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*Full Length Research Paper*

# Decreased $\beta_1$ Na<sup>+</sup> - K<sup>+</sup> ATPase subunit expression and defective focal adhesion kinase activation mediate disturbed cell polarity in autosomal recessive polycystic kidney disease

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**ARPKD is a developmental disease that results from fibrocystin loss of function mutation. It affects mainly renal collecting ducts and biliary system. The exact mechanism of how fibrocystin mutation results in the disease manifestations is not known as the exact functions of fibrocystin are also not known. However, this hypothesis explains one of the major mechanisms that may be involved in ARPKD development based on a group of proved observations in previous publications. The impaired epithelial cellular polarity seen in ARPKD might result from impaired  $\beta_1 / \beta_2$  Na<sup>+</sup>- K<sup>+</sup> ATPase expression together with impaired FAK activation and functions, with possible involvement of adhesion signaling in development of those interactions. Expression of  $\beta_1$  Na<sup>+</sup>- K<sup>+</sup> ATPase subunit is necessary for E-cadherin mediated establishment of epithelial cell polarity. Focal adhesion kinase interacts with adhesion proteins and is essential for establishment of polarized cell migration. Both together may explain how fibrocystin loss of function mutation can result in reversed epithelial cell polarity which proceeds to intracystic secretion in ARPKD.**

**Keywords:** ARPKD, Fibrocystin, FAK, Na<sup>+</sup> - K<sup>+</sup> ATPase, and epithelial cell polarity.

## Disclaimer

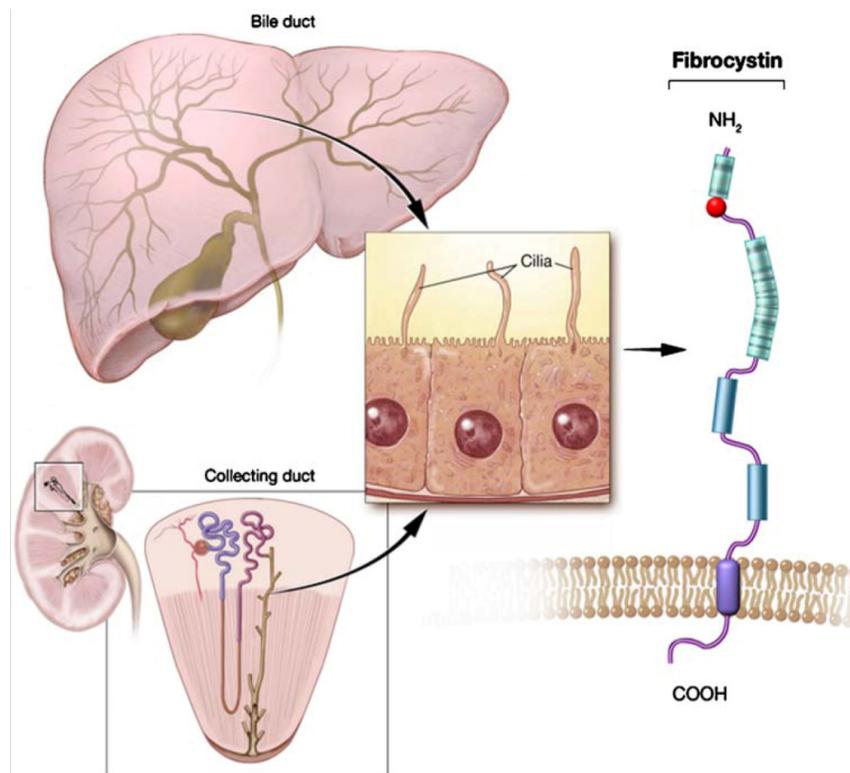
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All materials used in testing of the hypothesis were supplied by the department laboratory director.

## INTRODUCTION

Autosomal recessive polycystic kidney disease (ARPKD) is a developmental disease which affects 1/ 10000- 1/ 40000 live births and usually presents in the neonatal period with enlarged echogenic kidneys. At initial presentation, approximately 45% of infants have liver

abnormalities, including hepatomegaly, dilated intrahepatic (and occasionally extra hepatic) biliary ducts, and mildly increased echogenicity. Pulmonary hypoplasia resulting from oligohydramnios occurs in a number of affected infants (Katherine and Ellis, 2014).



