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DEPARTMENT OF PHYSIOLOGY – MEDICAL SCHOOL

Molecular pathways of polycystin-1 (PC1) implicated in the initiation and progression of tumors

Konstantinos A. Papavassiliou

PhD Thesis

Η παρούσα διδακτορική διατριβή εκπονήθηκε υπό τον ελληνικό τίτλο «Μοριακά μονοπάτια της πολυκυστίνης PC1 που ενέχονται στην ανάπτυξη και εξέλιξη των όγκων», στο Εργαστήριο Φυσιολογίας της Ιατρικής Σχολής του Εθνικού και Καποδιστριακού Πανεπιστημίου Αθηνών

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Μέλη Τριμελούς Συμβουλευτικής Επιτροπής

Μιχάλης Κουτσιλιέρης, Καθηγητής Ιατρικής Σχολής, Ε.Κ.Π.Α. (επιβλέπων)

Βασίλειος Γοργούλης, Καθηγητής Ιατρικής Σχολής, Ε.Κ.Π.Α.

Έλενα Γκόγκα, Καθηγήτρια Ιατρικής Σχολής, Ε.Κ.Π.Α.

Μέλη Επταμελούς Εξεταστικής Επιτροπής

Μιχάλης Κουτσιλιέρης, Καθηγητής Ιατρικής Σχολής, Ε.Κ.Π.Α. (επιβλέπων)

Βασίλειος Γοργούλης, Καθηγητής Ιατρικής Σχολής, Ε.Κ.Π.Α.

Έλενα Γκόγκα, Καθηγήτρια Ιατρικής Σχολής, Ε.Κ.Π.Α.

Πηνελόπη Κορκολοπούλου, Καθηγήτρια Ιατρικής Σχολής, Ε.Κ.Π.Α.

Μυρσίνη Κουλούκουσα-Γιαννιού, Αναπληρώτρια Καθηγήτρια Ιατρικής Σχολής, Ε.Κ.Π.Α.

Θεόδωρος Τρουπής, Αναπληρωτής Καθηγητής Ιατρικής Σχολής, Ε.Κ.Π.Α.

Αναστάσιος Φιλίππου, Αναπληρωτής Καθηγητής Ιατρικής Σχολής, Ε.Κ.Π.Α.

Πρόεδρος Ιατρικής Σχολής: Καθηγητής Πέτρος Π. Σφηκάκης

Curriculum vitae

KONSTANTINOS (KOSTAS) A. PAPAVASSILIOU

Date of birth:	November 29, 1988
Place of birth:	Manhattan, New York, NY USA
Citizenship:	Dual, Greek & American
Home address:	10 Makedonon Street, 11521 Athens, Greece Phone: 210-6461967 & 6975103701 (mobile) E-mail: kpapavassiliou@gmail.com

Education

1994 - 1996	Primary Education, “International School Heidelberg, A School for Peace” (Internationale Gesamtschule Heidelberg), Heidelberg, Germany
1996 - 2003	Primary and Secondary Education, “Arsakeio” High School, Patras, Greece
2003 - 2005	Secondary Education, Anatolia High School, Anatolia College, Thessaloniki, Greece
2003	Selected for Salem International College, Summer School, Überlingen, Germany [attended German Literature courses]
2005 - 2007	International Baccalaureate Program (IB), Athens Psychiko College, Athens, Greece
2008 - 2010	Undergraduate Studies, Boston University, Boston, MA USA
2010 - 2014	Bachelor of Liberal Arts (ALB) Program, Harvard University, Boston, MA USA
July 2014 - present	PhD candidate, Department of Physiology, Medical School, National and Kapodistrian University of Athens, Athens, Greece *Thesis Title*: «Molecular pathways of polycystin-1 (PC1) implicated in the initiation and progression of tumors» *Thesis Advisor/Supervisor*: Prof. M. Koutsilieris, MD, PhD

March 2015 - present	MD student, Medical School, National and Kapodistrian University of Athens, Athens, Greece Semester currently enrolled: 12th / Total semesters required for degree completion: 12

Academic Degrees

May 2014	Bachelor of Liberal Arts (ALB), Harvard University, Boston, MA USA

Scientific Publications

A. ***Peer-review journals*** **[Total citations: 454 / h index: 10]**

1. Coulocheri SA, Pigis DG, **Papavassiliou KA**, Papavassiliou AG. Hydrogen bonds in protein-DNA complexes: where geometry meets plasticity. ***Biochimie*** 2007;89(11):1291-303 [**citations: 76**]
2. Papachroni KK, Karatzas DN, **Papavassiliou KA**, Basdra EK, Papavassiliou AG. Mechanotransduction in osteoblast regulation and bone disease. ***Trends Mol Med*** 2009;15(5):208-16 [**citations: 207**]
3. Spyropoulou A, Gargalionis A, Dalagiorgou G, Adamopoulos C, **Papavassiliou KA**, Lea RW, Piperi C, Papavassiliou AG. Role of histone lysine methyltransferases SUV39H1 and SETDB1 in gliomagenesis: modulation of cell proliferation, migration, and colony formation. ***Neuromolecular Med*** 2014;16(1):70-82 [**citations: 55**]
4. **Papavassiliou KA**, Papavassiliou AG. Histone deacetylases inhibitors: conjugation to other anti-tumour pharmacophores provides novel tools for cancer treatment. ***Expert Opin Investig Drugs*** 2014;23(3):291-4 [**citations: 18**]
5. **Papavassiliou KA**, Papavassiliou AG. Bromodomains: pockets with therapeutic potential. ***Trends Mol Med*** 2014;20(9):477-8 [**citations: 16**]
6. Gargalionis AN, Korkolopoulou P, Farmaki E, Piperi C, Dalagiorgou G, Adamopoulos C, Levidou G, Saetta A, Fragkou P, Tsioli P, Kiaris H, Zizi-Serbetzoglou A, Karavokyros I, **Papavassiliou KA**, Tsavaris N, Patsouris E, Basdra EK, Papavassiliou AG. Polycystin-1 and polycystin-2 are involved in the acquisition of aggressive phenotypes in colorectal cancer. ***Int J Cancer*** 2015;136(7):1515-27 [**citations: 25**]

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8. Karamouzis MV,[‡] **Papavassiliou KA**,[‡] Adamopoulos C, Papavassiliou AG. Targeting Androgen/Estrogen Receptors Crosstalk in Cancer. ***Trends Cancer*** 2016;2(1):35-48 [**citations: 19**] [[‡]**equal contribution**]

   [**Featured on the cover** of the issue under the title "Orpheus and Eurydice The Interplay of Hormone Receptors". The cover depicts the painting "*Orpheus and Eurydice*" by the modern Greek painter and poet, Nikos Engonopoulos. Cover image legend in Contents/Editorial Board page: «On the Cover: "*Orpheus and Eurydice*", Nikos Engonopoulos, oil on canvas, 1958. Androgen (AR) and estrogen (ERβ) receptors are emerging key players in the development and progression of prostate and breast cancers. Molecular studies unveiled a complex crosstalk between these receptors. On pages 35–48 in this issue, Karamouzis *et al.* discuss the implications of receptor signaling crosstalk for the design of improved targeted therapies for hormone cancers. *Orpheus* is AR and prostate cancer and *Eurydice* is ERβ and breast cancer. **Concept by Kostas A. Papavassiliou**. Reproduced with permission from Erriete Engonopoulos.»]
9. Adamopoulos C, Mihailidou C, Grivaki C, **Papavassiliou KA**, Kiaris H, Piperi C, Papavassiliou AG. Systemic effects of AGEs in ER stress induction *in vivo*. ***Glycoconj J*** 2016;33(4):537-44 [**citations: 13**]
10. **Papavassiliou KA**, Papavassiliou AG. Transcription Factor Drug Targets. ***J Cell Biochem*** 2016;117(12):2693-96 [**citations: 11**]
11. Lepetsos P, **Papavassiliou KA**, Papavassiliou AG. Redox and NF-κB signaling in osteoarthritis. ***Free Radic Biol Med*** 2019;132:90-100 [**citations: 3**]
12. **Papavassiliou KA,** Zoi I, Gargalionis AN, Koutsilieris M. Polycystin-1 affects cancer cell behavior and interacts with mTOR and Jak signaling pathways in cancer cell lines. ***J Cell Mol Med*** 2019; 23(9):6215-6227

B. ***Translations***

PRINCIPLES OF CHEMISTRY – A MOLECULAR APPROACH. Author: Nivaldo J. Tro / Publisher: Prentice Hall-Pearson Education Inc., New Jersey USA 2010.

