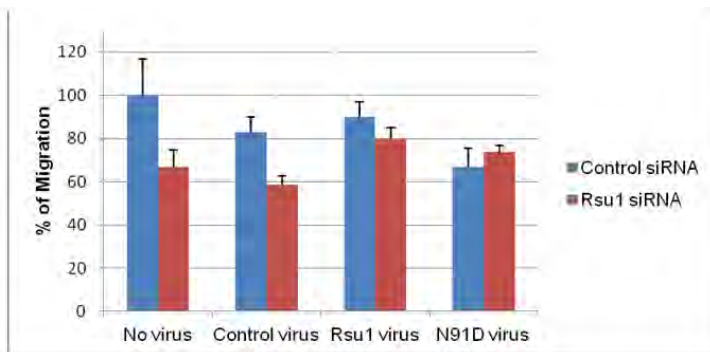


Figure 11. Rsu1 binding to the IPP complex is not required for cell migration. Control or Rsu1-depleted cells were plated in Oris^R migration plates containing an insert that was removed at 36 hours post lentivirus infection to initiate migration.

Quantitation was performed spectrophotometrically 24 hrs later using a template to define the field of migration. The results are reported as the mean of four wells for each condition. Error bars represent standard error. T test result: Rsu1 KD infected with control virus:Rsu1 or Rsu1N91D virus $p < 0.05$.

Control siRNA –non-specific siRNA. Control virus is empty vector.

Task 6. Modulating the specific levels and the phosphorylation of RIPP complex proteins in an *in vivo* model of wound healing. Mouse punch wounds will be exposed to 1) gel-encased siRNA (or morpholinos) to deplete or 2) viral vectors to elevate wildtype and phosphomimetic mutants of the PINCH1 and Rsu1 proteins and the effect on temporal wound closure will be assessed by image analysis of wound.

The studies on the effect of modulating PKC η and RIPP protein have not detected any effect. There are several potential contributing factors. The most important is the delivery method for the morpholinos. The plan to introduce these molecules into wound sites using plurotic gel was based on a study demonstrating the efficacy of this approach following inhibition of osteopontin expression (1). However, in the following three years since this study was published no additional reports of successful use of this technique for external wound healing have been published. While publications citing use of plurotic gel at several internal sites have been reported, it does not appear to be reproducibly effective for the introduction of nucleic acid compounds into mouse punch wounds. However, animal experiments that may produce a better design of delivery methods are ongoing.

Key Accomplishments:

- Rsu1 is phosphorylated in response to activation of PKC at consensus PKC sites.
- The association of Rsu1 and PINCH1 requires phosphorylation at consensus Protein Kinase C sites.
- Inhibitor of cell adhesion blocks Rsu1 and PINCH1 association.
- Depletion of Rsu1, PINCH1 or ILK inhibits cell migration *in vitro* and restoration of Rsu1 in Rsu1-depleted cells re-establishes migratory capability.
- Disruption of PKC signaling inhibits migration of HaCAT cells.
- Inhibition of PKA activity enhances migration.
- Activation of Epac enhances migration.
- Depletion of Rsu1 or PINCH1 elevates cAMP levels and enhances PKA activity.

Reportable Outcomes:

- "*Mammary Epithelial Cell Changes in Adhesion and Migration by Alteration of RSU1 Expression*" Reyda Gonzalez-Nieves, John Buckingham, ML Cutler. Abstract Annual Meeting, American Association for Cell Biology Meeting, December 2009, San Diego, CA.
- "*Regulation of Cell Migration in Mammary Epithelial Cells by Rsu1 and the IPP complex*". Reyda González-Nieves, Akiko DeSantis, Mary Lou Cutler. Poster presentation at Era of Hope Meeting in Orlando, Florida, August 2011.

Conclusions: The expression of the RIPP proteins is required for migration of human keratinocyte cell line *in vitro*. In addition, the phosphorylation of RIPP proteins contributes to their association and complex formation. For Rsu1- the sites include serine 264 and 268. Formation of the RIPP complex depends on appropriate signals including those that derive from cell adhesion. These signals appear to include those from PKC pathway and, hence, the inhibition of PKC blocks Rsu1-PINCH1 association, PINCH1-ILK association, and cell migration. The RIPP complex stabilizes cell adhesion. In the absence of a stable RIPP complex cell detachment occurs, a process accompanied by elevation of cAMP and activation of PKA. Detachment is reversed and restoration of migratory activity occurs by inhibition of PKA or by direct stimulation of Epac. The effects of depletion of Rsu1, a member of the RIPP complex, can be reversed by expression of viral-encoded versions of the protein including a mutant that does not bind to PINCH1, the RIPP partner of Rsu1. This suggests that the RIPP complex participates not only in the physical linkage of integrins to actin but also in regulating the signaling events that control adhesion and migration.

The potential for this information to enhance understanding of the signaling requirements for *in vivo* migration is significant.

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APPENDIX

Item 1.

Primers used for site-directed mutagenesis of Rsu1 consensus phosphorylation sites:

S4A: 5' CAGATTACGCTGGTTCCAAGGCACTGAAGAAGCTGGTG
3' CACCAGCTTCTTCAGTGCCTTGAACCAAGCGTAATCTG

S23G: 5' GGAAGTGGACATGGGTGACAGGGGTATCTCC
3' GGAGATACCCCTGTCACCCATGTCCACTTCC

T135A: 5' GGAAACTTCTTCTACCTCACCGCCCTGGCACTCTATCTAAGC
3' GCTTAGATAGAGTGCACGCAGGGCGGTGAGGTAGAAGAAGTTTCC.

S 163G: 5' GTTGCAGATACTCGGCCTCAGGGATAATGACC
3' GGTCATTATCCCTGAGGCCGAGTATCTGCAAC

T241A: 5' GCTTACAAGTACCTCTACGGCAGACACATGCAAGCGAAC
3' GTGTCTGCCGTAGAGGTAAGCTTCTGAACGAA

S264A / S268G:
5' AAACCAAAAAGATCGGCCGAAACCCCTAGCA
3' CGGCCGATCTTTTTTGGTTTGTGCTTATTCTTCTTTGG