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CALCIUM HOMEOSTASIS AND POLYCYSTIN-2 EXPRESSION IN T-LYMPHOBLASTS OF PKD SUBJECTS

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1. ABSTRACT

Mutations of polycystin-1 (PC1) and polycystin-2 (PC2), coded by PKD1 and PKD2 genes, account for approximately 85 and 15% of Autosomal Dominant Polycystic Kidney Disease (ADPKD) cases, a common and important inherited kidney disorder. PC2 is a calcium-permeable and -regulated channel, interacting with the membrane receptor PC1, both colocalizing in the 'primary cilium' of epithelial cells as a flow-sensitive mechanosensor involved in signal-transduction. Diagnostic tools to decrease ADPKD are little in progress. Renal ultrasound, the principal diagnostic procedure, not allows an early diagnosis. The molecular characterization of the mutations can have a prognostic value, the progression to ESRD occurring more rapidly in PKD1 patients. The genetic test is, however, very expensive, time consuming, many missense variants remaining without a known function.

This study is addressed to define whether PC1 and PC2 are expressed and play some functional role in T-lymphoblasts (HTL). These cells are easily obtainable from peripheral blood, differently from kidney cells and transgenic mice that represent canonical models for the study of PKD gene expression and function. In particular, attention has been focused on the possible relation between abnormal PCs levels and Ca^{2+} homeostasis in HTL obtained from ADPKD patients and thus carrying a mutation of either PKD1 or PKD2 gene. Expression studies have also considered fibrocystin/polyductin (FC1), mutated in the recessive form of the disease, known to be functionally related to PCs in vivo. Reduced cytoplasmic Ca^{2+} levels or other cellular abnormalities in ADPKD HTL will provide further information on the extrarenal role of PCs. Moreover, as HTL are easily and quickly obtainable from peripheral blood cells, positive results may lead to an ADPKD diagnosis based on quantitative and functional evaluation of PC1/PC2 channel complex.

Peripheral blood samples from 31 (13 F) controls and 34 PKD patients (21 F) including some genetically characterized ADPKD1 subjects, have been considered in the present study and compared to B-lymphoblastoid cell lines (LCLs) and human adult and embryonal kidney cell lines. Results obtained demonstrate that PC1 and PC2 are expressed in HTL, although at a lower level than in kidney cells. Since PC1 is not correctly quantifiable in HTL, no comparison between PC1 levels in

ADPKD HTL and controls is reported. On the contrary, the degree of PC2 expression in HTL, which appear comparable to that of B-LCLs, does not differ in ADPKD and non-ADPKD HTL (PC2 relative abitrary units, 0.572 ± 0.228 SD vs 0.553 ± 0.176 SD). Few cases with very low PC2 levels (lower than 2 fold the SD of ADPKD1 average values) and showing a less severe disease because entered in dialysis very late (over 70 y. o.), may carry a PKD2 mutation, which should be expected in 15% of ADPKD. Also the investigation of FC1, mutated in the recessive form of PKD, and of FCL, its homologue highly expressed in activated T-cells, does not reveal quantitative alteration in the level of combined FC1/FCL proteins in ADPKD HTL.

As far as the function of PC1/PC2 channel complex is concerning, in HTL average values of PAF-evoked [Ca^{2+}] are lower in ADPKD than in non-ADPKD (78.96 ± 9.67 SE nM vs 124.51 ± 16.87 nM, $p<0.05$), and a same reduction is present in ADPKD PBL (112.03 ± 13.87 nM SE vs 183.32 ± 20.54 nM, $p<0.01$). These results show that PCs have a functional role in T-cells. However, the large and overlapping distribution of the data in both PBL and HTL does not allow the use of [Ca^{2+}] differences to distinguish individual ADPKD and non-ADPKD cases. A further marked cellular abnormality observed in ADPKD HTL is consistent with the anti-apoptotic action of PC1: ADPKD HTL death is higher compared to normal HTL (after 2 days of culture the % of trypan blue positve cells is 7.6 ± 2.8 SD vs 24.1 ± 9.7, $p<0.05$). Moreover, the observation of a larger size of cell aggregates in ADPKD compared to control HTL, is consistent with the PCs-dependent positive control of cell scattering/migration of renal epithelial cells. Alterations, presumably located at membrane level, are also present in ADPKD neutrophils which are basically more activated, but less responsive to stimuli, than control cells.

Overall these findings indicate that aploinsufficiency of PKD1, the more probable involved gene in these patients, is detectable in HTL as an impairment of the PC1/PC2 channel, reduced cell survival and altered aggregation, thus playing PC1/PC2 a still undefined role in HTL. Aploinsufficiency in these cells seems, however, not sufficient to discriminate between unique ADPKD and not ADPKD subjects.

2. INTRODUCTION

2.1 POLYCYSTIC KIDNEY DISEASE

Polycystic kidney disease (PKD) is a genetic disorder characterized by the growth of numerous fluid-filled cysts in the kidneys, which filter wastes and extra fluid from the blood to form urine, and also regulate amounts of certain vital substances in the body. PKD cysts can profoundly enlarge the kidneys while replacing much of the normal structure, resulting in reduced kidney function and leading to kidney failure (Fig. 1).

When PKD causes kidneys to fail which usually happens after many years, the patient requires dialysis or kidney transplantation. About one-half of people with the most common type of PKD progress to kidney failure, also called end-stage renal disease (ESRD).

PKD affects 12.5 million people worldwide, making it twice as common as multiple sclerosis and five times as common as cystic fibrosis, and in the United States, it is the fourth leading cause of kidney failure.

Two major inherited forms of PKD exist. Autosomal Dominant PKD (ADPKD) is the most common form. Symptoms usually develop between the ages of 30 and 40, but they can begin earlier, even in childhood. About 90 percent of all PKD cases are autosomal dominant PKD. Autosomal Recessive PKD (ARPKD) is a rare form. Symptoms of ARPKD begin in the earliest months of life, even in the utero.

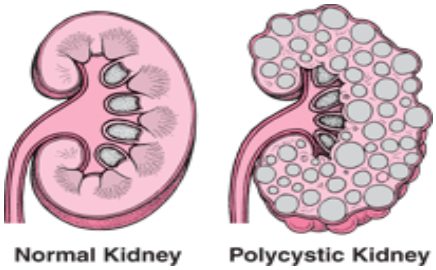


Figure 1: Schematic picture of normal and polycystic kidney

2.2 AUTOSOMAL DOMINANT POLYCYSTIC KIDNEY DISEASE

ADPKD (MIM 173900) is the most common Mendelian disorder of the kidney and affects all racial groups worldwide, with a frequency of 1:500 to 1000 (Gabow PA., 1993). It is characterized by focal and sporadic development of cyst formation in ductal organs, particularly kidney and liver, and by gastrointestinal, cardiovascular, and muscoskeletal abnormalities in an age-dependent manner. Typically, only a few renal cysts are detected in most affected individuals before 30 yr of age.

However, by the fifth decade of life, hundreds to thousands of renal cysts will be found in the majority of patients (Gabow PA, 1993). Renal cysts arise as a focal outpouch from any segment of the nephron and increase in size and number throughout the patient's life. The normal renal parenchyma is progressively replaced by ballooning, fluid-filled structures in a process that results in end stage renal disease in -50% by the sixth decade of ADPKD cases (Gabow PA, 1993).

Although the renal involvement is the most important clinical manifestation, the ADPKD can be defined a multisystem disorder presenting a considerable individual variation in the gene expression. Clinical symptoms generally begin in the third to fifth decades, although the diagnosis by utrasonography can occasionally be made in the children. However, cysts form at a later age in 34% of patients with negative utrasonographic analysis (Kimberling WJ et al, 1988).

Overall, ADPKD accounts for 5-10% of ESRD and represents a major health care burden in developed countries.

2.2.1 RENAL MANIFESTATIONS AND FUNCTIONAL ABNORMALITIES

The renal manifestations of ADPKD encompass structural, functional and endocrine abnormalities as well as complications (Gabow PA, 1993). The main

structural change is the cyst formation. In the early state of the disease less than 5% of nephrons are cystic. The renal cysts vary considerably in size and appearance from a few millimeters to a many centimeters and from clear to cloudy/chocolate-colored, suggesting hemorrhage. Other structural abnormalities are adenoma formation, which is found in 21% of the patients.

The earliest and most consistent functional abnormalities are a decrease of renal concentrating ability. Certain renal endocrine functions appear to be altered, as reflected by the increase in the secretion of both renin and erythropoietin.

Abnormal renin secretion results from the activation of the renin-angiotensinaldosterone system, presumably by stretching and attenuation of intrarenal vessel around the cysts, causing areas of distal ischemia (Gabow PA, 1993).

In ADPKD cysts derive from all-nephron segments, including occasional glomeruli. Morphologic studies examining individual cysts suggest that, in the early phase, cysts maintain continuity with the parental nephron, and then enlarge as a consequence of alterate proliferation forming the out-pocketing from the tubule wall. However, no evidence of tubule continuity can be demonstrated in larger cysts. Finally, the cysts lose their tubular connection and become isolated from the glomerulus, and the active secretion of cystic epithelium thus favors a further cyst expansion (Sutters and Germino, 2003).

Animal models of renal cystic disease, cultures of Madin Darby canine-kidney cells (MDCK) and cultures of human renal cystic epithelium demonstrate that alterations in cell growth, fluid secretion, and extracellular-matrix composition are all involved in cystogenesis.

Although some studies using passaged cells have not shown increased cell growth of cystic epithelium compared with controls, other semiquantitative studies have shown increased numbers of cell divisions of cyst epithelia in primary tissue culture compared with the normal renal tubular cells. Moreover, human ADPKD primary cultures have shown amplified proliferative response to growth factors such as epidermal growth factors (EGF) and decreased inhibitory response to inhibitors of proliferation such as tumor growth factor β (TGFβ). Thus, autocrine and paracrine systems might influence the growth of the cysts, but abnormal cell proliferation

might be not sufficient for cysts formation and enlargement (Gabow PA, 1993).

Evidence of increased proliferation in ADPKD came also from the ultrastructural identification of micropolyps and the determination that cyst's epithelium accommodated the gigantic degree of expansion not by stretch, but by an increased number of cells. In keeping with these anatomical observations, the proliferation-associated genes c-myc, Ki-67 (Lanoix J et al, 1996) and PCNA (Nadasdy T et al, 1995) were found to be significantly over-expressed in cyst epithelia of ADPKD subjects. Furthermore, over-expression of c-myc in transgenic mice was associated with the development of polycystic kidneys (Trudel M et al, 1991).

Apoptosis is extremely rare in normal tissues, but it has been observed in non-dilated, cystic tubules and glomeruli in ADPKD tissues. Markers of proliferation and apoptosis share a similar distribution in ADPKD and there appears to be a correlation between the degree of proliferation and the level of apoptosis (Sutters and Germino, 2003). These studies suggest that the two processes are in some way related. Further support for the idea that loss of polycystin pathways leads to increased proliferation and apoptosis has emerged from some studies demonstrating the potential for overexpressed full-length polycystin-1 to have precisely opposite effects (Bhunia AK et al, 2002; Boletta A et al, 2000).

Modified fluid secretion is also necessary in the cystogenesis. In vitro cyst formation from MDCK depends on whether proliferation occurs with or without secretion. Stimulation of cyclic AMP (cAMP) in this model produces net fluid secretion and enlargement of the cysts, suggesting a link between this second messenger and cystogenesis. Another potential mechanism responsible for fluid secretion may be the abnormal location of Na+/K+-ATPase mistargeted from its usual basolateral position to an apical location. Therefore, apical membrane localization of the Na+/K+-ATPase also reflects a relatively undifferentiated phenotype of epithelial cells lining collecting tubule cysts. In fact, these cells appear relatively flattened and lack the distintive microcavillous apical configuration typical of a mature renal tubular epithelium, supporting that they might be de-differentiated (Calvet JP, 1993). The persistence of the apical membrane Na+/K+-ATPase, as functional enzyme, results in the transport of sodium from the basolateral to the apical surface of cystic epithelia in monolayer culture. The reversed direction of

sodium transport could result in cyst growth by causing sodium and fluid to move into a cyst cavity rather than out of it (Avener ED et al, 1992).

Moreover, that regards extracellular matrix, an alteration in the composition of the extracellular matrix is suggested by abnormal appearance of the basement membrane synthesized by human renal cystic epithelium in culture as compared with that formed by normal human tubular cells. The cystic epithelium elaborates extracellular proteins not produced by normal renal cells (Wilson PD, 1997). Furthermore, abnormal basement membrane has been observed in biopsy specimens from subjects with hereditary ADPKD before the cysts can be detected. Later in the disease the basement surrounding the cysts is an abnormal, thickened mass of interwoven fibrils.

2.2.2 EXTRARENAL MANIFESTATIONS

ADPKD also is associated with significant comorbidities from its extrarenal complications, such as valvular cardiac defects, colonic diverticulosis, inguinal hernias, and intracranial arterial aneurysms (Gabow PA, 1993).

Cysts occur in other organs as liver, ovary, pancreas, spleen and central nervous system. Complications due to hepatic cystogenesis appear to be influenced by female steroid hormones. The main no cystic extrarenal manifestation is the presence of cardiac valvular abnormalities, as a mitral —valve prolapse, palpitation and non exertional chest pain. The aortic valve can also be abnormal and can require surgical replacement. Aortic and mitral valves may have myxomatous degeneration, suggesting a disordered matrix metabolism.

One of the devastating extrarenal manifestations is intracranial saccular aneurysm (berry aneurysm). These events support an abnormal matrix and perhaps altered cerebral vascular reactivity in ADPKD, suggested by high frequency of headaches.

The principal no cystic gastrointestinal manifestations are colon diverticula and colon perforation (Gabow PA, 1993).

2.3 GENES INVOLVED IN ADPKD

ADPKD exhibits considerable variability with respect to both its renal and extrarenal manifestations (Qian F et al, 1996). Genetic heterogeneity is likely to account for at least some of these differences. There are different forms of ADPKD, which are caused by mutations in at least three different alleles. Approximately 85% of ADPKD cases are caused by mutations in the PKD1 locus. The remaining of ADPKD cases are accounted for by mutations in the PKD2 and possibly the PKD3 loci (Malhas AF et al, 2002). So far only two genes responsible for the disease have been mapped: the PKD1 gene located at the chromosome 16p13.3 (Reeders ST et al, 1985) and the PKD2 gene at the chromosome 4q13-23 (Kimberling WJ et al, 1993; Peters DJ et al, 1993). Linkage studies in a small number of families have not yet mapped the disease-gene responsible for the third ADPKD gene (Bogdanova N et al, 1995; Daoust MC et al,, 1995; de Almeida S et al, 1995).

Mutations in the different genes have been shown to cause the same disease presenting with common features and symptoms that prevent one type from being distinguished from another on clinical ground. Nevertheless, it is a general agreement that type 2 ADPKD follows a milder course with end stage renal disease (ESRD) occurring approximately 10-12 years later than PKD1-related disease, about at 60-70 years (Koptides M et al, 2000).

2.4 PKD1 GENE AND ITS EXPRESSION

Linkage analysis in several ADPKD families demonstrates the Mendelian inheritance of the disorder. This analysis identified the first locus responsible for ADPKD, designed PKD1, mapping close to the α-globin cluster on the short arm of the chromosome 16 (Reeders ST et al, 1985). Subsequently, the position of the PKD1 locus was refined at chromosome band 16p13.3 (Breuning MH et al, 1990). An accurate long range restriction map located PKD1 locus in an interval of approximately 600-750 kb between the markers GGGI and SM7 (Harris PC et al,

1994, Germino GG et al, 1992, Somlo S et al, 1992). The density of CpG islands and the identification of many mRNAs transcripts indicated that this area is rich in gene sequences. Some studies estimated that the candidate region contains approximately 20 genes (Germino GG et al, 1992).

Disease-associated genomic rearrangements, detected by cytogenetics, gene sequences and pulsed-field gel electrophoresis (PFGE), have been instrumental in the identification of genes associated with many genetic disorders. Positional segregation of PKD1 locus in an unusual ADPKD family with contiguous gene syndrome presenting severe childhood onset polycystic kidney disease and tuberous sclerosis (TS) was useful to lie PKD1 gene immediately adjacent to tuberous sclerosis gene 2 (TSC2) (The European Polycystic Kidney Disease Consortium, 1994). This family presents a balanced translocation 16p13.3-22q11.21 and breakpoint analysis allowed to identify a novel gene named polycystic breakpoint protein gene (PBP) that lies adjacent to TSC2 (The European Polycystic Kidney Disease Consortium, 1994). Identification of deletions and point mutations in PBP gene confirmed its identity as the PKD 1 gene (The European Polycystic Kidney Disease Consortium, 1994; Schneider MC, 1996).

The characterization of the PKD1 gene has been more difficult than for other disorder genes because more than three quarters of the gene is embedded in a DNA region that is duplicated elsewhere on chromosome 16, exactly in 16p 13.1. A segment of 40-50 kb, present as a single copy in the PKD1 area (16p13.3), is reiterated as several divergent copies in the more proximal region 16p 13.1, termed the homologous gene (HG) area. The HG region encodes three large transcripts, HG-A (21 kb), HG-B (17 kb) and HG-C (8.5 kb), and although these have 3’ ends which differ from PKD1, they share substantial homology to the PKD1 transcript over most of their length: it is not known whether HG transcripts produce functional protein (The European Polycystic Kidney Disease Consortium, 1994).

Sequencing of cDNA contiguous revealed a total PKD1 transcript of 14.148 bp which extents ~ 52 kb of genomic DNA region (Hughes J et al, 1995). The entire transcript consists of 46 exons (GenBank accession number: L33243); the first 33 exons localize in the duplicated region (Watnick TJ et al, 1997). The PKD1 transcript shows an open-reading frame (ORF) of 12.912 bp, a 5' untranslated region (5' UTR) of 228 nucleotides and a 3' untranslated region (3' UTR) of 1.019 nucleotides (Hughes J. et al, 1995) (Fig. 2). This transcript encodes for a 4.302 amino acid membrane glycoprotein (as

described below) named polycystin-1 (PC 1).

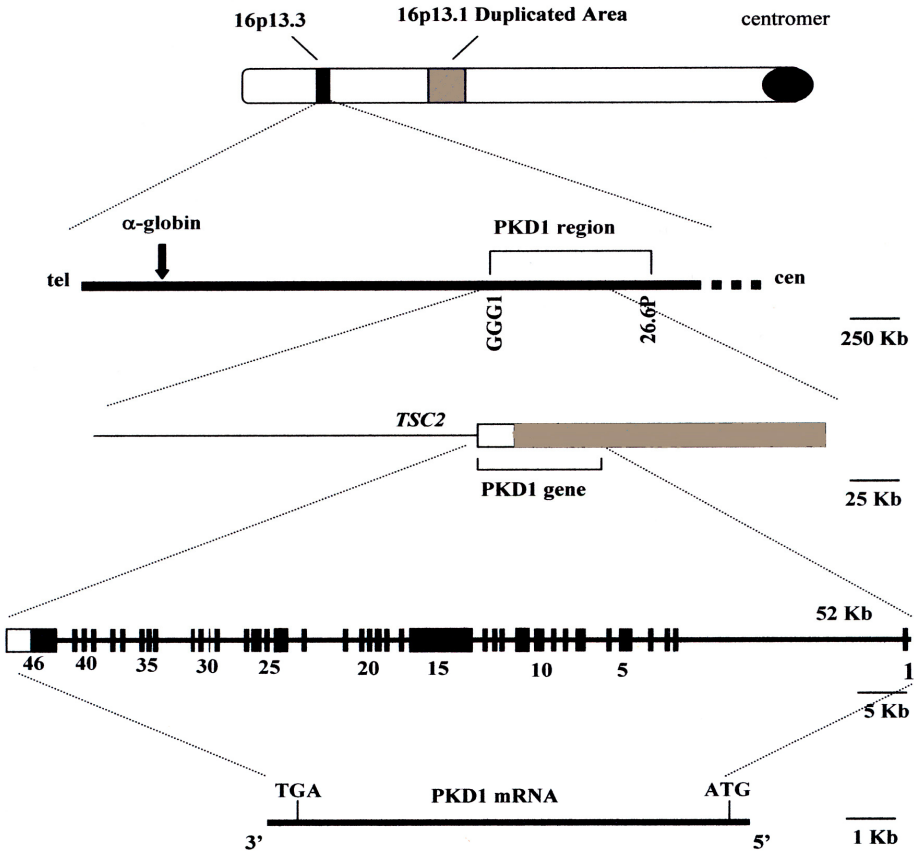


Figure2: Localization and genetic structure of the PKD1 gene

The analysis reported by the European Polycystic Kidney Disease Consortium (1994) showed a 14 kb PKD1 mRNA transcript identified by Northern blotting in several tissue-specific cell lines (Epstein Barr virus-transformed lymphocytes; K562 erythroleukemia; normal fibroblasts; HeLa cervical carcinoma; G401 renal Wilm's tumor; Hep3B hepatoma; SW13 adrenal carcinoma and G-CCM astrocytoma). The transcript was detected often at low levels; the highest expression levels were detected in fibroblasts and in the astrocytoma cell line. Significant PKD1 mRNA was also obtained in cell lines derived from kidney and liver.

By RNAse protection assay, using a single copy region of the PKD1 gene, the highest levels in the brain (cerebral cortex) and the lowest in the thymus (6- to 7-fold decrease) were found; kidney showed intermediate levels of decreased expression. These data were confirmed by RT-PCR performed using human cDNA libraries

(Buraczynska MJ. et al, 1995). A moderate level of PKD1 expression was found by analyzing kidney and liver of ADPKD subjects (2 fold respects with the normal subject). mRNA analysis showed that a widespread expression of PKD1 gene is consistent with the systemic nature of ADPKD (Ward CJ et al, 1996).

2.4.1 THE POLYCYSTIN 1 AND ITS FUNCTION

Polycystin-1 (PC1), the product of the *PKD1* gene, contains 4302 amino acids and has a molecular weight of about 500.000 D (Hughes J et al, 1995; International Polycystic Kidney Disease Consortium, 1995; Sandford R. et al, 1997).

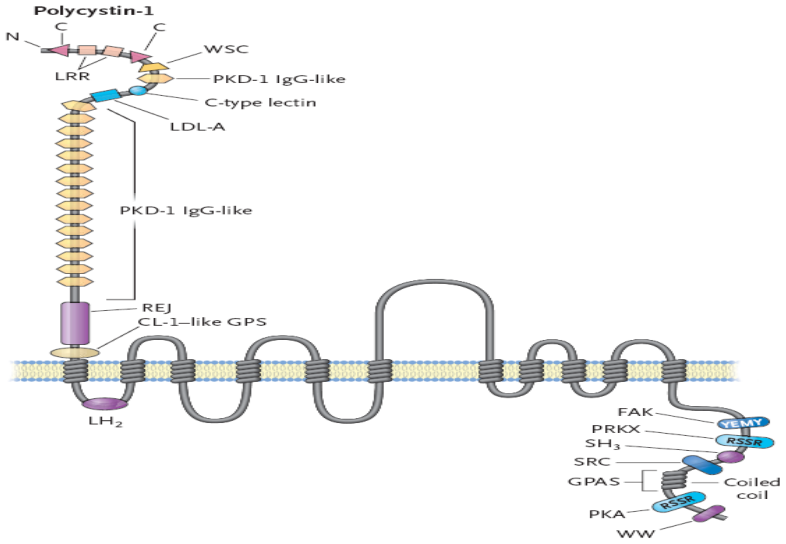


Figure 3. Structure of polycystin-1

As shown in figure 3, PC1 is an integral membrane protein that is predicted to contain 11 transmembrane segments. The large extracellular amino-terminal domain contains a set of distinct protein motifs, including two leucine-rich repeats flanked by cysteine-rich domains, a C-type lectin domain, a WSC domain, and 16 immunoglobin-like domains called PKD repeats. Many of these motifs are involved in protein-protein or protein- carbohydrate interactions, that leads to think that p PC1 may function as a receptor for an as yet unidentified ligand.