Papavassiliou KA: Translation of the textbook in Greek ("*Αρχές Χημείας – Μοριακή Προσέγγιση*", Copyright 2012, BROKEN HILL PUBLISHERS LTD, ISBN: 978-960-489-237-2)

[The book is currently the syllabus book used by first-year medical and dental students in the course 'Medicinal Chemistry' (1st semester) at the Medical School of the National and Kapodistrian University of Athens, Athens, Greece]

Meeting Presentations

- Gargalionis AN, Korkolopoulou P, Piperi C, Dalagiorgou G, Farmaki E, Adamopoulos C, Levidou G, Zizi-Serbetzoglou A, Karavokyros I, Fragkou E, Tsioli P, Saetta A, **Papavassiliou K**, Karamouzis M, Tsavaris N, Basdra EK, Papavassiliou AG. Investigation of the role of polycystin-1 and polycystin-2 in colorectal cancer. Abstract No e14592, Category: Gastrointestinal (Colorectal) Cancer, **2014 ASCO Annual Meeting** (May 30 - June 3, 2014) Chicago, IL, USA [poster presentation]
- **Adamopoulos C, Mihailidou C, Grivaki C,** Papavassiliou KA**, Kiaris H, Papavassiliou AG, Piperi C**. A NOVEL *IN VIVO* APPROACH TO STUDY ENDOPLASMIC RETICULUM STRESS INDUCTION BY ADVANCED GLYCATION END PRODUCTS. 12th International Symposium on the Maillard Reaction (12th ISMR)**, Tokyo, Japan (September 1-4, 2015) [poster presentation]**
- Papavassiliou KA. The role of polycystins 1 & 2 in cancer. **Molecular Medicine from Bench to Clinical Praxis: Challenges & Questions**, Athens, Greece (January 27-28, 2017) [oral presentation]
- Papavassiliou KA. Exploring the role of polycystins in tumor development. **Molecular Medicine from Bench to Clinical Praxis: Challenges & Questions**, Athens, Greece (January 26-27, 2018) [oral presentation]

Grants / Research Funding

2016: Research Program entitled "The polycystin 1 & 2 (PC1 & PC2) signaling pathway in carcinogenesis" from the Hellenic Society of Medical Oncology (HeSMO). Amount awarded: € 7.000

Awards

2018: Award of Excellence in 'Cardiology', First Department of Cardiology, Medical School, National and Kapodistrian University of Athens
2018: Award of Excellence in 'Differential Diagnosis', Third Department of Medicine, Medical School, National and Kapodistrian University of Athens

Experimental Skills

- Expertise in a variety of contemporary laboratory techniques (isolation and culture of primary cells/cell lines, DNA cloning/sub-cloning, transfection, PCR/RT-PCR, RNA isolation, immunohistochemistry, immunofluorescence, SDS-PAGE, western blotting, electrophoretic mobility-shift assay, proximity ligation assay) [courses in 'Principles and Techniques of Molecular Biology' (BIOS E-12) and 'Experimental Molecular and Cellular Biology' (BIOS E-176), both taken at Harvard University (Spring 2011 and January 2012, respectively)]
- Development of mechanical-stretch application devices in the context of a project with subject "Culture of bone cells in artificial matrices", Departments of Experimental Physiology & Biological Chemistry, Medical School, National and Kapodistrian University of Athens, Athens, Greece (01/2014 - 06/2014)

Languages

Greek (native); English (fluent); German (excellent read and write, B2 Mittelstufe Deutsch level)

ΌΡΚΟΣ του ΙΠΠΟΚΡΑΤΗ

"Ορκίζομαι στον Απόλλωνα τον Ιατρό και στον Ασκληπιό και στην Υγεία και στην Πανάκεια και σε όλους τους Θεούς επικαλούμενος την μαρτυρία τους, να τηρήσω πιστά κατά τη δύναμη και την κρίση μου αυτό τον όρκο και το συμβόλαιό μου αυτό. Να θεωρώ αυτόν που μου δίδαξε αυτή την τέχνη ίσο με τους γονείς μου και να μοιραστώ μαζί του τα υπάρχοντά μου και τα χρήματά μου αν έχει ανάγκη φροντίδας. Να θεωρώ τους απογόνους του ίσους με τα αδέλφια μου και να τους διδάξω την τέχνη αυτή αν θέλουν να τη μάθουν, χωρίς αμοιβή και συμβόλαιο και να μεταδώσω με παραγγελίες, οδηγίες και συμβουλές όλη την υπόλοιπη γνώση μου και στα παιδιά μου και στα παιδιά εκείνου που με δίδαξε και στους άλλους μαθητές που έχουν κάνει γραπτή συμφωνία μαζί μου και σε αυτούς που έχουν ορκισθεί στον ιατρικό νόμο και σε κανέναν άλλο και να θεραπεύω τους πάσχοντες κατά τη δύναμή μου και την κρίση μου χωρίς ποτέ, εκουσίως, να τους βλάψω ή να τους αδικήσω. Και να μη δώσω ποτέ σε κανένα, έστω κι αν μου το ζητήσει, θανατηφόρο φάρμακο, ούτε να δώσω ποτέ τέτοια συμβουλή. Ομοίως να μη δώσω ποτέ σε γυναίκα φάρμακο για να αποβάλει. Να διατηρήσω δε τη ζωή μου και την τέχνη μου καθαρή και αγνή. Και να μη χειρουργήσω πάσχοντες από λίθους αλλά να αφήσω την πράξη αυτή για τους ειδικούς. Και σε όποια σπίτια κι αν μπω, να μπω για την ωφέλεια των πασχόντων αποφεύγοντας κάθε εκούσια αδικία και βλάβη και κάθε γενετήσια πράξη και με γυναίκες και με άνδρες, ελεύθερους και δούλους. Και ό,τι δω ή ακούσω κατά την άσκηση του επαγγέλματός μου, ή κι εκτός, για τη ζωή των ανθρώπων, που δεν πρέπει ποτέ να κοινοποιηθεί, να σιωπήσω και να το τηρήσω μυστικό. Αν τον όρκο μου αυτό τηρήσω πιστά και δεν τον αθετήσω, είθε να απολαύσω για πάντα την εκτίμηση όλων των ανθρώπων για τη ζωή μου και για την τέχνη μου, αν όμως παραβώ και αθετήσω τον όρκο μου να υποστώ τα αντίθετα από αυτά".

ΒΑΛΕΝΤΙΝΟΣ: Αρχίζεις μαντεύοντας ποια θα μπορούσε να είναι η μελωδία. Προσπαθείς να την ξεχωρίσεις από το θόρυβο. Δοκιμάζεις τούτο, δοκιμάζεις τ' άλλο, κάτι αρχίζεις να πιάνεις – είναι μισερό, αλλά αρχίζεις να προσθέτεις νότες που λείπουν ή όχι ακριβώς τις σωστές νότες... και λίγο λίγο... ο χαμένος αλγόριθμος!

Tom Stoppard, *Αρκαδία*

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// Ευχαριστίες

Η παρούσα διδακτορική διατριβή είναι αποτέλεσμα συλλογικής προσπάθειας των τελευταίων ετών, για αυτό το λόγο θα ήθελα να ευχαριστήσω όλους αυτούς που συνέβαλαν καθοριστικά σε αυτή.

Αρχικά, ευχαριστώ πολύ τα μέλη της τριμελούς συμβουλευτικής επιτροπής της διατριβής: α) τον επιβλέποντα καθηγητή της διατριβής, Καθηγητή της Ιατρικής Σχολής του Ε.Κ.Π.Α. κ. Μιχάλη Κουτσιλιέρη, τόσο για την εμπιστοσύνη που έδειξε στο πρόσωπό μου από την πρώτη κιόλας στιγμή, όσο και για τη διαρκή καθοδήγηση που μου παρείχε, αποτελώντας την επιστημονική πυξίδα που πάντα συμβουλευόμουν, β) τον Καθηγητή της Ιατρικής Σχολής του Ε.Κ.Π.Α. κ. Βασίλειο Γοργούλη για την ενθάρρυνση και την επιστημονική του αρτιότητα που συνέβαλαν στην εκπόνηση της παρούσας διατριβής, και γ) την Καθηγήτρια της Ιατρικής Σχολής του Ε.Κ.Π.Α. κ. Έλενα Γκόγκα για τη διεύρυνση των ερευνητικών και γνωσιακών μου οριζόντων στην ογκολογία.

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Ένα πολύ μεγάλο και ειλικρινές ευχαριστώ θα ήθελα επίσης να εκφράσω προς τη βιολόγο Ιλιάννα Ζώη για την αμέριστη βοήθεια στην εκτέλεση των πειραμάτων καθώς και για την άψογη συνεργασία μας.

Η οικονομική στήριξη ήταν εξέχουσας σημασίας για τη διεξαγωγή της παρούσας διατριβής. Θα ήθελα, λοιπόν, να ευχαριστήσω την Εταιρεία Ογκολόγων Παθολόγων Ελλάδας (ΕΟΠΕ) για τη χορήγηση οικονομικής ενίσχυσης στα πλαίσια των Ερευνητικών Προγραμμάτων της ΕΟΠΕ για το έτος 2016.

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Εύχομαι η διατριβή αυτή να συνεισφέρει, έστω και ελάχιστα, στην κατανόηση των παθοβιολογικών μηχανισμών της καρκινογένεσης.

Abstract

Polycystic Kidney Disease (PKD), which is attributable to mutations in the *PKD1* and *PKD2* genes encoding polycystin-1 (PC1) and polycystin-2 (PC2) respectively, shares common cellular defects with cancer cells, including uncontrolled cell proliferation, abnormal differentiation, and increased apoptosis. Interestingly, PC1 regulates many signaling pathways such as Jak/STAT, mTOR, Wnt, AP-1, and calcineurin-NFAT that are also used by cancer cells for sending signals that will allow them to acquire and maintain malignant traits. Nevertheless, the molecular relationship between polycystins and cancer is unknown. In this study, we investigate the role of PC1 in cancer biology. Our *in vitro* results propose that PC1 modulates cell proliferation and migration and interacts with mTOR and Jak signaling pathways in colorectal (HT29), breast (MCF7), prostate (PC3), lung (A549) and glioblastoma (GOS3) cancer cell lines. Understanding the molecular details of how both polycystins PC1 and PC2 are associated with the pathogenesis and development of cancer may lead to the identification of novel potential therapeutic targets in cancer.

