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The Role of Rac1 Signaling in Tissue Polarity and Branching Morphogenesis During Salivary Gland Development

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The role of Rac1 signaling in tissue polarity and branching morphogenesis during salivary gland development.

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ABSTRACT

Salivary gland development in embryonic mouse occurs through the process of branching morphogenesis and differentiation to ultimately give rise to a fully functional, saliva-secreting adult gland. During branching morphogenesis, epithelial cells establish an apical and basal pole which determines the ultimate direction in which salivary fluid and proteins are secreted. Exactly how salivary tissue polarity is determined on a cellular and glandular level is not fully understood. Rac1, a member of the Rho GTPase family, is an intracellular signal transducer that has been shown in other cell types to regulate cell polarization. Rac1 is also important for the organization of apical adherens junction (e.g. E-cadherin) and tight junction (ZO-1, claudin-3) proteins. I hypothesized that Rac1 and its upstream activator, Tiam1, are required for salivary gland polarization. Previous data from our laboratory showed that chemical inhibitors of Rac1, NSC23766 and EHT1864, significantly inhibited growth and branching morphogenesis of embryonic E13 mouse submandibular salivary glands. Further, Rac1 inhibition also resulted in mislocalization of basement membrane matrix proteins to the apical side along with mislocalization of apical proteins, PAR3 and PAR6, to the basal side, hinting at a role for Rac1 during salivary gland branching morphogenesis and polarization. In this study, I found that inappropriate deposition of basement membrane proteins to the apical side was not due to aberrant endocytic vesicle trafficking upon Rac1 inhibition. I also confirmed that mislocalization and decreased expression of PAR proteins 3 and 6 and PKC zeta occurred with Rac1 inhibition, implicating Rac1 in mediating polarity since Rac1 inhibition deregulated the PAR, the master polarity regulator proteins. Further, I showed that the Rac1-specific guanine exchange factor (GEF), Tiam1, previously shown to be required for salivary gland branching morphogenesis, was not only strongly expressed by salivary gland cells, and localized basally, but it also decreased

with Rac1 inhibition suggesting a positive feedback loop with Rac1. The results of my study provide novel functions for Rac1 GTPase in the development and polarization of salivary glands and will eventually help us to understand the role of Rac1 in salivary gland disease conditions such as Sjögren's syndrome in which Rac1 expression is known to be deregulated.

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Sharon Sequeira, for all her help and guidance throughout my research experience and during the writing of this thesis. I would also like to thank my advisor, Dr. Melinda Larsen, for accepting me to her lab and her encouragement and support. Finally, I would like to thank everyone in the Larsen lab who made the working environment very comfortable and enlightening.

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The role of Rac1 in tissue polarity during salivary gland development.

LIST OF ABBREVIATIONS

AJ - adherens junction

BM - basement membrane

E - embryonic day

ECM - extracellular matrix

GAP - GTPase -activating protein

GEF - guanine exchange factor

HSPG - heparan sulfate proteoglycan

PAO - phenylarsine oxide

PBS - phosphate buffered saline

SMG - submandibular salivary glands

Tiam1 - T-lymphoma invasion and metastasis 1

TJ - tight junction

MATERIALS AND METHODS

Materials

Antibodies used were anti-Tiam1(Santa Cruz Biotechnology, sc-872), anti ZO-1 (Invitrogen, 33-9100), anti-claudin-3 (Invitrogen, 34-1700), anti-E-cadherin (BD Biosciences, 610182), anti-laminin-111 (Invitrogen), anti-collagen IV-(Millipore, AB756P), anti-PARD3 (Abcam, ab4840), anti-PAR6 (Abcam, ab45394), anti-integrin α6 (BD Biosciences, 555734), anti-PKC zeta (Santacruz Biotechnology, sc-7262), anti-PAR1b (Santa Cruz Biotech, sc-46607), DAPI and Rhodamine-phalloidin was from Molecular Probes. Phenylarsine oxide (PAO) was obtained from Invitrogen and (dissolved in DMSO). NSC23766 (Calbiochem) and EHT1864 (Sigma) were dissolved in DMEM-F12 phenol-red free media at a stock concentration of 10 mM.

Cell lines

SIMS, Sca-9, (adult mouse submandibular salivary gland ductal epithelial cells) and SMGC10 (adult rat submandibular salivary gland acinar epithelial cells) were cultured as previously described (Daley et al., 2009; Laoide et al., 1996; Quissell et al., 1997). All cells were grown at 37˚C in a humidified incubator in 95% air/5% CO_2.

Embryonic submandibular salivary gland culture

The day of discovery of a vaginal plug was determined as embryonic day 0 (E0). Timed pregnant adult female CD-1 mice (Charles River) were euthanized following protocols approved by the UAlbany IACUC committee, dissected, and their embryos are harvested at embryonic day 13 (E13). The submandibular salivary glands (SMGs) were removed from the embryos with a sterile

scalpel and fine forceps under a dissecting microscope. Whole SMGs were cultured as previously described (Daley et al., 2009). The SMGs were placed on porous Nucleopore Track-Etch membranes (0.1 μm) floating on 200 μl of DMEM/F12 medium supplemented with 150 μg/ml Vitamin C, 50 μg/ml Transferrin and 1X penicillin/streptomycin in 50 mm Mattek glass-bottom microwell dishes. Where indicated in the figures, Rac1 pharmaceutical inhibitors NSC23766 (25-100 μm) and EHT1864 (5-10 μm) were dissolved in DMEM:F12 media and added directly to the media under the glands. All plates were incubated at 37˚C in a humidified incubator in 95% air/5% CO_2 for the indicated time points. Brightfield images were captured using a Nikon TS-100 microscope with a digital camera at 4x magnification at the initial time point (2 hours) and every 24 hours thereafter.

Immunofluorescence staining and Confocal microscopy

Immunostaining was performed essentially as described (Larsen et al., 2003). SMGs or cells were prepared for immunostaining by fixation in 4% paraformaldehyde with 5% sucrose in 1 x phosphate buffered saline (PBS) for 20-30 minutes, washed in 1x PBS, 0.5% Tween-20 (1x PBS-Tween), then permeabilized with 0.1% Triton X-100 in 1x PBS for 15-20 minutes, washed in 1x PBS-Tween, and finally, blocked with 20% donkey serum in PBS-Tween containing 1 drop of M.O.M. blocking reagent (Vector Laboratories) for at least 1 hour. The SMGs or cells were incubated with primary antibodies (1:100) in a 200μl volume overnight at 4˚C with gentle shaking. The samples were washed 4x for 10 minutes in 1x PBS-Tween. They were then incubated with cyanine dye-conjugated secondary antibodies (1:200) for 2 hours at room temperature in 200 μl volumes in the dark with gentle shaking. Nuclei were stained with DAPI added to the secondary Ab solution at a dilution of 1:5,000. Actin was stained with Rhodamine-

phalloidin added to the secondary antibody solution at a dilution of 1:300. The samples were washed 4x for 10 minutes in 1x PBS-Tween. SMGs were mounted on glass slides with 70 μm Secure-Seal imaging spacers in 35 μL of mounting solution (Biomedia Gel Mount/PPD at a 1:100 ratio) and imaged on a Leica SP5 confocal microscope at 20X or 63X magnification. All confocal imaging was done by Dr. Sharon Sequeira from the Larsen laboratory. All confocal images within a given experiment were captured using the same laser intensity settings and the same gain settings so that intensities of each signal could be compared.

Western blot analysis

SMGs or cells were lysed by adding ice-cold RIPA buffer (including complete protease inhibitor cocktail and phos-STOP phosphatase inhibitor cocktail, Roche) (~10 μL/gland) on ice, vortexing every 5 minutes for 20 minutes and sonicating briefly with a probe sonicator. Cell debris was cleared by centrifugation at high speed (16,000 g) for 20 minutes. Protein concentration of the supernatants was assayed using 2-3 μL of obtained protein lysates using a Micro BCA assay kit (Pierce). Approximately 3-10 μg of protein was loaded per lane on 4-20% NuPAGE Bis-Tris SDS-PAGE Gels (Invitrogen). Proteins on the gels were electrophoresed for 1 hour and 30 minutes at a constant 150 volts, transferred to a PVDF membrane for 1 hour and 30 minutes at 30 volts on ice, blocked in 5% non-fat dry milk in TBS-Tween, washed (4X for 15 mins) then incubated in primary antibodies (1:500 - 1:1000 in 1% milk-TBS-T) overnight at 4˚C on a rotary shaker. Blots were washed the next day (4X for 15 mins), incubated in HRP-conjugated secondary antibodies (1:2,000 dilutions in 1% milk-TBS-T) for 2 hours at room temperature on a rotary shaker, then developed with ECL or SuperSignal chemiluminescent dection agent (Pierce) for 3 minutes and imaged on a film developer using X-Ray film. X-rays were scanned and

densitometric quantification of blots was done using ImageJ software (NIH) and graphed using MS-Excel. GAPDH, a house keeping gene, is used as a loading control (Barber et al., 2005). On same PVDF membrane, whole blot would be cut into smaller blots to separate the size of GAPDH and size of protein that was looked for. These sliced membranes were blotted with their specific primary antibodies. Protein expression of protein in question was found by dividing the densitometry of protein in question by that of GAPDH. Statistics performed using GraphPad (Prism 5.0) software.

Chapter 1. The role of Rac1 signaling in branching morphogenesis during salivary gland development.

INTRODUCTION

Salivary gland development

In order for complex ordered structures such as mammalian organs to be formed, many organs undergo the process of branching morphogenesis in which different types of cells and molecular programs have to interact. Several organs like the salivary glands, pancreas, lungs, kidneys and mammary glands initiate branching morphogenesis early during embryonic development (Tucker, 2007). The submandibular salivary gland (SMG) is one of three major salivary glands and is responsible for secreting the majority of salivary fluid produced in mammals. In the developing mouse embryonic submandibular gland (SMG), branching morphogenesis occurs when the oral epithelium thickens and a single bud grows on a stalk into mesenchymal connective tissue at around embryonic day 12 (E12). Clefts, or indentations in the basement membrane, start to form in the epithelial bud resulting in 3-5 epithelial buds at E13 with corresponding ducts. The gland undergoes repeated rounds of clefting and proliferation, duct elongation, and lumen formation until it forms a highly branched structure by E18. Concomitantly with changes in morphogenesis, the SMG also undergoes tissue polarization (development of apical and basal surfaces) and eventually cellular differentiation (saliva protein and fluid secretion). The hollow, single cell layer, secretory end pieces or ‘acini’ of the submandibular gland secrete the fluid and mucus components of saliva, which in humans can equal 1-1.5L per day, along with numerous salivary proteins, antibodies and enzymes. Together, these serve to lubricate the oral cavity, facilitate swallowing, aid in digestion and protect teeth

from dental cavities as well as the oral mucosa from microbial growth and diseases. The acinar secretions are carried into the mouth via tubular interconnected ducts made up of ductal cells which further modify the ionic concentrations of saliva (Patel et al., 2006). These early morphogenetic events are closely recapitulated in ex vivo-grown SMG cultures (**Figure 1**), which can therefore be used to study the molecular and signaling pathways controlling SMG branching morphogenesis.

Rac GTPase expression, signaling, and function

The small GTPase protein, Rac, is a key member of the Rho GTPase family of signaling proteins. There are three different Rac isoforms - Rac1, 2 and 3. Rac1 is ubiquitously expressed and its deficiency results in embryonic lethality in mice by E9.5, even before the salivary glands develop (Sugihara et al., 1998). Lack of Rac2 expression allows normal development but results in hematopoietic cell defects. Rac3 is highly expressed in the brain (Wennerberg and Der, 2004). Microarray data from the salivary gland mRNA database (available at sgmap.nidcr.nih.gov) shows that all three isoforms are expressed early in SMG development with Rac1 mRNA showing highest expression (**Figure 2A**) around E13-E17stages, which is, coincidentally, when SMG morphogenesis occurs. Further, Western blot analysis for total Rac 1/2/3 protein levels at different days of development confirms Rac protein expression in the SMGs (**Figure 2B, C**). Although there is abrupt disappearance of Rac protein expression at E17, this might be due to post transcriptional regulation of the protein. Nevertheless, Rac can be studied in SMGs because it is expressed. Whether Rac functions in mammalian SMG development is currently unknown; however, it was shown that Drosophila larval salivary glands fail to develop normally or migrate posteriorly compared to wild type flies if the Rac1 gene is knocked out (Pirraglia et al., 2006), suggesting that Rac1 plays an important role in salivary gland development.

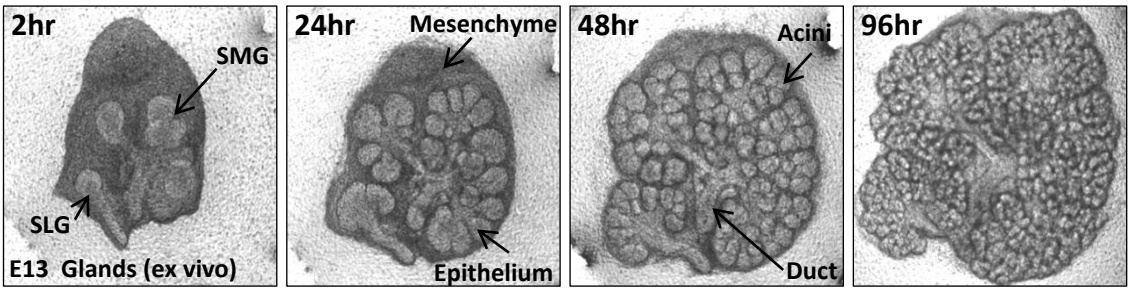

Figure 1. Embryonic mouse salivary glands continue to undergo branching morphogenesis in culture. Brightfield microscopic images of submandibular (SMG) and sublingual (SLG) salivary glands isolated from mouse embryos on embryonic day 13 (E13) and cultured for the indicated times. Glands show significant cell proliferation, bud clefting, and duct elongation during branching morphogenesis. Mesenchyme condenses around the epithelial cells as branching progresses. Epithelial cells eventually differentiate to form acini, which secrete saliva, and ducts that modify and transport the saliva into the oral cavity.

