

(#863) Rap1 GTPase is Involved in RPE Cell Barrier Function

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Introduction:

The most severe vision loss associated with neovascular age-related macular degeneration (AMD) is initiated when choroidal endothelial cells (CECs) originating from the choriocapillaris become inappropriately activated and subsequently migrate through Bruch's membrane and then across the retinal pigment epithelium (RPE). Once across the RPE barrier, choroidal neovascularization (CNV) within the neurosensory retina can occur.

Under normal conditions, it is believed that the RPE can effectively limit CNV by forming a physical barrier and by appropriate compartmentalization of angiogenic factors such as VEGF. However, in aging eyes, metabolic stresses, hypoxia, and inflammation can all increase angiogenesis and cause RPE barrier compromise. Better understanding of the proteins that regulate the RPE barrier may thus improve our understanding of why CNV occurs and lend insight into mechanisms to reduce its occurrence.

Signaling molecules such as the small GTPases of the Rho family have been implicated in cell-cell junctional formation and maintenance, as well as regulation of actin cytoskeleton remodeling during dynamic events, including junctional assembly and disassembly. Rap1, which is a member of the Ras superfamily of GTPases has been previously shown to be involved in regulating the assembly and permeability of both endothelial and epithelial cell junctions.

Like all GTPases, Rap1 acts as a molecular switch, cycling between an active (GTP-bound) and an inactive (GDP-bound) form. GTP binding and subsequent activation of GTPases is facilitated by guanine nucleotide exchange factors (GEFs), whereas inactivation occurs by hydrolysis of GTP to GDP and is catalyzed by GTPase-activating proteins (GAPs).

Purpose:

To determine whether the small GTPase Rap1 regulates the formation and maintenance of the retinal pigment epithelial (RPE) cell junctional barrier.

Methods:

We utilized ARPE-19 cells as an *in vitro* cell culture model to study RPE barrier properties. To dissect the role of Rap1 in this process, we used two techniques to inhibit Rap1 function: (1) Overexpression of RapGAP protein, which acts as a negative regulator of endogenous Rap1 activity, and (2) RNAi-mediated knockdown, with engineered adenovirally-transduced microRNAs. For some experiments we also utilized a specific Rap1-activating compound (8CPT-2'O-Me-cAMP, Biolog).

Transepithelial electrical resistance (TER) of RPE plated on Transwell filters was used as the standard measure of monolayer permeability (EVOM Voltohmmeter). For calcium-switch experiments, RPE were treated with EGTA (4mM, 30 min) to disrupt cell-cell junctions, and synchronous junctional reassembly was induced upon washout. Real-time cellular analysis (RTCA) was also used as a readout for monolayer barrier strength (xCELLigence System, Roche). This technology measures the electrical impedance of cells grown directly on gold micro-electrode plates. Changes in impedance (represented as "Cell Index") reflect changes in barrier function and permeability.

Immunofluorescence microscopy was used to visualize localization of cadherin and F-actin; under steadystate conditions, and also during junctional reassembly (calcium switch).

CEC transmigration across RPE monolayers grown inverted on Transwells was performed. ARPE were pre-treated with control or Rap1 knockdown virus to assess choroidal neovascularization under conditions of Rap1 inhibition in RPE.

Results:

Figure 1: Loss of Rap1 GTPase activity by expression of RapGAP (compared to GFP alone) reduces steady-state TER and decreases electrical impedance of RPE monolayers.

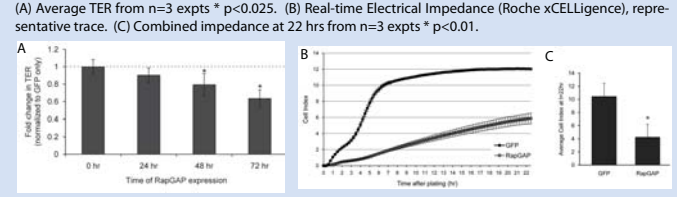


Figure 2: Conversely, activation of Rap1 GTPase by treatment with 8CPT-2'O-Me-cAMP increases electrical impedance of RPE monolayers.