Closer to the membrane, there is a region of homology to the sea urchin egg jelly receptor (Moy GW et al, 1996) and a potential proteolytic cleavage site (GPS

domain) (Mengerink et al, 2002; Ponting CP et al, 1999). Between the first and second transmembrane domains, there is a region of similarity to lipoxygenases (PLAT domain) (Ponting CP et al, 1999; Bateman A et al, 1999). The carboxy-terminus of PC1 is located in the cytoplasm and contains a coiled-coil domain that mediates protein-protein interactions as well as several potential sites of phosphorylation.

PC1 is expressed in many tissues, including the kidney, brain, heart, bone and muscle (Geng L et al, 1996). Several studies have been identified PC1 in the plasma membrane of tubular epithelial cells, especially in the distal nephron and collecting ducts (Geng L et al, 1996; Ibraghimov-Beskrovnaya O et al, 1997). In mature tubules it is in the lateral membrane at sites of cell-cell interaction (Foggensteiner L et al, 2000). Indeed, PC1 has been identified in cell junctional complexes, including adherens junctions and desmosomes (Xu GM et al, 2001; Bukanov NO et al, 2002). PC1 is a glycosylated protein (Boletta A et al, 2002) that exists in two pools: one is sensitive to endoglycosidase H (Endo H), and another, in the plasma membrane, is Endo H-resistant (Newby LJ et al, 2002).

The structure of PC1 is similar to a family of cell surface receptors that are involved in the acrosome reaction, an essential step in fertilization (Moy GW et al, 1996). The acrosome reaction is an exocytic process in which a large vesicle contained within the sperm head fuses with the plasma membrane and releases its contents into the extracellular medium. The acrosome reaction is triggered by the binding of a ligand, in the jelly surrounding the egg, to the egg jelly receptor on the sperm head. Activation of the egg jelly receptor results in increased cytosolic calcium and pH and in vesicle fusion. The homology between egg jelly receptors from sea urchin and mammals and PC1 (Moy GW et al, 1996; Mengerink et al, 2002; Hughes J et al, 1999).

Motifs that are conserved between PC1 and one or more egg jelly receptors are the REJ domain, transmembrane segments, C-type lectin domain, PLAT domain, GPS domain, and PKD repeats. The homology with the egg jelly receptors suggests that PC1 may also be a cell surface receptor and that the signaling pathways may be similar. Of note in this regard is the demonstration of proteolytic cleavage at the GPS domain in suREJ3, a PC1 homologue (Mengerink KJ, et al, 2002). This observation raises the possibility that proteolytic cleavage at the conserved GPS domain in PC1 has also a role in polycystin function in the kidney. Additionally, a role for PC1 in regulating exocytosis in kidney-derived cells has recently been proposed (Charron AJ

et al, 2000)

Another model organism that is providing clues to the function of polycystins is the nematode *Caenorhabditis elegans*. PC1 and polycystin-2 (the product of the PKD2 gene, see later) homologues in *C. elegans* are essential for the stereotyped mating behavior mediated by a specialized group of ciliated sensory neurons. In *C. elegans*, the polycystin proteins appear to function as mechanosensors (or chemosensors), and their appearance in cilia as well as intracellular membranes has prompted recent interest in the role of the former in mammalian kidneys (Barr MM et al, 1999, 2001). In fact, Nauli and collaborators have been proposed that PC1 functions as a mechanosensor (Nauli SM et al, 2003, 2004), as described later on.

The cytoplasmic carboxyl-terminal domain of PC1 has been shown to activate a number of *intracellular signaling pathways*. Transient transfection of the PC1 carboxyl-terminus activates the Wnt signaling pathway via stabilization of □-catenin and activation of TCF/LEF transcription factors (Kim E et al, 1999). Interestingly, one of the downstream targets for activation by this pathway appears to be the PKD1 gene itself (Rodova M et al, 2002).

The expression of β-catenin is downregulated in Pkd1 mutant mice, an effect that is corrected by the administration of PPARy agonists. Several studies have suggested that polycystin-1 regulates G protein signaling. The carboxyl-terminal domain of polycystin-1 can directly bind heterotrimeric G proteins and the regulator of G protein-signaling RGS7 (Kim E et al, 1999; Parnell SC et al, 1998). A study has shown that full-length PC1 behaves as a G protein-coupled receptor that activates $G\alpha_{i/o}$ and releases Gβγ subunits (Delmas P et al, 2002). Signaling though this pathway is independent of RGS proteins but is antagonized by polycystin-2. G protein signaling pathways regulate processes that are important in cyst formation, such as fluid secretion, proliferation, cell polarity, and differentiation (Grantham JJ et al, 2001). G proteins also appear to be involved in PC1 activation of c-Jun N-terminal kinase and transcription factor AP-1 (Arnould T et al, 1998; Parnell SC et al, 2002). A characteristic feature of cyst epithelial cells is an abnormally high rate of cellular proliferation (Nadasdy T et al, 1995; Grantham JJ et al, 1993; Murcia NS et al, 1999). Overexpression of full-length PC1 in MDCK cells inhibits cellular proliferation and suppresses cyst formation (Boletta A et al, 2000). Overexpression of the carboxyl-terminal domain of PC1 may increase FBS-dependent cell proliferation through a

PKCalpha-mediated Erk1,2 activation (Manzati E et al, 2005).

Moreover, Bhunia and coworkers (Bhunia AK et al, 2002) have shown that PC1 has a direct role in the regulation of the cell cycle by inducing cell cycle arrest at the G0/G1 transition. Progression through the cell cycle is controlled by cyclin-dependent kinases (Cdks), and it was found that polycystin-1 inhibits Cdk2 by upregulating its inhibitor, $p21^{CIP1/WAF1}$. PC1 activates the JAK/STAT signaling pathway in a process that requires polycystin-2 and that leads to activation of $p21^{CIP1/WAF1}$ (Bhunia AK et al, 2002).

2.5 PKD2 GENE AND ITS EXPRESSION

The second gene responsible for 15% of ADPKD cases was mapped to the chromosome 4 by linkage analysis of several large non-PKD1 families. Non-PKD1 patients have been diagnosed at an older age and had fewer cysts at time of diagnosis. These patients were less likely to have hypertension and lived longer (Peters DJM et al, 1993). Linkage analysis has defined the PKD2 genetic interval between the polymorphic markers D4S423 and D4S231. Then, positional cloning in yeast artificial chromosome (YAC) contigs and high-density sequence tag sites (STS) have mapped the PKD2 region (Mochizuki T et al, 1996).

The PKD2 gene, located at 4q21–q23 (Kimberling WJ et al., 1993; Peters DJM et al., 1993) and encoded in at least 15 exons spanning ~68 kb of genomic DNA (Hayashi T et al, 1997) (Fig.4), produces polycystin-2 (PC2).

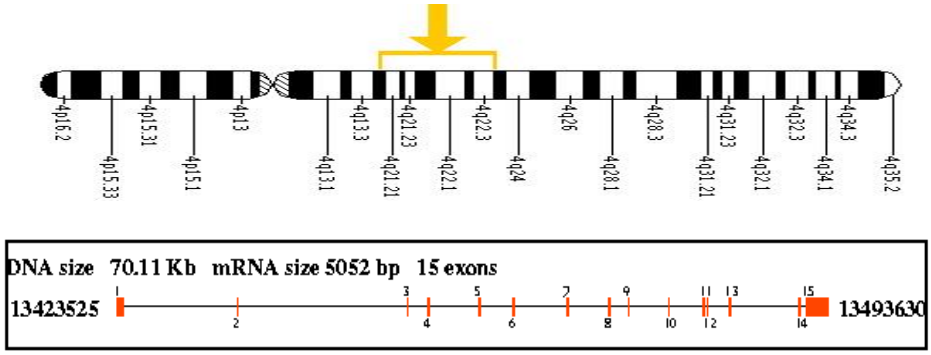


Figure. 4: Chromosomic localization of PKD2 gene, genomic structure and schematic representation of its transcript.

The first exon is at least 660 bp long, extending from the 5' end of the K1-1 cDNA clone (Mochizuki T et al., 1996) to the first splice donor site. This exon is very GC-rich and contains a pair of closely spaced NotI restriction sites in the open reading frame. Exon 15 contains 234 bp of the open reading frame, the TGA termination codon, and 2085 bp of 3' untranslated region. The range of exon sizes is from 99 (exon 10) to 2322 (exon 15) bp. All of the splice acceptor and splice donor sites conform to the AG/GT rule (Mount S. M et al, 1982; Shapiro and Senapathy, 1987). Introns 9, 11, and 14 are small (0.2, 0.3, and 0.5 kb, respectively) and the introns closer to the 5' end of the gene tend to be larger. A minimum of 40 nucleotides of intronic sequence beyond the splice junctions are provided, and these regions are likely to contain the branch site sequences. Mutations in branch sites can disturb splicing.

Seven of 14 exon junctions do not disrupt codons. Two pair of these occur in consecutive positions (exons 6–7 and 9–10), whereas another is the junction between the last two exons. In addition, exons 1 and 2 both disrupt the codon after the first nucleotide, and exons 4 and 5 both disrupt the codon after the second nucleotide. Therefore, deletion of exons 2, 5, 7, 10, or 15, if they occurred as either mutations or splice variants, would be predicted not to alter the open reading frame.

The transmembrane domains 1, 3, 4, and 6 are divided among two exons while transmembrane domains 2 and 5 are contained in single exons. The EF hand domain is contained in exon 12. The region of PC2 from codon 872 to the carboxyl terminal has been implicated in direct heterotypic interaction with PC1 (Qian F et al, 1997). This region is contained in exons 14 and 15. Of the three truncating mutations described in PKD2 (Mochizuki et al., 1996), two were found in exon 5 and the third in exon 11. The PKD2 transcript shows an ORF of 2904 bp, a 5' untranslated region (5' UTR) of 67 nucleotides and a 3' untranslated region (3' UTR) of 2086 nucleotides with polyadenylation signal (Mochizuchi T et al., 1996).

The Northern blot analysis revealed a ~5.4 kb transcript expressed in most fetal and adult tissues. PKD2 transcript is strongly expressed in ovary, fetal and adult kidney, testis, small and large intestine and fetal lung.

Alternative splicing has studied occurring PKD2/Pkd2 (human/murine) splice forms on the mRNA and protein levels (Hackmann K et al., 2005). Three major alternative transcripts of PKD2/Pkd2, PKD2/Pkd2Δ6, PKD2/Pkd2Δ7 and PKD2/Pkd2Δ9, and one minor splice form, PKD2/Pkd2Δ12–13, numbered according

to deleted exons or parts thereof, are identified. The minor Pkd2/PKD2 splice variant, lacking exons 12 and 13, generates intact proteins of 872/874 amino acids The transcript lacking half of exon 6 is produced through a splice junction next to the A473 codon in the murine and next to the A475 codon in the human PKD2 sequence, in a 100% conserved region, fusing the first 't' nucleotide of the next codon (F474/F476 and Pkd2/PKD2) to the beginning of exon 7. This splice variant results in a shift of the reading frame and generates a truncated protein of 481/483 amino acids for Pkd2/PKD2. Similarly, the alternative transcript resulting from full exclusion of exon 9 (Δ9) has also a frameshift after C630/C632 (Pkd2/PKD2) and code for a truncated Pkd2/PKD2 protein of 644/646 amino acids. The transcript lacking exon 7 (PKD2/Pkd2Δ7) generates significantly altered protein variant of 910 and 912 amino acids.

Using the PSORT II program, the models predicted for PC2Δ7 loss of the third transmembrane domain, generated from parts of exons 6 and 7 and its replacement through a new transmembrane span built out of relevant exon 6 and exon 8 regions. In comparison to PC2, PC2Δ7 had one predicted transmembrane domain less and as a consequence of this reversed topologies of the third and fourth extracellular/intracellular loops, as well as predicted reversed polarity (extracellular) of the C-terminus (cytosolic in PC2).

This PC2Δ7 protein appeares stable but apparently does not interact with PC1 which should be due to the reversed topology (extracellular) of the interacting C-terminus (intracellular in PC2). Pkd2Δ7 transcript is predominantly expressed in brain and amounted to 3–6.4% of Pkd2 transcripts. The inability to interact with PC1 expands further the PKD1-independent functions of PC2 forms (Hackmann K et al., 2005).

2.5 1 THE MUTATIONS OF PKD2 GENE

More than 100 mutations in the *PKD2* gene have been identified in people with polycystic kidney disease. Mutations in the *PKD2* gene include changes in single nucleotides and deletions or insertions. Most PKD2 mutations are predicted to result in the production of an abnormally small, nonfunctional version of the PC2

protein (Aguiari G et al, 1999).

In particular, the missense mutation in exon 5, where a T is replaced by a G in the mutated gene, results in the substitution of a glycine for a tryptophan at codon 414 of the protein. A tryptophan at this position is conserved between polycystin-2, polycystin-1, and its *C. elegans* homologue. This substitution by a glycine is located in the first extracellular loop. Glycine, which can adopt a much wider range of conformations than any other amino acid, may have an important effect on the folding of this loop, resulting in a hampered protein function. Alternatively, the tryptophan-414 could be essential for interactions with other proteins (Veldhuisen B et al, 1997).

2.5.2 THE POLYCYSTIN-2 (PC2): STRUCTURE AND GENERAL ASPECTS

The *PKD2* gene product, polycystin-2 (PC2), is a 968-amino acid sequence with a calculated molecular weight of ~110 kDa. Modeling with several hydrophobicity algorithms suggested that PC2 is a membrane-spanning protein containing six transmembrane domains (TM 1-6) and cytoplasmic N- and C-termini. In the C-terminus putative phosphorylation sites, an EF-hand motif, a homodimerization domain, and a coiled-coil domain (Mochizuki T et al, 1996) have been identified (Fig. 5).

Western blotting analysis, using anti-sera directed against PC2 cytoplasmatic domains, demonstrated that PC2 is an integral membrane protein that is widely expressed and highly conserved in several renal and nonrenal tissues (including small and large intestine, pancreas, liver, lung, bowel, brain, thymus, reproductive organs, placenta).

Glycosylation analysis showed that this protein is an Endo H-sensitive glycoprotein suggesting a pre-middle Golgi localization (Cai Y et al, 1999). Subcellular fractionation showed that heterogously expressed full- length PC2 is predominantly localized in the endoplasmic reticulum of cultured cells. Mutants truncated at or before amino acid Glu787, on the other hand, showed altered trafficking

with a portion of the expressed protein appearing on the plasma membrane. This finding was helpful to define a 34-amino acids region (Glu^{787}-Ser^{820}), in the cytoplasmic tail of PC2 necessary for exclusive ER retention/retrieval of the protein (Cai Y et al, 1999).

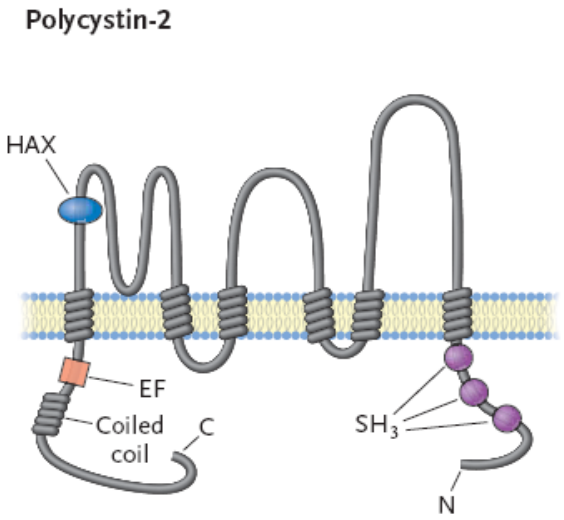


Figure 5. Structure of polycystin-2

Immunohistochemistry studies on formalin fixed native tissues have not provided adequate resolution to conclusively establish the PC2 subcellular localization in vivo. In some tissues, including the proximal tubule of the nephron, a cytoplasmic granular staining pattern has been observed, while in the distal nephron a more markedly staining and basal distribution was described. It is possible that provocative environments such a specific segments of the nephron or association with particular binding partners can effect re-localization of PC2 under special circumstances and that such re-localization may be functionally significant (Cai Y et al, 1999).

Nevertheless, it has been reported that PC2 co-immunoprecipitates with PC1, membrane protein localizing at multiple adhesion complexes in Chinese hamster ovary (CHO) cells co-expressing epitope-tagged full-length PC1 and 2. This suggested that PC2 in part is also able to localize in the plasma membrane forming heteromeric complexes in presence of heterogously expressed PC1 (Hanaoka K et al, 2000).

Using polyclonal antisera some reports indicated that PC2 is located in the

plasma membrane in MDCK cells as in other cell lines. PC2 expression was not restricted to the plasma membrane, but was also present in the Golgi apparatus and in the vicinity of a subset of filaments. Concordantly, upon subcellular fractionation on sucrose gradients, PC2 co-migrated in a first pool with ß1-integrin, a plasma membrane marker, and in a second pool with the Golgi apparatus, ER and cytoskeleton (actin). Heterologously expressed PC2 was directed to the plasma membrane only when a putative PC2 ER retention signal was mutated or when cells were co-transfected with PC1 (Hanaoka K et al, 2000; Cai Y et al, 1999, Aguiari GL et al, 2004). It has been also observed that localization of over-expressed full-length PC2 is in the ER of MDCK cells and, occasionally, in the plasma membrane, while untransfected cells did not show co-distribution with the ER. These data suggest that heterogously expressed PC2 does not reflect endogenous PC2 distribution in MDCK cells. Over-expression may influence protein folding and intracellular trafficking (Scheffers MS et al, 2002).

From the functional point of view, PC2 is a member of the transient receptor potential (TRP) superfamily of Ca^{2+}-permeable cation channels, which is likely involved in cell signaling events and/or Ca^{2+} transport in target tissues. The six transmembrane (6TM) domains of PC2 are homologous with the TRP channels and with Na^{+} and Ca^{2+} voltage-gated channels (Mochizuki T et al, 1996). Homology between PC2 and other TRP channels centers in a 270-amino acid stretch from TM2 through TM6 and intervening loops of PC2, particularly similar to the voltage-activated α_E-subunit of Ca^{2+} channels.

The similarity also extended to the COOH-terminal end of PC2 including the EF-hand domain present in some TRP channels, and the α-subunit of the L-type Ca^{2+} channel (Mochizuki T. et al, 1996). This is particularly relevant in light of the fact that the EF-hand provides a regulatory region of voltage-gated channel inactivation (Saimi Y et al, 2002). This homology allows to consider PC2 as the TRP-type (TRPP2) non-selective cation channel.

2.6 PC2 PROTEIN CHANNEL FUNCTION

Polycystin-2 shares a ~25% identity and a ~50% similarity with ~450 amino acids of PC1 and its *Caenorabditis elegans* homologue, ZK945.9. The similarity between PC2 and PC1 (and ZK945.9) extends over the first and sixth transmembrane region in PC2, but does not include the N- and C-terminal regions. The corresponding region of PC1 has been predicted to contain four transmembrane segments, three of these corresponding to transmembrane 1, 2 and 5 in PC2, and the fourth localizing between fifth and sixth span of PC2 (Mochizuki T et al, 1996).

As previously mentioned, the transmembrane spanning region of PC2 shares a significant sequence homology with voltage-activated Ca^{2+} and Na^{+} αl channel proteins. There is a comparable degree of similarity with ~270 residues of the voltage-activated Ca^{2+} channel α1E (VACCα1E-1). These channel proteins contain four homologous domains, each with six transmembrane spans, which are predicted to form the pore structure. The similarity of the COOH-terminal region includes the putative EF-hand domain, a specialized helix-loop-helix motifs that have Ca^{2+} binding activity in ~70% of the proteins in which they occur. Unpaired EF-hand sequences have been implicated in Ca^{2+}-sensitive inactivation of some forms of L-type VACCα1. EF-hand domains that do not coordinate Ca^{2+}, however, remain important to protein function. On the basis of the sequence homology to the channel proteins, it has been predicted that PC2 functions as an ion channel or pore modulating calcium homeostasis and other signal-transduction pathways in response to information derived from extracellular interaction (Mochizuki T. et al, 1996).

Again, the topology, similarity and homology that PC2 shares with the transient receptor potential channel (TRPC) superfamily span over a region that is conserved between PKD2 and PKD1, suggesting that not only PC2 is structurally related to TRPC proteins (Scheffers MS et al, 2002) and leading to a classification of polycystins into TRPP channel family (Birnbaumer L. et al, 2003) (Fig 6).

TRPP

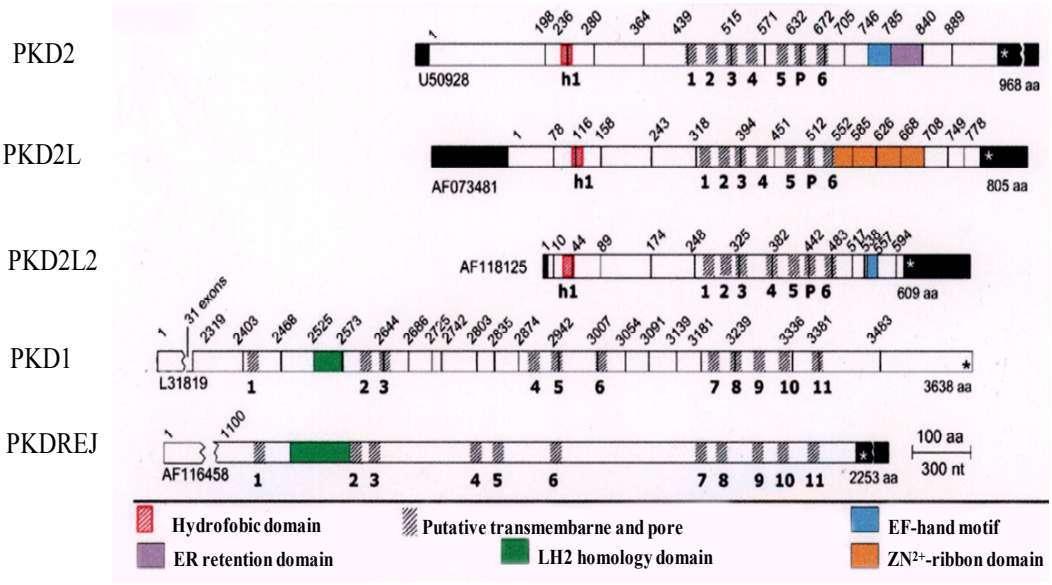


Figure. 6: Comparison of the exon boundary along the open reading frames of TRPP channels. Black boxes: 5' and 3' untraslated sequences; open boxes: coding exons; diagonally hatched rectangles: hydrophobic domains and pore regions; red hatched rectangles: hydrophobic domain of TRPCs and TRPVs.

It has been also possible by sequence comparison to construct the phylogenetic tree organizing the PKD protein (Fig. 7).

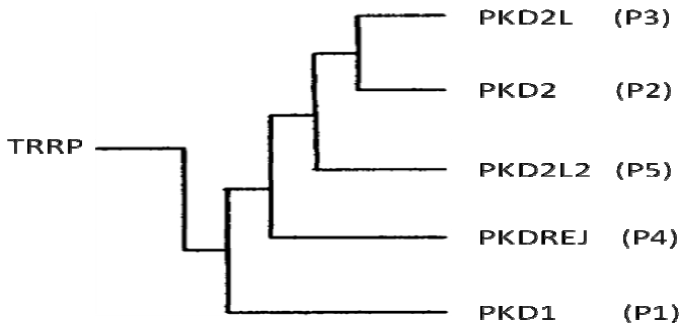


Figure 7: Phylogenetic tree of TRPP channels.