KEYWORDS

Cancer, Jak signaling, mTOR signaling, polycystins, PC1, cell proliferation, cell migration

Περίληψη

Η πολυκυστική νόσος των νεφρών (PKD), η οποία οφείλεται σε μεταλλάξεις στα γονίδια *PKD1* και *PKD2* που κωδικοποιούν την πολυκυστίνη-1 (PC1) και την πολυκυστίνη-2 (PC2) αντίστοιχα, έχει κοινές κυτταρικές βλάβες με τα καρκινικά κύτταρα, στις οποίες συμπεριλαμβάνονται ο ανεξέλεγκτος κυτταρικός πολλαπλασιασμός, η ανώμαλη κυτταρική διαφοροποίηση και η αυξημένη κυτταρική απόπτωση. Ενδιαφέρον αποτελεί το γεγονός ότι η PC1 ρυθμίζει πολλά σηματοδοτικά μονοπάτια όπως των Jak/STAT, mTOR, Wnt, AP-1 και calcineurin-NFAT που χρησιμοποιούνται επίσης από τα καρκινικά κύτταρα για την μεταβίβαση σημάτων που θα τους επιτρέψουν να αποκτήσουν και να διατηρήσουν κακοήθη χαρακτηριστικά. Παρόλα αυτά, η μοριακή σχέση μεταξύ πολυκυστινών και καρκίνου είναι άγνωστη. Σε αυτή τη μελέτη, ερευνούμε το ρόλο της PC1 στη βιολογία του καρκίνου. Τα *in vitro* αποτελέσματα μας, προτείνουν ότι η PC1 ρυθμίζει τον κυτταρικό πολλαπλασιασμό και την κυτταρική μετανάστευση και αλληλεπιδρά με τα σηματοδοτικά μονοπάτια mTOR και Jak στις καρκινικές κυτταρικές σειρές του παχέος εντέρου (HT29), του μαστού (MCF7), του προστάτη (PC3), του πνεύμονα (A549) και του γλοιοβλαστώματος (GOS3). Η κατανόηση των μοριακών λεπτομερειών του τρόπου με τον οποίο και οι δύο πολυκυστίνες PC1 και PC2 σχετίζονται με την παθογένεση και την ανάπτυξη του καρκίνου μπορεί να οδηγήσει στην αναγνώριση νέων πιθανών θεραπευτικών στόχων στον καρκίνο.

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ

Καρκίνος, σηματοδότηση Jak, σηματοδότηση mTOR, πολυκυστίνες, PC1, κυτταρικός πολλαπλασιασμός, κυτταρική μετανάστευση

GENERAL PART

CHAPTER 1

Polycystins

1.1 The family of polycystins

The term polycystins includes a relatively new family of proteins consisting of 8 protein molecules. The two representative members of the family are Polycystin-1 (PC1) and Polycystin-2 (PC2) detected in most tissues of the human body and encoded by the *PKD1* (Polycystic Kidney Disease 1) and *PKD2* (Polycystic Kidney Disease 2) genes, which are localized on human chromosomes 16p13.3 and 4q21-23, respectively. The rest of the family members are divided into two subcategories based on their functional and structural affinity to PC1 and PC2. The PC1 class includes Polycystin-REJ, expressed in testicles, and Polycystin-1L1, Polycystin-1L2 and Polycystin-1L3 proteins with relatively diffuse tissue expression. PC2 belongs to Polycystin-2L1 (PC2L1) and Polycystin-2L2 (PC2L2), which is limited to the heart and testicles. Due to the similarity of the PC2 subfamily in terms of sequence and topology with the transient receptor potential channel (TRP) family, the former is thought to belong to the family of the TRP channels (TRPP2 or PC2, TRPP3 or PC2L1, TRPP5 or PC2L2) [1].

PC1 was the first protein to be identified; this was done via cloning based on the topology of the PKD1 gene in 1994 [2]. The purpose of this study was to investigate the causes of autosomal dominant polycystic kidney disease (ADPKD), a common genetic disease that leads to the formation of polycystic kidneys and consequent renal impairment. Mutations in the PKD1 and PKD2 genes were causally associated with the disease, while rapid advances in the molecular genetics of ADPKD continuously facilitate the decipherment of the function of these proteins [3].

1.2 Polycystin-1

1.2.1 Structure

PC1 is translated from a 14.5 kb (kilobases) transcript, which has 228 nucleotides in the 5' sequence and 1019 nucleotides in the 3' untranslated region (UTR) sequence. The encoded polypeptide consists of 4304 amino acids and has a molecular weight of 462 kDa (kiloDalton). It has a large extracellular N-terminal end, eleven transmembrane domains and a smaller intracellular C-terminal end [2, 4, 5]. The extracellular domain consists of about 3000 amino acids and contains a unique combination of peptide regions: two leukine rich replicates (LRRs) flanked by two cysteine-rich regions, the region WSC (cell wall integrity/cell response component), a C-type lectin domain, a region homologous to low-density lipoprotein A (LDL-A), 16 PKD (polycystic kidney disease) regions, and a region of about 1000 amino acids called REJ (receptor for egg jelly) that is homologous to the protein detected in sea urchin eggs [7]. The LRR and C-type lectin regions are also commonly found in other structures that play a role in protein-protein interactions, cell adhesion and extracellular carbohydrate binding. The LDL-A region also functions as a binding point [2]. Of particular interest are the repetitive PKD structures, 15 of which are in sequence, have an immunoglobulin structure and provide mechanical support, elasticity and mechanically-induced ability to increase the extent of the N-terminus [7]. Regarding the REJ region, recent studies show that it consists of repeating β-sheet structures which are likely to be fibronectin III type regions [8].

Before the transmembrane domains, there is a proteolytic region of G-protein coupled receptor protein (G protein-coupled receptor protein) where PC1 undergoes functional cis-autoproteolysis and cleavage; this results in the N-terminal and C-terminal portions remaining non-covalently attached. The transmembrane domains contain 5 intracellular and 5 extracellular loops, while the

C-terminus consists of 225 amino acids and has the structure of a spiral coil and the capability of functional cleavage in different regions, as will be further elucidated [6].

1.2.2 Expression and tissue distribution

PC1 is expressed in a wide range of human tissues. It has been detected in cellular extracts and tissues in the thymus, duodenum, heart, tonsils, intestine, spleen, thyroid gland, lungs, testes, stomach, liver, vascular endothelium, etc. Its expression is usually limited to the epithelial cells of each tissue, such as urinary bladder, hepatobiliary, breast, and pancreas epithelium [9]. Very high expression levels have been observed in the cerebral cortex, whereas renal tissue levels are characterized by intermediate levels of expression [10].

Regarding embryonic kidney tissue, PC1 is detected in the earliest epithelial nephron precursors [11]. Expression in 5-6 week embryos is detected in the mesonephric tubules while high levels of expression are seen in the renal tubules near the nephrogenic zone, near the ureteric bud at 15 weeks, and in areas of the Bowman capsule in differentiated vascular glomeruli. The intensity of expression increases continuously during differentiation until the 24th week and then progressively decreases to result in a lower level in differentiated renal tissue [12]. Consequently, PC1 expression is localized in epithelial cells during kidney formation, which originate either from nephrogenic mesenchyme or from the ureteric bud [10].

In differentiated renal tissue, a pattern of expression appears to be established in the later stages of nephrogenesis [10, 11] in the renal tubules of the cortex around the glomerulus, the distal convoluted tubule, the loop of Henle, and the collecting tubules. Weak expression has also been observed in the vascular endothelium [9].

A thorough study of PC1 expression has been done in ADPKD, where expression is also limited to epithelial tubular cells and detected in 70-100% of cysts. The difference is that in several studies there is a clear increase in the intensity of expression in the epithelial cells that form the pathological cysts, although the intensity of the expression varies a lot among them [9, 10]. On the other hand, there are studies that indicate that the intensity of expression in epithelial cells that line the cysts is weaker than that in embryonic and differentiated normal renal tissue, while in some studies the expression is absent in 10-30% of the cysts [13]. This multiple differentiation in PC1 expression in epithelial cells that line the cysts also raises different theories about the mechanism of initiation of the disease, which will be analyzed in the description of PC1's biological involvement in the pathophysiology of ADPKD (see section 1.6) [11]. At the same time, two-thirds of the PKD1 gene has been shown to form a copy in a region close to that of the original gene on chromosome 16 and generate new transcripts leading to the formation of related proteins and making it difficult to detect PC1 due to the antigenic similarity in the extracellular end. This is a possible reason for the difficulties and differences that occur in various studies in determining the PC1 expression patterns [2].

1.2.3 Cell Localization

The vast majority of data regarding the detection of PC1 within the cellular structure are derived from renal tissue studies, where the main clinical manifestations of ADPKD are identified. The use of monoclonal antibodies against different protein epitopes in the extracellular loop of the transmembrane domain and its intracellular portion indicates that PC1 is expressed in the cell membrane, in the cytoplasm and is absent from the epithelial cell nucleus [14].