**Figure 1.** Diagrammatic demonstration for the ultra-structure of the fibrocystin (polycystin) molecule, which is expressed in the cilia of both the bile ducts of the liver and collecting ducts of the kidney (copied from *Pediatric Radiology*© Springer-Verlag 2008, Vol.10, 1007) (Baris Turkbey et al., 2008)

Approximately 30% of affected infants die in the neonatal period or within the first year of life mainly due to respiratory insufficiency and or superimposed pulmonary infections. More than 50% of affected children progress to end-stage renal disease (ESRD), usually in the first decade of life. With neonatal respiratory support and renal replacement therapies, the ten-year survival of those who live beyond the first year of life has improved to 82%. Fifteen-year survival is estimated to be 67%-79%, and may be improving. A minority present in older childhood or young adulthood with hepatosplenomegaly and evidence of portal hypertension (Katherine and Ellis, 2014).

ARPKD results from fibrocystin loss of function mutation. Fibrocystin is encoded by *pkhd1*, an extremely large gene that comprises 86 coding exons (Katherine and Ellis, 2014). The functioning protein is a large, trans-membrane, receptor-like protein of 500 kDa which is thought to be involved in tubulogenesis and/or maintenance of duct lumen of epithelium. It associates with basal bodies and primary cilia in renal epithelial cells (Figure 1) (Baris et al., 2008). Pathogenesis of ARPKD involves three major processes; proliferation of epithelial cells, dilatation of the lumen, and secretion of fluid into the lumen. Accordingly, epithelial cells of collecting ducts,

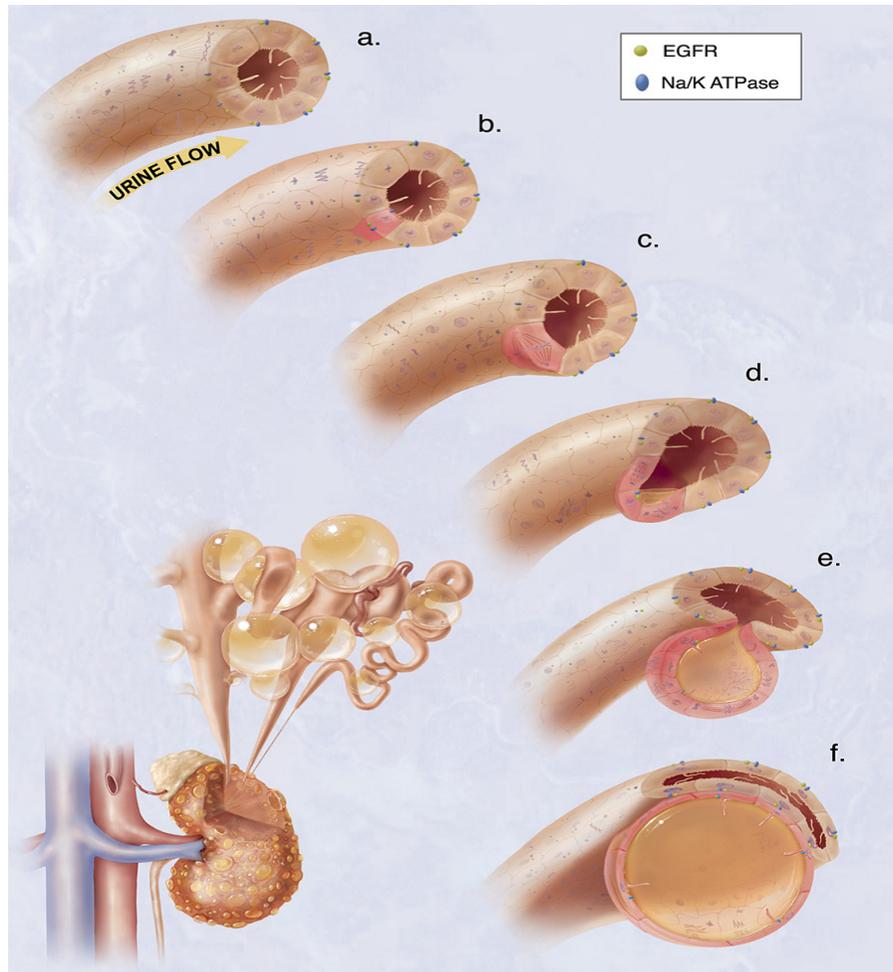
which line the growing cysts in ARPKD, secret fluid into cysts' lumina (Figure 2) (Christian et al., 2010).