Despite the homology with voltage-gated Ca^{2+} and TRP channels, PC2 exhibits distinct physiological features: it shows a large single conductance, permeability to the monovalent cations K^+ and Na^+, permeability to Ca^{2+}, and inhibition by Ca^{2+}, La^{3+}

and Gd^{3+}, and by a pH reduction. Further, PC2 was inhibited by amiloride, reflecting functional similarities with other cation-selective epithelial ion channels (Gonzàlez-Perrett S et al, 2001). Amiloride sensitive cation transport has also been observed in ADPKD renal cysts. Hetero-oligomerization of PC2 with other proteins, including PC1 (Qian F. et al, 1997; Tsiokas L. et al, 1997) has been described and perhaps it plays regulatory functions (Lakkis M., Zhou J., 2003).

Calcium imaging studies in epithelial cells over-expressing PC2 demonstrated enhanced inositol 1,4,5-triphosphate (IP3)-mediated calcium release from intracellular stores as endoplasmic reticulum (ER), in consequence of surface receptor stimulation (Koulen P et al, 2002). Taken together, the Ca^{2+}-dependent calcium channel activity, the ER localization and enhanced calcium release activity, indicate that PC2 may be a new type of calcium release channel with properties that allow it to mediate calcium-induced calcium release (CIRC) (Cahalan MD et al, 2002).

In vivo analysis demonstrated that the Ca^{2+} activation/inactivation of PC2 is modulated by the S812 residue controlling the transient duration and relative amplitude of channel opening (Cai Y et al, 2004).

2.6.1 FUNCTION AT THE PLASMA MEMBRANE

Functional expression of PC2 as an ion channel has been first reported by Germino and coworkers (Hanaoka K et al, 2000). In this study, it has been demonstrated that PC2 alone is unable to form a functional channel in CHO-K1 cells, but in association with PC1, it displayed channel activity. Some of the biophysical properties such as cation selectivity and regulation by Ca^{2+}, are in agreement with a previous study on the structurally related channel, PKD2L1 (Chen XZ et al, 1999). In the PC1-PC2 co-expression study (Hanaoka K et al., 2000), it has also been shown that PC1 facilitates the targeting of PC2 to the plasma membrane which is necessary for channel activity. In further support of the relevance of the interaction to ADPKD, naturally occurring mutations in PKD1 or PKD2 genes that would eliminate their interaction result in loss of channel activity. Therefore, this study set the stage for functional expression of PKD2 gene and indicates that the role of PC1 is simply to chaperone PC2 to the plasma membrane, where PC2 forms a constitutively active

channel (Pelucchi B et al, 2006).

Upon overexpression, PC2 forms a functional channel with constitutive activity in the plasma membrane. It allows non-selective passing of cations, with slightly higher selectivity for Ca2+ over Na+ and K+, but higher conductance in K+ (Cantiello HF et al, 2004). PC2 can function at the plasma membrane, but its activity is under complex regulation involving shuttling between ER and plasma membrane, protein–protein interactions, and modes of activation. Specifically, it has been shown that the amount of PC2 in the plasma membrane is dynamically regulated by interacting proteins, inluding PC1 (Hanaoka K at al., 2000, Delmas P et al., 2004), polycystin-2 interactor, Golgi- and ER-associated protein-14 (PIGEA-14) (Hidaka S et al., 2004). Regulation also occurs by posttranslational modifications (serine phosphorylation by casein kinase 2 (CK2) (Kottgen M et al., 2005) and glycogen synthase kinase 3 (GSK3) (Streets et al, 2006), and by interaction with other channel subunits in the plasma membrane including PC1, as previously mentioned (Hanaoka K, et al., 2000), (Delmas P et al, 2004; Newby LJ et al, 2002; Qian F et al, 1997), TRPC1 (Tsiokas L et al, 1999), TRPV4 (Li X et al, 2005) and, finally, activation secondary to cell surface receptor (epidermal growth factor receptor (EGFR) (Ma R et al, 2005).

2.6.2 FUNCTION AT THE ER

Cai and coworkers (1999) first have been reported that native or transfected PC2 is accumulated in the ER. Deletion studies have identified a cluster of acidic residues in the C-terminal cytosolic tail of PC2 that is responsible for the retention of the protein in the ER (Cai Y et al, 1999). Interestingly, the pathogenic mutant PKD2(742X) which lacked the ER retention signal was forwarded to the plasma membrane, but this mutant did not display channel activity (Hanaoka K et al., 2000). Moreover, it was shown that PC2 functioned as a novel intracellular Ca2+ channel which was activated in response to increases in intracellular Ca2+ concentration. In the kidney epithelial cell line, LLC-PK1, Ca2+ imaging experiments revealed that PC2 overexpression enhanced the amplitude and duration of G protein coupled receptor

(GPCR)-induced Ca2+ release transients. However, PC2 overexpression does not alter the Ca2+ content of the intracellular stores as the response to the SERCA inhibitor, thapsigargin, is identical between mock- and PC2-transfected cells (Koulen P et al, 2002). Moreover, PC2 activity is solely dependent on intracellular rather than extracellular Ca2+. Therefore, these data indicate that PC2 functioned exclusively as an intracellular Ca2+-induced Ca2+ release channel in kidney epithelial cells. PC2 enhances local intracellular Ca2+ concentration in response to an initial rise in Ca2+ and, therefore, it regulates intracellular Ca^{2+} concentration in a localized fashion. Others reports have been confirmed the role of PC2 in intracellular Ca^{2+} release and showed that it interacts with the isoform 1 of the IP3 receptor (IP3R1). However, PC2 overexpression augments mostly the duration rather than the amplitude of the Ca^{2+} release transient (Li Yat al., 2005).

Moreover, experiments in immortalized lymphoblasts (Aguiari G et al, 2004) from patients showed that PC2 plays a role in G protein coupled receptor-induced Ca^{2+} signaling, but the possibility that PC2 could have also contributed to Ca^{2+} signaling through Ca^{2+} influx is not clearly addressed in these studies.

Overall, despite the differences, there is significant gain- and loss-of-function evidence to suggest that, in addition to residing in the ER, PC2 also has a functional role in regulating intracellular Ca^{2+} release in response to localized changes in intracellular $Ca^{2+.}$

2.6.3 REGULATED TRAFFICKING BETWEEN ER AND PLASMA MEMBRANE

The ER and plasma membrane pools of PC2 is dynamically regulated and its activity is tightly controlled. In fact, Köttgen and coworkers showed that serine phosphorylation within the ER retention sequence by CK2 reconstituted a binding site for a group of proteins called phosphofurin acidic cluster proteins 1 and 2 (PACS 1 and 2) (Kottgen M et al, 2005). Binding of PACS2 to PC2 prevented its movement to the plasma membrane. The S812 is important for this function because its conversion to A812 within the acidic cluster in human PC2 resulted in increased expression in the plasma membrane of cultured kidney epithelial cells (Kottgen M et

al, 2005).

Moreover, constitutive phosphorylation of S76 of human PC2 by GSK3 is necessary for its targeting to the plasma membrane (Streets at al, 2006). Mutation of S76 to A (or S80 in zebrafish) results in PC2 accumulation in intracellular compartments and fails to rescue the cystic phenotype in zebrafish injected with a *pkd2*-specific antisense morpholino oligonucleotide. Interestingly, the S76/S80 mutation does not affect the ciliary expression of PC2 (see next paragraph). Moreover, PIGEA-14, an interacting protein with PC2, binds to PC2 to facilitate the movement of PC2 from the ER to the Golgi (Hidaka S et al, 2004).

In conclusion, the quantity of PC2 in the plasma membrane is regulated and, furthermore, this regulation could have some functional consequences.

2.6.4 FUNCTION AT THE PRIMARY CILIUM

All cell types have a monocilium, a microtubule-based structure protruding into the extracellular space. In the case of an epithelial cell, the primary cilium is localized in the apical surface. The cilium is formed from the basal body in growth arrested cell (Hagiwara H et al, 2004). In kidney epithelial cells, it was established that loss of ciliary function and/or formation results in cystic diseases pointing to a central role of this organelle in PKD pathophysiology (Zhang MZ, at al, 2004; Davenport JR et al., 2005). Because cilia function as sensory organelles in the olfactory and vision systems (Snell WJ et al, 2004), it is believed it may have a similar sensory function in the renal epithelial cells (Pazour GJ et al, 2003). Such a function could be fluid flow sensing (Praetorius HA et al, 2003). In MDCK cells, it has been shown that mechanical bending of the primary cilium or fluid flow resulted in increases in intracellular Ca^{2+} concentration which is dependent on extracellular Ca^{2+}, PLC activation and inositol 1,4,5 trisphosphate (IP_3)-induced release of intracellular Ca^{2+} stores (Praetorius HA et al, 2001, 2003a, 2003b). It is remarkable that bending-induced Ca^{2+} signaling is not affected by inhibition of ryonodine receptors. The expression of PC1 and PC2 in the primary cilium has shown by several groups (Luo Y et al, 2003; Pazour GJ et al, 2002; Geng L et al, 2006). Nauli and coworkers (Nauli SM et al, 2003) have demonstrated that renal epithelial cells

respond to fluid flow by an increase in intracellular Ca^{2+} concentration through the action of PC1 or PC2, and the cilium is absolutely required for fluid flow (Fig.8).

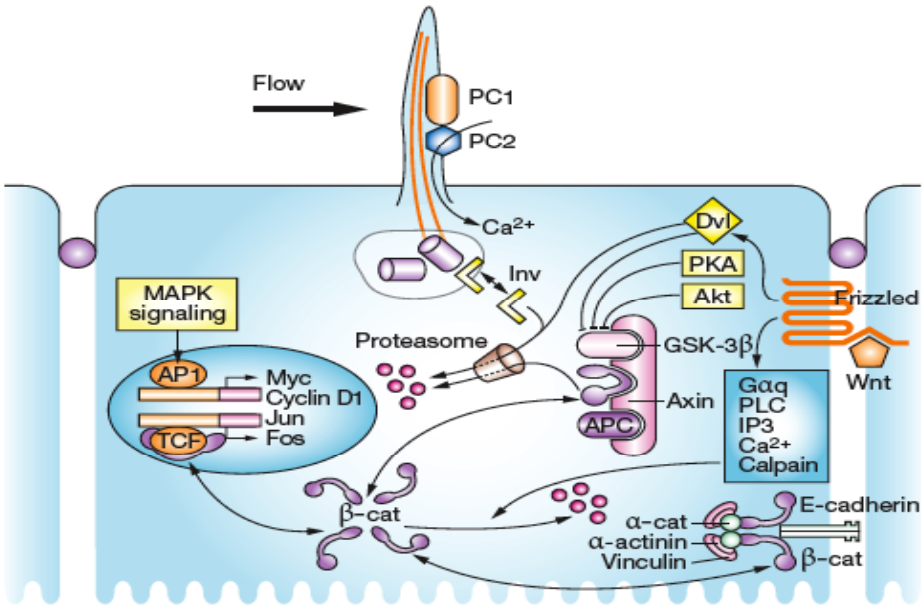


Figure. 8: Localization of polycystin-1 and polycystin-2 in the primary cilium and a schematic model of fluid flow mechanosensation. Akt, protein kinase B; AP1, activator protein 1; IP3, inositol triphosphate; MAPK, mitogen-activated protein kinase; β-catenin (β-cat); glycogen synthase kinase-3β (GSK-3β); adenomatous polyposis coli (APC) protein; T-cell factor (TCF1) family; Protein kinase A (PKA); Dishevelled (Dvl); PC1, polycystin-1; PC2, polycystin-2 (Torres VE and Harris PC, 2006).

In response to luminal flow, the primary cilium bends and initiates a signaling cascade through the PC1/PC2 complex, in concert with ryanodine receptors, that activates signaling pathways (Nauli SM et al, 2003; Yoder BK et al, 2002). Initial response to mechanical stimulation is the influx of Ca^{2+} across the plasma membrane. PC1, with its large extracellular domain, may sense the bending of the primary cilium induced by fluid flow, functioning as a mechano-fluid stress sensor and the resultant conformational changes of PC1 transduce the mechanical signal into a chemical response by activating tightly associated PC2 Ca^{2+} channel. In fact, using antibodies specific for PC2, it has also been shown that Ca^{2+} entry was blocked, suggesting a predominant role of PC2 in flow-induced Ca^{2+} influx (Nauli SM et al, 2003). This local Ca^{2+} influx in the cilium subsequently triggers intraorganellar Ca^{2+} release inside the cytoplasm through Ca^{2+}-induced Ca^{2+} release

and this changes in intracellular Ca^{2+} concentration may then alter various cell functions, including gene expression, growth, differentiation and apoptosis, thus altering tissue and organ development

2.6.5 FUNCTION AT THE MITOTIC SPINDLES AND CENTROSOME

PKD2 expression has been detected in the mitotic spindles (Rundle DR et al, 2004) and centrosome (Jurczyk A et al, 2004). However, its functional role in these two cellular structures is unclear. Rundle and coworkers propose that the presence of PC2 in the mitotic spindles may serve to ensure symmetric distribution of PC2 between mother and daughter cells and to regulate Ca^{2+} signaling during cell division.

Moreover, PC2 has been found to co-immunoprecipitate with pericentrin and other components of the intraflagellar transport (IFT) machinery. Knockdown of pericentrin resulted in loss of cilia. Therefore, it was proposed that PC2, pericentrin, and IFT proteins may be required for ciliary function and assembly (Jurczyk A et al, 2004). Moreover, this brings to think that PC2 may actually function upstream of ciliary formation, at the level of the centrosome.

2.7 PC2 ACTIVATION by EGF

Traditionally, channel activation is considered to occur through gating (opening and closing) of the channel. Therefore, an activating agent would prolong opening and/or reduce closing of the channel. The mechanisms underlying PC2 gating are currently unknown. However, it is reported that PC2 activity was induced in response to EGFR stimulation in the LLC-PK1 cell line (Ma R et al, 2005). EGF does activate PC2 through the activity of the γ2 isoform of PLC (PLC-γ2) and phosphoinositide 3-kinase (PI3K) and this activation is independent of the depletion of intracellular Ca^{2+} stores. EGF activated PC2 by releasing it from PIP_2-mediated inhibition, as PIP_2 would be expected to directly bind to the channel. Moreover, the

reduction of PIP2 is responsible for the EGF-induced activation of PC2. Therefore, this study showes that PC2 is activated under physiological conditions and this activation is necessary for the increase in intracellular Ca^{2+} concentration by EGF (Ma R et al, 2005). In this case, it may be supposed that PC2 may function downstream of other growth factors because *EGFR* deletion caused cystic dilatation, but *EGFR* knock out does not produce a phenotype as severe as in *pkd2* knock out animals. It has been, therefore, shown that PC2 channel activity produced by PC2 either acting alone or in association with other subunits was necessary for EGF and perhaps other growth factor-induced Ca^{2+} signaling. This type of Ca^{2+} signaling could be responsible for long term effects on cellular phenotypes such as differentiation, proliferation, etc. The mechanism of PC2 activation by EGF appears to be different from the mechanism by which EGF activated another TRP channel, TRPC5 (Bezzerides VJ et al, 2005). In the case of TRPC5, EGF acted through Rac1 to induce the activation of (PIP(5)Kα) and production of PIP_2, which in turn promoted the vesicular translocation of TRPC5 from intracellular compartments to the plasma membrane (Bezzerides VJ et al., 2005). It was proposed that both mechanisms may be functional in PC2 (Ma R et al, 2005). PIP_2 indirectly inhibits PC2 by recruiting to the channel a protein that regulates PC2 activity in a voltage-dependent fashion, that is to function as a negative regulator at negative potentials and an activator at positive potentials.

2.7.1 ACTIVATION THROUHT PC1

and coworkers were the first to show that PC2 activation occurs through PC1-mediated gating at the plasma membrane by a voltage independent way (Delmas P et al., 2004). They showed that ligand binding to the REJ domain of PC1 induced a conformational change in PC1 which somehow resulted in PC2 activation. Therefore, in addition to functioning as a chaperone, PC1 may also function as a gate for PC2. In addition they found that PC1 activation by the MR3 antibody activated the Gi/o subunits of G proteins. As a result, PC1 stimulation could modulate cell signaling through two independent modes, activation of Ca^{2+} entry through PC2 and also activation of G proteins. Then, PC2 activation has been shown to be induced by

EGF (Ma R et al., 2005), binding to PC1 (Delmas P et al., 2004) and/or intracellular Ca^{2+} (Koulen P et al, 2002). Gating and activation by PC1 has been documented in the plasma membrane and it is likely to occur in the cilium because of the presence of PC1 in the cilium, and the lack of the fluid flow response in cells lacking PC1 or PC2 (Nauli SM at al, 2003). Moreover, PC2 reduced the plasma membrane expression of PC1 by sequestering it in the ER (Grimm DH et al, 2003) but the function of PC1 complexed with PC2 in the ER is unknown.

2.7.2 PKD2-MEDIATED SIGNAL TRANSDUCTION

In regard to PC2-mediated signal transduction and its relevance to ADPKD, it was recently shown that PC2 regulated cell proliferation through a physical interaction with Id2 (Li X et al, 2005), a known inhibitor of the basic helix-loop-helix (bHLH) transcription factors. Binding of Id2 to the C-terminal cytosolic tail of PC2 resulted in the cytosolic sequestration of Id2 and upregulation of the activity of the cell cycle regulator, p21, resulting in reduction of proliferation through the downregulation of cyclin-E and CDK2 activities. PC1 overexpression somehow enhanced the phosphorylation state of PC2 and subsequently increased binding of PC2 to Id2. Therefore, Li and collaborators (Li Y et al, 2005) proposed that naturally occurring mutations in PKD2 would result in insufficient cytosolic sequestration of Id2 and subsequent increase in nuclear import of Id2. Similarly, PC1 mutations would enhance the nuclear translocation of Id2 by reducing the phosphorylation state of PC2 and thereby allowing Id2 to travel to the nucleus. This increased expression of Id2 in the nucleus was shown to result in increased proliferation in cells harboring pathogenic mutations of PC2 and/or PC1.

2.8 PC2 PROTEIN ASSOCIATIONS

The significant homology of PC2 with TRPC proteins (TRPC1-7) suggested its structural relation to the channel family. It is still unclear if all TRPC proteins form store-operated channels (SOC). There are evidences that TRPC1, TRPC4 and TRPC5

may encode some forms of SOC, however functional data pertaining to TRPC6 do not support this hypothesis. Data in regard to a direct role of TRPC3 in regulating Ca^{2+} entry in response to store depletion are still evolving. Such complexity may be explained by the idea that individual TRPC proteins may differentially associate with additional proteins to produce specific functions (Sheffers MS et al, 2002). Immunoprecipitation studies showed that PC2 can specifically interact with TRPCs through two distinct domains. Interactions between their C-terminal domains are sufficient, but not necessary, to mediate the association between PC2 and TRPC1; a transmembrane region is also able to mediate interaction independently of the C-terminal region. Moreover, PC2 is the first non-TRP protein described to specifically interact with TRPC 1 but not TRPC3. These data point to a specific role for PKD2 in Ca^{2+} transport and suggest that specific associations of TRPC proteins with PC2 and/or PC2-related molecules may contribute to the functional properties of these channels (Tsiokas L,1999).

It has been also demonstrated that PC2 co-operating with PC1 participates to the JAK-STAT signaling pathway (Bhunia A.et al, 2002). PC2 is also able to upregulate the AP1-dependent transcription in HEK293 cells involving activation of the Janus N-terminal kianse 1 (JNK1), p38 and PKCε (Arnould T et al, 1998). Importantly, target deletion of AP-1 disrupts normal organogenesis and it is embryo lethal, suggesting an important role of AP-1 in nephrogenesis (Arnould T et al, 1998). Gel shift and super-shift analysis showed that PC2 increased the binding of Jun-containing AP-1 to the TRE, increased the phosphorylated c-Jun and p38. Dominant-negative mutants of Cdc42, Rho-A and Rac-1 (small GTP-binding proteins) significantly reduced PC2 mediated AP-1 activation suggesting their involvement in p38 JNK activation. Proteins belonging to the small G proteins are known to regulate complex cellular programs, including the organization of the actin cytoskeleton, cell motility, shape, adhesion and polarity. Interestingly, Cdc-42-dependent p38 activation appears to inhibit cell cycle progression, arresting cells at the G1/S transition, suggesting that PC2 may modulate cell cycle. Moreover, PC1 and PC2 have been hypothesized to be component of a common signaling pathway and their signals converge on the AP-1 activation. The synergism between PC1 and PC2 C-termini seems to depend on the complementary activation of two different isozymes, PKCα interacting with PC1 and PKCε with PC2. Activation of PKCα inhibits c-Jun

phosphorylation on negatively regulatory DNA binding sites, but it fails to generate trascriptionally active AP-1. In contrast, PKCε alone induces a powerful AP-1 response in several tissues, although the precise signaling cascade remains unclear (Arnould T et al, 1999). Moreover, the combinatorial activation of PKCα and appears to cause induction of the cyclin-dependent kinases inhibitors $p21^{waf1/cip1}$ and $p27^{kip1}$, hyperphosphorylation of the retinoblastoma (Rb) protein, and an inhibition of the cell cycle progression in G1 of intestinal epithelial cells (Frey MR et al, 1997). Thus, the ability of PC1 and PC2 to activate PKCa and - ε, respectively, may inhibit cell cycle progression and promote the cellular differentiation of tubular epithelial cell during renal development (Arnould T et al, 1999).

PC2, like PC1, is found to interact with the *actin cytoskeleton*-associated proteins. Originally, at least two proteins were found interacting with PC2: the CD2-associated protein (CD2AP), an adapter protein mediating an indirect attachment to the cytoskeleton, and Hax-1, an actin cytoskeleton-associated protein (Arnould T et al, 1998). PC2 interacts with Hax-1, through their TM 5 and 6 domains, in a very specific way. Responsible for the specific interaction probably is a stretch of 8 amino acids, localized in the loop 5, immediately followed by a negatively charged (aspartic acid) residue, which is considered to be important for cation selectivity in Ca^{2+} channels. The PC2-related protein as PC2L that differs in this region did not interact with Hax-1. The PC2/Hax-1 interaction may have important functional implications since Hax-1 is associated with ER and mitochondria. Two models have been proposed considering PC2 integrated into a protein complex involved in cell-matrix interactions. In the first model integrins as integral plasma membrane proteins mediate the binding to the extracellular matrix; they signal through non-receptor tyrosine kinases to cortactin. Cortactin connects to Hax-1, which in turn binds to PC2. Because the loop 5 is the putative pore-forming region of PC2 and related proteins, Hax-1 may not only be an adaptor between cortactin and PC2, but in addition a key element to regulate the PC2 function. The association of Hax-1 with loop 5 of PC2 could either induce or block local ion fluxes originating from the ER. In the second model, it has been considered that PC2 itself is located in the plasma membrane and, through its interaction with PC1, is associated with the extracellular matrix. Signaling would occur by interaction between PC2 and Hax-1, which through its binding to cortactin would mediate the association with the actin cytoskeleton.

Moreover, both PC1 and PC2 may regulate monomeric G-proteins, and these, in turn, are necessary for the translocation of cortactin to the cell periphery (Gallagher AR et al, 1999).