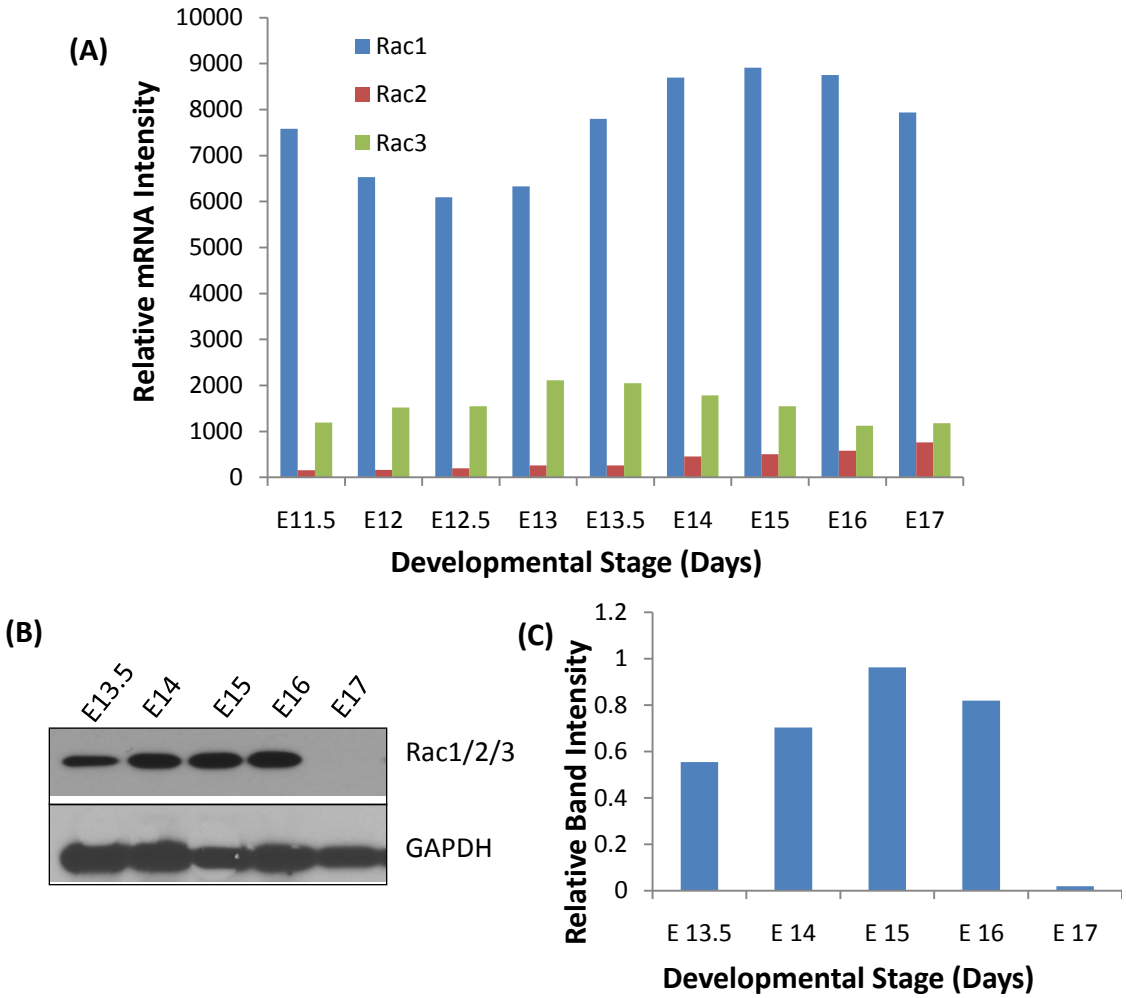

Figure 2. Rac GTPase is abundantly expressed during early SMG development. (A) Graph of Rac mRNA levels from the online salivary gland developmental expression database (http://sgmap.nidcr.nih.gov) shows all three Rac isoforms are expressed in SMG development with Rac1 showing highest expression at E13 -E17 stages. (B) Western blots for total Rac (1/2/3) protein from SMG lysates at different days of development. GAPDH was used as loading control. Panel B was performed by Dr. Sharon Sequeira, Larsen Lab. (C) Densitometric quantification of the Western blot in Figure 2B with Rac normalized to GAPDH.

Rac1 acts as a 'molecular switch' by existing in either an "ON" or activated conformation when it is bound to GTP or an "OFF" or inactivated conformation when it is bound to GDP (**Figure 3**). Activation of Rac is regulated by GEFs (guanine exchange factors) such as the Rac-specific GEF, T-lymphoma invasion and metastasis 1(Tiam1), while GTP hydrolysis and consequently Rac deactivation is promoted by GTPase -activating proteins (GAPs), such as α1-Chimaerin and *β*2-Chimaerin (Caloca et al., 2003; Marland et al., 2011). Rac1 regulates multiple cellular functions including cell-cell and cell-matrix adhesions, actin cytoskeleton organization, transcription, cell proliferation (Bosco et al., 2009), polarity (Iden and Collard, 2008), cell migration (Ridley, 2001), and endocytic trafficking (Ridley, 2006). Interestingly, all of these functions are also a part of branching morphogenesis but whether and how Rac1 may play a role in mammalian salivary branching morphogenesis is not known.

Tiam1 signaling and function

Tiam1 is a Rac-specific GEF which promotes the exchange of GDP for GTP, thus converting Rac1 to its active form. In epithelial cells, Tiam1 stimulates actin nucleation to create lamellipodia and filopodia (Georgiou and Baum, 2010). In keratinocytes, Tiam1 controls polarization of migratory cells (Pegtel et al., 2007). Microarray data from the online salivary gland mRNA database (available at sgmap.nidcr.nih.gov) shows that Tiam1 mRNA is expressed during SMG development (data not shown). Even though Tiam1 contributes to the activity of Rac1and functions in cell migration, polarization, and actin cytoskeleton organization, its function in salivary gland cells is not known. *I therefore hypothesized that Tiam1-mediated activation of Rac1signaling is required for embryonic mouse salivary gland branching morphogenesis.*

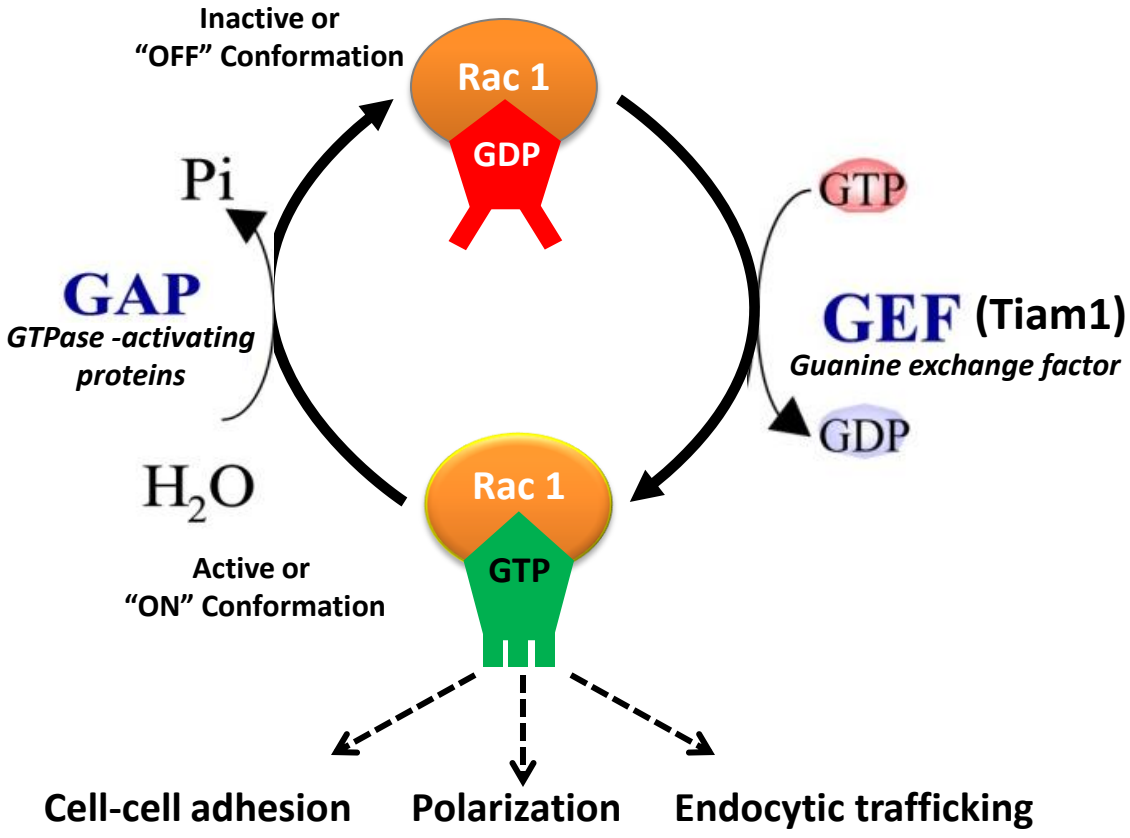

Figure 3. Rac1 GTPase activity is regulated by a 'molecular switch' mechanism. Rac1 is activated or in an "ON" or conformation when it is bound to GTP or an "OFF" or inactivated conformation when it is bound to GDP. Activation of Rac is regulated by GEFs (guanine exchange factors) such as the Rac-specific GEF, Tiam1 (T-lymphoma invasion and metastasis), while GTP hydrolysis and consequently Rac deactivation is promoted by GAPs (GTPase-activating proteins). Activated Rac has been shown in non-salivary cell types to regulate functions such as cell-cell adhesion and polarization and endocytic protein trafficking within cells.

Inhibition of Rac1 and Tiam1 signaling

To study Rac signaling, commercially available, structurally distinct chemical inhibitors of Rac, NSC23766 and EHT1864, were used for the experiments described in this thesis. NSC23766 (Gao et al., 2004) was shown to act by binding to the GTP site of Rac and inhibit GEF interaction thus maintaining Rac in an "OFF" or "inactive" conformation. EHT1864 (Shutes et al., 2007) binds to the GTP binding region of Rac and destabilizes the binding of GTP to Rac, thus inactivating the protein. NSC2377 is Rac1 GTPase-specific whereas EHT1864 not only bind to Rac1 but to other isoforms of Rac. Concentrations to be used for each Rac inhibitors were determined by dose curve done by Dr. Sharon Sequiera. IC50 of NSC23766 was about 50μM and effective dose was known be around 50μM to 100 μM. IC50 of EHT1864 was much lower than NSC23766. EHT1864 is about 10-fold more potent than NSC23766 (Desire et al., 2005). It was previously determined in the lab the concentration of inhibition used throughout the experiment inhibits the Rac without affecting the total levels of Rac expression and without affecting other Rho GTPase family activity. E13 mouse SMGs were isolated and cultured ex vivo in the presence or absence of each of these inhibitors to test the effects of inhibition of Rac signaling on processes involved in SMG branching morphogenesis. Since no chemical inhibitors specific for Tiam1 inhibition are commercially available, we used siRNA techniques to knockdown Tiam1 expression in E13 SMGs and tested the effect on SMG branching.

RESULTS

Pharmaceutical inhibitors of Rac activity block SMG branching morphogenesis

I first tested whether Rac1 activity was required for embryonic SMG branching morphogenesis. E13 glands were treated with two different pharmaceutical Rac-specific inhibitors, NSC23766 and EHT 1864, for 24 hours. For Rac1 to be activated, the catalytic domain of the GEF interacts with Rac1 to facilitate GTP binding to Rac1. These small molecule inhibitors target this interaction specifically, causing Rac1 to remain in its inactive conformation (Gao et al., 2004; Shutes et al., 2007). When the glands were treated with each of the two inhibitors, branching decreased within 24 hours of treatment (**Figure 4A**). Morphometric analysis was performed by counting the number of buds at 2 hours and again at 24 hours and dividing the latter by the former to find the fold change in the number of buds (**Figure 4B**). We found that both inhibitors decreased branching morphogenesis and this was statistically significant ($*p<0.05$, using ANOVA). Thus, I concluded that Rac activation is crucial for branching and overall growth in the embryonic SMGs. These inhibitors were not toxic to the glands but only deactivated Rac for the period of treatment because washout experiments (not shown here) previously performed by Dr. Sequeira indicate that glands initially treated with NSC23766 and EHT1864 with inhibited morphologies expected as in Figure 4, were capable of significant recovery following removal of the inhibitor for an additional 48 hours.