Much research work was done for investigating the role of fibrocystin mutation in the development of abnormal renal/ biliary epithelial cells proliferation/ apoptosis and tubular dilatation, which revealed involvement of many pathways (Figure 3) (Bo et al., 2011; Becker et al., 2010; Belibi et al., 2004; Zaika et al., 2013). However, till moment there is no clear explanation for how fibrocystin mutation can lead to intracystic secretion.

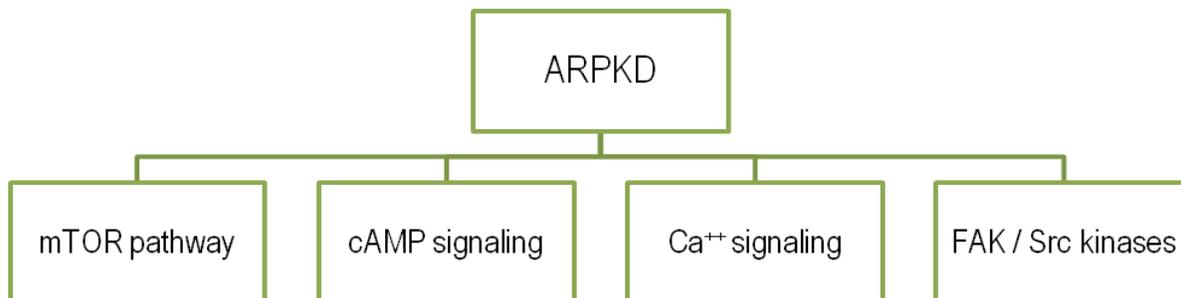
In ARPKD, the following findings have been observed;

- i) Loss of fibrocystin function leads to defective focal adhesion kinase activity (Israeli et al., 2010).
- ii)  $\beta_1$   $\text{Na}^+$  -  $\text{K}^+$  ATPase subunit expression is decreased for  $\beta_2$  subunit (Patricia, 2011).
- iii) Cellular polarization is disturbed and  $\text{Na}^+$  -  $\text{K}^+$  ATPase localizes to apical rather than lateral cellular borders, which contributes to a major role in disease pathogenesis (Krupinski and Beitel, 2009; Robert and Surya, 2008).

Under physiological conditions, both FAK and  $\beta_1$   $\text{Na}^+$  -  $\text{K}^+$  ATPase subunit localize to cell adhesions where both are suggested to be involved in controlling cellular polarization and intracellular signaling (Israeli et al., 2010; Patricia, 2011; Krupinski and Beitel, 2009).



**Figure 2.** Hypothetical model of cystogenesis. The illustration depicts mechanosensory function of renal tubular epithelial cilium. a. Each cilium plays an important role to transmit extracellular information, such as urine flow, into the cell. This message may provide critical signals to the cell regarding the direction of cell division along the tubule. b. Insults, such as genetic disorder or random mutation, will result in abnormal ciliary function to sense fluid movement. c. The functional abnormality in ciliary sensing may result in loss of planar cell polarity. d. Direction of cell division becomes randomized, resulting in increasing tubular diameter rather than tubular elongation. e. Budding of a cyst from the renal tubule and abnormal localizations of epidermal growth factor receptor (EGFR) and Na<sup>+</sup> - K<sup>+</sup> ATPase pump are typical characteristics of polycystic kidney. f. The cyst is eventually enlarged and isolated. Multiple cysts from the neighboring nephrons are illustrated on the bottom left corner. (Copied from *Frontiers in Bioscience* 2008, Vol.13, 4451-4466)



**Figure 3.** Pathways involved in ARPKD (Bo et al., 2011; Becker et al., 2010; Belibi et al., 2004; Zaika et al., 2013)

## HYPOTHESIS

The impaired epithelial cellular polarity seen in ARPKD might result from impaired  $\beta_1 / \beta_2$   $\text{Na}^+ - \text{K}^+$  ATPase expression together with impaired FAK activation and functions, with possible involvement of adhesion signaling in development of those interactions.