As previously mentioned, two-hybrid screens and reciprocal immunoprecipitation of the native proteins from renal epithelial cell lines shown that PC2 associates with CD2AP. This association is also proven by *in vivo* association staining. CD2AP adapter protein partially co-localizes with PC2 and interacts with CD2 and nephrin, both immunoglobulin superfamily member proteins involved in forming specialized cell adhesion. Furthermore, CD2AP has been implicated in regulation of spatial and temporal assembly of signaling complexes that link membrane proteins to the cytoskeleton, including the focal adhesion complex protein $p130^{CAS}$. Taking into account these data, it has been proposed that in the kidney tubule CD2AP mediates the association of PC2 with multimeric complexes including PC1, cytoskeleton components, components of the basolateral membrane targeting machinery, or signaling complexes such as those mediating PC2 induced AP-1 activation (Lehtonen S et al, 2000). Later on, it was demonstrated by *in vitro* and *in vivo* approaches, that PC2 is directly associated with the cytoskeleton via tropomyosin-1 (TM-1), a well-characterized component of the actin microfilament present in muscle and non-muscle cells. Using the yeast two-hybrid method to screen human kidney and heart cDNA libraries a new splicing isoform (TM-la) has been isolated. The interaction between the protein pair was enhanced by the presence of Ca^{2+}. Furthermore, experiments targeted the interacting domains to G821-R878 of PC2. Co-immunprecipitation studies using over-expression of PC2 and TM-1 protein in NIH3T3 cells and *Xenopus* oocytes indicated the overlapping subcellular localization of both proteins, with the former localized mainly in the ER and latter distributed in the cytoplasmic stress fibers, with abundance in the perinuclear region. Thus, TM-1 may act to anchor PC2 to the system for structural purposes and/or for stabilizing PC2 molecules in the membrane (Li Q et al, 2003). Two-hybrid method has been useful to identify tissue specific PC2 partners. Evidence supports that, in heart, PC2 associates with the cardiac and skeletal isoforms of troponin I (TnI), playing various functional roles, such as an angiogenesis inhibitor and Ca^{2+} channel modulator, in addition of being a constituent part of the troponin complex. It is well-known that TnI along with the troponin C (TnC) and T (TnT) makes up the troponin complex, which regulates muscle

contraction in a Ca^{2+} dependent fashion in concert with tropomyosin. *In vitro* biochemical method, including GST pull-down and microtiter binding assays, proven that PC2 specifically associated with Tnl3 by the R872-H927 interaction segment, that do not overlap with EF-hand domain (E754-D781), ER-retention domain (E787-S820) and the TM-1-association domain (G821-R878). It is possible that the function of PC2 or the PC2-PC1 complex is modulated by the actin cytoskeleton system through direct association of PC2 with TnI and TM-1, which is important for cell growth and capillary morphogenesis. Because the TnI serves as an angiogenesis inhibitor and TM-1 as a suppressor of neoplastic growth, altered TnI-PC2 and TM-1 interactions due to pathogenic PC2 or PC1 mutations may lead to undifferentiated cyst growth and angiogenesis in and around cysts in ADPKD (Li Q et al, 2003).

Upon the identification of the gene mutated in ARPKD and of its protein product (fibrocystin/polyductin, FC, see later), it was shown that PC2 was also able to interact with FC because FC completely or partially colocalize with PC2 at the plasma membrane and primary cilium and can be reciprocally coimmunoprecipitated (Wang S et al, 2007). Although incomplete removal of FC by small interfering RNA did not abolish flow-induced intracellular calcium responses, an antibody to extracellular epitopes of FC blocked cellular calcium responses to flow stimulation, suggesting that FC and polycystins share, at least in part, a common mechanotransduction pathway.

2.9 PC1 AND PC2 CO-OPERATE IN A COMMON COMPLEX

Since the two genes responsible for ADPKD codify for integral membrane proteins, it has been proposed that polycystins can co-work, as yet anticipated, in a common signal transduction cascade together regulating cellular events.

We have already mentioned that, by using the two hybrid system and *in vivo* and *in vitro* co-immunoprecipitation assays, PC1 C-ter was shown to interact with the PC2 C-ter forming heterotypic interactions (Qian F et al, 1997; Tsiokas L et al, 1997). This protein association supports the model through which the two gene products participate in a common signaling pathway to regulate cell proliferation and differentiation. Inspection of polycystin sequences using the COILS program identified

a probable coiled-coil structure at the position 4214-4248 (Qian F et al, 1997). Further two-hybrid analysis using deletions of PKD1 and PKD2 localized the interacting domain of PC1 to 40-amino acids region contained within the last 76 amino acids of PC1 (Tsiokas L et al, 1997). The domain of PC2 responsible for the binding of PC1 was found to reside in a region spanning the last 97 amino acids of PC2 protein (Tsiokas L et al, 1997). The PC2 proposed model (Mochizuki T et al, 1996) may self-associate forming an ion pore channel, similarly to the family of voltage Ca^{2+} and Na^{+} α_1 channel, by homotypic interactions of its coiled-coil structure. Homotypic associations may compete with the PC1/PC2 heterotypic interactions probably involved in the channel activity modulation (Qian F et al, 1997). Moreover, the PC1 C-ter contains an intriguing PEST motif that appears to target PC1 for rapid degradation. Deletion of this sequence, in transfection experiments using a PC1 chimeric fusion protein, resulted in increased proteins levels and stable up-regulated surface expression of PC1 in presence of PC2 (Tsiokas L et al 1997). It is possible that during tubulogenesis, stable expression of PC1 depends upon the presence of PC2.

We have already mentioned that in Chinese Hamster Ovary (CHO) cells transfected with PC1 and PC2, PC1 is required to co-assemble a polycystin complex and bring PC2 to the cell surface, where it is responsible for a channel activity. Confocal immunofluorescence images of full-length PC1 and PC2 transfected cells showed that PC2 localized into the cell, and not on the surface, when expressed alone. In contrast, when full-length PC1 and -2 were co-expressed, both polycystins co-localized at the plasma membrane, suggesting that PC1 is required for the recruitment of PC2 to the plasma membrane (Hanaoka K et al, 2000).

Electrophysiological studies demonstrated that PC1 recombinant peptides (lacking of N-ter 1811 residues) acts as a $G_{i/o}$-protein-coupled receptor and regulates the activity of ion channels via $G_{i/o}$ proteins. These findings confirm the hypothesis that PC1 is involved in signal transduction pathways and the ligand binding could be not necessary to initiate to G protein signals. The PC1 ability to activate Gprotein is abrogated by co-expression of PC2, suggesting that the constitutive activation of G-protein by PC1 is antagonized by PC2, thus regulating the untraditional G-coupled receptor activity (Delmas P et al, 2002)(Fig. 9).

The function of the PC1/PC2 channel complex may explain the secretory phenotype in ADPKD. The variety of signaling binding functions of PC1 C-ter tail led to

hypothesize that it might also regulate ion transport processes mediating or regulating cyst expansion. Expression of the PC1 C-terminal portion at the *Xenopus* oocyte surface up-regulates inward current that includes a major contribution from Ca^{2+}-permeable nonspecific cation channel.

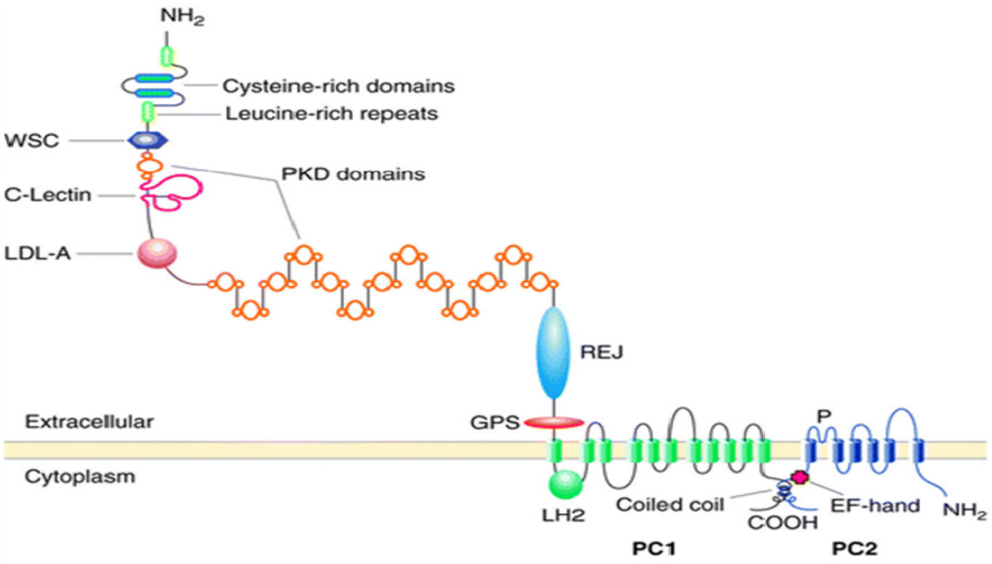


Figure 9: PC1 and PC2 interact by their coiled-coil domain located in the C-termini.

The function of the PC1/PC2 channel complex may explain the secretory phenotype in ADPKD. The variety of signaling binding functions of PC1 C-ter tail led to hypothesize that it might also regulate ion transport processes mediating or regulating cyst expansion. Expression of the PC1 C-terminal portion at the *Xenopus* oocyte surface up-regulates inward current that includes a major contribution from Ca2+-permeable nonspecific cation channel. Deregulation of these or similar channels in ADPKD may contribute to cyst formation or expansion (Vandorpe DH et al, 2001). Consistently, ADPKD mutations incorporated into the fusion protein containing PC1 C-ter attenuated or abolish encoded cation currents; point mutations and truncations showed that cationic current expression requires integrity of a region encompassing the putative coiled-coil domain of PC1 tail (Vandorpe DH et al, 2002).

Furthermore, some studies showed that PC2, expressed in endoplasmic reticulum (ER) of epithelial cells, is a functional Ca^{2+}-activated high conductance channel that is permeable to divalent cations and mediates intracellular Ca^{2+} release through its own channel activity (Koulen P et al, 2002). In porcine kidney (LLC-PK_1) cell lines calcium release activity mediated by PC2 did not require the co-expression of exogenous PC1, suggesting that PC2 channel function does not require co-assembly with PC1, although a function for low levels of native PC1 in these cells cannot be excluded. It is possible that PC2 itself forms the channel or require the assembling with other proteins. It is likely that PC1 interaction may occur *in vivo* to regulate the PC2 activity (Koulen P et al, 2002, Pelucchi B et al, 2006).

Evidence for the role of PC1 in the calcium homeostasis has been provided by study with the bioluminescent protein aequorin system, showing that the over-expression of PC1 C-ter tail in HEK293 and HeLa epithelial cells causes an increased level of ATP-evoked cytosolic Ca^{2+}, even in absence of extracellular calcium (Aguiari G et al, 2003). This suggests that the Ca^{2+} cytosolic increase is due to a release from intracellular stores mediated by PC1. These data support the concept that the coupling of ER Ca^{2+} release channels, as well as PC2, to the cell surface channel receptors, like PC1, may be an important mechanism to control cell behavior, as cell proliferation and differentiation (Aguiari G et al, 2003). Further data suggest that PC1 C-terminal tail prolongs the ATP-stimulated chloride conductance, probably as a consequence of up-regulation of SOC entry. Despite the controversial data about the calcium origin, in or outs, the data propose a relationship between increase in Ca^{2+} concentrations and subsequent activation of trans epithelial chloride secretion involved in cyst development and expansion (Wildman SS et al, 2003; Hooper KM et al, 2003).

In vitro reconstitution of lipid bilayer system indicates that PC1 activates and stabilizes the channel activity of wild-type PC2 (Arnould T et al, 1998; Xu GM et al, 2003). These data are a direct demonstration of electrophysiological significance of PC1/PC2 interaction, namely the activation and stabilization of PC2 channel activity by PC1 (Xu GM et al, 2003).

The resulting increase in channel activity by the polycystin complex may be critical for the activation of cation-dependent signaling pathways normally associated with various cell functions including cell cycle, vesicle trafficking and ion

transport. Functional interruption of this interaction may account for abnormalities in protein targeting, cell growth, and ion transport, characteristic of ADPKD.

Further biochemical experiments were performed to test the hypothetic PC1/PC2 common signaling pathway. In a lymphoblastoid cell line, CB33 (Shim et al., 1998), which had no detectable PC2 expression, exogenously expressed PC1 co-immunoprecipitated with JAK2, nevertheless is was unable to induce JAK2 phosphorylation and $p21^{waf1}$ up-regulation (Bhunia AK et al, 2002). Cross-immunoprecipitated experiments in JAK2 and PC2 co-transfected cells showed that PC2 is unable to precipitate JAK2 in absence of PC1. This data suggest that PC2 is not necessary for the binding of JAK2 to PC1, but is essential for its activation and the subsequent induction of $p21^{waf1}$. Since the PC2 channel has the highest permeability for calcium, one intriguing possibility is that calcium influx mediated by the channel could activate a signaling cascade, which in turn is responsible for the phosphorylation and activation of JAK2 (Bhunia AK et al, 2002).

These results support the previous studies suggesting that both PC1 and PC2 proteins are the partners of a common signaling pathway whose disruption results in the indistinguishable PKD1 and PKD2 phenotypes.

2.10 THE MUTATIONAL MECHANISM IN ADPKD

A major point of discussion has been why cysts only develop in a proportion of nephrons when all cells have the same germline mutation. It has been suggested that cyst development is a two hit process, analogous to Knudson's classic model for carcinogenesis, with a somatic second mutation to the normal PKD gene required before the cyst can develop (Reeders ST, 1992). By using technique minimizing other cell type contamination, it has been defined that renal cysts in ADPKD are of clonal origin. Moreover, somatic mutation of the germline normal PKD1 allele is the second, rate limitant step in the cystogenesis and it is likely to account for the focal formation of renal cysts (Qian F et al, 1996).

2.10.1 HUMAN STUDIES SUPPORTING THE TWO HIT MODEL FOR ADPKD

Because each cyst is composed of both epithelial and stromal cells, the contamination with the latter cells can mask the detection of important genetic events (monoclonality and somatic mutations) in the former cells. (Qian F et al, 1996). By analyzing cells carefully isolated from individual type 1 ADPKD cysts it has been demonstrated that ~20% of these showed a clonal deletion, defined as loss of heterozygosity (LOH), of the *PKD1* locus (Qian F et al, 1996). Subsequent studies documented intragenic *PKD1* mutations in ~30% of liver cysts from type 1 ADPKD patients (Badenas C et al, 2000). Somatic *PKD2* mutations were also found in cystic epithelia from patients with type 2 ADPKD, supporting the LOH at the PKD2 locus (Pei Y et al, 1999; Torra R et al,1999; Koptides M et al, 1999) also.

The necessity of LOH in cystic tissues suggests a molecular recessive mechanism of the disease similar to that seen in numerous tumor suppressor genes. In this model, however, the frequency of "second hits" in cystic epithelia must be extremely high to account for thousands of cysts that are observed. The very high number of somatic mutation predicted in the proposed model is surprising since no known dynamic elements have been identified within the genomic sequence of *PKD1* and since the adult kidney is thought to have a relatively low mitotic index. It has been previously reported an extremely unusual 2.5 kb polypirimidine tract within intron 21 that may be responsible for the gene's increased rate of mutation (The European Polycystic Kidney Desease Consortium, 1995). Similar but much shorter elements present within other genes have been shown to undergo triple-helix formation both *in vitro* and *in vivo.* The triplex formation induces mutagenesis in a mammalian cell culture system and this process appears to require the excision and transcription-coupled repair. It has been postulated that PKD1 may cause gratuitous and potentially error-prone repair, constituting endogenous source of genetic instability (Qian F et al, 1996).

Regarding to the type 2 ADPKD, it cannot be excluded a similar mechanism to type 1 ADPKD. Whereas the initial step is certainly a germline mutation of PKD2, the nature of the second event is not yet known. It is possible that uncharacterized unstable elements within the genome structure of PKD2 cause frequent somatic

inactivation in a manner analogous to that PKD1. The relative infrequency of ADPKD2 argues against this hypothesis, however. The phenotypic similarity of the disorders may offer an alternative hypothesis. It is possible that PC1 and PC2, known to interact in a common pathway, participate in a trans-heterozygous "two-hit" model, an additional mechanism of cystogenesis in ADPKD (Peters DJM, et al, 2001).

2.10.2 MOUSE STUDIES OF THE "TWO-HIT" MODEL FOR ADPKD

Target mutations of *Pkd1* and *Pkd2* have been used to generate mouse models for functional studies of these genes *in vivo*. Two Pkd1 "knock-out" models were originally created in which the mutant allele was truncated in the C-terminus (Lu W et al, 1997). By contrast, several *Pkd2'* "knock-out" models were created by crossing mice with either a true "null", or an unstable allele (Wu G et al. 1998 and 2000). In general, the renal phenotype of the *Pkd1* and *Pkd2* "knock-out" models was very similar. Heterozygous inactivation of either *Pkd1* or *Pkd2* resulted in focal renal and extra-renal cyst formation during late adult life. However, homozygous inactivation of *Pkd1* and *Pkd2* was associated with massive polycystic kidney disease and embryonic or perinatal lethality (Lu W et al, 1997; Wu G et al, 1998 and 2000). The *Pkd2* mouse studies derived from two different ES cell lines were especially informative. In one ES cell line, the targeted disruption created a true null allele *(Pkd2)* in which Pkd2 was disrupted at exon I. In a second ES cell line, the targeted disruption resulted in an unstable allele *($Pkd2^{ws25}$),* in which the targeting vector was inserted in tandem with the wild-type exon 1. This unstable allele could undergo intra- or intergenic somatic recombination to form either a wild-type or null allele. By crossing the mice from these two lines, "knock-out" mice with different combinations of the $Pkd2^{-}$ and $Pkd2^{ws25}$ alleles were created. It is of interest that PC2 expression was uniformly negative in the cystic linings of these animals, suggesting that complete loss of this protein was required for cyst development. By contrast, PC1 immunoreactivity was reported in only one of the Pkdl knock-out models and was noted to be variably expressed in some, but not all, of the cyst lining cells (Pei Y, 2001). In PKD2 mice models, the renal cystic phenotype correlated with the allelic series reflecting the frequencies of individual

epithelial cells that had undergone biallelic Pkd2 inactivation. Of the live-born Pkd2 mutant animals, the most severe disease occurred in Pkd2-/ws25, followed by Pkd2ws25/ws25, Pkd2+/- and Pkd2ws25/+ mice. These findings provide the most definitive evidence for the "two-hit" model of ADPKD. In addition, transgenic mice over-expressing Pkdl were found to have a cystic phenotype suggesting that perturbation of the Pkdl dosage could lead to cystogenesis (Pritchard L. **et al,** 2000). More recently, Lantinga-van Leeuwen et al (2004) generated a novel mouse model with a hypomorphic Pkd1 allele. These homozygous Pkd1 mice are viable, with bilaterally enlarged polycystic kidneys, thus showing that a reduced dosage of Pkd1 is sufficient to initiate cystogenesis and vascular defects. The use of inducible Pkd1-knockout mouse model lead also to conclude that Pkd1 inactivation is not sufficient to initiate the cell proliferation necessary for cyst formation (Takakura A et al, 2008); a paracrine mechanism may account for focal cell proliferation and regional disease progression. Therefore, an additional genetic or nongenetic "third hit" may be required for rapid development of cysts in polycystic kidney disease.

2.11 AUTOSOMAL RECESSIVE POLYCYSTIC KIDNEY DISEASE

Autosomal recessive polycystic kidney disease (ARPKD, MIM 263200) is a monogenic genetic disorder found mainly in infancy, with a prevalence of 1 in 20.000 newborns (Shaikewitz ST and Chapman A 1993, Zerres K et al, 1998).

Apart from the extrarenal phenotype, such as biliary dysgenesis, hepatic fibrosis, portal and systemic hypertension, oligohydramnios and pulmonary hypoplasia, bilateral enlarged polycystic kidneys are the major finding. Cysts in the kidney arise mainly from the collecting ducts. There is a fundamental structural difference between ADPKD cysts and ARPKD cysts: ADPKD cysts rapidly close off from urinary flow and continue to expand by transepithelial secretion. ARPKD cysts remain open with respect to the entire nephron, maintaining both afferent and efferent tubular connections.

The mortality rate thus far still reaches 30% of infants (Roy S et al, 1997). However, half of the children who survived the neonatal period finally developed

ESRD (Fonck C et al, 2001).

The clinical spectrum is variable and the majority of cases are identified late in pregnancy or at birth. As many as 30 to 50% of affected neonates die shortly after birth from respiratory insufficiency due to a critical degree of pulmonary hypoplasia. Renal failure is rarely a cause of neonatal demise.

2.11.1 *PKHD1* GENE AND ITS PROTEIN PRODUCT (FIBROCYSTIN/ POLYDUCTIN)

More than 250 mutations in the ARPKD disease gene, PKHD1 (polycystic kidney and hepatic disease 1), have been identified thus far (Guay-Woodford et al, 1995; Zerres K et al, 1998). PKHD1 is among the largest human genes characterized to date, on chromosome 6p12.2, extending over a genomic segment of at least 470 kb and including a minimum of 86 exons.

PKHD1 undergoes a complex pattern of alternative splicing, generating transcripts highly variable in size. Moreover, Northern expression analysis suggests the likelihood that splice forms of PKHD1 generate alternative proteins, including secreted products (Nagasawa Y et al, 2002; Onuchic LF et al, 2002). Moreover, multiple alternative variants of fibrocystin may provide a mechanism for regulating the temporal and spatial functions of fibrocystin isoforms in a tissue-specific manner (Onuchic LF et al, 2002; Ward CJ et al, 2002).

The longest open reading frame PKHD1 transcript contains 67 exons and the encoded protein, designated fibrocystin/polyductin (FC), is a large 4.074-aa protein with a calculated molecular mass of 447 kDa. As shown in Fig 10, FC represents a novel putative integral membrane protein with a short cytoplasmic C-terminal tail (~192 aa) containing potential protein kinase A phosphorylation sites (Nagasawa Y et al, 2002; Onuchic LF et al, 2002; Ward CJ et al, 2002), a single transmembrane (TM)-spanning segment, and a very long extracellular amino terminus.

The longest ORF of the mouse ortholog (Pkhd1) encodes a protein of 4059 aa; the mouse and human protein sequences are 73% identical overall and 55%

identical in the carboxyl-terminal tail (Nagasawa Y et al, 2002). The putative ~3.860 aa extracellular portion contains several immunoglobulin-like, plexin, transcription factor (IPT) domains that can be found in cell surface receptors and in the Rel family of transcription factors.

Between the IPT domains and the TM segment, multiple parallel beta-helix 1 (PbH1) repeats are present, a motif that can be observed in polysaccharidases. Based on the structural features of the deduced protein and on the human ARPKD phenotype, FC might be involved in cellular adhesion, repulsion, and proliferation. In addition, the domain and structural analyses suggest that the PKHD1 potential products may be involved in intercellular signaling and function as receptor, ligand, and/or membrane-associated enzymes.

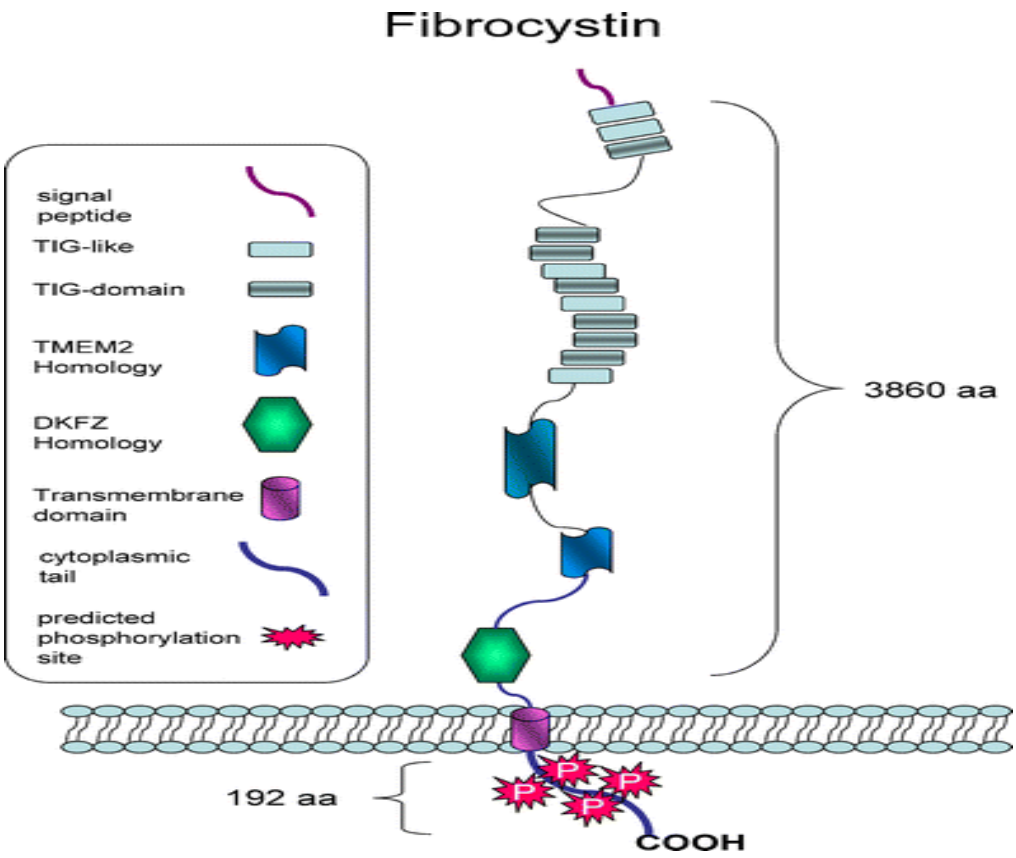


Figure 10: Predicted structure of fibrocystin. Fibrocystin is a large integral membrane protein with the largest known open reading frame translating a 4074-amino-acid novel peptide (4059 amino acids in mouse).