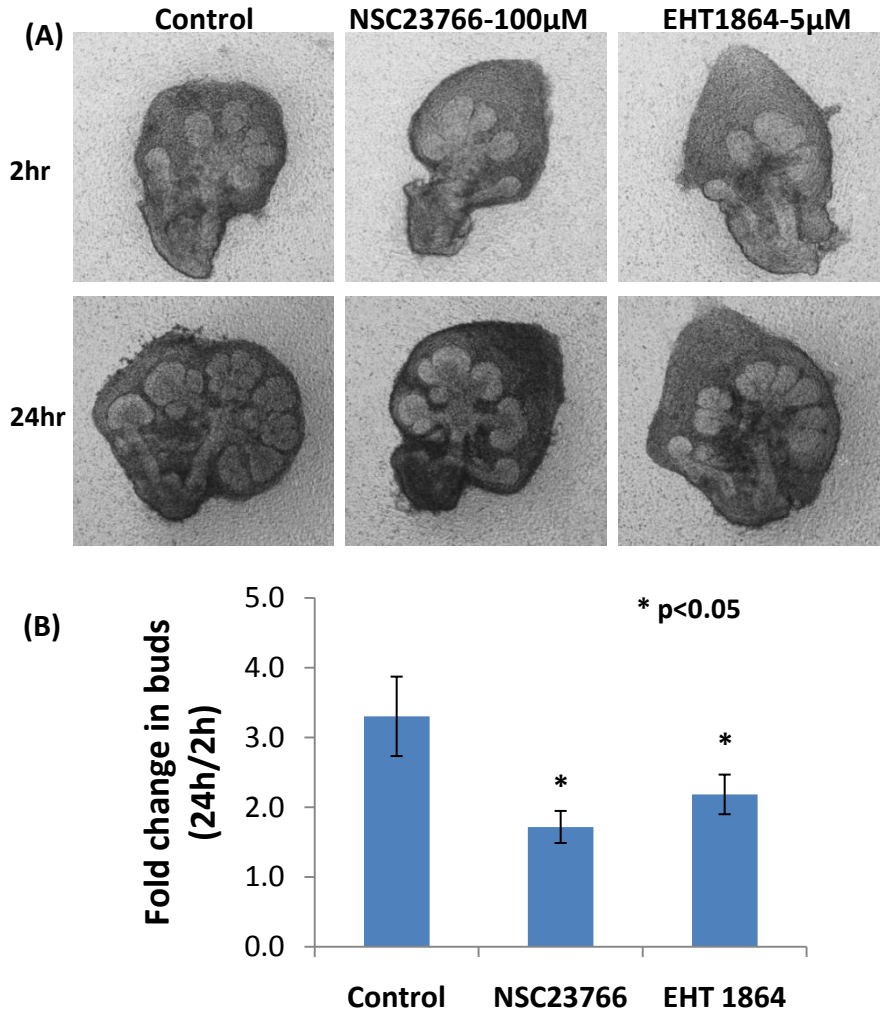

Figure 4. Pharmacological inhibition of Rac1 blocks SMG branching morphogenesis. (A) Brightfield microscopic images of live mock control-treated E13 SMGs or treated with inhibitors NSC23766 and EHT1864 for 24 hours shows decreased branching with inhibitors. (B) Quantification of the number of buds (normalized to 2 hrs) reveals significant decrease in branching in the presence of both Rac1 inhibitors, *p<0.05 using one-way ANOVA, bars are mean SEM of two independent experiments.

Tiam1 is expressed in salivary gland cells and is located basally.

The Rac-specific GEF, Tiam1, can activate Rac and initiate downstream signaling (Munro, 2006). Tiam1 function has not been studied in salivary glands. The online salivary gland mRNA database indicates that Tiam1 mRNA is highly expressed during E11.5 to E16, so we tested expression and localization of Tiam1 protein by immunostaining and confocal imaging in SIMS cells (**Figure 5**) and primary E13 SMGs (**Figure 6**). In SIMS cells and other cells capable of achieving apical-basal polarization, ZO-1 is an apical tight junction protein. Therefore, ZO-1 was used to label the apical membrane to allow evaluation of the location of Tiam1 on either the apical or basal cell surfaces. We found that Tiam1 localized largely in the cytoplasm but the localization was more intense on the basal sides both in the cells (Figure 5) and in the SMGs (Figure 6). In addition, we also observed that Tiam1 staining intensity seemed to decrease with Rac inhibition (Figure 5B, 5C, 6). This was further confirmed using Western blotting analysis for Tiam1 levels (**Figure 7**). Previous data by Dr. Sequeira using siRNA to knockdown Tiam1 showed that SMG branching morphogenesis is significantly decreased when Tiam1 expression is decreased. Therefore, Tiam1 is not only expressed during but is also required for SMG branching morphogenesis. Since Rac1 and Tiam1 are important to branching morphogenesis, their role in other functions, such as cell-cell adhesion and polarization that are also a component of branching morphogenesis, can be tested.

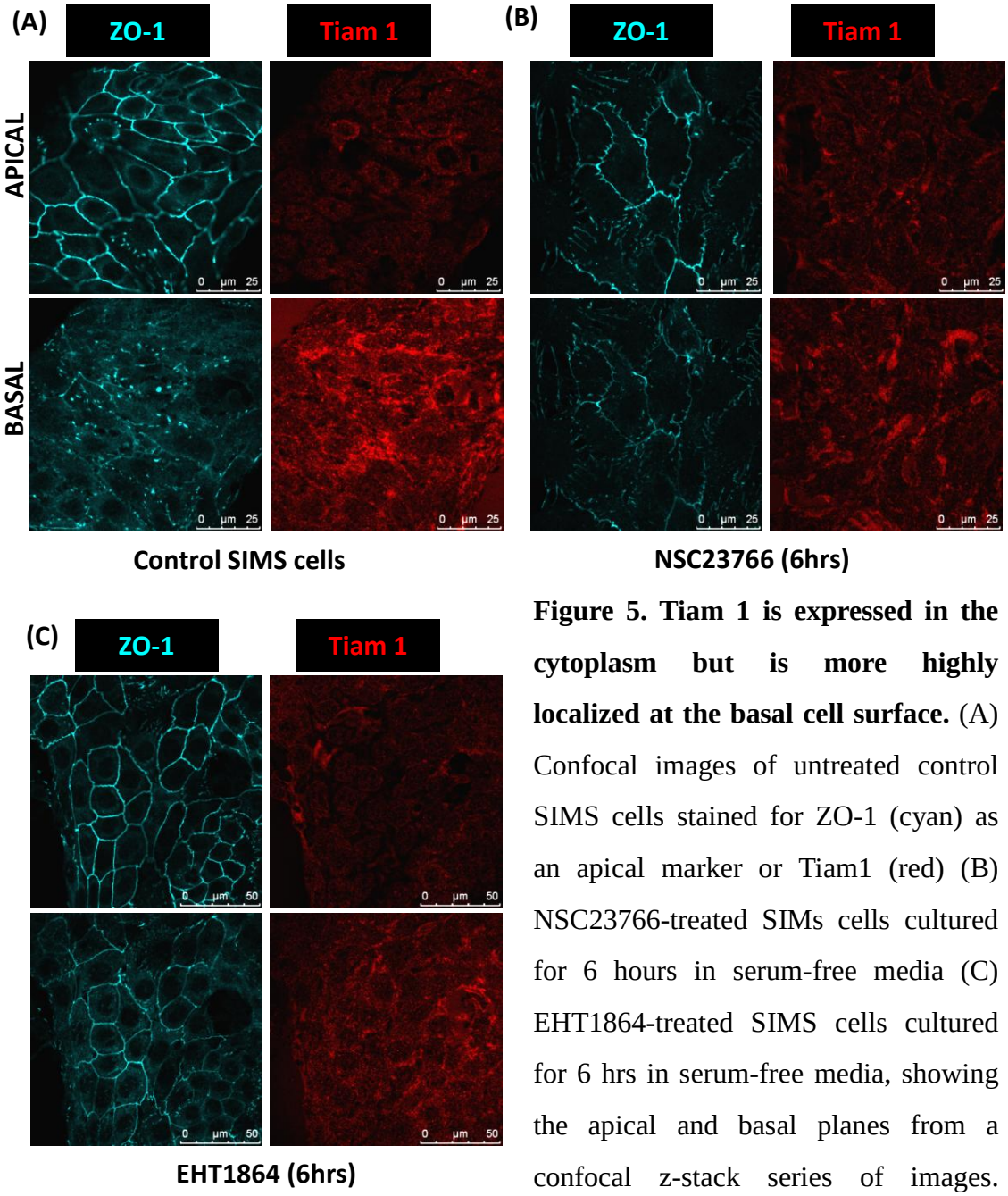

Figure 5. Tiam 1 is expressed in the cytoplasm but is more highly localized at the basal cell surface. (A) Confocal images of untreated control SIMS cells stained for ZO-1 (cyan) as an apical marker or Tiam1 (red) (B) NSC23766-treated SIMs cells cultured for 6 hours in serum-free media (C) EHT1864-treated SIMS cells cultured for 6 hrs in serum-free media, showing the apical and basal planes from a confocal z-stack series of images. Tiam1 is intensely stained at the basal surfaces in control cells and this is decreased in Rac-inhibited cells.

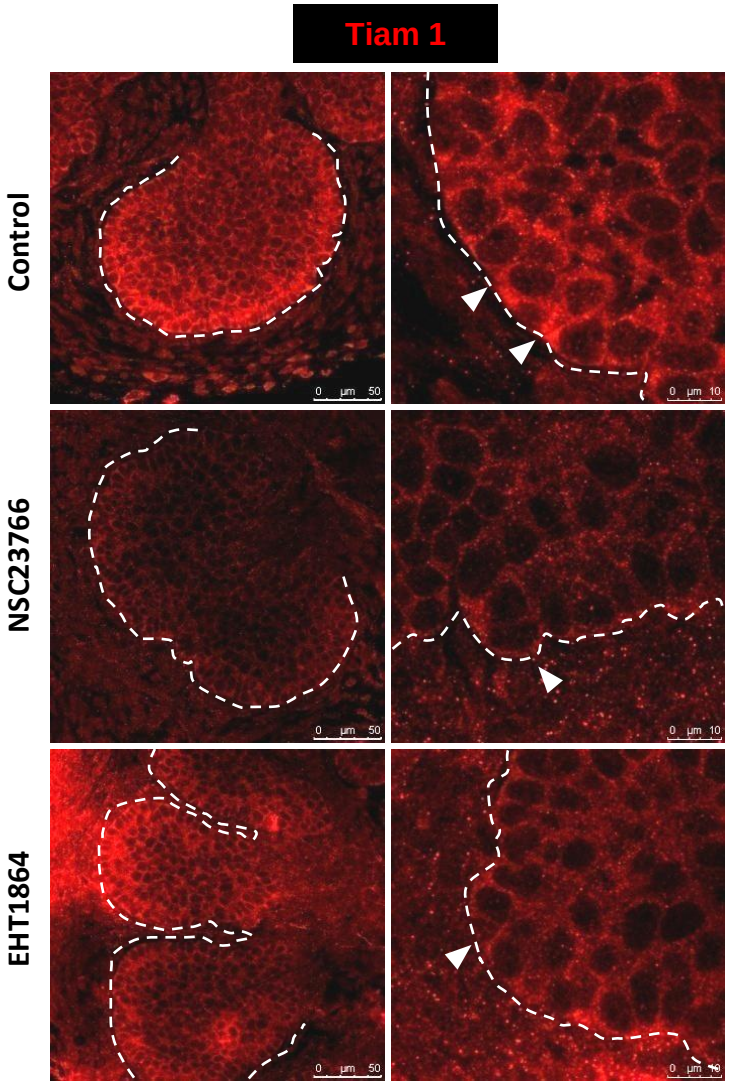

Figure 6. Tiam1 is expressed in SMGs more intensely to the basal cell surface. Confocal images (left, 20x magnification, right, 63x magnification) of 24 hour-inhibitor treated SMGs stained for Tiam1 (red), indicating that Tiam1 is expressed in the cytoplasm and along cell membranes of SMGs and localizes basal side of the gland (arrowheads). Basal and overall expression of Tiam1 is decreased in Rac-inhibited glands. Dashed white line outlines the basal edges of the buds.

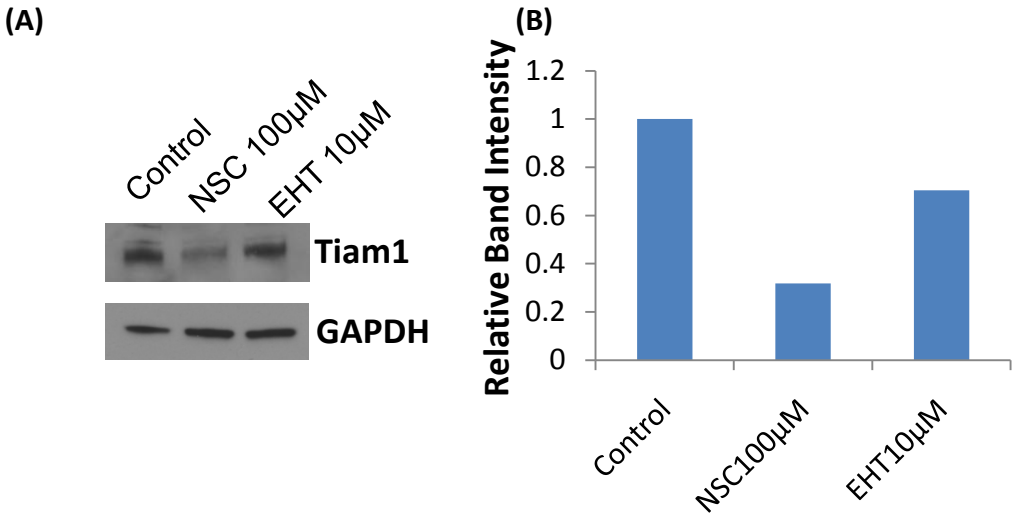

Figure 7. Tiam1 expression decreases upon Rac inhibition. (A) Western blot of control untreated or Rac inhibitor-treated SMG lysates (24 hours) shows the Tiam1 protein band. GAPDH was used as loading control (B) Densitometric quantification of the western blots in (A), normalized to GAPDH levels, shows decreased expression of Tiam1 with Rac inhibition.

DISCUSSION

The mammalian salivary gland attains a highly branched structure by undergoing the process of branching morphogenesis. This process is essential for maximal production and unidirectional secretion of saliva. It is therefore important to study the molecules that mediate salivary gland branching morphogenesis. In this study we found a role for the GTPase Rac1 and its upstream activator, Tiam1, in submandibular salivary gland branching morphogenesis.

Rac1 is required for branching morphogenesis because the glands treated with Rac inhibitors showed a significant decrease in bud growth, consequently affecting branching morphogenesis. In past literature, it has been shown that Rac1 plays a role in many functions that are also a part of branching morphogenesis. Not only is Rac1 important for those functions but also for branching morphogenesis. Therefore, Rac1 may control some of these cellular activities during branching morphogenesis.