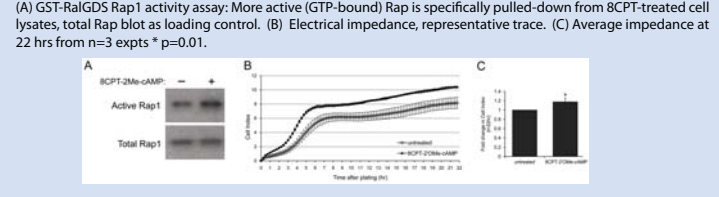


Figure 3: Efficient RNAi-mediated knockdown of Rap1 expression using an engineered microRNA.

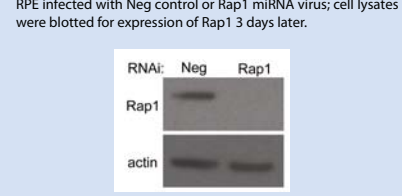


Figure 4: Loss of Rap1 protein by RNAi or inhibiting its activity by expressing RapGAP disrupts both cadherin junctional localization (A), and induces stress fiber formation with loss of junctional actin (B).

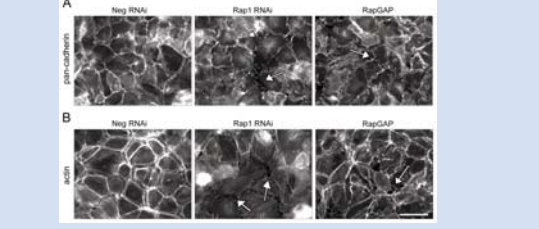


Figure 5: Loss of Rap1 protein by RNAi reduces TER of RPE monolayers over time. Graph shows fold change in TER of RPE knockdown cells, normalized to Neg control=1 at each timepoint. TER from n=3 expts * p<0.05.

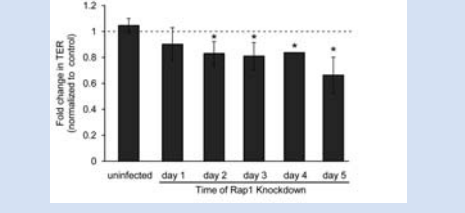


Figure 6: TER recovery following calcium switch (junctional reassembly) is delayed in RPE lacking Rap1 protein (RNAi) or activity (RapGAP expression). Representative experiment, n=avg of 4 wells, * p<0.02.

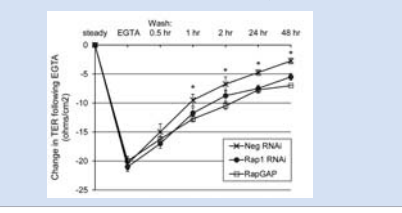


Figure 7: Knockdown of Rap1 by RNAi inhibits cadherin relocation to cell junctions following calcium switch.

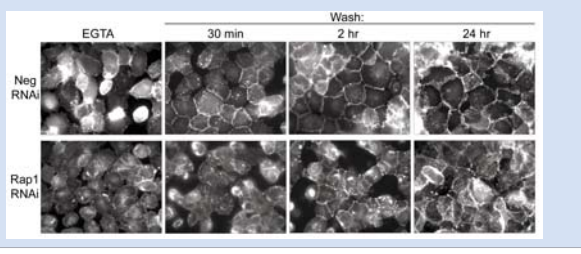
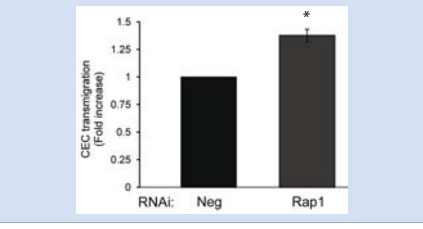


Figure 8: CEC transmigration is greater across RPE monolayers that have Rap1 knockdown. Combined fold change from n=3 expts, * p<0.05.



Conclusions:

1. Loss of Rap1 or inhibition of its activity in RPE reduces steadystate TER and electrical impedance of ARPE-19 cell monolayers.
 2. This loss of RPE barrier function is also correlated with mislocalization of cadherins and formation of gaps within the monolayer.
 3. Junctional reassembly following calcium switch is also affected after loss of Rap1 protein; cadherin recruitment back to cell-cell contact sites, and TER recovery are both delayed
 4. CEC transmigration is significantly higher in Rap1-knockdown ARPE-19 monolayers compared to control.
- Therefore: Rap1 GTPase is required for maintenance and formation of RPE cell junctions and regulation of barrier function. Our observation that RPE monolayers lacking Rap1 allow greater transmigration of CECs suggests a possible role for potentiating choroidal neovascularization during the pathology of neovascular age-related macular degeneration.

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