## SUGGESTED METHODOLOGY

(A) Using Madin-Darby canine kidney (MDCKII) cells that form tubular structures and/ or cilia to allow discrimination of apical and baso-lateral borders by fluorescent microscopy;

1. Localization of  $\text{Na}^+ - \text{K}^+$  ATPase by Immunofluorescence, using anti  $\alpha_1$  monoclonal antibodies.

2. Assessment of FAK and FAK Y397 phosphorylation by WB using specific antibodies.

3. Assessment of  $\beta_1$  and  $\beta_2$   $\text{Na}^+ - \text{K}^+$  ATPase subunits expression by WB using specific antibodies and quantitative PCR.

4. Knockdown of fibrocystin using specific siRNA followed by repetition of steps 1- 3.

5. Knockdown or inhibition of FAK (using specific siRNA or FAK-14 inhibitor) followed by repetition of steps 1- 3.

(B) Using ARPKD primary cells and cloned myroslated FAK (myr FAK, a constitutively active FAK);

1. Localization of  $\text{Na}^+ - \text{K}^+$  ATPase by IF, using anti  $\alpha_1$  monoclonal antibodies.

2. Assessment of FAK and Y397 phosphorylation by WB using specific antibodies.

3. Assessment of  $\beta_1$  and  $\beta_2$   $\text{Na}^+ - \text{K}^+$  ATPase subunits expression by WB using specific antibodies and quantitative PCR.

4. Expression of myr FAK in ARPKD cells, followed by repetition of steps 1- 3.

## DISCUSSION AND CONCLUSION

The localization of transport pumps determines the physiological function of the duct epithelium. When electrolyte pumps, of which  $\text{Na}^+ - \text{K}^+$  ATPase is the most important, localize to the basolateral borders of the duct epithelial cells, this physiological localization results in pumping of the  $\text{Na}^+$  ions in the interstitial spaces and sub-epithelial tissue. Accordingly, water follows the higher osmolarity and the epithelium is absorptive (Krupinski and Beitel, 2009).

$\text{Na}^+ - \text{K}^+$  ATPase is a transmembrane protein complex composed of  $\alpha$  and  $\beta$  subunits.  $\alpha$  subunit is the channel with ATPase activity, while  $\beta$  subunit has a regulatory function. Naturally occurring isoforms for every subunit include  $\alpha_1$  &  $\alpha_2$  and  $\beta_1$  &  $\beta_2$  (Patricia, 2011). In ARPKD, it was found that  $\text{Na}^+ - \text{K}^+$  ATPase shows mislocalization