Because Tiam1 is a specific upstream activator of Rac, Tiam1 was studied to understand its potential effect on Rac-mediated control of SMG branching. We found that Tiam1 was highly expressed in developing SMGs and localized in the cytoplasm with more intense staining on the basal cell surfaces in both cells and in whole SMGs. In line with our findings, recent studies have suggested that Tiam1 might associate with basal integrins to drive cell motility (O'Toole et al., 2011) which is required for SMG branching (Larsen et al., 2006). Surprisingly, although Tiam1 is a GEF that acts as an upstream activator of Rac, when we inhibited Rac, we found that Tiam1 levels were lowered, suggesting a positive feedback loop from Rac to Tiam1. Thus we speculate that Rac activation might be maintained through this loop given its critical role in SMG branching. Consistent with this hypothesis, we have previously observed that siRNA knockdown

of Tiam1 significantly decreased branching morphogenesis by about 40% (Sequiera, S. and Larsen, M., unpublished data). Taken together, our results suggest a novel and indispensable role for Rac1-Tiam1 mediated signaling in salivary gland branching morphogenesis.

Chapter 2. The role of Rac1 in tissue polarity during salivary gland development.

INTRODUCTION

Salivary gland tissue polarization

In the developing salivary glands, early establishment of apico-basal cell polarity is critical for unidirectional secretion of saliva during later stages of development. Moreover, as a consequence of salivary tissue polarization aid in this process, asymmetric distributions of signaling molecules and cell-cell adhesion molecules occur (Fukata et al., 2003). Apically localized adherens junction (AJs) and tight junction complexes (TJs) are key molecules involved in the establishment of SMG polarization. E-cadherin is AJ proteins and ZO-1 and claudin-3 are TJ proteins among many others. TJs not only serve to maintain a barrier between the apical and basolateral cellular plasma membrane but also regulate paracellular permeability which is maintained by polarized salivary epithelial cells, which is characteristic of an exocrine tissue (Lourenco et al., 2007; Maria et al., 2008; Tran et al., 2005). Salivary epithelial cells also derive important polarization and signaling cues from the basal extracellular matrix (ECM) produced by the mesenchymal cells and can themselves produce, secrete basally, and respond to basement membrane (BM) proteins (a specialized type of extracellular matrix) (**Figure 8**) (O'Brien et al., 2001). The BM matrix layer is composed of laminins, collagen IV, fibronectin and heparan sulfate proteoglycans (HSPGs) (Sequeira et al., 2010), which not only provide signaling cues to direct salivary epithelium but also provide a structural scaffold. BM is important for cleft formation during branching morphogenesis (Daley et al., 2009; Sakai et al., 2003). Importantly, deregulated expression and synthesis of BM proteins is a characteristic feature of salivary gland diseases such Sjogren's syndrome and adenoid cystic carcinomas (Shirasuna et al., 1993).

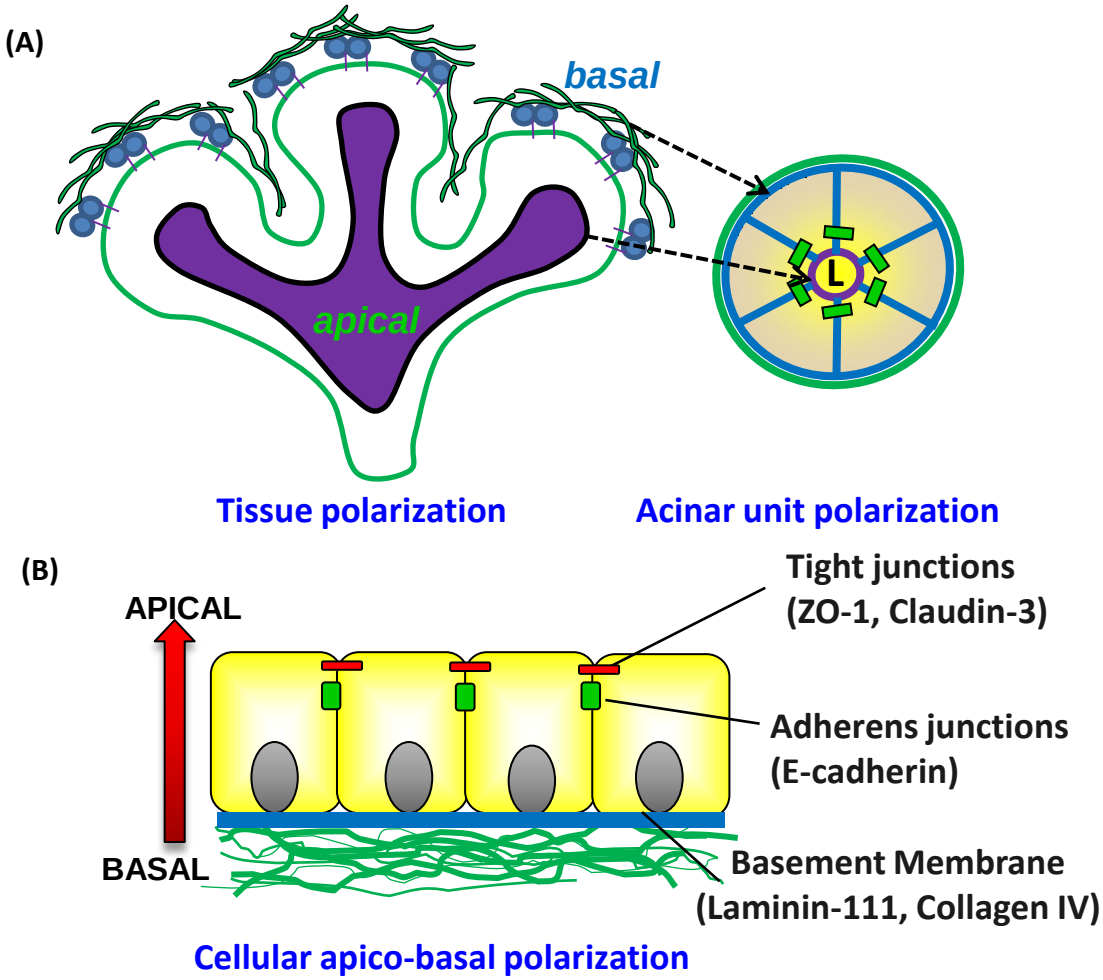

Figure 8. Tissue polarization results in asymmetric apico-basal division of membrane proteins. (A) In the developing salivary gland, there is no hollow lumen, but an apical side is developing in the center of the gland (purple). In the acinar units of developing salivary glands, the cell membrane surface that is facing the central hollow lumen (L) is the apical side and the opposite face is the basal side, adjacent to the basement membrane (green). (B) At the cellular level, tight junctions and adherens junctions proteins are asymmetrically divided, separating the apical side of the cell membrane from the basal side, outside of which basement membrane and extracellular matrix proteins are secreted. The red arrow represents the direction of saliva and protein secretion.

PAR complex proteins are master polarity regulators

It is known that cells can sense spatial cues from the extracellular matrix and activate intracellular Rho GTPase-mediated signaling pathways to propagate polarizing signals (O'Brien et al., 2001). The partition-defective (PAR) family is a group of proteins that are not only essential for asymmetric cell division but also they themselves are asymmetrically localized within the cell (Kemphues et al., 1988). In polarized mammalian epithelial cells, PAR3 and PAR6 are localized to the junctional complexes that divide the apical and basolateral surface, whereas PAR1b localizes to the basolateral surface (Bohm et al., 1997; Cohen et al., 2004). This apico-basal localization function of PAR proteins is required for overall polarization of the cell (Munro, 2006). PAR proteins do not work alone but instead work in complexes. Atypical PKC proteins (aPKC) bind to PAR3 and PAR6 to form a complex that accumulates at opposite poles to PAR1b. This antagonistic interaction between PAR-aPKC systems establishes complementary membrane domains, asymmetric distribution, and asymmetry of cellular functions (Suzuki and Ohno, 2006).

Rac -PAR protein interactions

Recently, connections between PAR complex and Rho GTPase proteins have been emerging. The asymmetric cytoskeletal organization of the apical PAR complex can occur through microtubule organization controlled by T-lymphoma invasion and metastasis (Tiam1) via atypical PKC (aPKC) (Bryant et al, 2008; Malliri et al, 2004). PAR3 can also directly bind the Rac GEF, Tiam1 to regulate cell–cell junctions. PAR proteins thus interact with Rac signaling by first binding to Tiam1. When Tiam1 is recruited to PAR3, this can lead to activation of Rac. The GTP-bound active form of Rac then specifically interacts with PAR6 to activate

aPKC (Munro, 2006). Thus there are reciprocal interactions between the Rac and PAR proteins and between Tiam1 and PAR proteins, suggesting that Rac1 and Tiam1 are important for cell polarization (**Figure 9**).

Previous studies in our laboratory suggested a possible role for Rac1 signaling in mouse embryonic SMG branching morphogenesis and polarization. Chemical inhibitors, NSC23766 and EHT1864, which blocked Rac1 activity, altered apico-basal distribution of apical adherens and tight junction complex proteins and distribution of basement membrane matrix proteins (S. S., and M. L., data not shown).

Based on this preliminary data, *I hypothesized that Rac1 signaling is required for the establishment and maintenance of polarity as well as the apical distribution of adherens junctions, tight junctions, and the basal secretion of basement membrane (BM) matrix proteins.* I further hypothesized that Rac1's role in basal polarity and altered deposition of BM proteins may be due to aberrant endocytosis or interaction with PAR proteins when its activity is inhibited. To test these hypotheses, I proposed to investigate the following: 1) determine if Rac inhibition causes any mislocalization or decreased expression of adherens, tight junctions, and BM matrix proteins, 2) analyze whether Rac inhibition causes aberrant endocytic trafficking of basal BM matrix proteins to the apical surface, and 3) test if Rac inhibition disrupted PAR protein localization and possibly expression in SMGs.

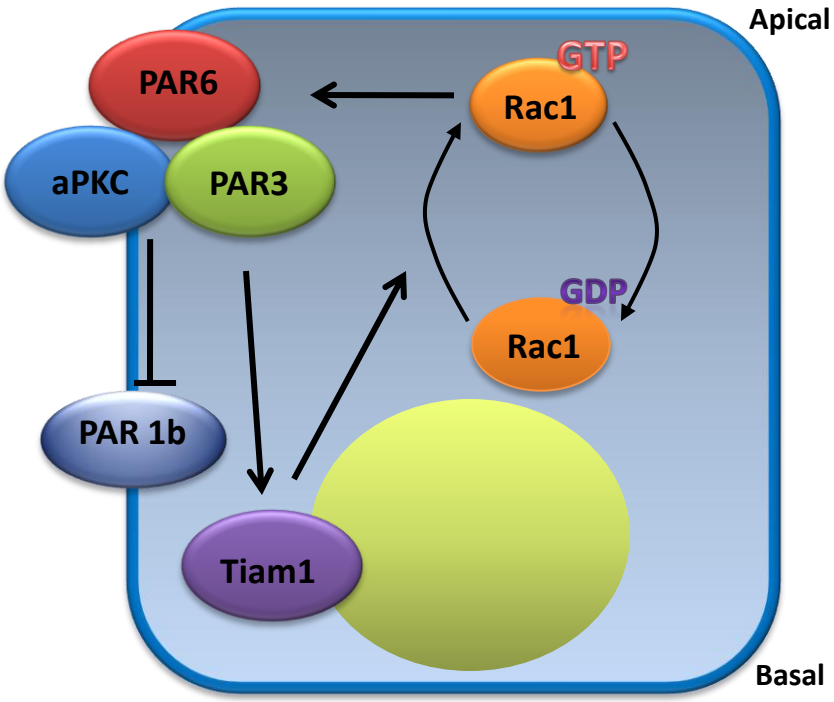

Figure 9. Model depicting interaction between the PAR polarity complex and Tiam1-mediated activation of Rac1. The apical PAR complex is localized to the apical membrane and restricts PAR1b localization to the basolateral side of the cell membrane. PAR complex proteins can regulate Rac activation and signaling by first binding to Tiam1. When Tiam1 is recruited to PAR3, this can lead to activation of Rac1. The GTP-bound active form of Rac1 then specifically interacts with PAR6 to activate aPKC.

RESULTS

Apical tight junction (TJ) and adherens junction (AJ) complex formation requires Rac1 activity.

Previous literature has defined a role for Rac in establishing cell-cell adhesions (Bosco et al., 2009). Cell-cell adhesion occurs via the formation of tight (TJ) and adherens (AJ) junction complexes between cells at the apical cell membranes. Previous data by Dr. Sequeira and an undergraduate student, Daniel Leonard, identified alterations in the expression and localization of cell-cell adhesion proteins, such as claudin-3, ZO-1 and E-cadherin, upon Rac inhibition (Sequiera, S., Leonard, D., Larsen, M., unpublished data). In this study, I confirmed these results by immunostaining mouse salivary epithelial cells (SIMS) for the TJ proteins, claudin-3 and ZO-1 (**Figure 10**) and the AJ protein, E-cadherin (**Figure 11)**. In control cells, these markers clearly outline the cell membranes more strongly on the apical side, as expected, and are low or diffuse on the basal side (Figure 10A, 11A). In contrast, we found that inhibition of Rac with NSC23766 (100μM for 24 hours), results in more diffuse or missing membrane staining patterns of claudin-3, ZO-1, and E-cadherin on the apical side along with a less intense staining intensity (Figure 10B, 11B). This effect was also observed with EHT1864 (10μM for 24 hours) (Figure 10C, 11C), as ZO-1 was also mislocalized to the basal side of the cell (Figure 10C). Together, this data suggests that Rac activity is required for apical positioning of cell-cell adhesion molecules.

Basal basement membrane matrix protein secretion requires Rac1 activity.

