Appendix A: Localization of Insulin receptor in primary airway cells

NOTE: the data in Appendix A.1.3 are included in a manuscript due to be submitted to the journal *Procedures of the National Academy of Sciences* in March of 2016 (I will be third author).

A.1.1 Brief Introduction:

As described in Section 1.3, CF-related diabetes (CFRD) is a common co-morbidity of CF that is correlated with accelerated lung function decline [1]. There is some evidence that CFRD is correlated with increased glucose levels in the airway lumen [2-4]. This glucose may serve as a nutrient source for pathogenic bacteria [5]. In collaboration with the Koval lab at the Emory University Department of Cell Biology, we have investigated the mechanisms of glucose regulation in the airway. We have found evidence that cultured primary airway cells exhibit insulin-dependent glucose uptake and that this is blunted in cells from CF patients. Interestingly, confocal microscopy of immuno-stained primary airway epithelial cells performed by myself and others has localized the insulin-dependent glucose transporter (GLUT4) to the *apical* side of the airway, suggesting that insulin-dependent glucose uptake may be a mechanism for removal of glucose from the airway lumen, which the correlating functional glucose uptake data suggest is defective in CF.

To determine where the insulin receptor (IR) is localized, I optimized and performed immunofluorescence experiments for this protein on polarized primary airway cells cultured on Transwell filters. In addition to the IR, the cells are concurrently stained for nuclei (DAPI) and for a junctional marker that allows the visualization of cell borders (β-catenin).

A1.2 Materials (make fresh for each experimental day):

1. DPBS: Sterile DPBS + Ca2+ ­and Mg2+
2. TX-100: 0.5 % Triton X-100 in DPBS
3. 4% PFA: 4% paraformaldehyde diluted from fresh ampule of 20% PFA
4. DPBS + GS: 2% Goat serum in DPBS
5. TX-100 + GS: 2% Goat serum + 0.5 % Triton X-100 in DPBS
6. AB solutions: 2% Goat serum in DPBS + prescribed antibody
   1. Primary Insulin receptor AB mix: (rIR) (Abcam cat # ab5500) 1:250 in AB solution
   2. Primary β-catenin AB mix: (mBCAT) (BD Biosciences cat # BD610153) 1:200 in AB solution
   3. Secondary AB mix: rCy2 1:1000 + rCy3 1:1000 in AB solution.
7. DAPI solution: dilute Hoescht 33258 1:1000 in DPBS

A1.2 Protocol:

1. Receive Transwells containing polarized airway epithelial cells maintained at air-liquid interface ≥ 3 weeks from the Cell Cultures Core of the Emory + Children’s Center for CF and Airways Disease Research (contact [mhkoval@emory.edu](mailto:mhkoval@emory.edu) ; [s.a.molina@emory.edu](mailto:s.a.molina@emory.edu)).
2. Rinse each filter 4 X with DPBS
3. Visualize filters by light microscope; verify that all mucous (dark gray mass) has been washed off of the cells.
4. Wash each filter 3 X for 5 minutes with DPBS at room temperature
5. Fix each filter with 4% PFA 10 min at room temperature
6. Wash each filter with 3 X for 5 minutes in DPBS at room temperature
7. Permeabilize each filter for 5 minutes in TX-100 at room temperature
8. Block/permeabilize by washing each filter 2 X for 5 minutes with TX-100 + GS at room temperature
9. Make up primary insulin receptor AB (0.75 ml of each per filter)
10. Add primary insulin receptor AB (0.25 mL above filter, 0.5 mL below filter), incubate O/N at room temperature
11. Wash 3 X for 5 minutes in DPBS + GS at room temperature
12. Make up primary β-catenin AB (0.75 ml of each per filter)
13. Add primary β-catenin AB (0.25 ml above filter, 0.5 mL below filter), incubate 2 hours at room temperature
14. Wash 3 X for 5 minutes with DPBS + GS at room temperature
15. Make secondary AB mixture (0.75 ml of each per filter)
16. Add secondary AB mixture, incubate 1 hour at room temperature
17. Wash 3 X for 5 minutes with DPBS + GS at room temperature
18. Incubate in DAPI solution for 10 minutes
19. Wash 3 X for 5 minutes with DPBS + GS
20. Wash 3 X for 5 minutes with DPBS.
21. Carefully cut the filters from Transwells using a sterile razor blade
22. Mount filters cell side up on labeled microscope slides with Slow-fade mounting media.
23. Image through the Z-stack of the filter via confocal microscopy utilizing 100x oil immersion objective and 0.25 µM slices. For presentation, the Z-stack can be reconstituted as a 3-D pavement view of the epithelium, as shown in Figure A1.1



Figure A1.1. The above is a 3D projection reconstituted from a z-stack of primary human bronchial epithelial cells from a CF patient. The insulin receptor (green in top panel, isolated in the second from bottom panel) shows strong signal at cell borders, including the apical junctional complex (white arrowheads). Beta catenin protein (red in top panel, isolated in second from top panel) serves as a cell-cell junctional marker. A special thanks to Darryl Hanover from the Santangelo lab (Georgia Institute of Technology) for assistance in imaging the airway epithelium filters.

References

1. Marshall, B.C., et al., *Epidemiology of cystic fibrosis-related diabetes.* J Pediatr, 2005. **146**(5): p. 681-7.

2. Philips, B.J., et al., *Glucose in bronchial aspirates increases the risk of respiratory MRSA in intubated patients.* Thorax, 2005. **60**(9): p. 761-4.

3. Brennan, A.L., et al., *Airway glucose concentrations and effect on growth of respiratory pathogens in cystic fibrosis.* J Cyst Fibros, 2007. **6**(2): p. 101-9.

4. Baker, E.H., et al., *Hyperglycemia and cystic fibrosis alter respiratory fluid glucose concentrations estimated by breath condensate analysis.* J Appl Physiol (1985), 2007. **102**(5): p. 1969-75.

5. Garnett, J.P., et al., *Elevated paracellular glucose flux across cystic fibrosis airway epithelial monolayers is an important factor for Pseudomonas aeruginosa growth.* PLoS One, 2013. **8**(10): p. e76283.