In line with the proposed role of PC1 as a mediator of intercellular communication, its main distribution is located on the basolateral surfaces of the cell membrane of tubular epithelial cells [15]. A similar localization appears in cell cultures of renal tubular cells and HUVEC (human umbilical vein endothelial cell), showing co-localization with cell adhesion molecules such as platelet endothelial cell adhesion molecule 1 (PECAM-1) or CD31 [9] . Electron microscopy data reveal that the majority of PC1 cytoplasmic molecules are associated with various structures across the cell membrane [14]. In Madin-Darby canine kidney (MDCK) cells, PC1 is localized to cell adhesion complexes in desmosomes and is in contact with intermediate filament proteins participating in cellular support and stabilization [16]. In contrast, PC1 has been detected on the apical surface of the cell membrane in embryonic kidney tissue, which is consistent with its co-localization with E-cadherin and β-catenin in the cell adhesion structures near the apical surface [14, 15]. PC1 is also found in the endoplasmic reticulum (ED) where its expression varies according to the expression levels of PC2 [17].

The topology of PC1 within the cell is affected by various types of mutations associated with ADPKD, mainly preventing the stable binding of transmembrane receptors 10 and 11 to the cell membrane [18]. Also, the transfer of PC1 to the lateral surface is interrupted in cells deficient in the *Tuberous sclerosis complex 2* (*TSC2*) gene, limiting PC1 to the Golgi apparatus, which is reversed by TSC2 re-expression [19].

1.2.4 Function

PC1 functions as a mechanosensory molecule that perceives extracellular, mechanical stimuli, and modulates cellular response accordingly by regulating key cellular features such as proliferation, differentiation, and apoptosis. PC1 mainly functions as an atypical receptor

coupled to G proteins. When PC1 is expressed alone, it appears to activate signaling pathways by direct linkage to and activation of the heterotrimeric Gai/o proteins. This is followed by the modification of cation selective channels for calcium and potassium GIRK (G protein-coupled inwardly-rectifying potassium channels) through the release of Gβγ subunits [21,22]. Based on this mechanism, it appears to be a protagonist in intercellular interactions and interactions with the extracellular matrix, and has been found to form polyprotein complexes with focal adhesion proteins, such as p130cas, focal adhesion kinase (FAK), paxillin, p-p60c-src (phospho p60 cellular src) and intercellular adhesion molecules such as E-cadherin, β- and γ-catenin [23].

PC1 forms functional complexes with protein kinases and phosphatases by modifying, via phosphorylation, its activity. PC1 has been found to be phosphorylated at its C-terminus by protein kinase A (PKA) [24]. Also, the first PKD region of PC1 interacts with the Ig (immunoglobulin) region of the receptor protein tyrosine phosphatase σ (RPTPσ) and the C-terminal domains with the receptor protein tyrosine phosphatase γ (RPTPγ). In this way, RPTPγ dephosphorylates PC1 at position Y4237 and modifies protein interactions, e.g. with PC2, and induced signaling pathways [25]. Similarly, PC1 interacts with and is dephosphorylated by protein phosphatase-1a (PP-1a), and this interaction is significantly attenuated in ADPKD-associated mutations of PC1 [26].

PC1 is also expressed in vascular endothelial cells where it plays a major mechanosensitive role. It appears that the homeostatic regulation of PC1, in terms of its topology and function, is a prerequisite for the conduction of extracellular shear stress due to flow, its transformation into intracellular signaling by altering the calcium concentration, and the biochemical synthesis of an important vasodilator, namely nitric oxide (NO) [27].

PC1 regulates cellular functions by activating key transcription factors. PC1 activates the transcription factor signal transducer and activator of transcription 3 (STAT3) via the janus activating kinase 2 (JAK2), leading to the phosphorylation of tyrosine residues and activation of transcription. However, the proteolytic cleavage of the carboxy-terminus of PC1 and its migration to the nucleus prevents the direct activation of STAT3, while the cleaved C-terminal tail acts as a STAT3 co-activator with the aid of cytokines and growth factors. Consequently, PC1 plays a dual role in the mechanism of activation of this particular transcription factor [28]. A different example of regulation of gene expression via PC1 is the activation of the Runx2 transcription factor. Overexpression of the C-terminal ends activates the Runx2 promoter in osteoblasts by regulating the intracellular calcium concentration [29]. A study by our research lab also showed that PC1, in osteoblastic type PDL cells subjected to mechanical stimulation, activates the gene expression of Runx2 via the calcineurin/nuclear factor of activated T-cells (NFAT), thus reinforcing the role of PC1 in skeletogenesis and regulation of bone growth [30]. The significance of the carboxy-terminus of PC1 in protein function is important. In vivo experiments in transgenic mice showed that PC1 in the renal tubular cells, under the application of mechanical stimuli, undergoes proteolytic cleavage at its C-terminus, which migrates to the nucleus and triggers signaling processes [31]. Transfection experiments with a plasmid encoding the last 193 amino acids of the C-terminus in the MDCK cell line showed that the recombinant protein could modify different phenotypes associated with total protein activity. In cells with endogenous expression of PC1 the introduction of the truncated form substantially mimicked the normal function of the protein. In contrast, in cells with PC1 overexpression the truncated form inhibited the effects of PC1 on cell functions, suggesting the existence of feedback mechanisms

of endogenous PC1 from its C-terminus [32]. The C-terminal end of PC1 also contains motifs that affect the microtubule and endoplasmic reticulum (ED) pattern and topology [33].

1.3 Polycystin-2

1.3.1 Structure

PC2 is a membrane protein consisting of 968 amino acids and has a predicted molecular weight of about 110 kDa. It is the protein product of the *PKD2* gene, which is located on chromosome 4q21-23 and has been estimated to have a sequence of about 5057 base pairs [34]. The *PKD2* gene has a 21% identical and 46% homologous sequence with amino acids 3688-4109 of *PKD1* [35]. PC2 has six transmembrane domains, a N-terminal and a C-terminal end. The loops 1, 3 and 5 of the transmembrane regions extend into the ER lumen when PC2 is located in the ER membrane or in the extracellular space when PC2 is located in the cell membrane, while loops 2 and 4 extend into the cytoplasm. This arrangement also favors the idea that the two ends of the protein are within the cytoplasm [36].

Of particular interest is the carboxyl terminus of PC2, for which biophysical analyses and molecular modeling have shown that it consists of three functional regions. The first is the so-called EF hand (PC2-EF), a well-known helix-loop-helix type domain specific for calcium ion binding. The second is a flexible linker and the third is a spiral coil that is also the catalytic domain of dimerization of the protein. Therefore, the PC2-EF region is a sensor for calcium ions and undergoes structural changes that depend on its specific function [37].

Compatible with the incorporation of PC2 into the TRP family of channels is its ability to form homo- or hetero-polymeric complexes. In addition to PC1, with which PC2 forms heterodimers, PC2 forms heterodimers with different members of the TRP family, such as the TRPC1 channel.

The formation of heteropolymers with the TRPC1 pump has a distinct function compared to PC1/PC2 heterodimers, since it has been shown to be activated by the corresponding activation of G protein coupled receptors in mIMCD3 epithelial renal cells [38]. However, apart from the region that catalyzes the dimerization of the protein in the coil coil region of the C-terminus, a new dimerization domain has been identified and functionally characterized at the N-terminus of PC2 (NT2-1-223, L224XH). This finding has formed a model of aggregation of PC2 molecules as a functional homo-tetramer, which depends on the function of both amino- and carboxy-terminal dimerization domains of the protein [39]. A recent study confirmed the tetrameric configuration of PC2 as a stable and functional structure of the protein, irrespective of the presence or absence of calcium ions, and showed that only the C-terminal oligomerization domain is capable of forming the tetramer [40].

1.3.2 Expression and tissue distribution

PC2 has been detected in most embryonic and differentiated tissues of the human body. In differentiated tissues the highest mRNA expression is observed in myocardium, kidney and pancreas. In embryonic tissues, expression is high in the lungs and kidneys [12]. On day E6, the first stage of growth being studied, intense PC2 expression was observed in the exoderm, the parietal endoderm, the cylindrical cells and the exo-embryonic endoderm. Up to day E9.5, PC2 is detected in the somite mesenchyme and myocardial cells [41]. In particular, embryonic kidney tissue showed pronounced expression of PKD2 transcripts between the 5th and 6th week in the mesonephros, metanephric mesenchyme and ureteric bud. From the 10th week onwards, all developing structures of the outer cortex and the branches of the ureteric bud showed expression at the mRNA level. In the underlying parenchyma, all tubular segments showed expression of

PKD2, but stronger expression was found in the distal tubules and the ascending loop of Henle. Until the 36th week the intensity of expression was high in all structures of the outer cortex, arteries and smooth muscle cells. After the 36th week, however, there is a rapid decrease in the level of expression, which almost disappears in proximal tubes after their differentiation [12]. In differentiated renal tissue, expression is detected in the distal tubules and collecting ducts, the ascending loop of Henle and the blood vessels. Similar was the pattern of expression at the protein level by Western blotting [12]. Immunohistochemical detection in differentiated renal tissue sections showed the same pattern of expression, being absent in the proximal tubule, the interstitial tissue, the glomerulus and the vasculature [42]. The immunohistochemical investigation in the cysts of PKD1 polycystic kidneys showed detection in the majority of cells that line the cysts with PC2 expression being associated with that of PC1 separately in each cell [43].