By in situ hybridization in mouse metanephros, it has been shown that Pkhd1 transcripts are not expressed in metanephric mesenchyme but are strongly

expressed in the branching ureteric bud. Moreover, in postnatal kidney tissue strong Pkhd1 expression was found in collecting ducts, with lower levels in proximal and distal tubules (Nagasawa Y et al, 2002). Immunofluorescence studies have revealed that, like the ADPKD proteins PC1 and PC2, FC co-localizes in centrosomes, basal bodies or primary apical cilia in renal epithelial cells (Menezes LF et al, 2004, Wang CJ et al, 2004; Ward CJ et al, 2003; Zhang MZ et al, 2004) and in the cilia of cholangiocytes of intra-hepatic biliary ducts (Masyuk TV et al, 2003).

2.11.2 FC REGULATES PC2 EXPRESSION

As previously mentioned, recent evidence suggests that FC, PC1 and PC2 are all localized at the plasma membrane and primary cilium, where PC1 and PC2 contribute to fluid flow sensation, and may function in the same mechanotransduction pathways (Wang S et al, 2007). A further study indicates that loss of FC down-regulates PC2 expression in vivo but that Pkd2 mRNA levels remain unchanged suggesting that FC expression may be essential for PC2 protein stability (Kim I et al, 2008 a).

Very recently, Ingyu Kim et al (2008 b) through the use of deletion and mutagenesis strategies, identified a PC2-binding domain in the intracellular C-terminus of FC and an FC-binding domain in the intracellular N terminus of PC2. The proximal portion of the N terminus of PC2, but not the distal portion, is involved in the binding between FC and PC2. Moreover, they also demonstrated that the peptide Asp90–Arg139 of PC2 may function as an FC-binding domain, and that the PC2-binding domain in the intracellular C terminus of FC is Gln3903–Glu3963. These binding domains provide a molecular basis for the physical interaction between PC2 and FC. In addition, they also found that physical interaction between the binding domains of PC2 and FC is able to prevent down-regulation of PC2 induced by polycystic kidney disease gene, encodes a loss of FC (Kim I et al, 2008b).

2.11.3 PKHDL1 ENCODES A RECEPTOR WITH INDUCIBLE T-LYMPHOCYTE EXPRESSION

It has been also described that a homolog of the ARPKD gene, PKHDL, exists and it express fibrocystin-L (FCL), a protein with low but highly significant identity and similarity to FC1 over the entire length of the protein, except the extreme C-terminal region (Hogan M et al, 2003).

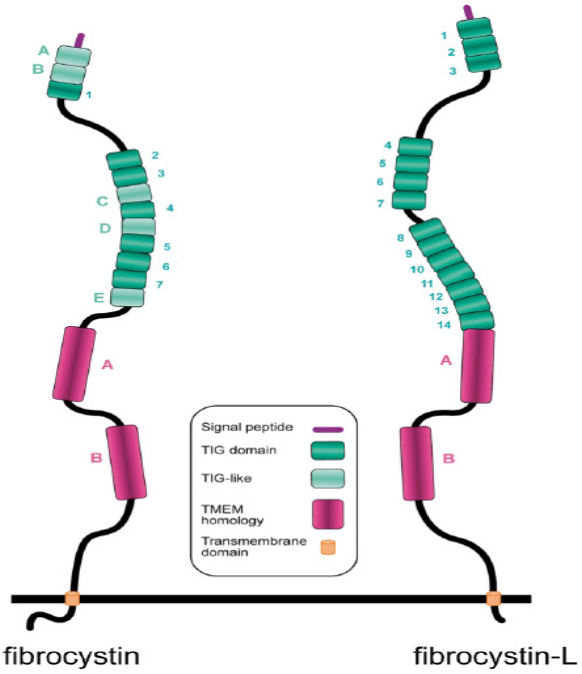


Figure 11: Comparison among the predicted structure of fibrocystin (FC) and fibrocystin-L (FCL). Fibrocystin-L (FCL) shows a highly significant identity and similarity to FC1 over the entire length of the protein, except the extreme C-terminal region.

Though the similarity of PCL to FC1 and evidence of expression in kidney and liver and data from ARPKD families, the lack of association of PKHDL1 with ARPKD is consistent with the major sites of expression of this gene in blood cell lineages. A preliminary analysis of highly purified cell populations from murine lymphoid organs raises the intriguing possibility that fibrocystin-L is up-regulated in T-cells following activation and, therefore, may serve a specific function in cellular immunity (Hogan M et al, 2003).

3. AIM OF THE WORK

Diagnostic tools to decrease ADPKD are little in progress. Renal ultrasound, the principal diagnostic procedure, not allows an early diagnosis. The molecular characterization of the mutation can have a prognostic value, the progression to ESRD occurring more rapidly in PKD1 patients. The genetic test is, however, very expensive, time consuming, many missense variants remaining without a known function. To explain either normal or mutated functions of PC1 and PC2, kidney cells and animal models are generally used. The identification of other cell systems suitable for investigating polycystin functions may be, therefore, of crucial importance in the field of both pathology and biology. PKD1 and PKD2 genes have been, in fact, costitutively expressed, though at low levels, in several human cell types and still without a clear function (Sutters M. and Germino GG, 2003).

A previous study (Aguiari G. et al, 2004) showed that PC1 and PC2 are expressed in B-lymphoblastoid cells (LCL) and play a role in Ca^{2+} homeostasis and signaling in these cells. In particular, in B-LCLs obtained from subjects with PKD2 mutations a reduction in PC2 and Ca^{2+} levels were found. Moreover, Ca^{2+} levels were also reduced in cells obtained from subjects with PKD1 mutations (Aguiari G et al, 2004). Then, PKD B-LCls with reduced Ca^{2+} levels and reduced PC2 levels highlight the presence of a PKD2 mutation, known to be associated to the milder ADPKD with important impact on diagnosis and prognosis.

Because T-lymphoblasts (HTL) and, even more, peripheral blood lymphocytes (PBL) are obtainable from peripheral blood more easily and quickly than B-LCLs, this study was addressed to define whether 1. PC2 is detectable in PBL and HTLs, and 2. reduced cytoplasmic Ca^{2+} levels are associated with ADPKD-derived cells. Results obtained from this study will provide further information on the extrarenal role of PCs and may lead to a diagnosis of ADPKD based on quantitative and functional evaluation of PC1/PC2 channel complex.

4. MATERIALS AND METHODS

4.1 PATIENTS AND CONTROLS

20-30 ml of peripheral blood, in vacuum blood collection tube EDTA, were obtained from 34 ADPKD (21 F) and 31 non-PKD patients (13 F). Informed consent was obtained from all patients before collection of blood.

4.2 PERIPHERAL BLOOD LYMPHOCYTE (PBL) PREPARATION

Blood was diluted with an equal volume of D-Phosphate Buffered Saline (D-PBS) (Celbio). Then, in a 50 ml centrifuge tube, 2 volumes of the diluted blood were stratified on 1 volume of Lympholyte®-H (Cedarlane Laboratories) and then centrifuged at 800g for 20 minutes at room temperature. Cells were carefully removed from the interface and transfered to a new centrifuge tube. The cells were diluted with D-PBS to reduce the density of the solution and centrifuged at 800g for 10 minutes. Pellet of lymphocytes were washed twice with D-PBS and centrifuged at 800g for 5 minutes.

Peripheral blood lymphocytes (PBL) were resuspended in RPMI 1640 (Celbio) supplemented with 10% fetal bovine serum (FBS) (Celbio), 0.1 mg/ml of penicillin and 0.2 mg/ml streptomycin (complete medium) and purified from PBMC by 2 hours of adherence to remove monocytes. After twice washing of PBL with RPMI 1640 they were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Celbio), 0.1 mg/ml of penicillin and 0.2 mg/ml streptomycin, supplemented with 2 μg/ml phytohemagglutinin (PHA). Stimulation to T-lymphoblast (HTL) was carried out for 72 hours; afterward, the medium was discarded, cells were washed three times with RPMI 1640 and the concentration was adjusted to 5×10^5 cells per ml of complete medium supplemented with 50 U/ml interleukin-2 (IL-2) (NOVARTIS).

4.3 CELL CULTURE

HTL were grown in RPMI 1640 (Celbio) supplemented with 10% fetal bovine serum (FBS) (Celbio) and supplemented with 50 U/ml interleukin-2 (IL-2).

B-LCLs, developed from peripheral blood cells using Epstein Barr virus transformation (EBV), were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 0.1 mg/ml of penicillin and 0.2 mg/ml streptomycin.

SV40-transformed human kidney tubular epithelial cell (4/5), gently provided by Dr. Peter Harrys from Mayo Clinic, were grown in 50% Dulbecco's modified Eagle's/50% Ham F12 (DMEM/F12), supplemented by 10% FBS, 0.1 mg/ml of penicillin and 0.2 mg/ml streptomycin.

Human embryonic kidney (HEK293) cells were grown in minimum essential medium (MEM) supplemented with 10% South America fetal bovine serum (FBS) (Celbio).

4.4 PKD2 siRNA EXPRESSING CELLS

One siRNAs for PC2 was constructed according to a published method (Aguiari et al, 2008). Briefly, 64 nt primers were designed to include a 19-nt PKD2 sequence, its complement, a spacer region, 5′BglII site and 3′HindIII site.

The forward PKD2 primer sequence: 5′-GATCCCCTGTGGAGGTGCTACTACAGTTCAAGAGACTGTAGTAGCACCTCCACATTTTTGGAAA-3′ and the reverse primer sequence: 5′-AGCTTTTCCAAAAATGTGGAGGTGCTACTACAGTCTCTTGAACTGTAGTAGCACCTCCACAGGG-3′.

Annealed double strands of DNAs were cloned into the pSUPER plasmid vector following the Oligoengene procedure.

HEK293 cells were maintained and cultured as previously described (Manzati et al. 2005). Wild-type (pSUPER) and the recombinant construct (pSsiPKD2) were co-transfected with pCDNA3 (ratio 1:10, respectively) by the

calcium phosphate method, in HEK293 cells (Pelucchi et al. 2006), and G418-resistant clones (HEK293^{pSsiPKD2}) were screened by Western blotting analysis. One clone with about 50% reduction of PC2 in comparison to HEK293^{pSuper} and HEK293^{pSsiPKD1} previously prepared clones (Aguiari et al, 2008), was used as kidney control cell line.

4.5 CELL PROLIFERATION ANALYSIS

Cells were seeded in 200 µl at low density (25000 cell/ml) in 96 multiwell plates. Then, cells were cultured for two-days in complete medium supplemented with 50 U/ml interleukin-2 (IL-2) and the cell number was determined using manual counting with a Burker counting chamber and staining for trypan blue exclusion after 4, 24 and 48 hours of culture. Growth curves were generated performing three different cell countings in at least three separate cell cultures.

4.6 SIZE OF CELL AGGREGATE ANALYSIS (image acquisition)

Cells were seeded in 2 ml at density of 7,5 x 10^5 cell/ml in 24 multiwell plates. Then, cells were cultured for one days in complete medium supplemented with 50 U/ml interleukin-2. Size of HTL clump was evaluated using an inverted phase-contrast microscope (Nikon Eclipse TE200, Melville, NY). The images were acquired with a CCD camera COOLSNAP (RS Photometrics, Tucson, Arizona, USA) and analyzed with PHOTOSHOP 5.5.

4.7 WESTERN BLOTTING ANALYSIS

Western blotting through the detection of the endogenous PC2 protein was performed on cell total lysates and membrane fraction.

4.7.1 CELL LYSATES

To obtain total cell extracts, cells were washed twice with D-PBS containing protease inhibitors, then they were centrifuged at 1600 rpm for 5 minutes, 4°C. The pellet was resuspended in a single detergent lysis buffer (10 mM TRIS-HCl pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 1% v/v triton X-100 and complete protease inhibitor cocktail for 30 minutes on ice. After centrifugation at 10000 g for 5minutes 4°C, an amount of cell lysate was assayed for protein content by Bradford method.

4.7.2 MEMBRANE PREPARATION

Membrane fraction subjected to electrophoresis was prepared according to *Nagano J et al, 2005*. Cells were harvested in buffer containing 20 mM Hepes pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA pH 8, protease inhibitors, and homogenized with 30 strokes of a Dounce B homogenizer. Homogenates were centrifuged at 1500 rpm for 10 minutes at 4°C to yield a post-nuclear pellet. The resultant supernatants were centrifuged at 100000 g for 1 hours at 4°C to yield crude membrane and cytosol fractions. Membrane pellets were resuspended in buffer containing 20 mM Hepes pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA pH 8, protease inhibitor and protein contents were measured by Bradford colorimetric method.

4.7.3 ELECTROPHORESIS AND IMMUNOBLOTTING

The membrane fractions and/or total cell lysates (80-100 μg), premixed with 6X loading buffer (60 mM Tris pH 6.8, 1.8% SDS, 6% v/v glycerol, β-mercaptoethanol, 0.002% p/v bromophenol blue), were subjected to 6% SDS–PAGE and proteins were transferred to nitrocellulose membrane (Pierce) for 2 hours in transfer buffer (25 mM Tris, 192 mM Glycine, 20% methanol pH 8.3). Blots were blocked overnight at 4°C in 5% non-fat dry milk in D-PBS, 0.05% Tween20®. Then membranes were processed for immunoblotting with different primary antibody:

monoclonal antibody anti-FC1 14A N-Ter gently provided by Dr. Peter Harris, Mayo Clinic, diluted 1:1000 in 5% non-fat dry milk in D-PBS, 0.05% Tween20®; polyclonal antibody anti-PC2 C-ter (YCC2) gently provided by Dr. Yiqiang Cai, Yale University, diluted 1:10000 in 5% non-fat dry milk in D-PBS, 0.05% Tween20®; monoclonal antibody anti-PC1 (SantaCruz), and monoclonal antibody anti β-ACTIN (Sigma-Aldrich).

After 2 hours of incubation, three washes with D-PBS 0.05% Tween20® were performed and then the nitrocellulose membranes were incubated with the respective HRP-coniugated secondary antibody (anti mouse or rabbit) (1:50000). After three washes with D-PBS, 0.05% Tween20®, immuno-bands were visualized by autoradiography with the enhanced chemioluminescence system (SuperSignalFemto, Pierce).

Band intensity was quantitatively analyzed by model GS-700 Imaging Densitometer (BioRad).

4.8 CALCIUM MEASUREMENTS

4.8.1 SOLUTIONS

Fluorescence measurements were performed either in saline solution containing 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM Na_2HPO_4, 5.5 mM glucose, 5 mM $NaHCO_3$, 1 mM $CaCl_2$ and 20 mM HEPES (pH 7.4 with NaOH), or in a Na^+-free saline solution containing 300 mM sucrose, 1 mM $MgSO_4$, 1 mM K_2HPO_4, 5.5 mM glucose, 1 mM $CaCl_2$ and 20 mM HEPES (pH 7.4 with KOH).

4.8.2 FURA-2/AM Ca^{2+} MEASUREMENTS BY INVERTED MICROSCOPE

Cytoplasmic Ca^{2+} and platelet-activating factor (PAF)-evoked calcium were measured in cells loaded with the fluorescent indicator Fura-2/AM. Cells (2 X 10^5) were seeded on poly-L-lysine (100μg/ml; Sigma-Aldrich)-coated coverslips and loaded with fura-2/AM (3 μM/30 min/37°C), and transferred to the thermostated stage of a Zeiss Axiovert 200 inverted microscope, equipped with a Sutter filterwheel and 340/380 excitation filters. Cells were stimulated with 2 μM PAF. In order to calibrate the system, cells on coated coverslip were perfused prior with a saline solution added with 2 μM Ionomicine and then with a 50 mM EGTA solution to chelate all released calcium.

Recording was performed for 15 min, acquiring 1 image every 1 sec or 3 sec, respectively. Signals were computed into relative ratio units of the fluorescence intensity of the different wavelengths (340/380 nm).

4.8.3 FURA-2/AM Ca^{2+} MEASUREMENTS IN CUVETTE

Changes in the intracellular free Ca^{2+} concentration ([Ca2+]i) were measured with Fura-2/AM, using an LS50 Perkin Elmer fluorometer (Perkin Elmer Ltd., Beaconsfield, UK). For fura-2/AM loading, cells (3 x 10^6/ml) were resuspended in the saline solution containing NaCl, in the presence of 1 μM fura-2/AM and 250 μM sulfinpyrazone (Sigma-Aldrich). Incubation was performed at 37°C for 30 min. Cells were then washed and resuspended in the Na^+-free saline solution. [Ca^{2+}]i changes were determined in a thermostated, magnetically stirred cuvette, with the 340/380 excitation ratio at an emission wavelength of 505 nm. Cells were stimulated with 2 μM PAF (Sigma-Aldrich) and, after that, added with 2 μM Ionomicine and successively with a 250 mM EGTA (Sigma-Aldrich) solution to chelate all calcium released.

4.8.4 FURA-2/AM Ca^{2+} MEASUREMENTS IN PLATE

Changes in the intracellular free Ca^{2+} concentration ([Ca2+]i) were also measured, always with the fluorescent indicator fura-2/AM, using Victor^3 multilabel counter Perkin Elmer (Perkin Elmer Ltd., Beaconsfield, UK). For fura-2/AM loading, cells (3 x 10^5/ml) were resuspended in the NaCl containing saline, in the presence of 1 μM fura-2/AM and 250 μM sulfinpyrazone (Sigma-Aldrich). Incubation was performed at 37°C for 30 min. Cells were then washed and resuspended in the Na^+-free saline solution and [Ca^{2+}]i changes were determined in a 96 wells plate at room temperature, with the 340/380 excitation ratio at an emission wavelength of 505 nm. Cells were stimulated with 2 μM PAF and, after that, added with 2 μM Ionomicine and with a 250 mM EGTA solution to chelate all calcium released.

4.9 DNA EXTRACTION

DNA extraction was carried out from peripheral blood by using the QIAGEN BioRobot Universal System (GmbH, Hilden, Germany).

4.10 PCR AMPLIFICATION OF PKD2 AND PRODUCTION OF DHPLC (Denaturing High Performance Liquid Cromatography) AMPLICONS

The oligonucleotides used for the amplification of PKD2 exons 1-15 were constructed to comprehend about 80-100 nucleotides upstream and downstream of each exon in order to pick up mutations possibly adjacent to coding regions (Table 2).

PCR amplifications were performed by using the AmpliTaq Gold (Applied Biosystems) kit. Each sample contained: 2.5 µl Buffer 10X, 2.5 µl MgCl (25 mM), 2.0 µl dNTPs mix (10 mM), 0.3 µl Taq Polymerase Gold (5 U/µl), 1.5 µl Forward/Reverse Primers each, 100 ng (1 µl) DNA solution,13.7 µl H_2O. 5% DMSO was added to the amplification solutions in order to increase the reaction product specificity. The PCR touch-down protocol was conducted in a 2720 Thermal Cycler (Applied Biosystems) and amplicons were analysed by agarose gel electrophoresis. A touch-down protocol with an elevated number of cycles, where the first pairing temperature is of 64 ºC and maintained at 1 minute for two cycles, was adopted because of different pairing temperatures of primers. The next temperatures, each maintained at 1 min for 5 cycles, decreased of 2 ºC up to 58ºC, this last temperature being maintained for 10 cycles. The next 15 cycles were carried out at 56ºC. In table 1 the amplification protocol is shown.

Temp. °C	***Time***	
94ºC	15'	1
94ºC	30"	
64ºC	1'	2
72ºC	45"	
94ºC	30"	
62ºC	1'	5
72ºC	45"	
94ºC	30"	
60ºC	1'	5
72ºC	45"	
94ºC	30"	
58ºC	1'	10
72ºC	45"	
94ºC	30"	
56ºC	1'	15
72ºC	45"	
72ºC	5'	2
4ºC	8	

Table 1: PCR touch-down protocol.

4.11 PCR PRODUCT ANALYSIS BY DHPLC

DHPLC analysis was carried out through the WAVE 4500 Genetics Analysis System (Transgenomic, Crew, UK). The melting temperature was calculated by using the Wavemarker program (table 2). The reagents used were: Buffer A (0.1 M Triethylammonium acetate buffer (TEAA) pH 7.0, Buffer B (0.1M TEAA pH 7.0, 25% acetonitrile), Buffer D (25% H_2O, 75% acetonitrile).

4.12 PURIFICATION OF PCR PRODUCTS

When DHPLC analysis of DNA amplified fragments showed an anomalous profile in comparison with that of the wild type, PCR amplifications were repeated and PCR products were purified by using the Microcon Centrifugal Filter Devices (Millipore) and submitted to sequencing.

4.13 SEQUENCING REACTION

The reaction mix was prepared by using the BigDye® Terminator v3.1 Cycle Sequencing kit: 2 µl 5X Buffer, 1 µl Sequecing Mix (dNTP, ddNTP, enzymes), 1 µl Forward or Reverse Primer (2,5 µM), 5 µl H_2O, 100 ng (1 µl) DNA. Reaction conditions were: 40 cycles of two step, 96 ºC for 10'' and 60 ºC for 4' (see table 2)

Table 2: Details of PKD2 amplicons.

Exonic fragment	**Size *bp***	**FORWARD&REVERSE**	**DHPLC conditions**	
			Temp. °*C*	**Initial % buffer B**
2	377	CCTACAAAACCAGTTTCTCAGTTGC		54,6
		CCCTCTGGTGCATACACACTTCC		
3	289	GCTGGTATGTGAATGTGTGCCGG	57,2- 58,2- 59,2	56,4- 56,4- 54,4
		GCAGGTCCTGTCGATACTCATG		
4	417	GGTTATGCAAACGATGCAGGCAGG	59,3	59,3
		GTGTCAGGGAATGAATGAATGAATGG		
5	441	CTTGTAATTGCCTCAAGTGTTCCAC	57,2- 58,8	59,7- 58,7
		CCTTCAGTTTTTACCCCTCCC		
6	416	GTAATGCATGAACAGAACAGATGG	55,5-56,4	59,3- 59,3
		GGAATATCAAGATCCACAATGCTGAGG		
7	403	GGTGAGCCCTTATAATTAATACATTGG	52,4- 54,4- 55,4	59,1- 58,1- 56,1
		GTAAATATTGAGCAGCTTTGGCTGG		
8	446	GTCAGAATTTAATTTCTTTTCAGGATG	55,5- 57,5	55,8- 53,8
		GCATGGGTAGATCAATGTTTTGGG		
9	312	GGCAGCTACCTATACTGCTAAAAGG	54- 55	57,1- 56,1
		GTGTGAGAGAAAAGAGAAGACAAGG		
10	313	GTCTTCATAAAGCACTCAGATTAGG	54- 55,5	57,1- 56,1
		GGATCAATGTTGATTTGAAAATCTGGG		
11	296	GGTTTGTAGTAGTTACTACTGTGAATGG	55,5- 57,7	56,6- 53,6
		GGCTAGAAATACTCTTATCACCAGG		
12	301	GTCTCTGTGTTGAGGGTGAACTGG	57,9	59,4
		AGAGTGTGGTGGGGAATGGG		
13	316	GTCCTTGGTGAGGCTTCTGTGG	59,3- 59,8	57,2- 56,2
		GGAGTGAATTCAGAGAGATGAGG		
14	324	CTTAGCACTTCCTTTTGAAAAGCC	59,4- 61,4	57,4- 56,4
		CTTCAATACTTCAAATACAACTGTCAGC		
15	449	CCTTACCAAACTACAGATTATTTGGTCC	57,8- 58,8- 59,8	59,8- 58,8- 56,8
		CAATCGGTCACAAAGACTAGCATCC		

4.14 SEQUENCE PURIFICATION

Purification was carried out with the Montage SEQ96 Sequencing Reaction Clean kit (Millipore). To one volume of the purified product two volumes of formamide were added. DNA was denatured a 94°C for 2 min and submitted to sequencing.