Previous data in the Larsen lab has indicated that Rac inhibition can affect basement membrane (BM) protein secretion (Sequiera, S., unpublished data). Whole mount immunocytochemistry and confocal imaging of primary SMGs for the ECM and BM proteins, laminin-111 and

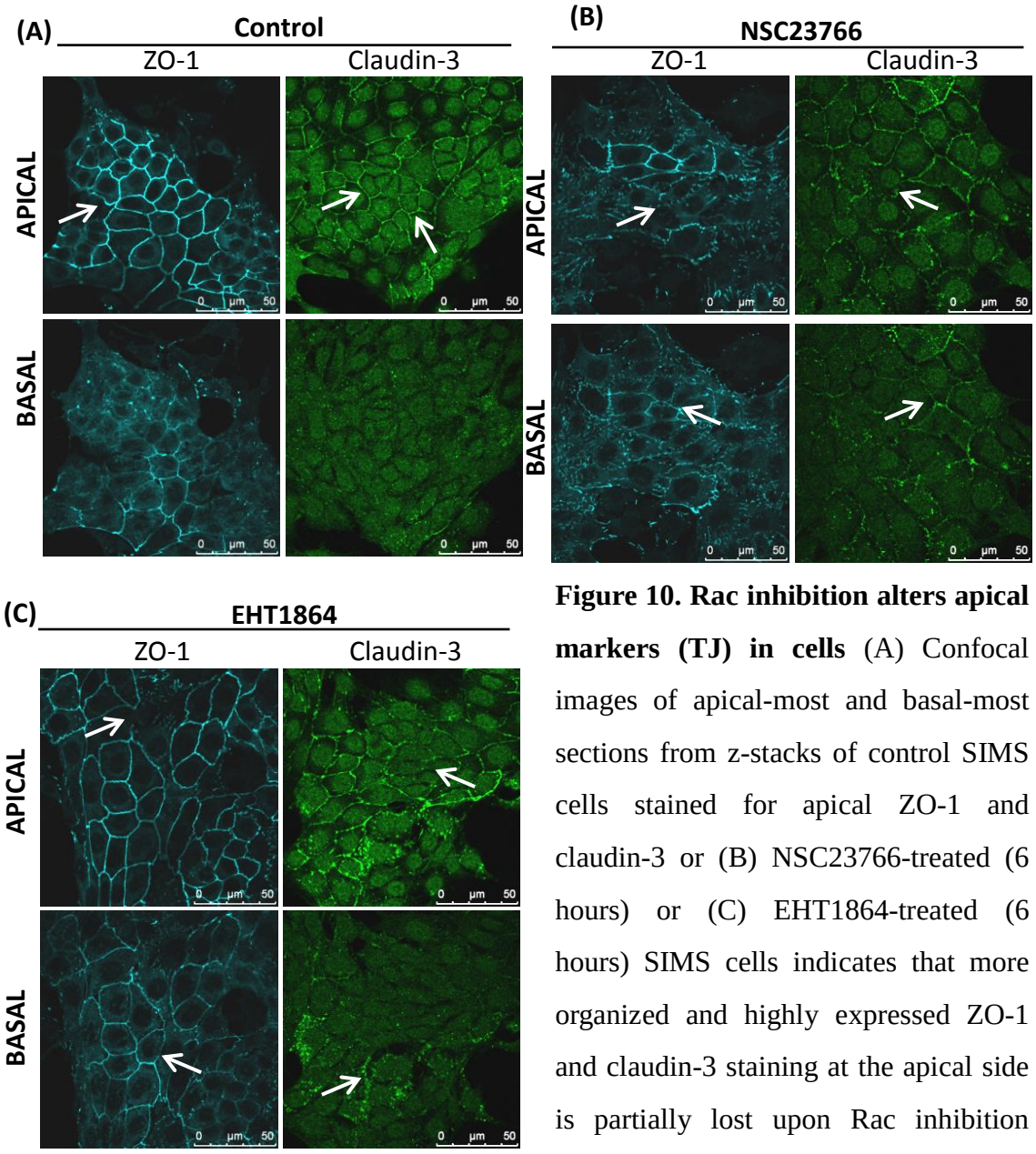

Figure 10. Rac inhibition alters apical markers (TJ) in cells (A) Confocal images of apical-most and basal-most sections from z-stacks of control SIMS cells stained for apical ZO-1 and claudin-3 or (B) NSC23766-treated (6 hours) or (C) EHT1864-treated (6 hours) SIMS cells indicates that more organized and highly expressed ZO-1 and claudin-3 staining at the apical side is partially lost upon Rac inhibition (indicated by white arrows) and some basal localization is gained. Each panel is from a different region of cells.

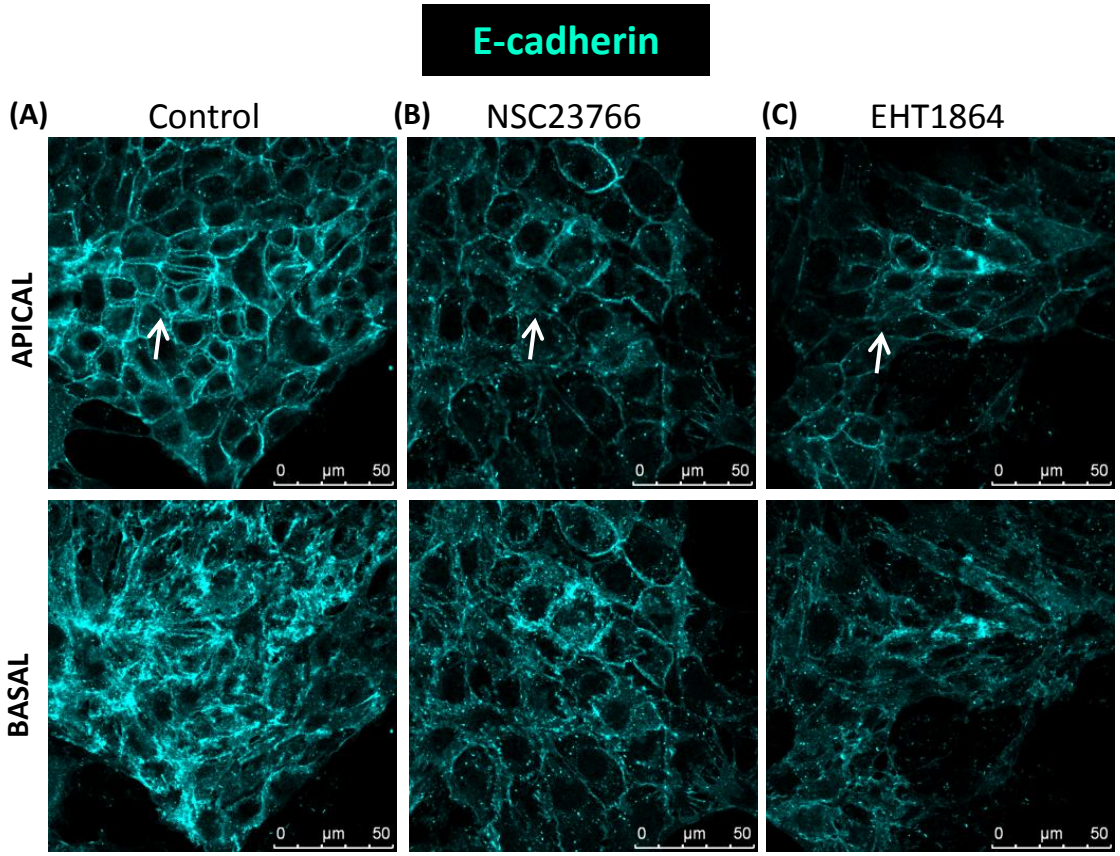

Figure 11. Rac inhibition alters apical markers (AJ) in cells (A) Confocal images of apical-most and basal-most sections from z-stacks of control SIMS cells stained for apical E-cadherin or (B) NSC23766-treated (6 hours) or (C) EHT1864-treated (6 hours) SIMS cells indicates that more organized and highly expressed E-cadherin staining at the apical side is partially lost upon Rac inhibition (indicated by white arrows) and some basal localization is gained. Each panel is from a different region of cells.

collagen IV (**Figure 12**) shows that, in untreated SMGs, these BM proteins are secreted towards the basal side, along the outside of the buds as normally expected. However, in Rac-inhibited glands, these proteins were relocated to the apical side and showed decreased basal expression compared to untreated glands. Thus, these results suggest that Rac inhibition alters basal secretion and localization of BM proteins.

Basal matrix protein deposition is not altered due to aberrant endocytosis upon Rac inhibition.

BM proteins are suggested to be trafficked to the basal cell surfaces via endocytosis (Denef et al, 2008). To test the hypothesis that Rac inhibition causes aberrant endocytic trafficking of basal BM matrix proteins to the apical surface, we immunostained E13 mouse embryonic submandibular glands (SMGs) for laminin-111 and collagen IV. When SMGs were treated with Rac inhibitor, NSC23766 (100 µM), basal matrix protein deposition was altered. Collagen IV, a BM protein that is localized basally in the control gland, was aberrantly distributed and localized at the apical side of the cell in NSC23766-treated glands (**Figure 13**). Treatment of the glands with phenylarsine oxide (PAO), an endocytosis inhibitor, for 10 hours did not significantly alter matrix protein secretion. This was also true for glands treated with a combination of NSC and PAO (1µM) for 10 hours. Thus, even when endocytosis was inhibited, basal matrix protein was localized in the apical side. These results suggested that aberrant apical basement membrane matrix distribution is not due to altered endocytic vesicular trafficking of basement membrane proteins upon Rac inhibition.

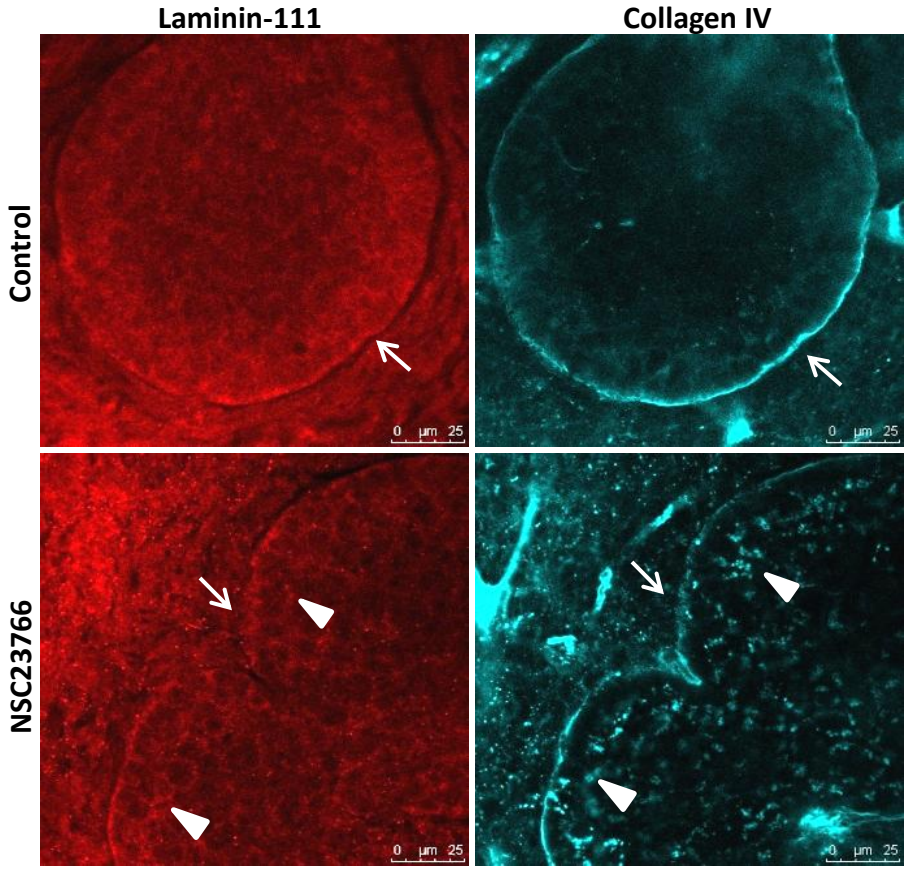

Figure 12. Rac inhibition affects basal secretion and localization of basement membrane (BM) matrix proteins. Confocal images of SMGs immunostained for laminin-111 (red) and collagen IV (cyan) in untreated SMG and NSC23766-treated (100µM) for 24 hours shows strong basal localization of BM proteins (arrows) at the outer edges of buds in control glands but reduced localization at the basal side and highly aberrant localization of BM proteins to the apical side (arrowheads) and around interior cells of the buds in Rac inhibitor-treated glands.

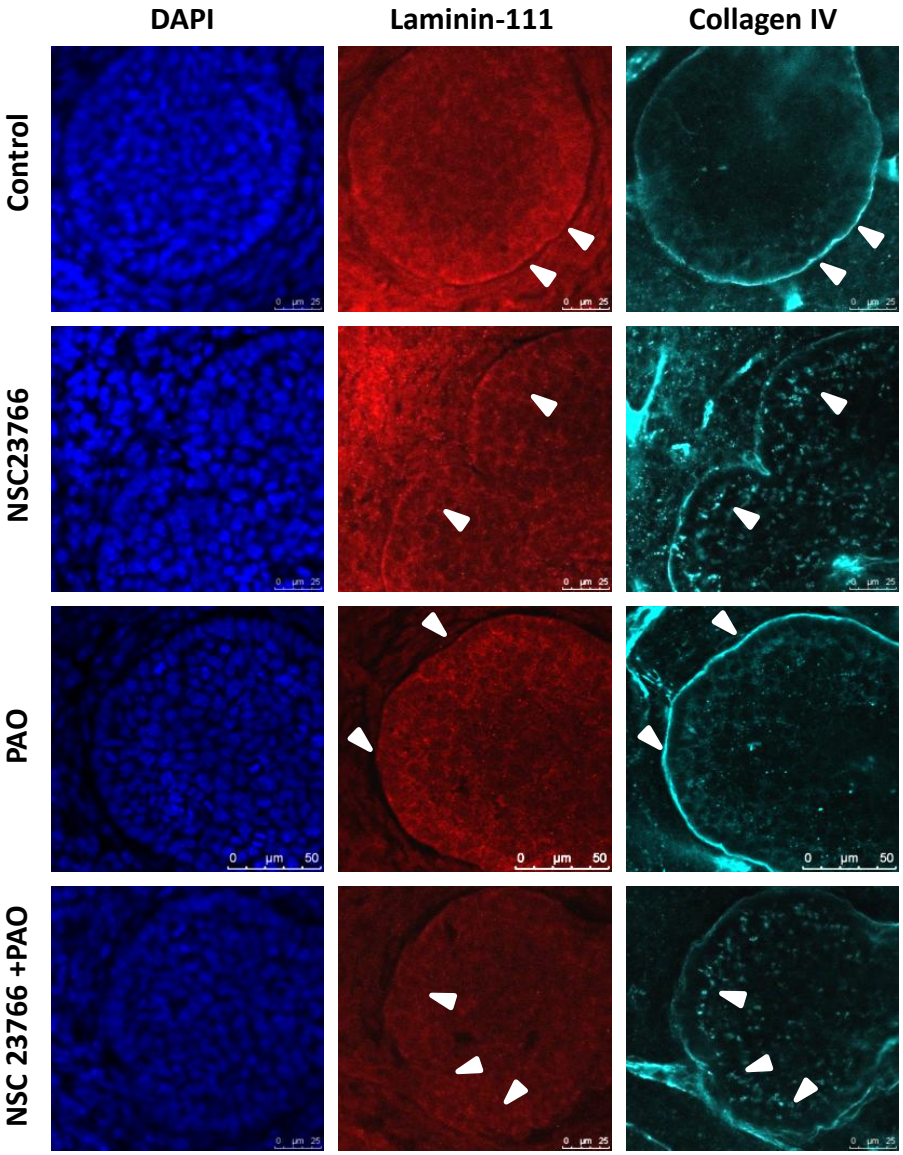

Figure 13. Aberrant apical basement membrane matrix distribution is not due to altered endocytic vesicular trafficking of basement membrane proteins upon Rac inhibition. Top panel of confocal images shows E13 SMGs stained for BM proteins laminin-111 and collagen IV localized to the basal surface of the buds. Treatment with NSC23766 (100µM) causes redistribution to the apical surfaces within buds. Treatment with phenylarsine oxide (PAO), an endocytosis inhibitor (1µM), and the Rac inhibitor, NSC23766, did not rescue this effect.