Regarding extrauterine tissues during embryogenesis, between weeks 5 and 6, PC2 is diffusively expressed in a variety of tissues, especially in the neural tube, nerve ganglia, liver and myocardium, while at week 16 the expression is more pronounced in anterior roots of the spinal cord [12]. Immunohistochemistry detected PC2 in various types of epithelial cells, such as the epithelial cells of the developing bronchial gland at 14 weeks, with strong expression in the trachea epithelium, the chondrocytes adjacent to the developing bronchi and the squamous epithelial cells in the esophagus at 20 weeks [42].

1.3.3 Cell Localization

PC2 is mainly found in the ER, cell membrane and primary cilia [15]. Also, PC2 has been detected in the mitotic spindle and the centrosome in dividing cells and in resting cells

respectively [44, 45]. Experiments of detecting PC2 by immunohistochemistry in differentiated renal tissue, as well as confocal microscopy experiments in cell lines, have shown that PC2 is mainly found on the basolateral surfaces of epithelial cells [42]. Immunofluorescence studies in cell lines transfected with the *PKD2* gene showed a distinct cytoplasmic and perinuclear expression pattern consistent with the localization of PC2 in the ER. The fact that PC2 is primarily located in the ER was confirmed by double immunofluorescence against PC2 epitopes and the ER-specific protein disulfide isomerase (PDI). The findings showed a significant overlap of the two proteins, demonstrating the specific location of PC2 in the ER, with its C-terminus proving to be a functional regulator of this specific localization [46, 47]. This is explained by the fact that the carboxy-terminus of PC2 carries a PC2 binding motif to the ER (ER retention motif), while a series of studies with truncated forms of PC2, lacking the corresponding motif, indicate that the protein migrates and is expressed in the cell membrane. The differences in the findings regarding PC2 expression appear to be due to its tissue-specific expression, the different types of cells studied and the different stages of cell differentiation [15].

The migration of PC2 into different subcellular compartments depends on protein-protein-like interactions that dynamically regulate and closely control PC2 molecules, mainly in the ER and the cell membrane [15]. For example, the adaptor proteins PACS-1 and PACS-2 (Phosphofurin acidic cluster sorting protein 1, 2) recognize an acidic complex at the carboxy-terminus of PC2 and direct the topology of PC2. Its binding to PACS-1 and PACS-2 depends on the phosphorylation of PC2 at serine 812 (Ser^{812}) by protein kinase CK2 (casein kinase 2), while mutant forms of PC2 that do not bind to the adaptor proteins, as well as the inhibition of CK2, lead to the transfer of PC2 to the cell membrane, where it functions as a calcium ion channel. The process of migration of PC2 to the cell membrane appears to be a two-stage process; the first

stage is controlled by PACS-2 in the ER and the second stage by PACS-1 in the Golgi apparatus [15, 48, 49]. The cellular distribution of PC2, however, also depends on its phosphorylation at the Ser^{76}/Ser^{80} site of its N-terminus by glycogen synthase kinase 3 synthase kinase (GSK3), which is important in vivo and in vitro to maintain a normal glomerular and tubular morphology of the kidney [50]. Also, PIGEA-14 (polycystin-2 interactor, Golgi and endoplasmic reticulum-associated protein) is another protein recognized as a regulator of the intracellular topology of PC2 that directs the transfer of PC2 from the ER to the Golgi apparatus [51].

1.3.4 Function

PC2 belongs, as mentioned, to the TRP family, which at the cellular level function under phospholipase C (PLC) as cell sensors, activated by a wide range of intracellular and extracellular stimuli [52]. PC2 differentiates its function according to the compartment of the cell where it is located each time. In the ER, PC2 was first found to function as a novel calcium ion channel, which is activated in response to the increase in calcium intracellular concentration but not extracellular. In the same study, in the renal epithelial cell line LLC-PK1, it appears that overexpression of *PKD2* enhances the intensity and duration of transient calcium ion release induced by GPCRs [53]. Other studies confirmed this view, since PC2 was found to interact with isoform 1 of the IP3R1 (inositol trisphosphate receptor 1) calcium ion channel by enhancing the duration rather than the intensity of transient calcium release [54]. Therefore, these data show that PC2 functions exclusively as a calcium release channel induced by the intracellular concentration of calcium ions.

PC2 is functionally expressed in the cell membrane, where it appears that it does not have the ability to form alone a functional channel except with the help of PC1 [55]. The contribution of

PC1 is not limited to channel formation, but it is the chaperone protein that facilitates the transfer of PC2 to the cell membrane [55]. Despite the small differences between the findings of the studies that followed, PC2 is able to form a functional channel in the cell membrane that is continuously activated when it is overexpressed, allowing the non-selective cation passage with slightly increased selectivity for calcium ions relative to potassium and sodium ions and a higher conductivity for potassium [56, 57]. PC2 requires the presence of PC1 also at the primary cilia, where both proteins are needed to trigger signaling via calcium ions induced by fluid flow in the renal tubules [58]. Different studies, however, show that PC2 can function independently of PC1 at the primary cilia, which is consistent with the absence of expression of PC1 in the primary cilia of embryonic cells [59]. The transfer of PC2 to mitotic spindles by the diaphanous related formin1 (mdia1) protein through physical interactions suggests the potential role of PC2 in calcium signaling in dividing cells while its detection in the centrosome suggests its function as a regulator along with factors of the intraflagellar transport (IFT) system in the formation of primary cilia [44, 45].

PC2 is activated and receives transfer signals to the cell membrane not only from PC1 [60], but also from other factors such as the epidermal growth factor (EGF). PC2 is activated in response to epidermal growth factor receptor (EGFR) receptor stimulation in the LLC-PK1 cell line [61]. EGF activates PC2 through the activity of the γ2 isoform of PLC (PLC-γ2) and phosphoinositide 3-kinase (PI3K), as well as the parallel decrease of phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) [61]. In addition, mice with homozygous EGFR gene deletion ($EGFR^{-/-}$) showed cystic enlargement in the collecting ducts indicating disturbed calcium signaling induced by growth factors as a possible cause of cyst formation [62].

1.4 The function of polycystins as a complex

It is now accepted that PC1 and PC2 physically interact with each other through their C-termini and form heterodimeric complexes in vivo in the cell membrane. This results in the formation of non-selective cation channels that are permeable to calcium ions. It appears that PC2 remains in the cytoplasm in the absence of PC1 and migrates to the cell membrane only when PC1 is present [55, 63]. For a long time, it was questioned whether PC1 acts as a functional component of this complex or simply plays the role of a chaperone companion to transfer PC2 to the membrane. Further studies have shown that PC1 activates and stabilizes PC2. Mutations in the interaction domains between the two proteins - namely the mutation R742X in PC2 and the mutation in the coil coil motif in PC1 - result in mutual effects that regulate and stabilize the two proteins [64]. For example, PC2 antagonizes the activation of G proteins by PC1. PC1 functions, as we have mentioned before, as an independent activator of Gi/o type proteins by releasing the Gβγ subunits. The expression of whole PC2 together with PC1 in nerve cell cultures prevented this effect suggesting that potential mutations in the interaction domains can lead to unintentional activation of G proteins by PC1 [21].

The interaction of the two proteins on the cell surface, however, has not been shown to exist as single repeat heterodimers. Using biochemical and crystallography methods, the composition of the subunits in living cells was elucidated and PC2 was found to form homotrimers (3 molecules of PC2) via a spiral coiled motif in the protein, and the homotrimer is in turn linked to 1 PC1 molecule through the spiral coiled pattern of PC1. Mutations that disrupt the PC2 homotrimer lead not only to its breakdown but also to the breakdown of the PC1-containing complex, as well as to the restriction of the expression of both proteins on the surface of the cell [65]. In addition, a second model has been proposed according to which the interaction of the two proteins exists

as a di-trimer in which one trimer consists of 3 PC2 molecules and the second of 2 PC2 molecules and one PC1 molecule again through interaction of the spiral coiled motifs [66]. Also, PC2, through its C-terminus, influences and stabilizes the cleavage of the carboxy-terminus of PC1 that migrates to the nucleus while favoring PC1 cleavage in the GPS region, processes which are independent of PC2's function as a calcium ion channel [67, 68]. It appears that the differences in the expression pattern of PC1 within the cell are influenced in part by the expression levels of PC2. When PC1 is expressed alone it is detected in the cell membrane and the ER, while co-expression with high levels of PC2 leads to the exclusive expression of PC1 in the ER together with PC2 [17].

Recent data show that between the two proteins there is probably a competitive relationship in which each protein modulates the other. PC2 carries a large number of phosphorylation sites, and one of these, the Ser^{829} site, is targeted by PKA and Aurora A (AurA) kinase, both of which regulate the migration of PC2 to various parts of the cell. Based on these data, a model is proposed, especially in ADPKD, which includes the disruption of the phosphorylation and dephosphorylation of PC2 in the Ser^{829} site and which is ultimately associated with the disruption of functional PC1 levels and PC1 dephosphorylation [50,69].

Finally, PC1 and PC2 show common regulatory elements in the promoter regions of the two genes encoding them. Comparison between different species in the promoter sequences showed that there are homologous and well conserved regions with transcriptional binding sites in both genes. The transcription factors E2F, EGRF, Ets (E-twenty six), MZF1 (myeloid zinc finger 1), Sp1 (Specificity protein 1), and ZBP-89 (zinc-binding protein-89) have emerged as potential regulators of the expression of both *PKD1* and *PKD2* [70].