4.15 SEQUENCING

DNA was submitted to sequencing with the ABI PRISM 3130 (Applied Biosystems). Sequences were analysed with the Chromas™ Software and compared with a wild-type sequence by using the BLAST 2 Sequence program (http://ncbi.nlm.nih.gov/BLAST).

4.16 PREPARATION OF HUMAN NEUTROPHILS

From the blood cells of three PKD and two non-PKD subjects, neutrophils were purified by the standard techniques of dextran (Pharmacia, Uppsala, Sweden) sedimentation, centrifugation on Lympholyte®-H (Cedarlane Laboratories) and hypotonic lysis of contaminating red cells. The cells were washed twice, resuspended in Krebs–Ringer–phosphate containing 0.1% w/v glucose (KRPG), pH 7.4, at a final concentration of $50x10^6$ cells/ml and kept at room temperature until used. The percentage of neutrophils was >98% pure and >99% viable as determined by the trypan blue exclusion test.

4.16.1 RANDOM LOCOMOTION

Random locomotion was evaluated with a 48-well microchemotaxis chamber (BioProbe, Milan, Italy), using the method of Zigmond and Hirsch (Zigmond SH and Hirsch JG, 1973), by estimating the distance (in μm) which the leading front of the cell migrated after 90 min of incubation at 37°C. A 3 μm pore size filter (Millipore, Rome, Italy) was used to separate the upper and lower compartments. Assays were carried out in triplicate under each experimental condition.

4.16.2 CHEMOTAXIS

Chemotaxis was studied by adding the chemoattractant to the lower compartment. The chemotactic factors used were: fMLP (Sigma, St Louis, MO, USA). The fMLP was diluted from a stock solution [10^{-2} M in dimethylsulphoxide, DMSO, Sigma)] with KRPG-A (KRPG-A, Sigma) containing 1 mg/ml of bovine serum albumin and used at concentrations ranging from 10^{-12} to 10^{-5} M. Assays were performed in duplicate under each experimental condition. The DMSO did not interfere with any of the biological assays performed. Data were expressed in terms of chemotactic index (CI), which is the ratio: (migration toward test attractant minus migration toward the buffer)/(migration toward the buffer).

4.17 STATISTICAL ANALYSIS

Where applicable, statistical errors of averaged data are given as means ± SE and statistical significance was assessed by Student's t-test.

5. RESULTS AND DISCUSSION

5.1 ANALYSIS OF PC2 LEVELS IN KIDNEY CELL LINES AND BLOOD DERIVED CELLS

Previous studies indicated that the expression of PKD2 and PKD1 genes is detectable in blood derived B-LCLs, though at a lower levels than in kidney cells (Aguiari G. et al, 2004). In order to investigate a possible functional role of PCs in HTL, we initially studied wether PC2 was detectable in HTL cells by immunoblot experiments. Western blot analysis of PC2 was performed in both total cell lysates and cell membranes of HTL, and compared with that found in B-LCLs, in embryonal and adult kidney cell lines (Figure 12).

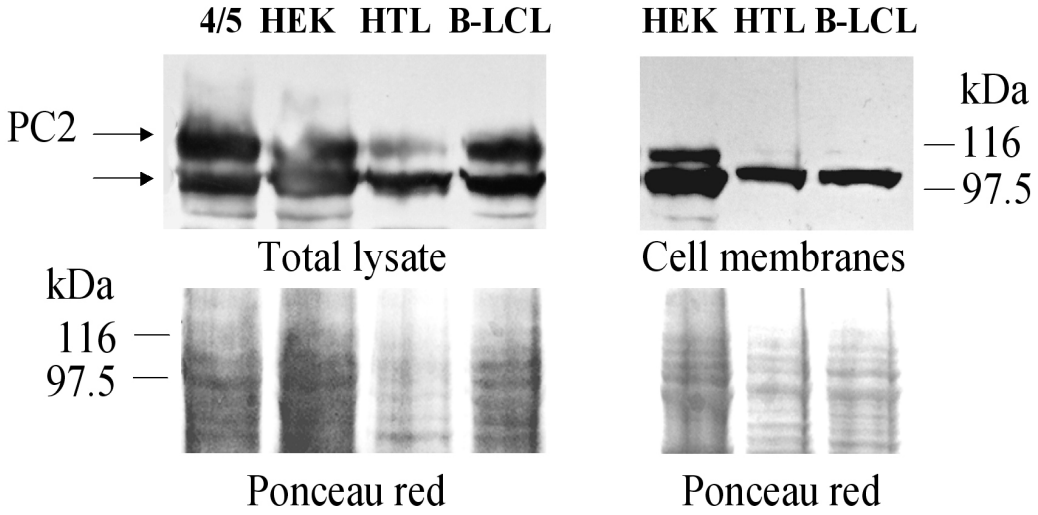


Figure 12: Western Blotting of PC2. Total cell lysates (100 μg) and membrane fractions (50 μg) were prepared and probed as described in Methods. 4/5 and HEK293 cells were human normal kidney cell lines obtained from adult (4/5) and embryonal kidney tubular cells; HTL and B-LCL were obtained from normal subjects. Protein levels were normalized to the intensity of Ponceau red stained bands.

The anti-PC2 YCC2 antibody, specific for the C-terminal peptide of the protein (Cai Y. et al, 1999), recognized two major bands of ~100 and 110 kDa in total

lysates of both kidney and blood derived cells. On the contrary, in membrane fractions the band of ~100 kDa was still detectable in all cell types, while that of ~110 KDa was clearly detected in kidney cells, but faintly in blood derived cells. Thus, PC2 expression in human HTL resulted comparable to that of B-LCLs, which are known to have a prevalent distribution in the endoplasmic reticulum compared to plasma membranes (Aguiari G. et al, 2004).

The expression of PC2 was, then, investigated in HTL isolated from ADPKD and non-ADPKD subjects, and further compared with that of kidney cell lines. The next Figure 13 shows the analysis of PC2 in 5 ADPKD HTL and 3 controls. In particular, HEK293 kidney cells have been used to identify the specificity of PC2 positive bands, expressing HEK293^{siPKD2} and HEK293^{siPKD1} cells siRNA either against PKD2 (siPKD2) or PKD1 (siPKD1). PC2 should be, therefore, downregulated only in PKD2 siRNA expressing cells.

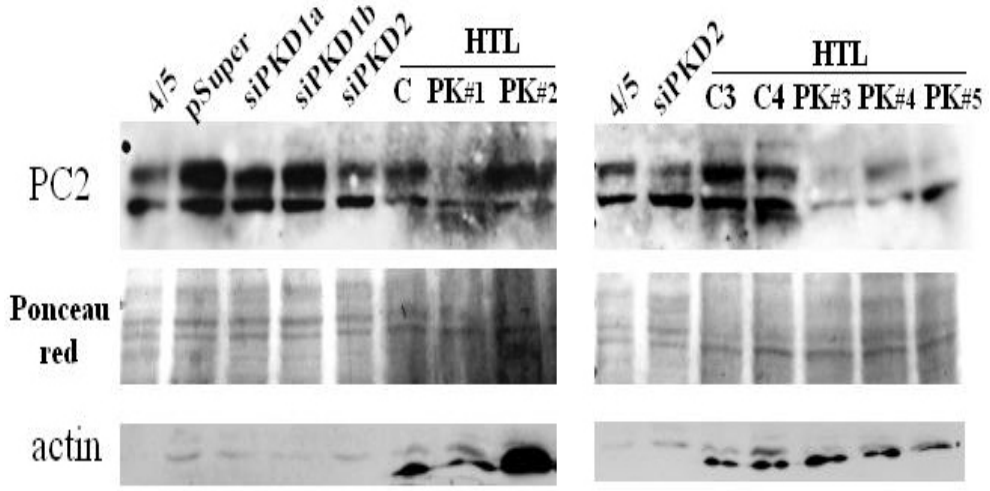


Figure 13: Immunoblot analysis of PC2 in HTL and in PKD1 and PKD2-downregulated HEK293 cells. Total cell lysates (30-100 μg) were prepared as described in Methods. 4/5 and HEK293 (30 μg) are adult and embryonal kidney cells, respectively. HEK293 cells were stably transformed with pSuper vector wild type (HEK293^{pSuper}, pSuper) and recombinant for PKD2 (HEK293^{siPKD2}) or PKD1 (HEK293^{siPKD1}) siRNA; HTL (100 μg) were from 3 normal- (C) and 5 ADPKD (PK) subjects. Protein levels were normalized to the intensity of Ponceau red stained bands or actin.

Results showed that levels of the 110 kDa band in HEK293$^{siPKD2.1}$ (siPKD2)

cells were lower (~ 66%) than in HEK293^{pSuper} (pSuper) and PKD1-downregulated (HEK293^{siPKD1a} and HEK293^{siPKD1b}) control cells. Therefore, the 110 kDa band was the specific one, and thus the band considered to quantify PC2 expression in cell samples. Moreover, PC2 in HTL was less (about 3.5 fold less) expressed than in kidney cells.

In order to better compared PC2 expression in HTL and B-LCLs, we analyzed 4 B-LCLs samples (obtained from one non-ADPKD subject, and from two ADPKD2 and one ADPKD1 genetically-characterized subjects) and 5 HTL samples (from one control and four ADPKD, including a case genetically characterized as PKD1) (Figure 14).

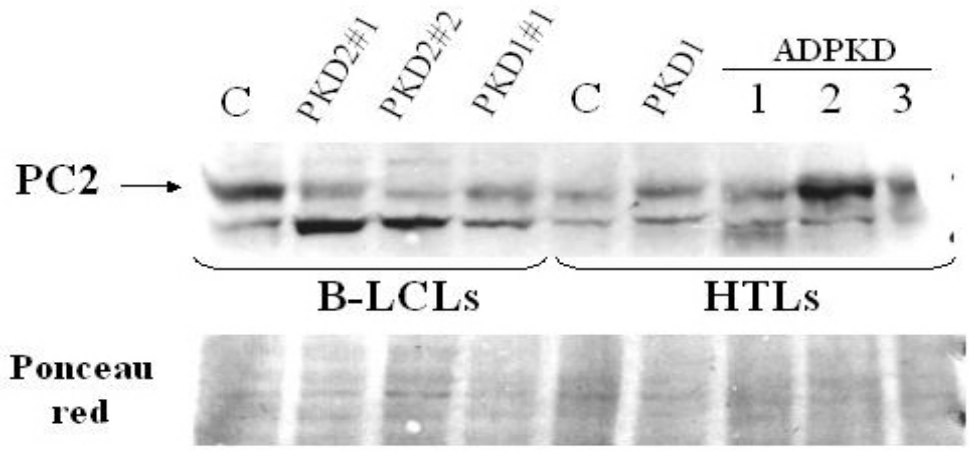


Figure14: Comparison between PC2 expression in HTL and B-LCLs. Total cell lysates (~ 100 μg) were prepared as described in Methods. B-LCLs were produced by transformation with the Epstein Barr virus of PBMC from non ADPKD and ADPKD affected subjects; HTL were one normal (C) and 5 ADPKD (PKD) subjects, one with a genotypically characterized PKD1 mutation (PKD1).

Overall, the degree of PC2 expression in HTL did appear comparable to that of LCLs. The apparently aspecific 100 kDa band appears more marked in PKD2 B-LCLs.

PC2 levels were measured in HTL samples obtained from ten ADPKD and five non ADPKD subjects and results obtained by relating PC2 to actin levels are shown in the Figure 15.

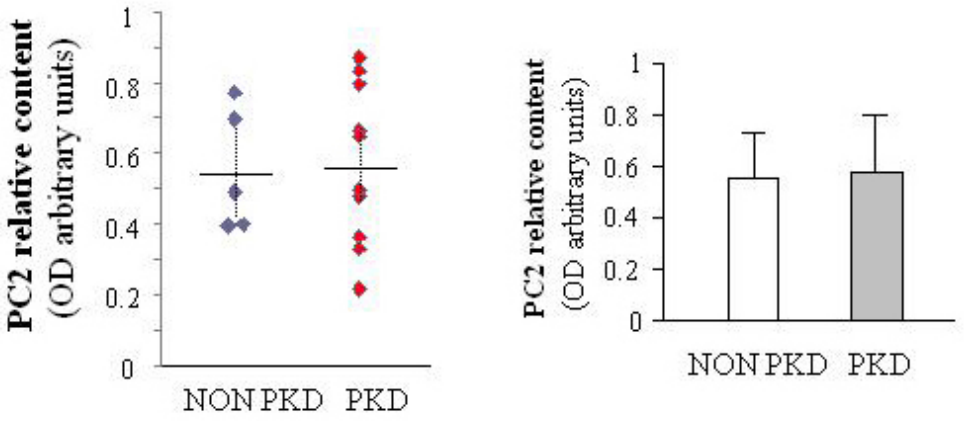


Figure 15: PC2 relative content in ADPKD HTL. Relative amounts of PC2 were obtained by comparing densitometric values of PC2 bands to those of actin. Histogram values are means ± SD in ten ADPKD and five non ADPKD HTL. Averages values were 0.572 ± 0.228 SD in ADPKD and 0.553 ± 0.176 SD in non ADPKD HTL.

No differences were found between the averages values of ADPKD and control HTL (0.572 ± 0.228 SD in ADPKD and 0.553 ± 0.176 SD in non ADPKD). Moreover, the four samples obtained from genetically characterized PKD1 patients have an average value of 0.490 ± 0.131 SD. One ADPKD case had a PC2 value lower than the double of SD of ADPKD1 average value. Considering that this subject was 71 y.o. when entered in dialysis, it may represent a possible ADPKD2 patient.

5.2 ANALYSIS OF PC1 LEVELS IN KIDNEY CELL LINES AND BLOOD DERIVED CELLS

The expression of PC1 was investigated in HTL isolated from ADPKD and non-ADPKD subjects and compared with that of kidney cell lines. As shown in the Figure 16, a PC1 positive broad band of more than 400 kDa was detected in HEK293 control cells and very slightly in the cells expressing PKD1 siRNA (siPKD1a and b). The same band, but even more smeared, was detected in both ADPKD and non ADPKD HTL. The abnormal migration pattern of PC1 in HTL, perhaps due to the

high concentration of loaded proteins (100 μg), did not allow to correctly measure PC1 in ADPKD HTL.

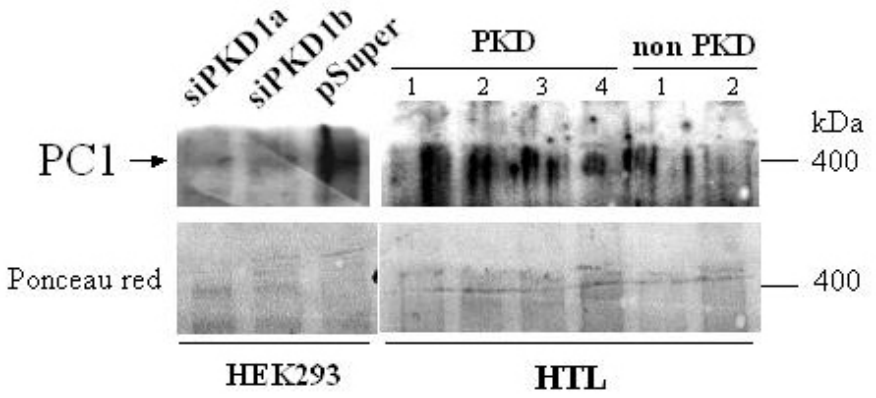


Figure 16: Immunoblot analysis of PC1 in HTL and HEK293 cells. Total cell lysates were prepared as described in Methods. HEK293 (50 μg) were stably transformed with pSuper vector wild type (HEK293^{pSuper}, pSuper) and PKD1 siRNA plasmid (HEK293$^{siPKD1a\ and\ b}$); HTL (100 μg) were from two normal- (C) and 4 ADPKD (PKD) subjects. Filter was probed with the anti Cter PC1 rabbit polyclonal antibody (Santa Cruz).

Nevertheless, since the tested HTL should carry PKD1 mutations because of their high incidence (85% of ADPKD), the presence of marked PC1 signals in ADPKD HTL suggested that PKD1 aploinsufficiency in HTL, if it exists, could not be detected by PC1 blotting analysis.

5.3 THE HOMOLOGUE OF THE PKHD1 GENE PRODUCT, FYBROCYSTIN/POLYDUCTIN, IS EXPRESSED IN HUMAN T-LYMPHOBLASTS

It has been reported that deficiency of fibrocystin/polyductin (FC1) increases the severity of cystic disease in Pkd2 mutants and downregulates PC2 *in vivo* (Kim I. et al, 2008). These findings imply a strong functional relationship between FC1 and PCs in vivo. Therefore, we investigated a possible, expression of FC1 in HTL from ADPKD patients, even if FC1 should be expressed at low level in extra renal-hepatic tissues. It is also known that FCL, the FC1 homologue, is

expressed at a low level in most tissues but only up-regulated specifically in T lymphocytes, suggesting a role in cellular immunity. Moreover, PKHD1 expression is almost undetectable in HEK293 cells (Hogan M.C. et al, 2003). We, therefore, investigated FC1 and FCL expression in HTL and kidney cells to explore possible molecular interactions between FC1/FCL and PCs proteins, as such interactions could be defective in presence of PCs mutations.

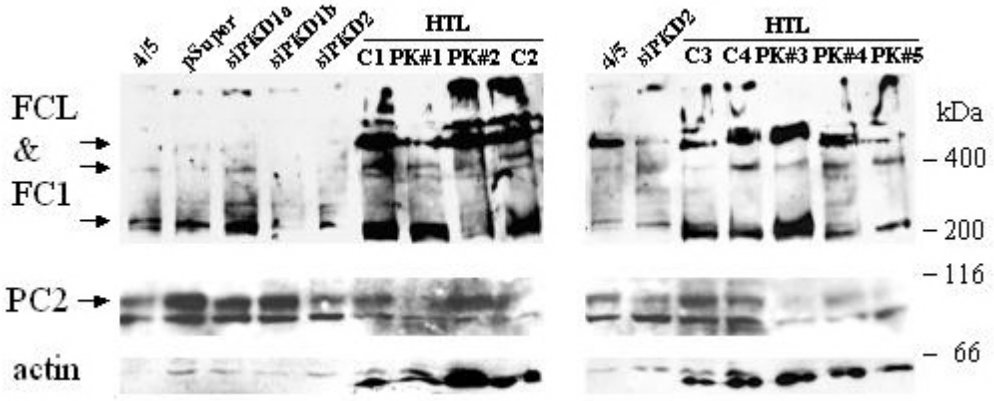


Figure 17: Comparison between FCL&FC1 expression in HTL and kidney cells. Total cell lysates from HTL (~ 100 μg) and from kidney cells (25 μg) were prepared as described in Methods. 4/5 and HEK293 (pSuper, siPKD1a, siPKD1b and siPKD2) cells are kidney tubular epithelial cells. HTL were 4 normal- (C1, C2, C3, C4) and 5 ADPKD HTL (PKD), including a genotypically characterized as PKD1 (PK#2).

As expected by the high homology between FC1 and FCL, the anti-FC1 antibody did recognize in HTL four major bands (of very high MW as possible aggregates, of ~ 500, ~ 400 and ~ 200 kDa), likely corresponding to FCL, while it highlighted three lighter bands (of ~ 500, ~ 200 kDa and one more light of ~ 400) in kidney cells (Figure 17). However, due to the variability of the bands inside the same samples, it is difficult to attribute the identity of the FCL and FC1 bands. We have considered, therefore, the overall expression of all combined bands to see for possible quantitative differences. The values normalized for the total protein content is represented in the next figure (Figure 18). Blood derived cells resulted to have higher values of FC1&FCL than LCLs kidney cells. Moreover, as expected by

data of PKHDL RNA already reported (Hogan M.C. et al, 2003), FC1&FCL positivity in B-LCLs was lower than in HTL. However, no differences were present between non-ADPKD and ADPKD HTL.

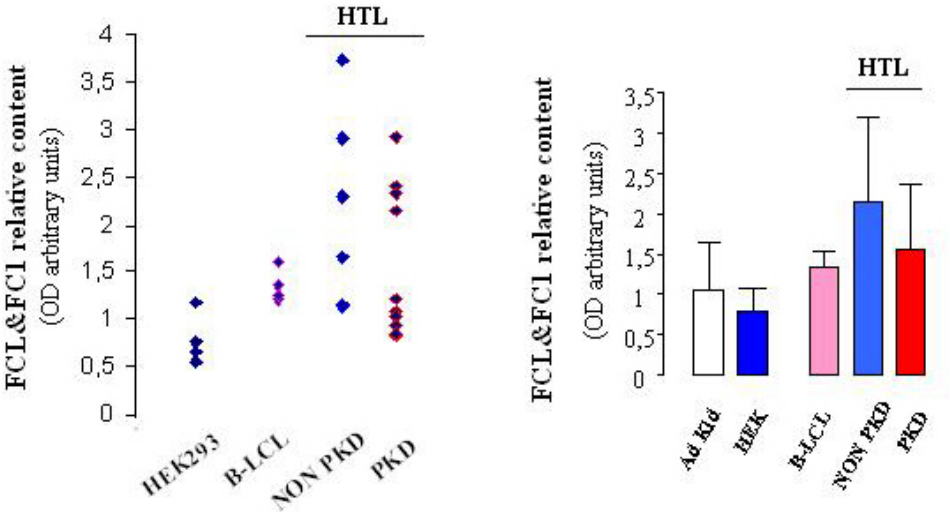


Figure 18: FCL& FC1 relative content. Relative amounts of FCL& FC1 were quantified by densitometry of FCL& FC1, Ponceau red stained bands. Histogram values are means ± SD of at least five separate experiments In ten ADPKD and five non ADPKD HTL, no differences were found. Adult (Ad) and embryonal (HEK) kidney cells are compared to blood derived cells.

5.4 PLATELET ACTIVATING FACTOR (PAF)-EVOKED CALCIUM RELEASE IN HTL

The possible functional alteration of the PC1/PC2 calcium channel in HTL obtained from ADPKD subjects, was ascertened by measuring Ca^{2+} release induced by the platelet-activating factor (PAF), a known agonist of Ca^{2+} release in B-LCLs (Aguiari G. et al., 2004). Initially, we performed an analysis on single cells by measuring Ca^{2+} release with an inverted microscope. Fura-2AM-loaded HTL seeded on coverslips (see Methods for technical details) were stimulated with PAF (2μM) which was able to cause an increase in cytoplasmic Ca^{2+} levels (see next figure 19).