Apical distribution of PAR6 polarity protein is altered upon Rac inhibition in SMGs.

Knowing that both apical and basal proteins are mislocalized when Rac is inhibited and that these alterations were not dependent on aberrant endocytosis, we questioned whether salivary gland tissue polarity was affected, since polarity is characterized by asymmetric distribution of cellular proteins to the apical or basal membrane domains. Because PAR (partitioning-defective) proteins are master polarity regulators, the relationship between Rac1 and PAR proteins was tested. SMGs were immunostained for PAR6 and confocal images indicate that in control glands PAR6 proteins were enhanced on the apical cell membrane domains (**Figure 14**), as expected (Suzuki and Ohno, 2006). However, when Rac1 was inhibited, we observed that PAR6 was redistributed and accumulated on the basal side of the cell membrane as well (Figure 14). Previous data by Dr. Sequeira showed that PAR3 showed the same mislocalized pattern when Rac is inhibited. Together, these data suggest that Rac1 activity is required for correct apical deposition of PAR3 and PAR6 proteins and raised at the possibility that altered SMG polarity may be due to altered PAR protein deposition when Rac1 is inhibited.

Apical distribution of PAR3 polarity protein is altered upon Rac inhibition in lungs.

Because lungs start to develop earlier than salivary glands and go through polarization at an earlier stage than the salivary gland, PAR proteins localization upon Rac inhibition was also examined in the lungs. Actin is an apical protein marker in polarizing epithelial cells and was used as an apical marker to compare with the location of PAR3. The lungs were immunostained for PAR3 and examined by confocal imaging. Confocal images indicate that in control glands PAR3 protein was located preferentially on the apical cell membrane domain (**Figure 15**) as expected. However, when Rac1 was inhibited, we observed that this protein lost expression in

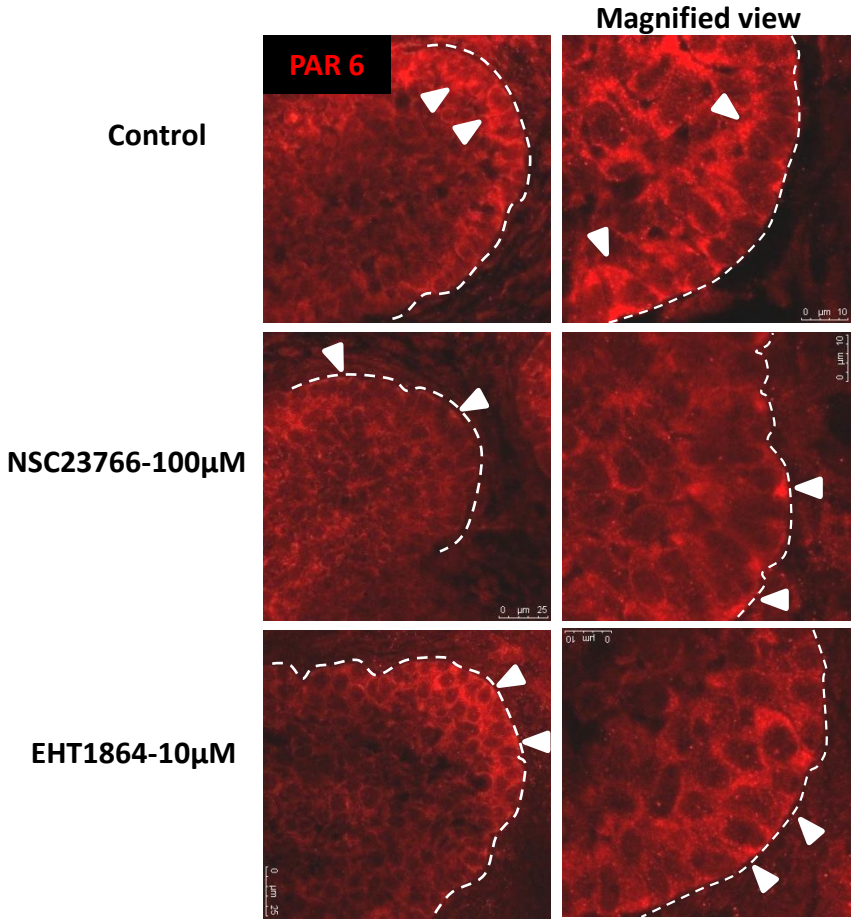

Figure 14. Apical distribution of PAR6 polarity proteins is altered upon Rac inhibition in SMGs. Top panel shows confocal images through the equatorial plane of control SMGs immunostained for PAR6 (red). Middle panel shows confocal images of NSC23766-treated (100µM) SMGs, while the bottom panel shows confocal images of EHT1864-treated (10µM) SMGs immuonstained as in the top panel. Instead of localizing primarily at the apical cell surfaces as in control (arrowheads), PAR6 proteins were also expressed at the basal side (denoted by white dashed lines and arrowheads) upon Rac inhibition.

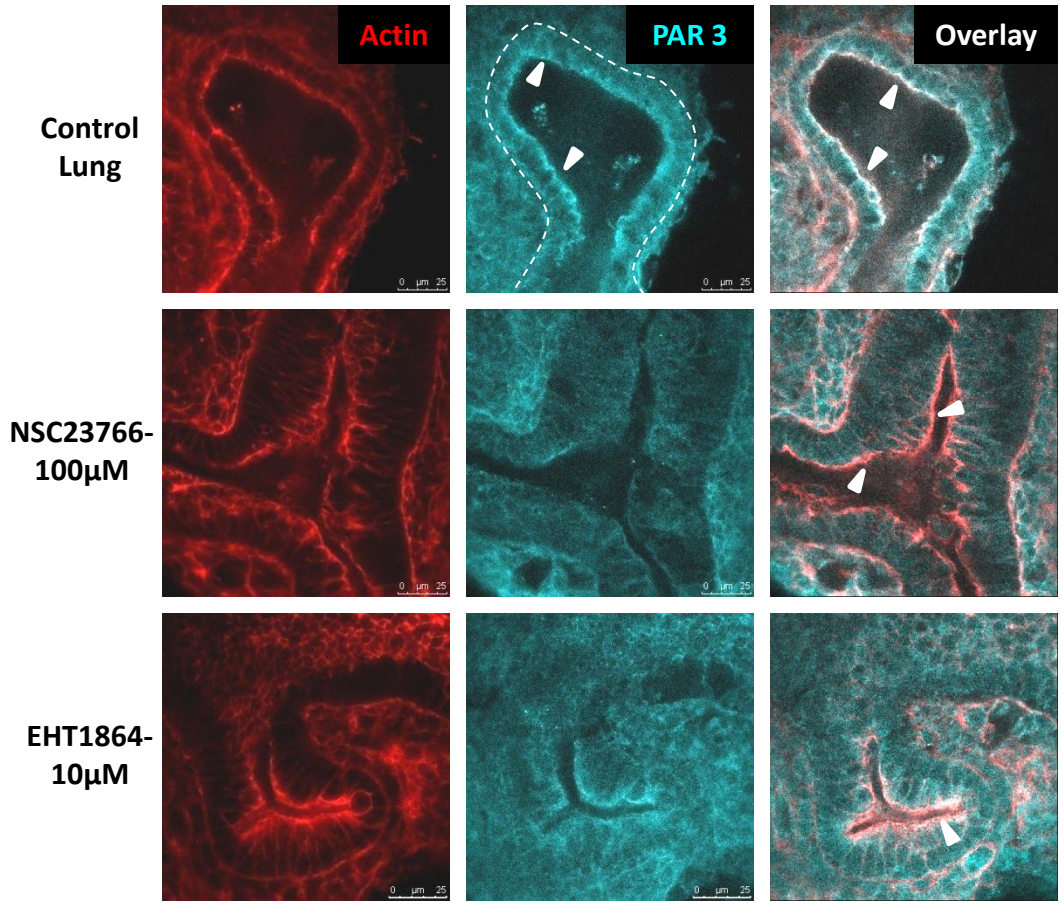

Figure 15. Apical distribution of PAR3 polarity proteins is altered upon Rac inhibition in lungs. Top panels show confocal images through the equatorial plane of control lungs immunostained for actin (red) as an apical marker or PAR3 (cyan). The middle panels show confocal images of NSC23766-treated (100µM) lungs while the bottom panels show confocal images of EHT1864-treated (10µM) lungs immuonstained as in the top panel. Decreased PAR3 protein localization was observed at the apical side (denoted by white colored overlay with Actin and PAR3) in NSC23766-treated lungs, compared to the control. In EHT1864-treated lungs, some apical PAR3 persisted in the reduced luminal space.

apical side (Figure 15). In NSC23766-treated lungs, loss of apical localization of PAR3 was more apparent than in EHT1864-treated lungs. In EHT1864-treated lungs, there was loss of lumen. Together, this data indicates that Rac1 activity is required for localization of PAR3 in the lungs as well and is required for proper lumen structure.

Apical distribution of PAR6 polarity proteins and basolateral distribution of integrin α6 are altered upon Rac inhibition in lungs.

The expression patterns of PAR6 and integrin a6 were examined in developing lungs in the presence and absence of Rac inhibition to identify Rac1-mediated effects on cell polarization. The lungs were immunostained for PAR6 and examined using confocal imaging. Confocal images indicate that in control lungs PAR6 protein was located on the apical cell membrane (**Figure 16**), as expected. However, when Rac1 was inhibited, we observed that this protein lost its localization at the apical cell membrane domain (Figure 16). Confocal images of integrin α6 indicate that in control lungs integrin α6 was located on the basolateral side of the cell membrane (Figure 16), as expected. However, when Rac1 was inhibited, we observed that this protein was relocated to the apical cell membrane domains (Figure 16). Confocal images of control lungs immunostained for integrin α6 demonstrated clearly organized single layers of epithelial cells surrounding the lumen, whereas in confocal images of Rac inhibited lungs immunostained for integrin α6, this organization was disrupted and luminal filling was observed (Figure 16). This indicated that Rac1 activity is required for correct deposition of PAR6 and integrin α6 and for the organized single cell layer structure of epithelial cells in the embryonic lungs.

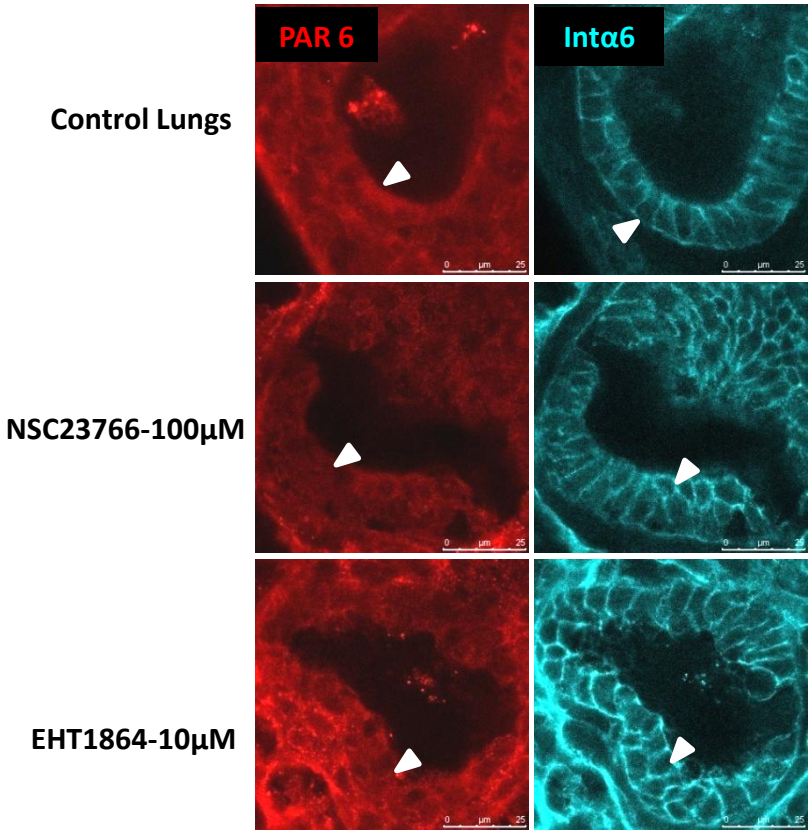

Figure 16. Apical distribution of PAR6 polarity proteins and basolateral distribution of integrin α6 are altered upon Rac inhibition in lungs. The top panel shows confocal images through the equatorial plane of control lungs immunostained for integrin α6 (red) as a basolateral marker or PAR6 (red). The middle panel shows confocal images of NSC23766-treated (100µM) lungs while the bottom panel shows confocal images of EHT1864-treated (10µM) lungs. Most strikingly, in the presence of the Rac inhibitors, the cellular organization was changed from a single polarized layer to a multiplayeed structure. Instead of localizing at the apical cell surfaces as in control (arrowheads), PAR6 proteins lost its localization at the apical side (arrowheads) upon Rac inhibition and was more broadly distributed. Instead of localizing at the basolateral cell surfaces as in control (arrowheads), integrin α6 proteins were also detected at higher levels on the apical side (arrowheads) upon Rac inhibition.