1.4.1 Polycystins and primary cilia

Cilia are well-preserved organelles that have been known for over 100 years. These antenna like structures are divided into two types: motile and non-motile/primary cilia. Relatively recently, it has been revealed that primary cilia play an important role in the pathogenesis of various human diseases, the so-called ciliopathies, including polycystic kidney disease, nephronophthisis and a multitude of pleiotropic syndromes. This role is expected due to the wide cellular distribution and involvement in the paracrine intercellular signal transduction. Inside cilia is a microtubule-based cytoskeleton called the axoneme. The axoneme of primary cilia typically has a ring of nine outer microtubule doublets (called a 9+0 axoneme), and the axoneme of a motile cilium has two central microtubules in addition to the nine outer doublets (called a 9+2 axoneme). Cilia do not produce proteins and as a result cells have developed an intraflagellar transport mechanism, which allows proteins to be carried up and down the microtubules [71].

PC1 and PC2 are detected in primary cilia, adjusting their sensory functions [73]. The first indication that this function may be due to the perception of mechanical forces due to extracellular fluid flow was that the mechanical bending of primary cilia using micropipettes led to an increase in intracellular calcium concentration [74, 75]. It then turned out that the flow of fluid leads to the influx of calcium ions into MDCK kidney cells, a function that requires intact primary cilia, PC1 and PC2. The calcium ion influx itself triggers an even greater increase in calcium concentration from the ER through the receptors of ryanodine and IP3 receptors [58, 76, 77]. In addition, PC1 has been shown to be a key molecule regulating calcium-dependent cell signaling as well as the production of a major vasodilator agent, nitric oxide (NO), by endothelial cells in response to changes in the extracellular shear stress [27].

The normal function of polycystins in primary cilia is important because defects in the mechanism of intracellular calcium regulation can contribute to polycystic kidney disease. Reduced calcium ion concentration in mutant renal epithelial cells is consistent with reduced clearance of cyclic adenosine monophosphate (cAMP). cAMP activates the mitogen-activated protein kinase (MAPK) pathway and, consequently, increases cell proliferation and defective secretion [78]. Dysfunctional PC1 and PC2, as seen in primary cell cultures from the cystic epithelium of ADPKD patients, lead to altered expression of the two proteins in the primary cilia and inability of cilia to respond to fluid shear stress; these data emphasize the active and essential role of PC1 and PC2 in the normal function of cilia [79].

1.5 Biological role of polycystins in cell homeostasis

1.5.1 Embryonic morphogenesis and differentiated tissue regeneration

A series of studies have shown that the deletion of the *PKD1* and *PKD2* genes in animal models leads to embryonic or perinatal mortality with the development of cysts during the second half of embryogenesis [80, 81]. These findings indicate that polycystins are not necessary during mesenchymal-epithelial transition but play a role in subsequent stages of differentiation and maturation of tubular epithelial cells. In particular, the carboxy-terminus of PC1 is capable of triggering branching morphogenesis by activating protein kinase C-α (PKC-α) [82]. The localization of PC1 in the cell membrane is also essential for the physiological differentiation of the tubules [83]. Correspondingly, inhibition of PC1 function in developing mouse kidneys appears to disrupt the generation of branches in the ureteric bud [84]. PC2 also regulates the morphogenesis of the branches in the renal epithelial cells. In cell models where PC2 expression is absent, the process of branching is more pronounced suggesting the possible involvement of

PC2 in the formation of renal tubules [85]. Also, PC1 and PC2 are essential for normal placental growth, as demonstrated in mutant mouse models that lack both alleles for PC1 ($Pkd1^{-/-}$) or by selective inactivation of *PKD1* or *PKD2* in endothelial cells [86].

All mammals' intestines and vasculature have left-right asymmetry. The event that primarily defines L-R asymmetry in the fetus is the left-handed flow of fluid at the embryonic node as a result of the right-handed rotation of the polarized and posteriorly tilted nodal cilia. The "two cilia" hypothesis suggests that in conjunction with the motile cilia that generate the flow, the immotile mechanically sensitive cilia within the node are displaced and respond only to the flow of the left rather than the right part of the node via a PC2 dependent mechanism [87]. PC1/1 (Pkd1-related locus Pkd1|1) which is associated with the genetic locus of PC1 was identified as the protein "partner" of PC2 in this process [88].

The role of polycystins during embryogenesis also involves their participation in the regulation of major properties of epithelial cells, such as planar cell polarity (PCP) and oriented cell division (OCD). During the development of renal epithelial cells the sensation of the flow from the primary cilia is accompanied by the activation of the protein inversin. This change results in the transition from the normal Wnt pathway to the non-canonical Wnt pathway known as the PCP pathway and affects planar cell polarity. In particular, PCP is the polarity at the level of an epithelial monolayer parallel to the basement membrane and perpendicular to the apicobasal polarity. It represents the ideal arrangement of cells in space or of the subcellular structures at the epithelial level [89]. The PCP pathway is inextricably linked to the orientation of cell division during growth and, due to PCP, the mitotic spindles of epithelial cells are aligned along the axis of the tubules so that the addition of the daughter cells occurs in such a way that the renal tubules

grow longitudinally and not transversely [90]. It has been demonstrated in vivo that the absence of PC1 is associated with the disorientation of cell division [91].

It is very likely that the complex interaction of these processes (PCP, oriented cell division, primary cilia signaling, etc.) plays an important role during the regeneration of the renal tubular epithelium. PC1 regulates signaling pathways that are associated with these processes, such as the mammalian/mechanistic target of rapamycin (mTOR) and the transcription factor signal transducer and activator of transcription 6 (STAT6). In normal tissue PC1 acts as the "brake" in the activation of both mTOR and STAT6 signaling. During tissue regeneration after damage, the proteolytic cleavage of the carboxy-terminal tail of PC1 allows the activation of these two molecules in order to accelerate the proliferation of epithelial cells and to repair the damaged tissue [90].

To ensure proper tubular morphogenesis during tissue repair, all angles of the mitotic spindle must be parallel to the longitudinal tubular axis [89]. A *PKD1*$^{-/-}$ model showed disoriented cell division during regeneration [91]. At the same time, the centrosome location could be used as an indicator of homeostatic PCP regulation in non-dividing cells. In a different *PKD1*$^{-/-}$ model an ectopic position of the centrosome was observed after the completion of the repair process, reinforcing the relationship between PC1 and PCP in these processes [92]. PC2 also plays a role in regulating cell viability, repair and remodeling in differentiated renal tissue. In an ischemia-reperfusion-type monomeric lesion model, the kidneys with a heterozygous *Pkd2* deletion were more susceptible to damage with elevated inflammatory and fibrotic elements, as well as an increased proliferation and a decreased expression of p21 [93].

1.5.2 Cell cycle regulation

Polycystic disease has been characterized as a " neoplasia in disguise" demonstrating increased cell proliferation and defective apoptosis. The aberrant regulation of these functions leads to the hypothesis that polycystins have a role in controlling cell proliferation, cell differentiation and apoptosis. The key signaling pathways through which polycytins regulate the cell cycle are the following three: the JAK-STAT pathway, the inhibitor of DNA binding (Id) pathway and the mTOR pathway [1].

The signaling pathways of PC1 include the STAT1 and STAT6 transcription factors. In renal cells overexpressing the whole PC1 protein, the JAK2 kinase is activated and thus STAT1. In turn, this activation induces the expression of the inhibitor of cyclin-dependent kinases (CDKs) p21 and disrupts the cell cycle at the G0/G1 phase. As it has been shown, PC2 is a necessary cofactor in this process [94]. In a different study, the expression of the cleaved carboxy-terminus of PC1 activated STAT6 by direct binding to the P100 co-activator. This same study suggested the binding of STAT6 by PC1 at the primary cilia in a normal renal tubular lumen with fluid flow and normal expression of PC1. In cases of absent urine flow, absence of PC1 or overexpression of mutant PC1, STAT6 migrates to the nucleus and activates transcription [95].

The Id family includes four relatively new transcription regulators that belong to the helix-loop-helix transcription factor superfamily. Id proteins inhibit the differentiation of particular cell lines and can activate proliferation. PC2 appears to correlate directly with Id2 and to control cell cycle progression. In particular, membrane-bound phosphorylated PC2 binds Id2 in the cytoplasm via direct binding and prevents its migration to the nucleus. Overexpression of PC1 leads to an increase in PC2 phosphorylation and enhances the PC2-Id2 interaction, whereas

mutations of polycystins in animal models or in patients with ADPKD lead to increased nuclear accumulation of Id2 [96].

Also, there is a strong relationship between polycystins and the mTOR pathway, a serine/threonine kinase that is the main regulator of cell growth and metabolism in all eukaryotic cells [97]. The phosphorylated/activated form of mTOR, as well as the mTOR effector molecule S6 kinase, have been found to be increased in the cystic epithelial cells of ADPKD patients and *PKD1*$^{-/-}$ mice. Moreover, inhibition of mTOR by rapamycin reduced the cystic phenotype in animal models [98].

PC1 also regulates apoptosis through the nuclear factor-activated-B cells (NF-κB) transcription factor. In the renal cell line HEK293, overexpression of the C-terminus of PC1 resulted in NF-κB activation by the PKC kinase and suppression of cellular apoptosis [99]. Protection against apoptosis appears to be provided by PC1 via activation of the PI3K-Akt pathway, and in a similar fashion PC1 induces resistance to apoptosis in MDCK cells [100, 101]. A recent study also showed that PC1 and PC2 protect renal epithelial cells from apoptosis as a result of a mechanical stress response and this function is mediated by the opening of the mechanosensitive potassium channels K_{2p} type. Consequently, there emerges a direct relationship between mechanostimulation and mechanoprotection against apoptosis with the main mediators being the polycystins [102]. Finally, PC2 protects against apoptosis by reducing intracellular calcium concentration in the ER [103].