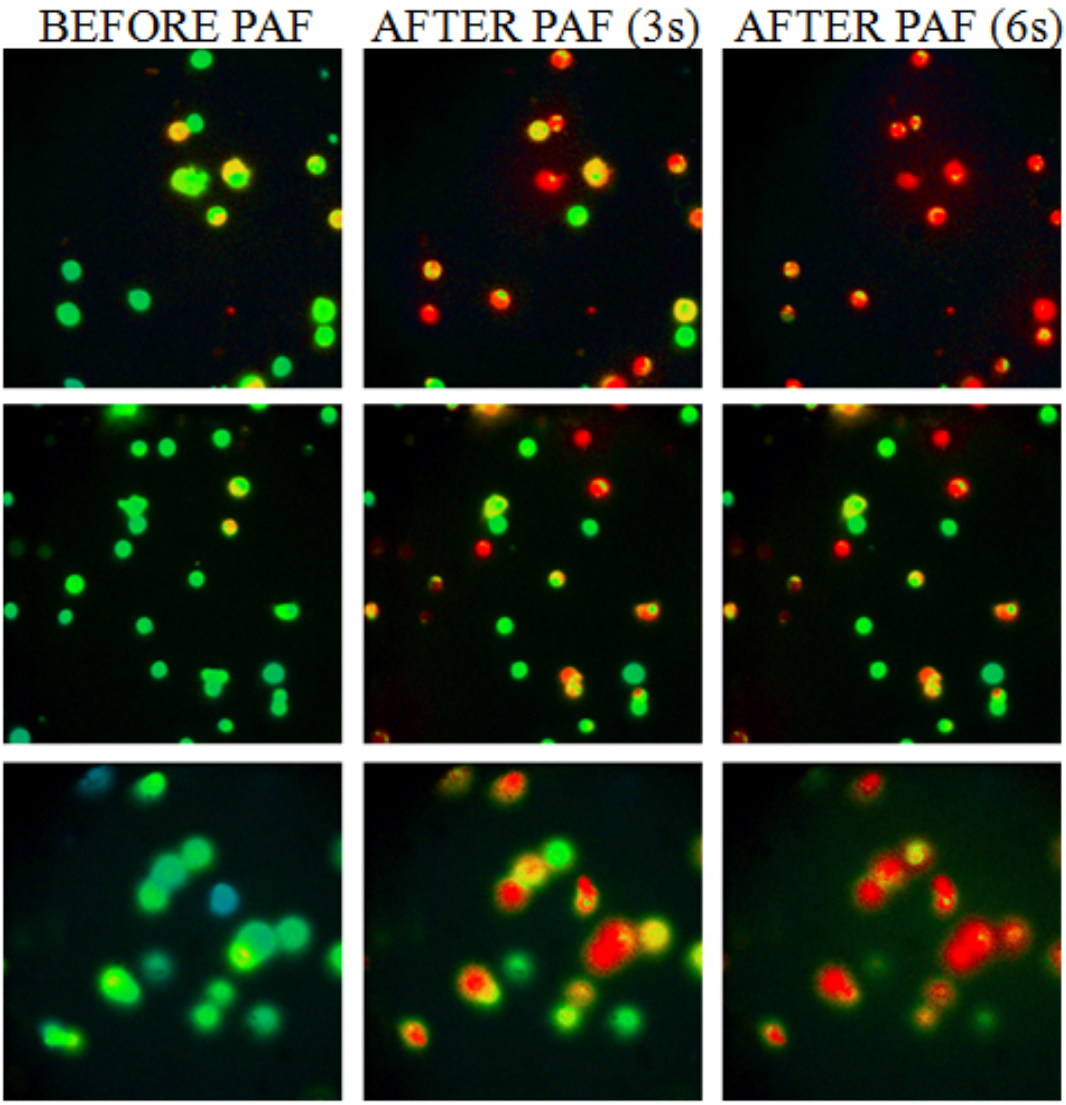


Figure 19: **Images of PAF-stimulated HTL**. HTL (2 X 10^5/ml) were loaded with 3μM Fura-2AM and seeded on poly-L-lysine-coated coverslips. Cytoplasmic Ca^{2+} and PAF-evoked calcium were measured by inverted microscope and were analyzed by MetaFluor Analyst software. Cells at different experimental times (before PAF, three and six seconds after 2 μM PAF stimulation) in three different experiments (one experiment/line) were shown. Non stimulated cells were green while PAF- stimulated cells became red indicating the increase of cytoplasmic Ca^{2+} levels.

As shown by the representative images, the cytoplasmic Ca^{2+} increase induced by PAF addition was indicated by green color turning to the red. Few cells did not respond. By these experiments, we have ascertained that the majority of HTL were responsive to PAF, although at different levels and times. This may occur because the microscope chamber was not a flow cell and the PAF distribution was not uniform.

5.5 MEASUREMENTS OF PAF-EVOCKED Ca^{2+} CONCENTRATION IN DIFFERENT BLOOD-DERIVED CELLS

In order to measure the changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]i$) by PAF, we set up a protocol. by initially testing B-LCLs from control subjects. Fura 2-AM-loaded cells $[Ca^{2+}]i$ were analyzed with the Perkin Elmer spectrofluorimeter. After the stabilization of Ca^{2+} basal values (Figure 20), the addition of 3 μM PAF to (2μM) fura 2-AM loaded cells produced a rapid and sustained increase in $[Ca^{2+}]_I$: $\Delta[Ca^{2+}]_I$ ~120 nM (basal 88.5 and PAF evoked Ca^{2+}= 207).

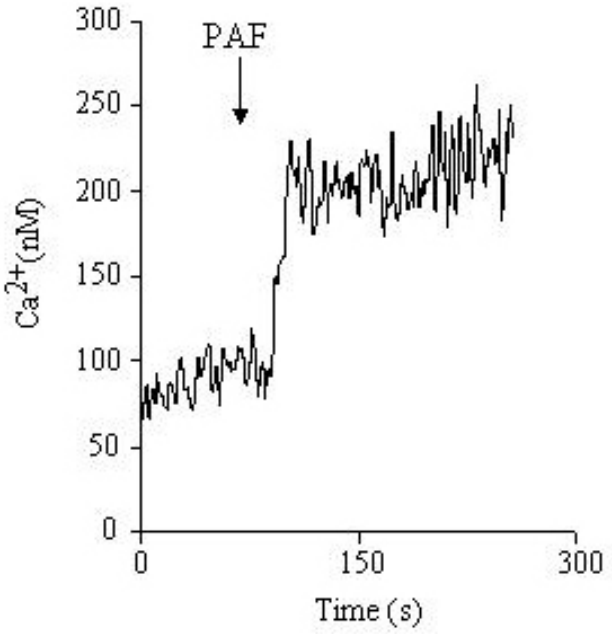


Figure 20: Example of PAF-evoked Ca^{2+} response in B-LCLs. Cells (3x 10^6/2 ml) were loaded with 3μM Fura-2AM, and $[Ca^{2+}]_I$ was assessed by spettrofluorimeter (Perkin Elmer). 2 μM PAF (arrow) evoked an increase in $[Ca^{2+}]_I$ of ~123 nM.

The concentrations of 1, 2 and 4 μM fura 2-AM did not produce in B-LCLs significant differences in the average agonist-induced $\Delta[Ca^{2+}]_I$. Similarly, differences were not found between 2 and 3 μM PAF that produced a similar ~ 100 nM $[Ca^{2+}]_I$ increase.

We next performed similar tests on HTL (Figure 21) to determine appropriate concentrations of fura-2AM and PAF for $[Ca^{2+}]$i measurements. Comparable results were obtained. For instance, HTL, loaded with 2μM Fura-2AM and stimulated by 2 μM PAF, increased cytosolic Ca^{2+} concentration with a $\Delta[Ca^{2+}]_I$ ~100 nM. Same results were obtained using 1μM Fura-2AM and 2 μM PAF ($\Delta[Ca^{2+}]_I$ ~135 nM).

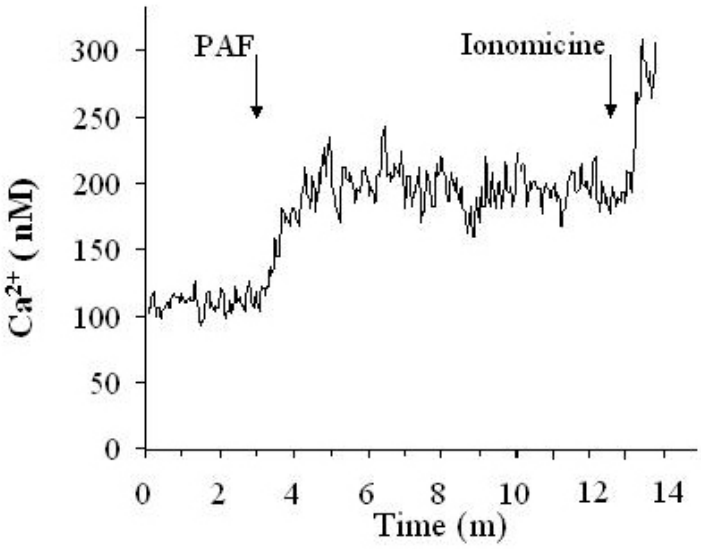
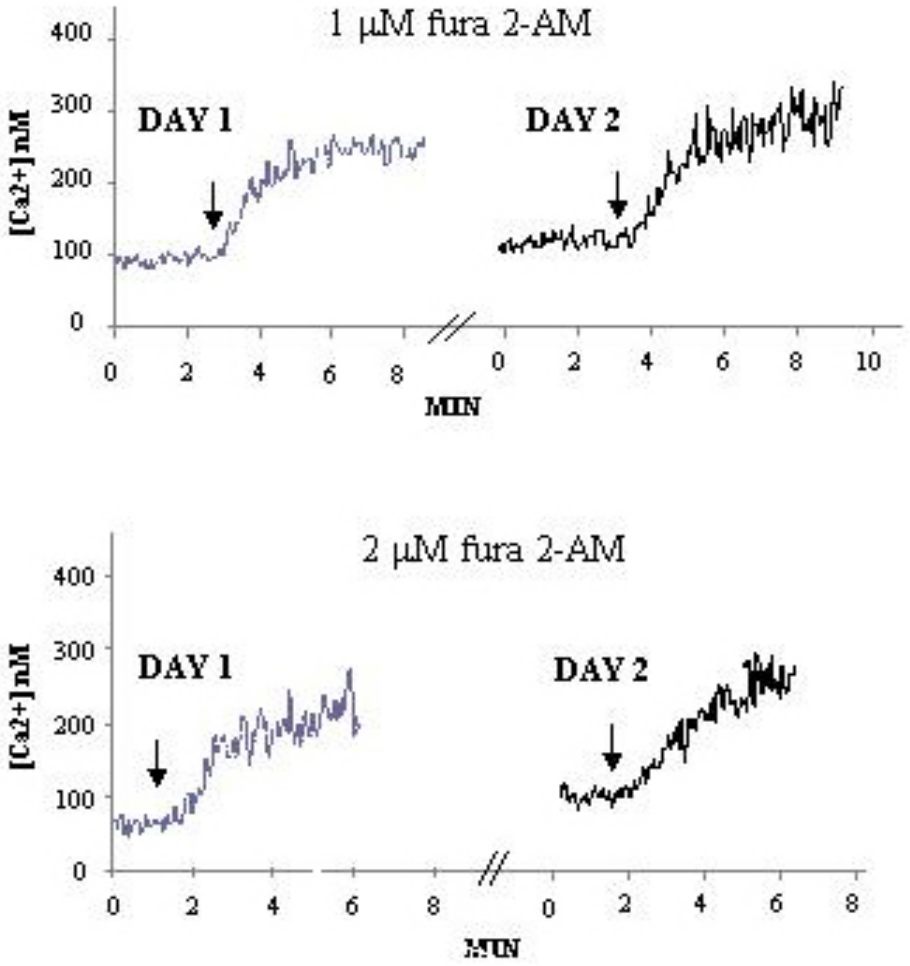


Figure 21: Example of PAF-evoked Ca^{2+} response in HTL. Cells (2 x 10^6 in 2 ml) were loaded with 2μM Fura-2AM, and $[Ca^{2+}]_I$ was assessed by spettrofluorimeter (Perkin Elmer). 2 μM PAF (arrow) evoked an increase in $[Ca^{2+}]_I$ of ~100 nM. 2 μM ionomicine was then added to measure fura 2-AM cell loading.

Overall, these results indicate that HTL are able to respond to PAF as B-LCLs do (Aguiari G. et al, 2004).

Then, in order to ascertain the possible use of peripheral blood lymphocytes (PBL) for Ca^{2+} measurements, we performed same experiments with PBL. These were tested immediately and one day after their isolation from peripheral blood, after loading with either 1 μM or 2 μM fura-2AM and stimulation with 2 μM PAF.



PAF-evocked $[Ca^{2+}]_I$ in PBL. Cells (3x 10^6 cells in 2 ml) were loaded either with 1 μM or 2 μM fura-2AM, and $[Ca^{2+}]_I$ was assessed by spettrofluorimeter (Perkin Elmer). DAY 1 shows values obtained with cells tested immediately after their preparation, DAY 2 those with cells tested the day after. In the first day, the PAF (arrows)-evocked increase in $[Ca^{2+}]_I$ was ~123 nM and ~130 nM, with 1μM and 2μM Fura-2AM, respectively. In the second day, the increase in $[Ca^{2+}]_I$ was ~ 136 nM and ~ 134 nM, with 1 μM and 2 μM Fura-2AM, respectively.

Also in this case, there was not substantial difference between the two loadings and two different times of analysis. Therefore, PBL could be tested as HTL, and both Ca^{2+} indicator and agonist were used at the minimal doses of 1 μM fura-2AM and 2 μM PAF.

To test a possible automatic system for Ca^{2+} measurement, some experiments in HTL were also performed by using a fluorometer multilabel plate counter.

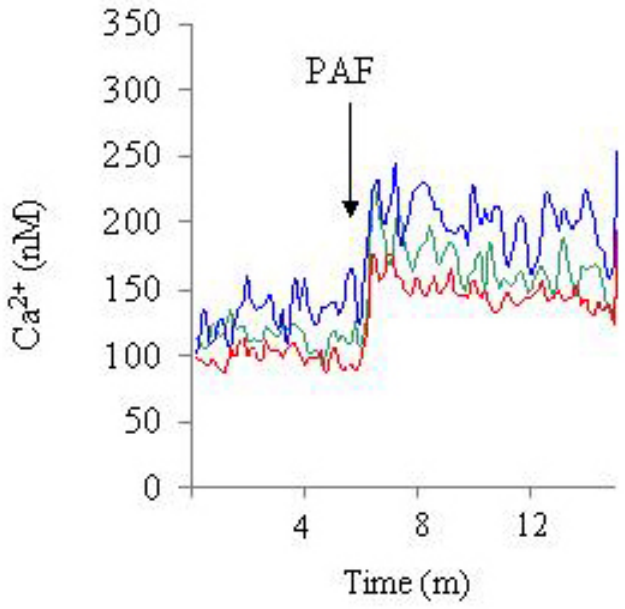


Figure 22: Ca^{2+} measurement by automatic multiplate fluorimeter. $[Ca^{2+}]_I$ was assessed by Victor (Perkin Elmer) in 1μM fura-2AM-loaded cells (in blue, 2 x 10^5; in green, 3 x 10^5; in red, 5 x 10^5 cells in 200 μl) and stimulated with 2 μM PAF.

The experiment shown in the previous figure with three different amounts of fura 2-AM-loaded HTL cells (2, 3 and 5 x 10^5) showed comparable results with the three different aliquots, but with a lower basal value at the highest cell number. Although measurements appear feasable, results obtained in repeated experiments were not always comparable with those obtained with the non automatic fluorimeter. The Victor apparatus did not result particularly suitable for Ca^{2+} measurements perhaps because of the lack of stirring, of a filter specific for the fura 2-AM and of termostatation.

5.6 PAF-EVOKED Ca^{2+} LEVELS ARE REDUCED IN HTL FROM ADPKD SUBJECTS

As previously mentioned, in B-LCLs obtained from subjects with PKD2 or PKD1 mutations, a reduction in PAF-evoked Ca^{2+} levels were found (Aguiari G. et al., 2004). In the current study PAF-evoked Ca^{2+} levels in HTL from ADPKD and

non ADPKD cases were measured by using the non automatic spectrofluorometer after addition of 2 μM PAF in HTL loaded with 1 μM fura 2-AM.

As shown in the next figure, the PAF-induced increase in $[Ca^{2+}]_I$ in ADPKD HTL was smaller than in control HTL. Results obtained in 31 non-ADPKD and 29 ADPKD subjects are shown in the histogram (Figure 23).

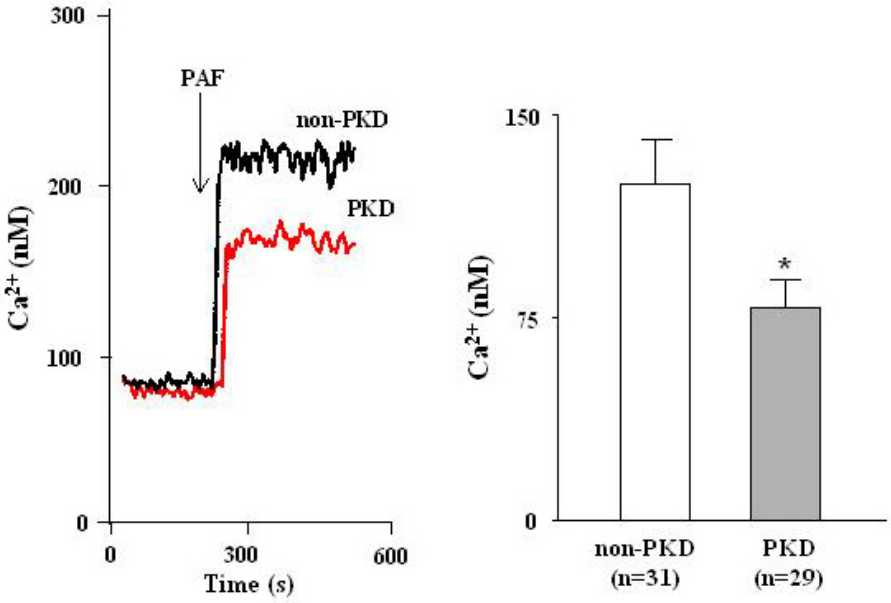


Figure23: Reduction of PAF-induced Ca^{2+} response in ADPKD-HTL. Curves of PAF-evoked Ca^{2+} response in HTL obtained from a non-ADPKD (non-PKD) and ADPKD (PKD) subjects. Cells were loaded with 1 μM fura 2-AM, stimulated with 2 μM PAF at the time indicated by the arrow, and analyzed as described in Methods. PAF-evoked Ca2+ levels were estimated as differences between peak and basal $[Ca^{2+}]_I$. Bars show results (average ± SE) from analysis of 31 non-APKD, 29 ADPKD obtained in duplicate (*, p<0.05 PKD vs non-PKD).

A statistically significant difference was observed in the average of PAF-evoked $[Ca^{2+}]_i$ elevation between ADPKD and non-ADPKD HTL (78.96 ± 9.67 SE in ADPKD, n=29, vs 124.51 ± 16.87 SE in non-ADPKD, n=31; p = 0.025).

The difference between the means was 45.55, and the medians were 58 and 92.92 in ADPKD and non-ADPKD HTL, respectively. No difference was found in Ca^{2+} basal values (82.8 ± 4 SE in ADPKD vs 86.2 ± 3.53 SE non-ADPKD).

Due to the high incidence of PKD1 forms (85%) in ADPKD, these results allowed to conclude that PKD1 mutations show aploinsufficiency in HTL as a reduction in

PAF-evoked Ca^{2+} levels.

5.7 PAF-EVOKED Ca^{2+} LEVELS ARE ALSO REDUCED IN PBL FROM ADPKD SUBJECTS

Ca^{2+} measurements were performed in PBL obtained from 28 non-ADPKD and 34 ADPKD subjects and levels evoked by 2 μM PAF treatment are shown in the next Figure 24.

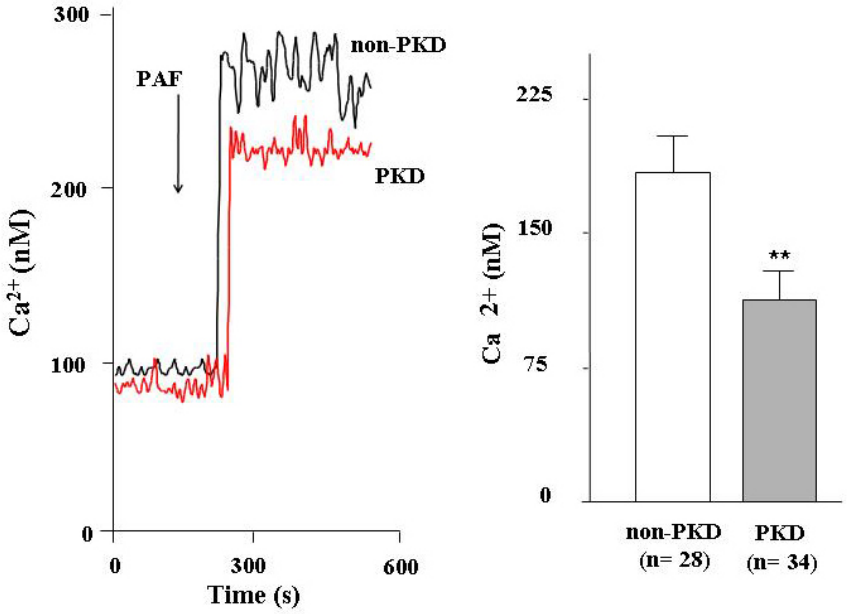


Figure24: PAF-induced Ca^{2+} release was reduced in ADPKD PBL. Example of curves of PAF-evoked Ca^{2+}. Bars (means ± SE) show the results of analysis of 28 non-ADPKD and 34 ADPKD obtained in duplicate (**, $p<0.01$).

There was a statistically significant difference in the average elevation in $[Ca^{2+}]i$ between ADPKD and non-ADPKD PBL (112.03 ± 13.87 SE in ADPKD, n=34, vs 183.32 ± 20.54 SE in non-ADPKD, n=28; $p = 0.0044$).

The difference between the means was 71.29, and medians were 84.05 and 174 in ADPKD e non-ADPKD PBL, respectively. Moreover, there was no difference in the Ca^{2+} basal values (87.74 ± 3,76 SE in ADPKD vs 92.40 ± 3.52 SE in non-ADPKD).

Therefore, variations in calcium homeostasis associated to ADPKD in HTL were also detectable in PBL. However, as shown in the next figure, data from ADPKD and non-ADPKD subjects are largely superimposable, thus making these data not useful for diagnostic criteria.

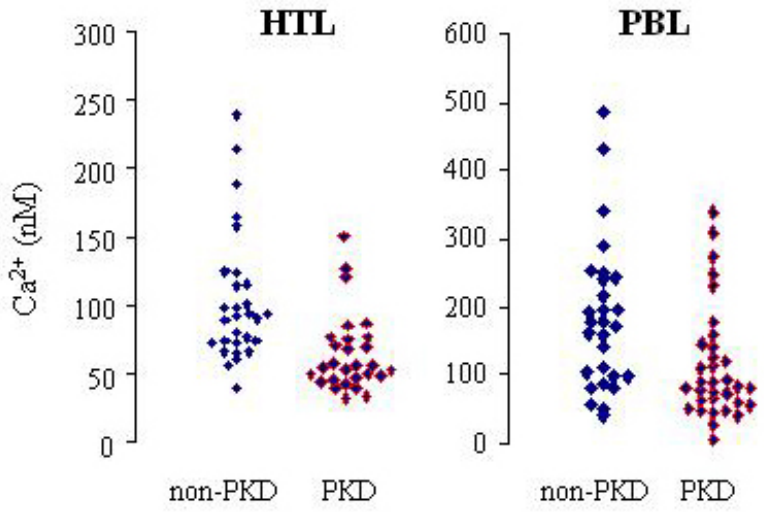


Figure 25: Distribution of PAF-evoked Ca^{2+} levels in HTL and PBL. $[Ca^{2+}]_I$ was measured in cells loaded with 1 μM fura 2-AM and stimulated with 2 μM PAF. quantified by densitometry of PC2, actin. Values were the results of at least a duplicate test in 29 ADPKD and 31 non ADPKD HTL, and in 34 ADPKD and 28 non ADPKD HTL.