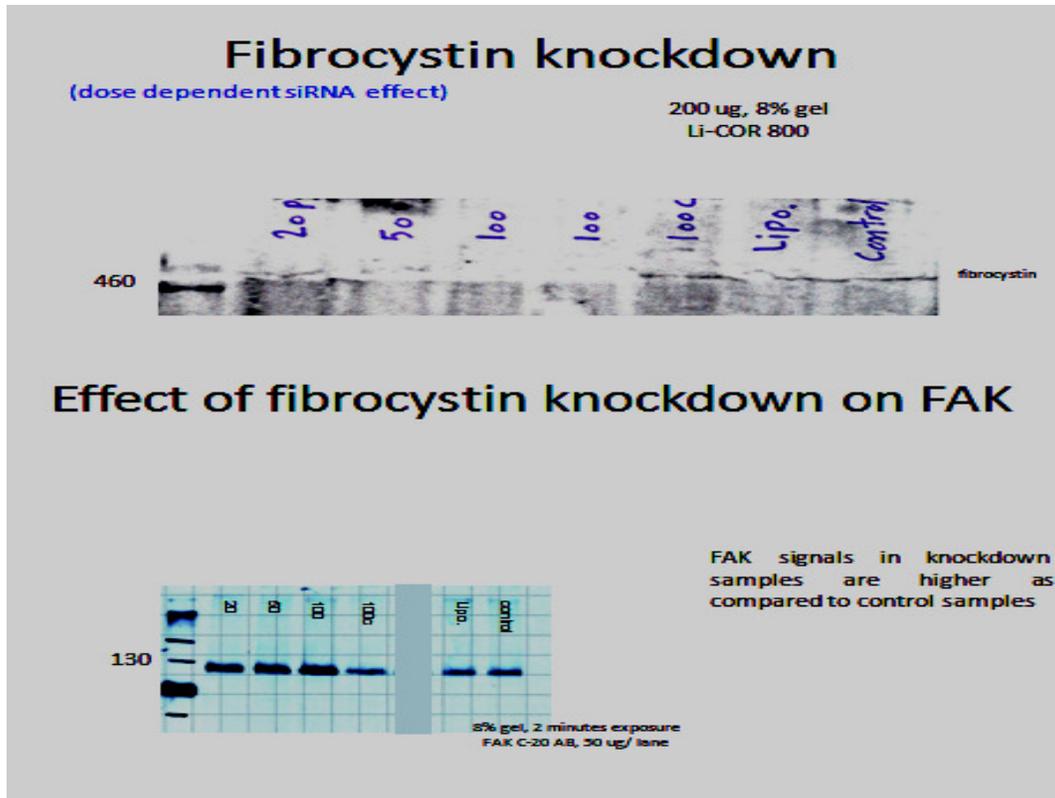
from basolateral to apical epithelial cellular borders (Figure 2), which coincided with a switch from  $\beta_1$  subunit expression to enhanced expression of  $\beta_2$  subunit. This pattern of expression and localization physiologically seen during nephrogenic development but not in mature developed nephrons (Patricia, 2011; Krupinski and Beitel, 2009; Robert and Surya, 2008).

According to this abnormal localization,  $\text{Na}^+$  ions are secreted into the lumen with the water following and the epithelium becomes secretory. Physiologically localized (basolateral)  $\text{Na}^+ - \text{K}^+$  ATPase was found to be essential for fusion of the multiple developing lumina into one lumen during renal tubulogenesis, and for maintenance of normal tube diameter through maintaining normal fluid flow. Block of  $\text{Na}^+ - \text{K}^+$  ATPase leads to dilatation of the tubules with epithelial proliferation secondary to dilatation (Krupinski and Beitel, 2009). However, how fibrocystin loss of function mutation can be involved in the process of  $\text{Na}^+ - \text{K}^+$  ATPase mislocalization!

During development, epithelial cellular borders cannot be marked into apical and baso-lateral due to absence of tight junctions between adjacent cells, which coincides with increased expression of  $\beta_2$   $\text{Na}^+ - \text{K}^+$  ATPase subunit. However, at late developmental stages, with the development of tight junctions, epithelial cells polarize into apical and baso- lateral borders, with  $\text{Na}^+ - \text{K}^+$  ATPase localizes to lateral cellular borders under physiological conditions, which coincides with increased expression of  $\beta_1$  subunit (Patricia, 2011).  $\beta_1$  subunits were found to localize to tight junctions and focal adhesions, where interactions with adhesion proteins signaling cannot be excluded (Madan et al., 2007; Irina et al., 2014).

The cell adhesion molecule E-cadherin has been implicated in development and maintaining of polarity of epithelial cells. Transformed MDCK cells with reduced levels of E-cadherin and  $\beta_1$   $\text{Na}^+ - \text{K}^+$  ATPase subunit showed disturbed polarization and increased migration. Expression of E- cadherin in these cells, without re-expression of  $\beta_1$   $\text{Na}^+ - \text{K}^+$  ATPase subunit, fail to re-establish cellular polarization. While re- expression of both proteins resulted in re-establishment of polarity and reduced cell motility. Which remarks a very important role of  $\beta_1$   $\text{Na}^+ - \text{K}^+$  ATPase subunit in regulating cell polarization (Irina et al., 2014; Rajasekaran et al., 2001; Sigrid et al., 2005).