Inhibition of Rac1 decreases the expression of PAR and PKC complex proteins in salivary epithelial cells.

To test whether Rac1 inhibition also affects the expression of Par and PKC complex proteins in salivary epithelial cells, I performed Western blot analysis in both Sca9 and SMGC10 salivary epithelial cells treated with increasing concentration of Rac1 inhibitors (**Figure 17A and C**). In SMGC10 cell lines, PAR3 and PAR6 protein expression decreased as NSC23766 or EHT1864 concentrations increased. In Sca-9 cell lines PKC zeta protein expression decreased as Rac inhibitor concentration increased, as shown by the normalized graphs of these Western blots (**Figure 17B and D**). There was an apparent decrease in the amount of PAR proteins and PKC zeta in the presence of NSC23766 and EHT1864.

Inhibition of Rac1 decreases the expression of PAR and PKC complex proteins in whole salivary glands.

Protein levels of PAR-PKC proteins were also examined in intact embryonic salivary glands. Western blot analysis in E13 embryonic salivary submandibular glands treated with Rac1 inhibitors (**Figure 18A and B**) showed that PAR3 and PAR6 protein expression was decreased in NSC23766- or EHT1864-treated glands, consistent with data from cell lines. This suggests that Rac1 activity is required for PAR and PKC complex protein levels in developing salivary glands.

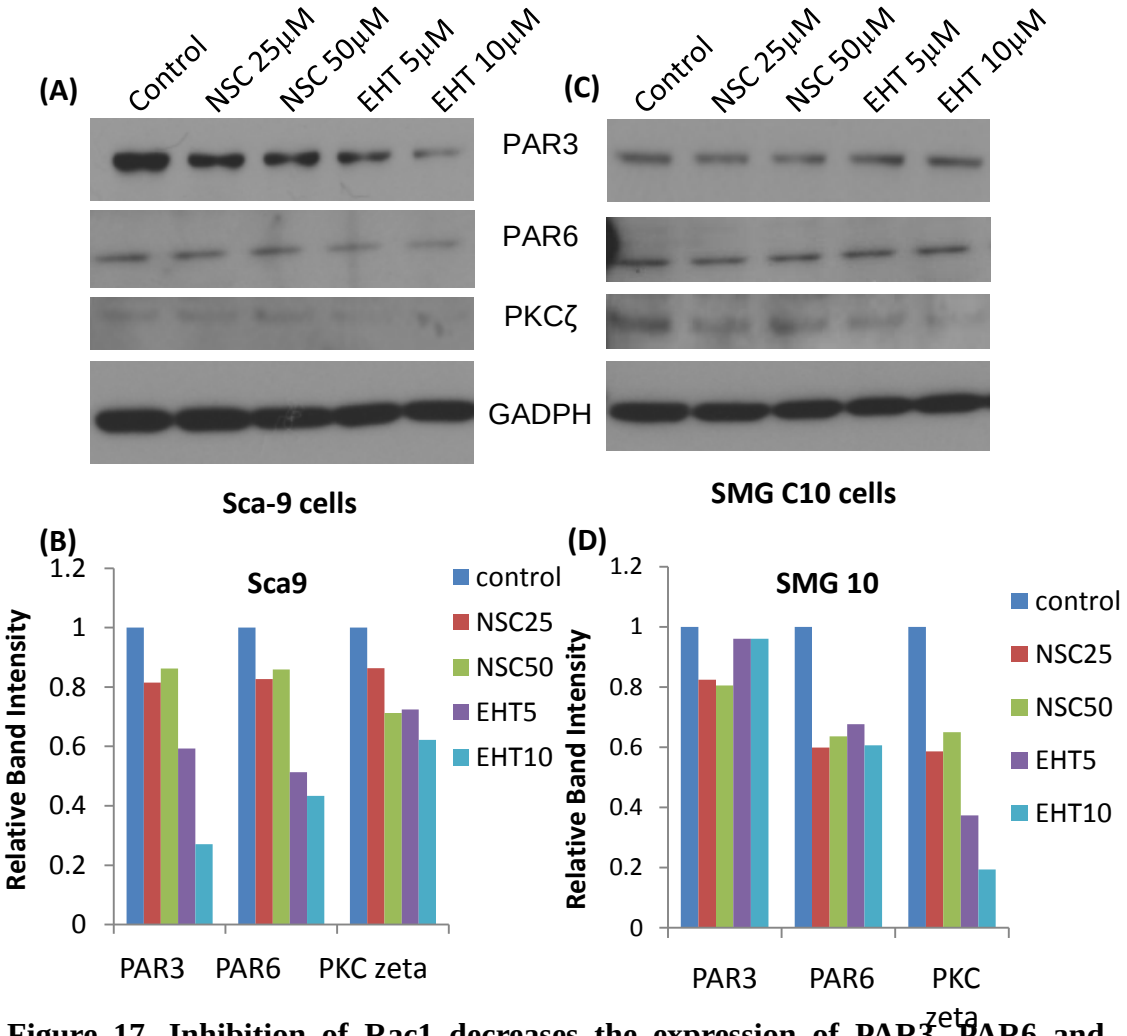

Figure 17. Inhibition of Rac1 decreases the expression of PAR3, PAR6 and aPKC complex proteins in salivary epithelial cells. (A) Western blot of SMGC10 cell line for PAR3, PAR6, and PKC zeta with and without Rac inhibition for 6 hours in two doses. (B) Densitometric quantification of the Western blots in A. (C) Western blot of Sca-9 cell line for PAR3, PAR6, and PKC zeta with and without Rac inhibition in two doses. (D) Densitometric quantification of the Western blot in C. Treatment with Rac inhibitor decreased the levels of all PAR proteins with the exception of PAR3 when SMGC10 cells are treated with the EHT inhibitor. All protein levels were normalized to GAPDH levels as loading control.

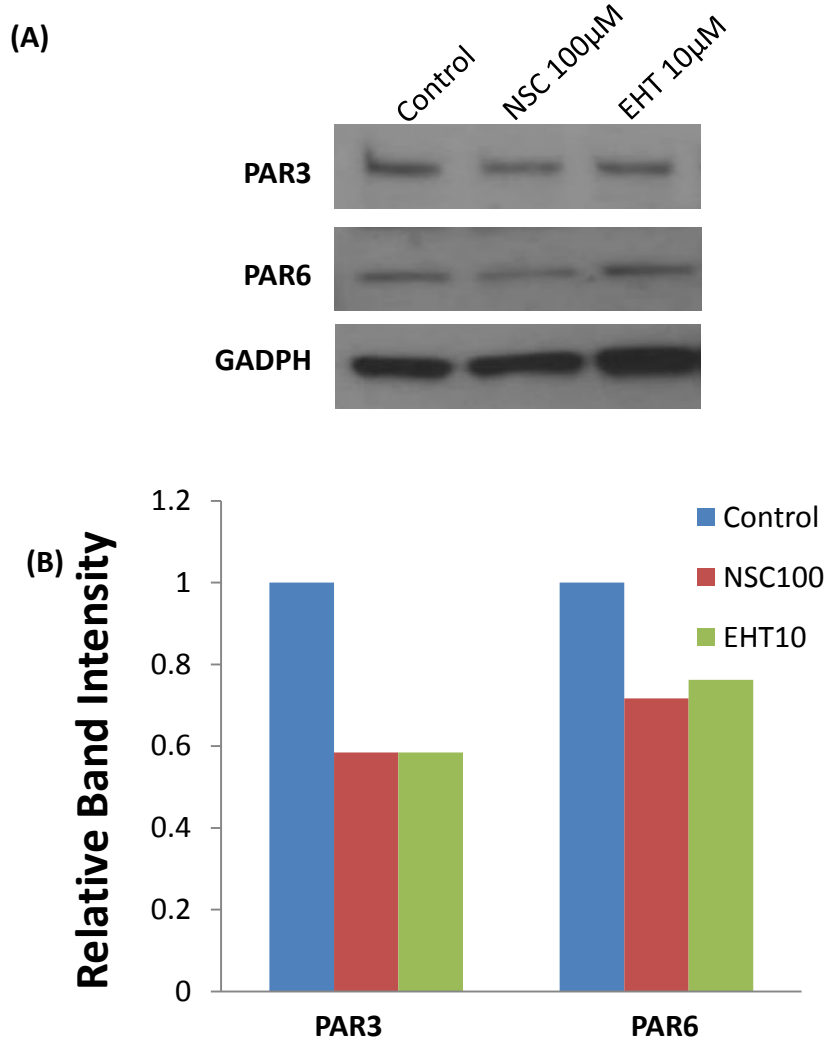

Figure 18. Inhibition of Rac1 decreases the expression of PAR3 and PAR6 proteins in embryonic SMG organ cultures. (A) Western blot of E13 SMG untreated or treated with 100μM NSC23766 or 10μM EHT1864 for 24 hours, using anti-PAR3 or anti-PAR6 antibodies. (B) Densitometric quantification of the Western blots in (A). All protein levels were normalized to GAPDH levels as loading control.

Basolateral distribution of Par1b polarity proteins remains unchanged with Rac inhibition.

Since the apical PAR3/PAR6/aPKC complex can restrict PAR1b to the basolateral side of the cell (Suzuki and Ohno, 2006) to maintain apico-basal asymmetry, we also tested whether PAR1b localization was affected by Rac1 inhibition. Confocal images of SMGs immunostained for PAR1b indicated a basolateral membrane localization especially evident in the outer polarized columnar layer of cells. This localization persisted even when Rac was inhibited by NSC23766 and EHT1864 (**Figure 19**). This suggests that Rac1 inhibition might only affect polarity through regulation of PAR3/PAR6/aPKC complex but not via regulation of PAR1b.

Inhibition of Rac1 does not affect the expression of PAR1b in salivary glands.

Protein levels of PAR1b were also examined in intact salivary glands treated with Rac inhibitors, using Western blot analysis (**Figure 20A**). There seems to be two bands for PAR1b, which may be alternatively spliced forms. Treatment of SMGs with NSC23766 or EHT1864 did not affect PAR1b protein expression compared to the untreated SMGs. Levels were quantified and are depicted by the normalized graphs of Western blots (**Figure 20B**). This data indicates that Rac1 inhibition does not affect the protein levels of PAR1b, and is consistent with the idea that Rac1 inhibition may affect polarity through the apical PAR3/PAR6/aPKC complex but not via the basal PAR1b protein.

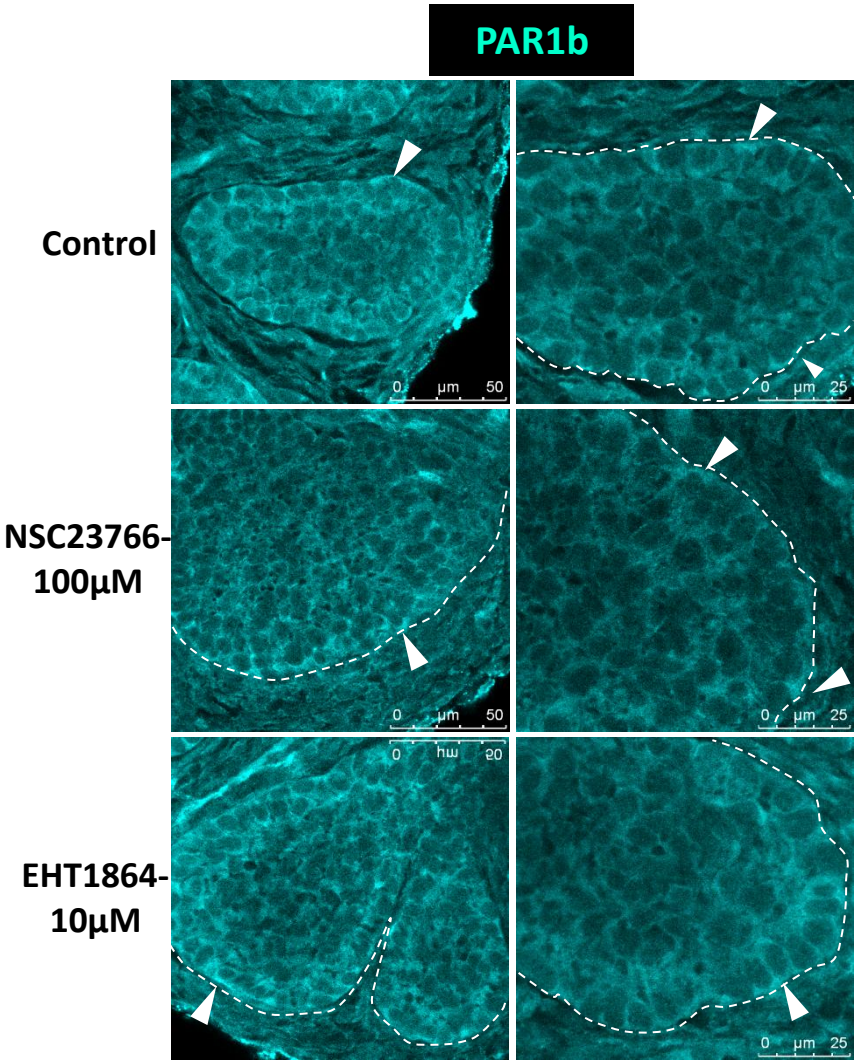

Figure 19. Basolateral distribution of PAR1b polarity protein is not affected by Rac inhibition. The top panels show confocal images through the equatorial plane of control E13 SMGs immunostained for PAR1b (cyan). The middle panels show confocal images of NSC23766-treated (100µM) SMGs for 24hrs, while the bottom panels show confocal images of EHT1864-treated (10µM) SMGs.