Nevertheless, we do not have yet analyzed cases with known PKD2 mutations, which may have the lowest values, as expected from the previous B-LCL study (Aguiari G. et al, 2004). In particular, in B-LCLs the differences between average values in ADPKD and control cases were higher: 271 ± 17.9 in controls, 149.8 ± 16.9 in ADPKD1 and 104.8 ± 8.2 in ADPKD2 LCLs. In the case in which the PAF-evoked $[Ca^{2+}]$i in ADPKD HTL were lower than control values, and the PC2 levels were lower than control values, we should expect the presence of a PKD2 mutation, and we could directly analyze the PKD2 and not the PKD1 sequence.

5.8 DHPLC ANALYSIS OF THE PKD2 GENE

In order to analyze the PKD2 sequence of cases with characteristics before mentioned (low PC2 levels and low PAF-evoked $[Ca^{2+}]_i$), a previous analysis of PKD2 amplicons by DHPLC was perfomed. All 15 exons were amplified as fragments of 289 to 449 bp. Due to the high content in GC, the amplicon of exon 1 resulted not analysable on the HPLC column. The quality of amplicons was examined by agarose gel electrophoresis before DHPLC analysis (Figure 26). In particular, 2 controls and 5 ADPKD subjects, two of which were genetically-characterized as carriers of PKD2 mutations (PKD2-FE.5 and PKD2-FE.15) (Aguiari G. et al, 1999), were analyzed.

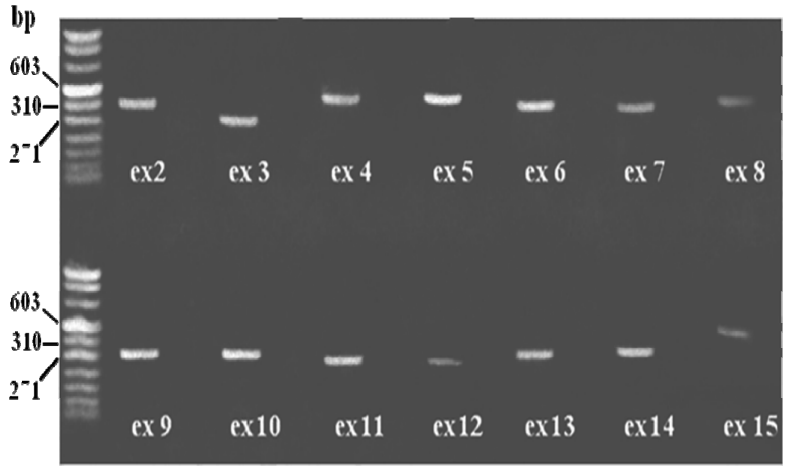


Figure 26: PKD2 PCR products resolved on a Trisacetate-EDTA 2 % agarose gel.

DHPLC chromatograms and sequence electroferograms with nucleotide variations detected in PKD2 amplicons are shown in next figures. An aberrant DHPLC profile that was found in amplicon 3 of a non genotypically characterized subject, was caused by a heterozygote nucleotide variation (843+56T→C) inside the intron 3 at the nucleotide 28743 (Figure 27).

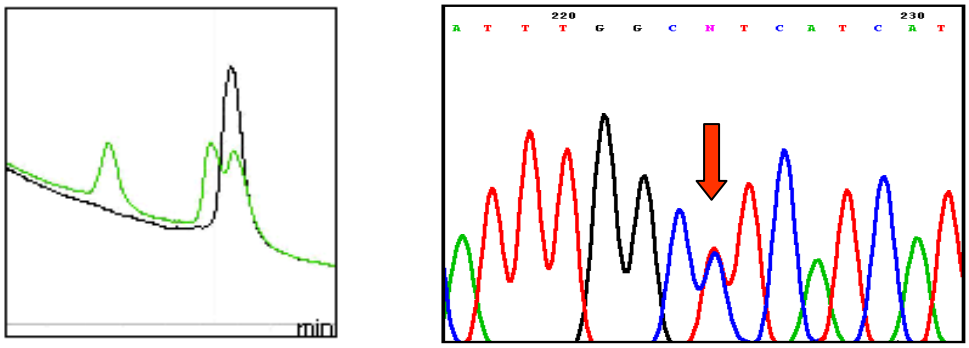


Figure 27: Aberrant DHPLC profile (black profile = reference wt sample) obtained from analysis of PKD2 exon 3 and its flanking sequences with the corresponding sequence electroferogram.

A further heterozygote nucleotide variation was found in amplicon 4 of a PKD2-FE.15 subject at the nucleotide 30562, corrispondig to a G/A substitution in intron 3, 22 bases before the 3' splice site (844-22G→A) (Figure 28), which was already detected in the same subject (Aguiari G. et al, 1999).

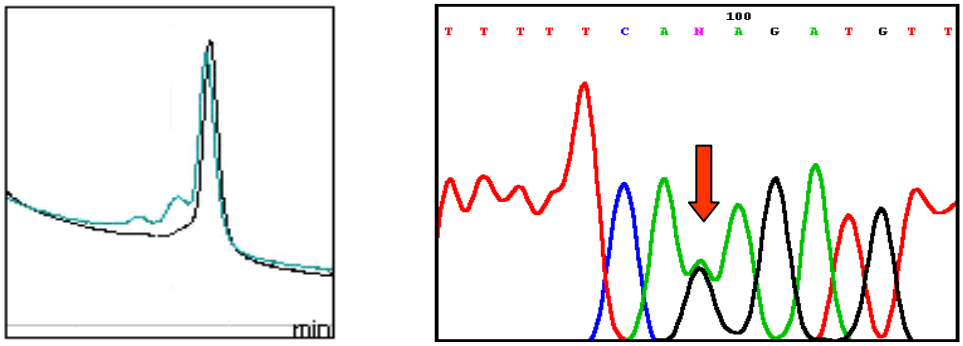


Figure 28: Aberrant DHPLC profile (black profile = reference wt sample) obtained from analysis of PKD2 amplicon 4 and its sequence electroferogram.

As previously found (Aguiari G. et al, 1999), an heterozygote base deletion (858delC) at level of the nucleotide 30597 in codon 286 was found in a PKD2 affected subject of the PKD2-FE.5. The mutations caused a frameshift, thus introducing a 29 novel amino acids before reaching the new stop codon (Figure 29).

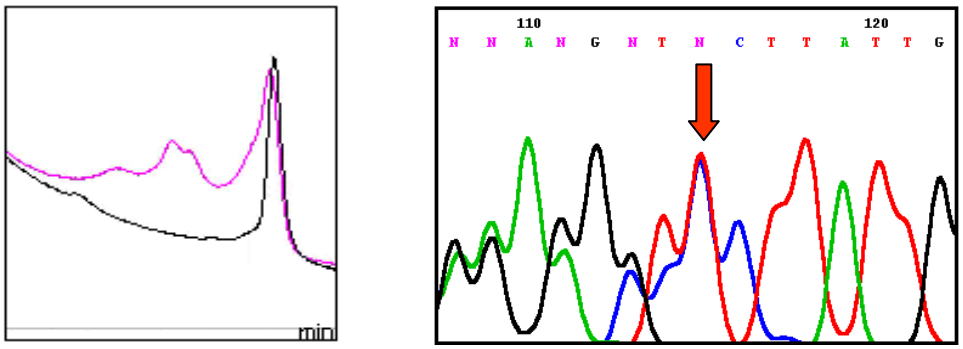


Figure 29: Aberrant DHPLC profile (black profile = reference wt sample) obtained from analysis of PKD2 amplicon 4 and its sequence electroferogram.

Finally, in exon 5 an abnormal DHPLC profile was found in the PKD2 affected subject of the PKD2-FE.15. The heterozygote mutation was a nucleotide transversion T→A at level of the nucleotide 35629 (1158T→A) producing a stop mutation in codon 386 (Figure 30).

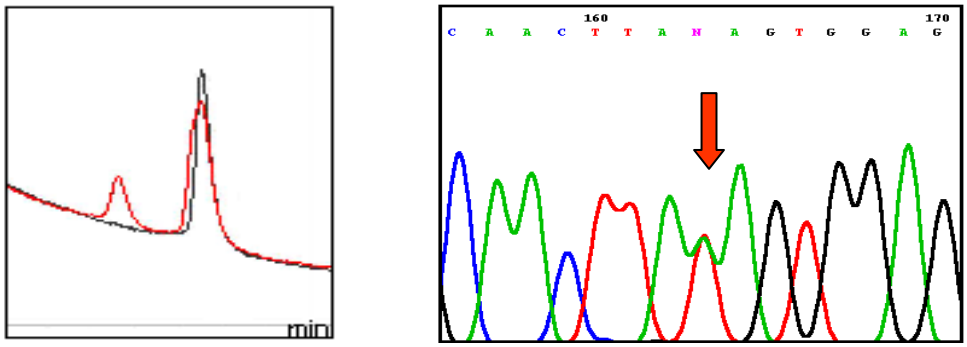


Figure 30: Aberrant denaturing high-pressure liquid chromatography (DHPLC) (black profile = reference wt sample) profile obtained from analysis of PKD2 exon five and its sequence electroferogram.

All these results showed that amplification and denaturating protocols, with the exception of exon 1, were suitable for the mutation analysis of PKD2 gene.

5.9 CELL PROLIFERATION

It is well accepted that polycystins play a role in the control of cell proliferation and apoptosis (Boletta A. et al, 2000). It is possible that a quantitative defect of either PC1 or PC2 in HTL may result in an alteration of the proliferation rate. Therefore, cell proliferation was investigated in HTL obtained from three ADPKD and three non-ADPKD subjects. Cell number was assessed after 4, 24 and 48 hours of culture. Results of trypan blue-negative cells are shown in the next figure (Figure 31).

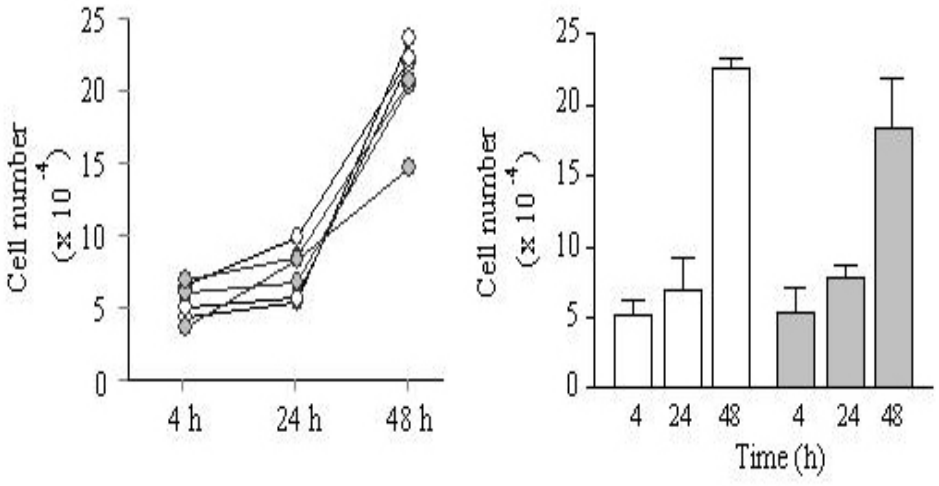


Figure 31: Cell proliferation of ADPKD (white) and non-ADPKD (grey) HTL. Cells (4 x10^4/ml) were seeded in RPMI 10% PBS, and trypan blue negative cells were counted after 4, 24 and 48 h of culture. Growth rates of ADPKD and non-ADPKD HTL (left). Bars (right) show means ± SD of two experiments in duplicates with 3 ADPKD and 3 non-ADPKD HTL.

We found that cell proliferation of HTL of ADPKD patients was not significantly reduced in comparison to that of non-ADPKD patients (PDK/non-PKD = 1.12 ± 0.008 and 0.94 ± 0.17, after 24 and 48 h, respectively). However, the percentage of trypan blue-negative ADPKD HTL after 48 h of culture, was significantly lower than that of control HTL (92.4 ± 2.8 SD in controls vs 75.9 ± 9.7 in ADPKD, p = 0.047). These findings did indicate that aploinsufficiency of PKD1,

the more probable involved gene in these patients, also appeared as a small alteration in the proliferation of 10% FCS-treated HTL.

Since PCs have been known to play a role in the control of cell proliferation, but also of cell apoptosis (Boletta A. et al, 2000, Qian Q. et al, 2003), a preliminary analysis of cell death was performed in HTL. The number of dead cells was assessed by counting trypan blue-positive cells (Figure 32).

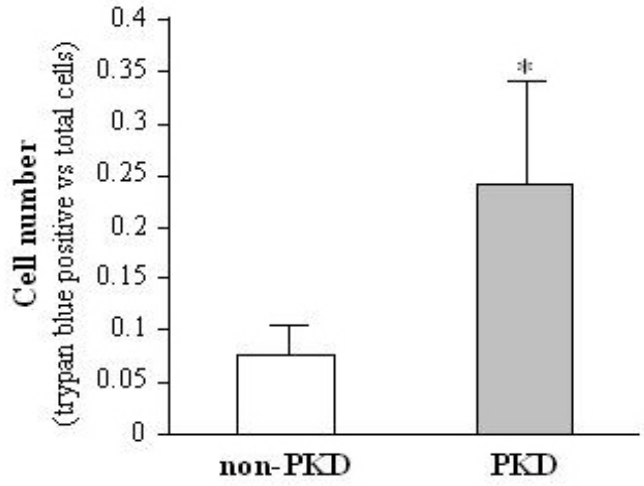


Figure 32: Number of trypan blue positive cells of ADPKD (PKD) and non-ADPKD patients. Bars show cell death in non-ADPKD and ADPKD HTL after 48 hours of culture in RPMI 10% FBS (*, $p<0.05$).

Surprisingly, the number of trypan blue-positive cells in ADPKD-derived HTL was significantly and clearly increased in comparison with control HTL (24.1 ± 9.7 SD in ADPKD vs 7.6 ± 2.8 in controls, $p<0.05$). The higher death value in ADPKD HTL was in line with the anti-apoptotic role of PC1 reported in kidney cells (Boletta et al, 2000) and in PKD2-mutated vascular muscle cells (Qian Q. et al, 2003).

5.10 CELL MORPHOLOGY

Since PC1 is reported to play a role in the interaction between cell-cell and cell-matrix, its quantitative defect may result in alteration of cell adhesion, possibly displayed by alterations in the aggregates of growing T-cells. HTL from ADPKD and control subjects were, therefore, studied for the size of their aggregates. After 24 h culture the morphology of clumps of four ADPKD and four non-ADPKD HTL were analysed by an inverted phase-contrast microscope (Figure 33)

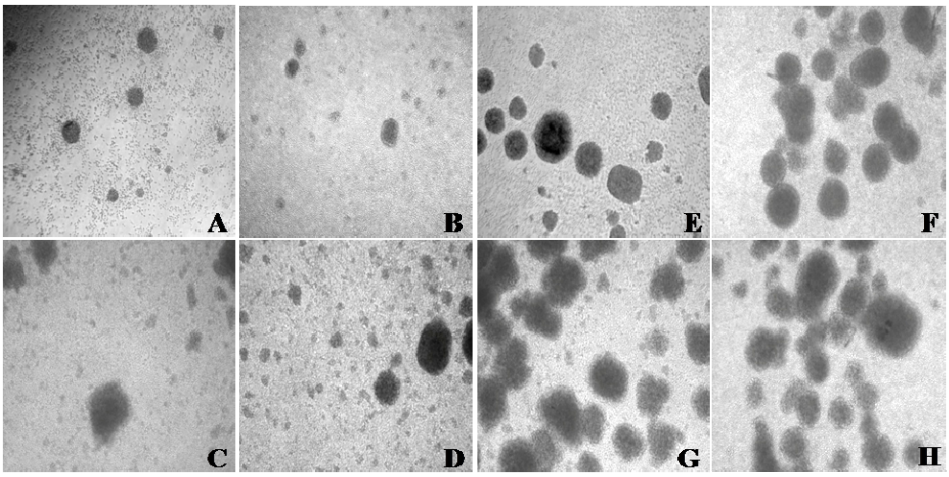


Figure 33: Phase-contrast images of clumps of four non-ADPKD (A, B, C, D) and four ADPKD (E, F, G, H) HTL (10X).

Clumps larger than about 3 mm in diameter have been counted in each image. Surprisingly, the average number of clumps in ADPKD HTL (14.75 ± 3.4) was greater in comparison to the clumps of non-ADPKD HTL (3 ± 1.4). This was a preliminary observation which suggested a difference in the aggregation capacity of normal and ADPKD-derived HTL.

5.11 NEUTROPHIL MOTILITY OF ADPKD SUBJECTS

Since some characteristics observed in ADPKD HTL have been shared with ADPKD B-LCLs, it is possible that other blood cell types could show abnormal features in ADPKD. PC1 and PC2 are known to be involved in the regulation of the actin cytoskeleton in renal epithelial cells: PC1 induces cell scattering and cell migration (Boca M. et al, 2007), and PC2 binds some cytoskeletal components (Li Q. et al, 2003). Because of the association between PCs, membranes and cytoskeleton, neutrophils from ADPKD subjects were investigated for their motility. Since fMLP is able to influence a directed migration in neutrophils, this molecule was used to investigate wether the motility of neutrophils obtained from ADPKD subjects was abnormal. Neutrophils from four ADPKD and four non-ADPKD were tested (Figure 34).

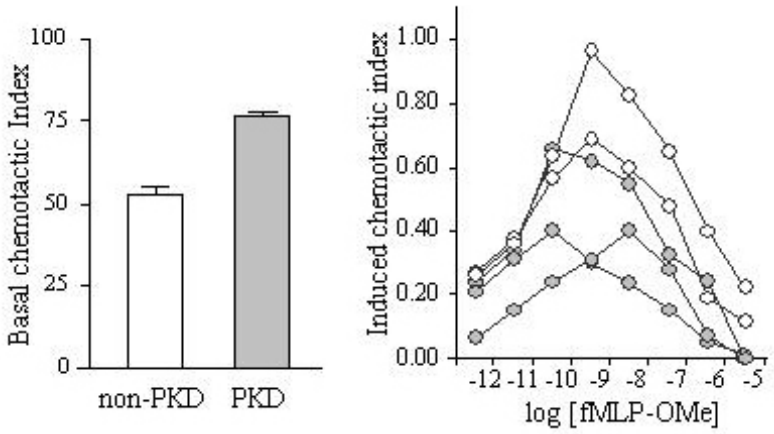


Figure 34: Chemotactic activity in ADPKD- (PKD) and non-ADPKD (non-PKD) -derived neutrophils. The activity was measured in presence of bovine serum albumin (1mg/ml) alone (left) or in association with the chemotactic factor fMLP (right) at concentrations ranging from 10^{-12} to 10^{-5} M. Neutrophils ($2x10^{-5}$/well) were treated as described in Methods section. Bars represent the mean ± SD of duplicate experiments (n= 4 ADPKD, n=4 non-ADPKD). Curves show the response to fMLP in three ADPKD (grey simbols) and two control samples (white simbols) .

Results showed that albumin-induced basal chemotactic activity (bars on the left) is higher in ADPKD-derived neutrophils than in controls. By contrast, the fMLP-induced chemotactic activity was in average lower in ADPKD-derived neutrophils (curves on the right). At each fMLP concentration, in fact, the

chemotactic index was lower in ADPKD cell samples, with the exception of one sample showing at 10^{-10} M fMLP the same chemotatic index as controls. Overall, these results indicated that also ADPKD-derived neutrophils show functional features different from non ADPKD cells.

6.CONCLUSIONS

Data presented in this thesis show that PKD1 and PKD2 expression is detectable at protein level in HTL. Furthermore, functional defects of the PC1/PC2 channel complex in HTL obtained from ADPKD patients are revealed as a decrease in PAF-evoked cytoplasmic Ca^{2+} levels. A further cellular alteration in ADPKD HTL is represented by the increase in the number of dead cells. These alterations represent an interesting characteristic of ADPKD cells because, as discussed herein, they may lead the way to a novel diagnostic approach for ADPKD.

As far as PC1 is concerning, its expression as a protein of > 400 kDa in size has been detected, but not correctly quantified in Western blotting conditions used in this study. Therefore, no comparison between PC1 levels in ADPKD HTL and controls has been performed. Dot blot or cytofluorimetric approaches, and possibly different specific antibodies may lead to efficient quantification of PC1 in these cells. PC2 expression is easily detected in HTL and its quantification does not differ between the 6 control and 10 ADPKD investigated cases. This is not surprising because of the high incidence of PKD1 mutations (85%) in ADPKD. However, one case shows constant values lower than the double value of SD of ADPKD1-genetically characterized HTL. This may represent an ADPKD2 case. Unfortunately, ADPKD2-genetically characterized patients have not yet examined for PC2 levels. Therefore, a quantitative defect of PC2, which is apparent in ADPKD B-LCL (Aguiari G. et al, 2004), is not yet demonstrated in ADPKD2 HTL.

Beside PC1 and PC2, also the investigation of FC1, mutated in the recessive form of PKD, and of FCL, its homologue highly expressed in activated T-cells (Hogan MC et al, 2003), does not reveal quantitative alteration in the level of combined proteins in ADPKD HTL. Certainly, the high level of FCL expression can make very difficult to highlight FC1 defects. In addition, if an interaction between FC1/FCL would exist only with PC2, the lack of ADPKD2 characterized cases does not allow to find such a possible difference. Considering the high expression of FCL in activated T-lymphocytes, a study using HTL at low activation levels may produce

lower FCL levels and, possibly, allow to evidence differences between ADPKD and control HTL.

Differently from PCs levels, analysis of Ca^{2+} homeostasis reveals a reduction in average levels of PAF-evoked Ca^{2+} in 29 ADPKD HTL. Similar reduction is detected in 34 ADPKD PBL. Therefore, functional impairment, although markedly lower than the 50% expected in the heterozygous condition, is apparent in ADPKD T-cells. Since ADPKD1 should exceed in investigated ADPKD cases, the Ca^{2+} reduction reveals the functional aploinsufficiency of PC1. However, the high scattering of the data does not allow the use of this parameter for the diagnosis. Again, the lack of ADPKD2-genetically characterized cases does not allow to define average Ca^{2+} levels in presence of PC2 defects, which, in B-LCLs, were associated to the lowest Ca^{2+} values (105 ± 8.2 SE in ADPKD2 vs 271 ± 17.9 SE in controls, Aguiari G. et al, 2004).

Differently from what observed in ADPKD B-LCLs where the defect in Ca^{2+} homeostasis was associated with a clear reduction in cell proliferation (Aguiari G. et al, 2004), a small difference exists in HTL proliferation between control and ADPKD cases. On the contrary cell death is clearly increased in ADPKD HTL. This is an interesting observation because of the anti-apoptotic action of PC1 (Boletta A. et al, 2000). More investigation should be therefore done with apoptosis markers to better define this abnormalities.

An additional difference between ADPKD and controls is the average size of cell aggregates which are larger in ADPKD HTL. Since PC-1 regulates cytoarchitecture and migration of renal epithelial cells (Boca M. et al, 2007), it may be that in clumps of ADPKD HTL a functional/quantitative reduction in PCs may impair leakage/scattering of cell from clumps. This alteration, presumably located at membrane level, is consistent with the alteration observed in ADPKD neutrophils which are basically more activated, but less responsive to stimuli, than control cells.

All together, the results suggest that immune cells might be a cellular model for studies with ADPKD patients considering PC1 and PC2 functions. Efforts need to be done to investigate the motive of the decreased functions of these cells expressing PC1/PC2 in patients with ADPKD. Furthermore, the increase in the number of control and ADPKD, including the ADPKD2, patients may lead to the definition of

parameters that could be used in the diagnosis. In particular, the cases with PC2 and Ca^{2+} levels expected in ADPKD2 cases, will be directly considered for DHPLC analysis and PKD2 sequence by using the protocols already set up.

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