Focal adhesion kinase (FAK) is a very potent and important intracellular tyrosine kinase which plays many important roles in adhesion signaling, cellular motility, cellular survival and growth. FAK interacts with Src tyrosine kinase and form a complex that exerts a group of synchronized tyrosine phosphorylation actions which mediates many vital cellular processes. Phosphorylation of FAK at different tyrosine residues results in various activities. FAK phosphorylation at Y397 results in auto-phosphorylation and auto-activation (Israeli et al., 2010; Mitra and Schlaepfer, 2006). It was described that



After optimization of the western blot protocol, the 500 kDa fibrocystin bands were detected and knockdown experiment was done using specific siRNA and lipofectamine reagent (Invitrogen).  $0.5 \times 10^6$  cells were seeded in 6 wells plate and incubated overnight till 70% confluency. The used siRNA concentrations for treatment of cells were 20, 50, 100 pmol/ ml, in addition to 100 pmol/ ml control non-specific siRNA treatment, lipofectamine only treatment and control non-treated cells. Cells were incubated after treatment for 48 hours before they were washed twice with cold PBS and scraped, on ice, in 0.5 ml RIPA buffer supplemented with proteases inhibitors. After sonification, cellular extracts were centrifuged to get rid of debris and protein concentration of each sample was determined according the laboratory protocol. FAK bands were detected using FAK C-20 antibody (Santa Cruz). Analysis of the results was done using Licor system software. Dose dependent fibrocystin knockdown resulted in corresponding dose dependent induced FAK

fibrocystin mutation or knockdown leads to defective phosphorylation of FAK at Y397 resulting in inappropriate FAK activity (Israeli et al., 2010). Meanwhile, RNAi studies established that FAK is important for polarized cell migration (Mitra and Schlaepfer, 2006; Alok et al., 2009). FAK deficient fibroblasts fail to establish proper polarized migration even with increased Src expression. This role of FAK is mediated through signaling interactions with other cytoskeletal and focal adhesion proteins (Alok et al., 2009). All those findings support the notion that FAK might be involved in regulation of cell polarity.

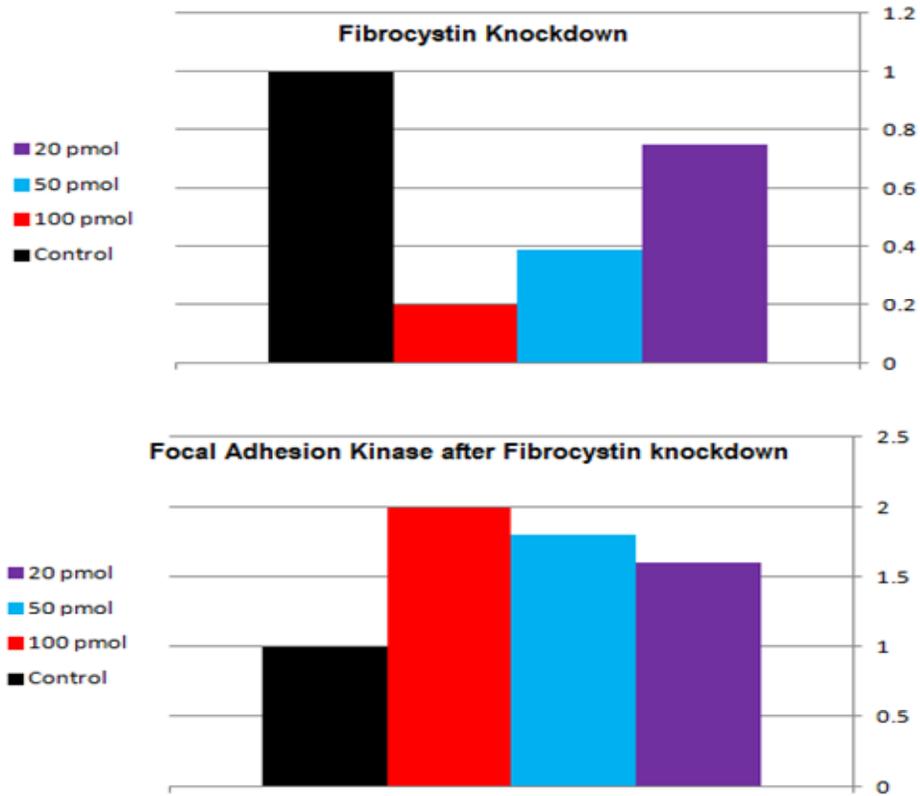
Decreased expression of  $\beta_1$  Na<sup>+</sup>- K<sup>+</sup> ATPase subunit, which is necessary for E- cadherin mediated establishment of cell polarity, and decreased activation of FAK, which interacts with adhesion proteins and is essential for establishment of polarized cell migration, both together may explain how fibrocystin loss of function mutation can result in reversed epithelial cell polarity which proceeds to intracystic secretion in ARPKD.