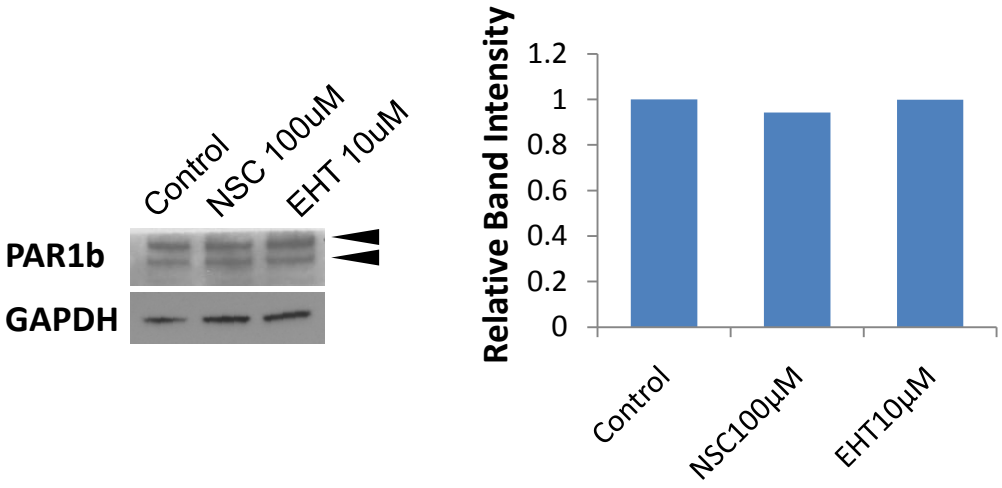

Figure 20. Inhibition of Rac1 did not affect PAR1b protein levels in SMGs. (A) Western blot of SMG for PAR1b, both with and without Rac inhibition for 24 hours with100μM NSC23766 or 10μM EHT1864. (B) Densitometric quantification of the Western blots in A. All protein levels were normalized to GAPDH levels as loading control.

DISCUSSION

While undergoing branching morphogenesis, salivary gland epithelial cells concomitantly attain apico-basal polarity. Polarity is also essential for unidirectional secretion of salivary proteins and fluid towards the apical lumen within acinar units of the adult gland. It is therefore important to study the molecules that mediate salivary gland apico-basal polarization to understand how salivary glands attain their function. In this study we identified a role for the GTPase Rac1, in polarization.

Previous research has pointed to a role for Rac1 in polarization of non-salivary gland cell types (Chen and Macara, 2005; Ewald et al., 2008; O'Brien et al., 2001; Pegtel et al., 2007). We found that Rac inhibition in both primary salivary gland embryonic tissue and immortalized salivary epithelial cell lines caused mislocalization of normally apical adherens (E-cadherin) and tight junction (ZO-1 and claudin-3) proteins. Expression of these proteins was reduced apically or presented at the basal side in inhibitor-treated cells. This led us to conclude that Rac1 is required for formation of cell-cell adhesion complexes and also for their localization.

Basement membrane proteins are located basally because they function to anchor an epithelial cell layer, separate the epithelial tissue from the connective tissue compartment, and provide growth and differentiation signals to the epithelium (Sequeira et al., 2010). In Madin-Darby canine kidney (MDCK) epithelial cysts, Rac1 was required for laminin-111 organization into BM and polarity establishment (O'Brien et al., 2001). We report here that, in mammalian mouse embryonic salivary gland, normal basal secretion of BM proteins was affected, leading to apically-deposited matrix proteins in the NSC23766- treated SMGs. These data are consistent with a function for Rac1 in basal localization of BM proteins.

BM protein secretion on the basal side of epithelial cells is thought to occur via

endocytosis, where by proteins are engulfed and deposited(Denef et al., 2008; Sorrosal et al., 2010). I hypothesized that one mechanism for relocation of BM matrix proteins to the apical side when Rac signaling was inhibited might be through endocytosis. If this was the case, when endocytosis was blocked through chemical inhibition with phenylarsine oxide, a potent endocytosis inhibitor, then we would expect that this altered deposition would be rescued and BM proteins would not be trafficked to the apical side. However, we observed that treatment with NSC23766 and PAO together did not rescue the apical deposition. Therefore, it can be concluded that aberrant apical deposition of BM matrix proteins did not involve endocytosis.

Rac1 has previously been shown to be an activator of the PAR3/PAR6/aPKC polarity complex, and Rac can bind PAR6 to activate aPKC (Munro, 2006). Because Rac activates the PAR proteins which are responsible for establishing polarity to account for the asymmetrical distribution of apical and basal membrane domains, a mechanism that might explain the aberrant deposition of BM/ECM proteins to the apical side could involve Rac-mediated effects on the levels of PAR protein complex proteins or their localization (Munro, 2006). Indeed, our results showed that Rac inhibition affected both PAR protein localization and protein levels since PAR3 and PAR6, which are known to be located in the apical side, were localized on the basal side upon Rac inhibition. This loss of apical position of PAR3 and PAR6 was also shown in NSC23766-treated lungs. This apical localization was not obvious in EHT1864-treated lungs. However, we observed that EHT1864-treated lungs lost lumen structure. This difference in effect of inhibitors must be due to their distinct mechanisms of inhibition (Gao et al., 2004; Shutes et al., 2007). NSC23766 may inhibit Rac1 more specifically than EHT1864 since it targets the activity of GEF binding to the GTP site. Although we have no proof for this yet, we just see that they have different effects and that may be due to different GEFs being targeted. NSC23766 is a

more widely used inhibitor than EHT1864. Rac inhibition also resulted in disorganization of the outer epithelial cells in the lungs. Expression of PAR3, and Par 6, and PKC zeta were also decreased upon Rac inhibition. This data suggests that Rac1 is responsible for correct membrane distribution of these PAR proteins, and altered SMG polarity may be linked to the alteration in PAR proteins distribution pattern. Interestingly, we also found that Rac1 might be required for normal expression of PAR protein levels as well since PAR3, PAR6, and PKC zeta protein expression decreased with Rac inhibition.

Because polarization is reported by others to be due to an antagonistic interaction between PAR3/PAR6/aPKC polarity complex (Suzuki and Ohno, 2006) and PAR1b, and PAR1b is located opposite of the complex, we believed it was important to study PAR1b with Rac inhibition. However, the results indicate that loss of polarization upon Rac inhibition cannot be mediated through PAR1b because normal PAR1b protein levels and localization at basolateral membrane domain persisted with Rac inhibition. This finding is interesting because it suggests that PAR 1b is not required for Rac-dependent basement membrane localization. Further studies will be required in this area.

Taken together, this work has shown that Rac1 is required for maintaining the localization and maintaining protein levels of cell-cell adhesion molecules, BM proteins, and apical PAR complex proteins PAR3 and PAR6. Regulation of these PAR proteins may be responsible for the apical localization of the apical tight junction and adherens junction proteins and for the localization of BM proteins to the apical membrane. The regulation of PAR proteins was identified in embryonic salivary gland organ cultures. Importantly, this function for Rac1 in regulation of cell polarity is not restricted to salivary gland, since we found that Rac1 also regulates polarity in the developing lung.

CONCLUSIONS AND SIGNIFICANCE

The findings from our study implicate Rac1 signaling as a critical requirement for mammalian SMG branching morphogenesis and tissue polarity (**Figure 21**). This work provided evidence that Tiam1-Rac1 signaling pathway is required for salivary gland branching morphogenesis. Rac1 is required for apical distribution of adherens and tight junction proteins and basal deposition of BM proteins. This regulation of polarity of Rac might be due to altered PAR protein localization and regulation of protein levels since we showed that Rac inhibition decreased the levels and the apical localization of the PAR3 and PAR6. Because it has been shown that Tiam1 directly interacts with PAR proteins in non-salivary cell types (Munro, 2006), this Rac-Par relationship might be mediated through Tiam1. In the future we will test if inhibition of Tiam1 alters PAR protein localization, and if PAR proteins can directly bind to Tiam1 or Rac1 in salivary gland cell extracts by performing co-immunoprecipitation experiments.

Knowing the molecular mechanisms by which the GTPase Rac1 and its GEF, Tiam1, influence signaling pathways that drive normal salivary gland tissue polarity and development will help us gain a better understanding of how these proteins are misregulated in non-curable diseases such as Sjogren's syndrome and adenoid cystic carcinomas. Xerostomia, or dry mouth, that occurs from salivary gland diseases, radiation therapy for head and neck cancers, or as a side effect of certain medications, results in fissured tongue, caries and loss of teeth, thick and stringy saliva, and fungal infections (Aframian and Palmon, 2008). Since there is no cure for xerostomia and all of the current treatments are short-term, our future goal is to engineer an artificial salivary gland that might serve as an effective replacement tissue in patients suffering from the symptoms of xerostomia. Therefore, understanding how Rac1 signaling contributes to gland formation and polarization will bring us one step closer to understanding how differentiation and saliva

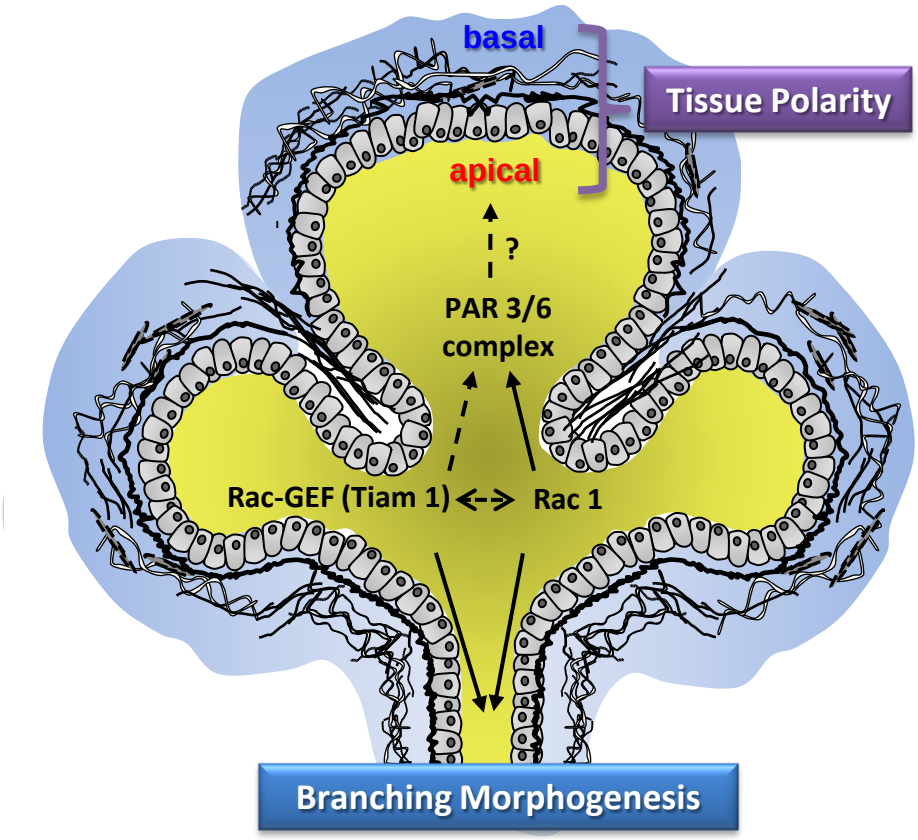

Figure 21. Rac is required for branching morphogenesis and tissue polarity in salivary gland development. The Tiam1-Rac1 signaling pathway is required for salivary gland branching morphogenesis. Rac1 is also required for apical distribution of adherens and tight junction proteins and basal secretion of BM matrix proteins, indicating that Rac modulates apical-basal polarity in the salivary gland. This regulation of polarity of Rac might be due to altered localization and expression of PAR proteins since decreased levels of PAR3 and PAR6 were detected, and they were mislocalized at basal surfaces. Because it has been shown that Tiam1 directly interacts with Par proteins in other studies, this Rac-PAR relationship might be mediated through Tiam1, but requires further investigation.

secretion are achieved during organ formation and further aid in the design of therapies to promote gland function.

FUTURE DIRECTIONS

Although we found that Tiam1 is required for branching morphogenesis, how it does this is not fully understood. Since Rac is required for polarization and Tiam1 is the connector of the feedback loop between PAR3/PAR6/aPKC complex and Rac activation, we would like to find the link between Tiam1 and Par polarity complex signaling. Future experiments are aimed at inhibiting Tiam1 using siRNA and testing whether PAR protein localization and protein levels are similarly affected as observed with Rac1 inhibition. If Tiam1 interacts with PAR proteins and how Rac might affect this interaction can be tested using immunoprecipitation, to see if PAR proteins are pulled down with anti-Tiam1 antibodies, both with and without Rac inhibitors. Further experiments will be needed to determine the molecular domains required for any interactions that are identified and any post-translational modifications that may be required. It is also interesting that protein levels are reduced in the presence of Rac inhibition. This finding suggests that Rac1-mediated regulation of PAR proteins is complex and that either a transcriptional regulation or protein degradation mechanism is involved in addition to regulation of protein localization and complex formation. Understanding the complex regulation of PAR proteins by Rac1-mediated signaling in establishment of epithelial polarity will require further investigation. In addition, besides the experiments that showed the pharmaceutical inhibitors of Rac activity block SMG branching morphogenesis, Rac1's role in adheren, tight junction, and BM proteins, and inhibition of Rac1 mislocalizes PAR complex proteins, all other experiments were done only once. It is hard to make a concrete conclusion on one experiment. Therefore, it is crucial to repeat some experiments to gain statistical significance in the future.

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