1.5.3 Intercellular interactions - interactions with extracellular matrix

In cell and animal models of ADPKD there has been detected an overproduction of extracellular matrix proteins, increased adhesion to type I and IV collagen, decreased cell migration in

response to growth factors, and altered expression of cell adhesion integrin receptor subtypes. Also in gene deletion studies in mice there was evidence that mutations in genes responsible for cell adhesion and ECM, such as laminin-5 and tensin, lead to the formation of cysts. At the same time, the extrarenal manifestations of ADPKD suggest a direct link between polycystins and the ECM. Intracranial/aortic aneurysms and various types of abdominal hernias indicate that there is a disorder of homeostatic regulation in ADPKD in terms of the mechano-induced response of vascular smooth muscle cells and a defect in the normal production of ECM proteins in the vascular wall layers and the abdominal wall. While these findings suggest that polycystins regulate intercellular interactions and ECM synthesis, it is difficult to identify the cases where polycystins have a primary regulatory role which leads to the formation of cysts and the cases where changes in the ECM are simply the result of progression of the disease [104].

PC1 is found in multiple focal adhesion structures which are the primary cellular structure mediating cell communication with the ECM. Thus, PC1 is found in complexes with talin, tensin, vinculin, α-actinin and with signaling proteins such as focal adhesion kinase (FAK), Src, p130cas and paxillin, in epithelial and smooth muscle cells [105]. At the same time, PC1 is found in complexes at intercellular communication sites along with PC2 and proteins such as E-cadherin and β-catenin [106].

In these localizations, PC1 is post-translationally modified by phosphorylation of tyrosine residues, indicating that it is capable of regulating/being regulated by cell interactions with ECM [23]. Expression of the carboxyl terminus of PC1 in cells of the medullary collecting duct resulted in phosphorylation and interaction of FAK and paxillin with each other and in the formation of focal complexes [108]. In ADPKD it appears that phosphorylation of PC1 leads to the breakdown of the PC1 / PC2 / E-cadherin / β-catenin complex [106]. On the other hand, the

N-terminus of PC1 carries multiple cell adhesion motifs such as the LRR, C-type lectin and repeating PKD regions. These regions have been found to bind directly to ECM proteins, such as collagen I and IV, through calcium signaling, fibronectin, laminin and to behave as binding domains for collagenolytic proteases [109-111]. A study of polycystin function in a zebrafish model where embryos had their *Pkd1* and *Pkd2* copies deleted showed ectopic and persistent expression of multiple collagen-related mRNAs, suggesting a loss of negative retrograde regulation that normally restricts collagen gene expression [112].

The question that arises is why do polycystins differ from the integrins and what is their uniqueness in cell adhesion processes. The answer is that the uniqueness of polycystines is probably related to the ability of PC1 to behave as a mechanosensitive protein [104]. A characteristic of mechanosensitive proteins, such as PC1, is their ability to undergo changes in their conformation due to mechanical force/pressure. This results in the unmasking of new, previously "hidden" binding sites, new amino acid residues for phosphorylation or the activation of novel enzymatic activity [113].

1.6 Polycystins and Polycystic Kidney Disease

Autosomal polycystic kidney disease (ADPKD) is a hereditary systemic disorder with severe renal manifestations and, in some cases, abnormalities in the liver, pancreas, brain, arterial blood vessels, or a combination of these organs. Every year, 300,000-600,000 people in the United States are diagnosed, regardless of gender or race, at a frequency of about 1:1,000 internationally, and 50% of patients progress to end-stage renal failure when reaching 60 years of age [114,115]. In 5% of cases, ADPKD appears as a spontaneous mutation, while in ¼ of new cases, no family history of the disease is reported. Affected patients have numerous cysts in the

kidney that can become a site of infection or hemorrhage after injury [115]. 85% of cases are due to mutations in *PKD1*, while the rest are due to mutations in the *PKD2* gene [114]. Specifically, in our country, a study in 53 Greek families revealed 8 possible mutations responsible for the disease, of which 5 were deletions and 3 were amino acid substitutions in the REJ region of *PKD1* [116].

In the cellular biology of ADPKD, aberrant polycystins are mainly involved in disturbing the regulation and activation of the same signaling pathways involved in the maintenance of cellular homeostasis (**Figure 1.1**).

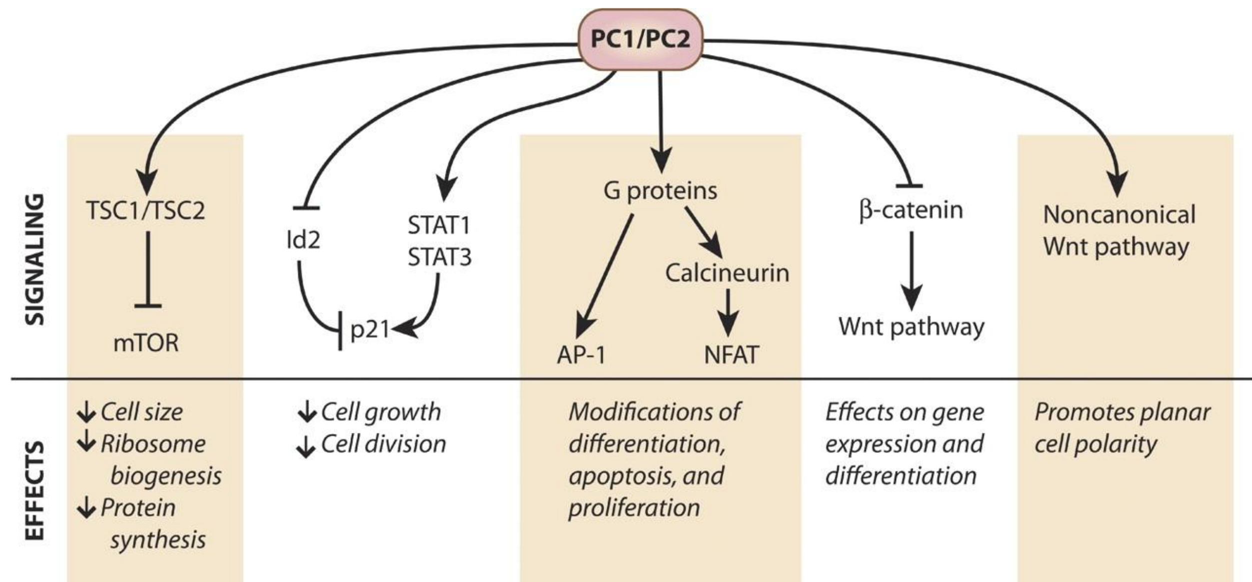


Figure 1.1 PC1 and PC2 affect multiple signaling pathways in ADPKD. A summary of the effect of polycystins on intracellular signal transduction associated with ribosome formation and protein synthesis (mTOR), cell growth and cell division (STAT, Id, p21), differentiation, apoptosis and proliferation (G proteins, AP-1, NFAT, Wnt) and planar cell polarity (noncanonical Wnt) in ADPKD [114].

The kidneys of a patient bearing the mutant gene will develop normally during a person's life. At some point, the cysts will be generated and then the cells that line the cysts will have lost both copies of the functional *PKD1* or *PKD2* genes. This suggests that a second hit is necessary, that is to say that a second somatic mutation will lead to the loss of the second allele [114]. Loss of the function of PC1 and PC2 during kidney development causes more severe cystic disease, indicating that polycystins play an important role during the increased cell proliferation and continuous cell division characterizing the neonatal period [117, 118]. Also, ADPKD appears to be a dose-dependent process that displays more and more severe phenotypic characteristics as the degree of PC1 functionality decreases [119].

Therefore, it appears that cysts can occur when the expression level of polycystins is lower or higher than a critical threshold required to maintain normal renal tubular formation. In these cases, it seems unlikely that only a single somatic mutation of the normal allele is required for the onset of cyst generation, and the theories of the effect of "threshold expression" and "third hit" which are related to stochastic factors such as renal damage or ischemia seem valid [120]. In differentiated and mature renal tissue the loss of heterozygosity would be an event without any consequences if it was not followed by the so-called "third hit" of ischemic and nephrotoxic damage as well as by the compensatory renal hypertrophy, thus triggering the onset of cystogenesis. These observations also partially reveal the unknown role of polycystins in mature renal tissue; the orchestration of proper tissue regeneration in response to kidney damage [120].

1.7 Polycystins and cancer

Among the biological properties characterizing cancer cells is the continuous transmission of signals that favor proliferation, resistance to apoptosis and activation of mechanisms of invasion

and metastasis [121, 122]. The involvement of polycystins in key cellular functions, such as proliferation, apoptosis, mediation of intercellular interactions, communication with the extracellular matrix and cell orientation suggest the potential involvement of both PC1 and PC2 in the biology of cancer initiation and progression.