## TESTING OF HYPOTHESIS

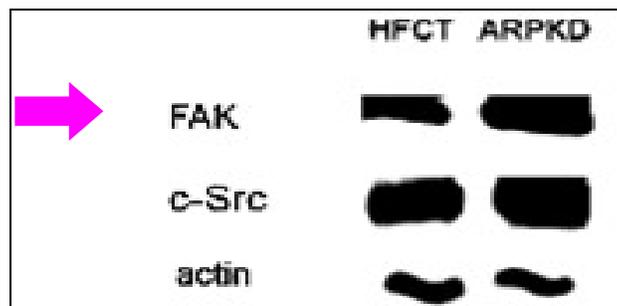
It was observed by Israeli and his colleagues that fibrocystin knockdown resulted in defective FAK activity, however, without corresponding increase in the FAK band as detected with immunoblotting (Israeli et al., 2010).

That would indicate that the defective FAK activity may be non-relevant for ARPKD development, because in any living cell if an enzymatic activity was blocked or defective, the cell will try to overcome this block with different mechanisms, including activation of other enzymatic activities that would overcome the point of block. However, with persistent enzymatic defect and with failure of the cell to bypass this enzymatic block, the cell will compensate with induction of the enzyme protein. With persistence of the cause of enzymatic defect, disease phenotype results.

Application of this simplified model to collecting duct epithelial cells, if the fibrocystin mutation/ knockdown-



**Figure 4.** Effect of fibrocystin knockdown on FAK bands in MDCK II cells. With 100 pmol/ml siRNA, 80% fibrocystin knockdown was achieved, with corresponding double fold increase in FAK band density.



**Figure 5.** increased FAK band density in ARPKD cells in comparison to age matched human fetal collecting tubules (HFCT) cells. (Copied from Israeli S, Amsler K, Zheleznova N, Wilson PD (2010). Abnormalities in focal adhesion complex formation, regulation, and function in human autosomal recessive polycystic kidney disease epithelial cells. *Am J Physiol Cell Physiol.*298 (4): C831-C846)

induced FAK defective activation was overcome by the cells so that it is not incorporated in disease development, the cell would not induce FAK and accordingly there would not be significant increase in FAK band in immunoblotting (as stated by Israeli et al).

To make sure of this point, three independent repeats of fibrocystin knockdown on MDCK II cells were performed and FAK bands were assessed with immunoblotting (Figure 4). The results showed double

fold increase of FAK in response to fibrocystin knockdown. However, confirmation with further repeats and assessment of mRNA expression would be essential.

In addition, FAK bands were obviously denser in cells of ARPKD renal collecting tubules than in cells of age-matched human fetal renal collecting tubules (HFCT), as presented by Israeli et al (Figure 5) (Israeli et al., 2010). This would prove the involvement of defective FAK activity in the development of ARPKD.

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