Very few studies have studied the role of polycystins in the biology of cancer. PC1 overexpression in hepatocellular carcinoma, lung and colon cancer cell lines led to the promotion of intercellular and ECM interactions and the inhibition of tumor cell invasion and migration via the Wnt pathway suggesting that PC1 acts as a potential tumor suppressor protein [123]. In addition, reduction of PC2 expression using silencing RNA (siRNA) resulted in significant suppression of intercellular adhesion in B16 mouse melanoma cells [124]. These two studies show that both PC1 and PC2 affect cancer cell adhesion and communication with the ECM. Furthermore, overexpression of PC1 in the same cell lines led to a significant increase in apoptosis and a disruption of the cell cycle at the G0/G1 phase, indicating its involvement in cell cycle regulation in cancer cells [125]. Finally, a study with *in vivo* and *in vitro* data has shown that PC-1 and PC-2 are involved in the acquisition of aggressive phenotypes in colorectal cancer [126].

CHAPTER 2

Polycystins and the mTOR and Jak pathways

2.1 Polycystins and the mTOR pathway

The first evidence of the relationship between polycystins and the mTOR pathway came from individuals who had an early onset of ADPKD symptoms. Many of these individuals carry a major deletion on chromosome 16 that includes both *PKD1* and *TSC2* [127]. A mutation in *TSC2* prevents the migration of PC1 from the Golgi apparatus to the cell membrane, thereby enhancing the formation of cysts [19]. Enhanced cystogenesis in individuals with both *PKD1* and *TSC2* deletions suggests that the two proteins function in a common pathway during the formation of the cysts. Given the well known association of TSC2 with mTOR, the logical conclusion was that mTOR mediates the involvement of polycystins in ADPKD [128]. Indeed, activation of mTOR was demonstrated in animal models of ADPKD carrying mutations in *PKD1* [98]. Together with mTOR, its substrate, p70S6K, is activated in the cystic epithelium and adjacent normal tissue [129,130], and so are other effectors downstream of mTOR, such as the mTORC2 target Akt, 4E-BP1 and S6rp [131]. Apart from animal models with polycystic disease, the mTOR pathway is also activated in human tissues with ADPKD , demonstrating that this activation is a common feature of the disease independent of the underlying mutation.

The molecular mechanisms of mTOR activation with regard to the function of polycystins are not fully elucidated. A suggested mechanism is that the carboxy terminus of PC1 interacts with TSC2 so as to prevent the phosphorylation of TSC2 by Akt at serine 939, thus maintaining TSC2 in the cell membrane. This results in an increase in the interaction of TSC2 with TSC1 in order to inhibit mTOR activation [132]. Moreover, overexpression of PC1 in cell cultures inhibits

p70S6K, 4E-BP1 and S6rp, whereas PC1 silencing causes their activation, demonstrating the functional association between mTORC1 and PC1. This second mechanism of mTOR modulation by PC1 is also dependent on TSC2 via inhibiting its ERK-induced phosphorylation at serine 664 by PC1 [133]. This mechanism is independent of the presence of primary cilia and fluid flow, which strengthens the relationship between polycystins and the mTOR pathway in epithelial cell types where the primary cilia are absent.

2.2 Polycystins and the Jak pathway

PC1 signaling pathways involve at least two STATs (STAT1 and STAT6). One study used full-length PC1-stable cell lines to show that PC1 induces STAT1 activation by direct association and activation of JAK2, which in turn induces p21 expression and modulates the cell cycle. However, the activation of JAK2 by PC1 requires PC2 because the R4227X truncation mutant of PC1 was able to bind but not activate JAK2, and full-length PC1 was unable to activate JAK2 in cells lacking PC2 [94]. Another study showed that the expression of the cleaved C-tail of PC1 activated STAT6 by directly binding to P100 [95]. In immune cells, STAT6 is activated by JAK1 or JAK3. Because PC1 does not bind to JAK1 and its interaction with JAK3 was not tested, the authors proposed that JAK3 may be involved in PC1-dependent activation of STAT6. Phosphorylation of STAT6 by PC1-activated JAK2 is not excluded. In this study, a pathological role of STAT6 was suggested. The authors examined the localization of STAT6 in human ADPKD kidneys and found increased expression of STAT6 in the nucleus of cyst-lining epithelial cells in ADPKD kidneys. These investigators proposed that, in normal renal tubular lumen with fluid flow and normal PC1 expression, STAT6 is sequestered in the cilia by PC1. In the absence of urine flow or PC1 or overexpression of mutant PC1, STAT6 translocates from the

cilia to the nucleus to initiate STAT6-dependent transcription [95]. Although several factors such as fluid flow, calcium influx, and cytokine stimulation have been speculated to facilitate the activation of the JAK-STAT pathway, the mechanism of STAT6 upregulation and activation in ADPKD remains unclear and requires further study. In addition, a study reports that PC1 is capable of affecting the activity of several STAT transcription factors by two distinct mechanisms. First, membrane-anchored PC1 can activate STAT3 by JAK2-dependent phosphorylation. Second, the proteolytically cleaved, soluble PC1 tail undergoes nuclear translocation and can coactivate STAT1, -3, and -6, which have been previously activated by tyrosine phosphorylation, e.g., by cytokine signaling. Hence, PC1 is a membrane protein that can both activate a STAT at the membrane and then coactivate the STAT in the nucleus after its own cleavage. This dual mechanism on multiple STATs suggests that PC1 can integrate diverse signals and orchestrate different biological responses to these inputs [134]. Finally, PC1 has been associated with the Jak pathway in bone biology activating the latter [135].

AIM OF STUDY

The aim of the present study was to examine the *in vitro* role of polycystin-1 (PC1) in cancer biology using cancer cell lines derived from five different types of human cancer (brain – GOS3, lung – A549, prostate – PC3, colon – HT29, breast – MCF7).

Specifically, this study sought to evaluate whether PC1 affects cancer cell behaviour and interacts with signaling pathways that are commonly deregulated in cancer cells such as the mTOR and Jak cascades.

SPECIFIC PART

CHAPTER 1

MATERIALS AND METHODS

2.1 Cell Cultures

MCF7, PC3, A549, HT29 and CACO2 cell lines were cultured in DMEM containing L-glutamine, 4,5 g/l D-glucose and pyrophosphate sodium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (10,000 U/mL penicillin-10,000 mg/mL streptomycin). GOS3 cancer cells were cultured in RPMI 1640 medium GlutaMAX supplemented with 10% FBS, 1% penicillin-streptomycin (10,000 U/mL penicillin-10,000 µg/mL streptomycin). CHLA-259 cells were grown in a base medium of Iscove's Modified Dulbecco's Medium supplemented with 20% FBS, 4mM L-Glutamine, 1X ITS (5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenous acid). HBEC3-KT cells were cultured in Airway Epithelial Cell Basal Medium (ATCC PCS-300-030) supplemented with Bronchial Epithelial Cell Growth Kit (ATCC PCS-300-040). MCF10A cells were cultured in DMEM/F12 plus 5% horse serum supplemented with penicillin, streptomycin, L-glutamine, 20 ng/ml epidermal growth factor, 0.5 µg/ml hydrocortisone, 10 µg/ml insulin and 100 ng/ml cholera toxin. HPrEc cells were cultured in Prostate Epithelial Cell Basal Medium (ATCC PCS-440-030) supplemented with Prostate Epithelial Cell Growth Kit (ATCC PCS-440-040). All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2- 95% air.

2.2 PC1 Knockdown

MCF7, PC3, A549, HT29, and GOS3 cancer cells were transfected with Dharmacon's chemically synthesized siRNA SMARTpools [human PC-1, L-007666-00-0005, ON-

TARGETplus Human PKD1 (5310) siRNA - SMARTpool, 5 nmol] and non-targeting siRNA for control cells (D-001210-01-05, siGENOME Non-Targeting siRNA #1, 5 nmol), in dilution 1:20 in 1x siRNA buffer, using DharmaFECT 2 Transfection Reagent, 0.2mL (Dharmacon) in dilution 1:50 in DMEM (Gibco,Thermo Fisher Scientific) according to literature [10]. Cell starvation was performed for 6 hours before transfection in order to achieve proper cell cycle synchronization.

2.3 Antibodies

The following primary antibodies were used for Western blot analysis: Polycystin-2 (sc-10376 Santa Cruz Biotechnology), p70-S6K (sc-230 Santa Cruz Biotechnology), phospho-p70-S6K (sc-8416 Santa Cruz Biotechnology), phospho-mTOR (5536 CST), phospho-4E-BP1 (2855 CST), PTEN (9559 CST), Akt (9272 CST), phospho-Akt (9271 CST), actin (MAB1501 Millipore), polycystin-1 CT2741 (kindly provided by the Baltimore Polycystic Kidney Disease Research and Clinical Core Center), mTOR (701483 Thermo Fisher Scientific), 4EBP1 (AHO1382 Thermo Fisher Scientific), JAK2 (ab37226 Abcam), phospho-JAK2 (ab32101 Abcam). The following secondary antibodies were used: goat anti- mouse IgG HRP-conjugate (AP124P Millipore), goat anti-rabbit IgG HRP-conjugate (AP132P Millipore), donkey anti-goat IgG HRP-conjugate (A00178 GenScript). The IgPKD1 inhibitory antibody was a generous gift from Dr O. Ibraghimov-Beskrovnaya and H. Husson (Genzyme Co., Boston, MA).

2.4 Semi-quantitative PCR and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from cultured cells using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PrimeScript RT reagent kit-Perfect Real Time

(Takara Bio, Japan) for RT-PCR was used for cDNA synthesis according to the manufacturer's protocol.

For semi-quantitative PCR, the produced cDNA was amplified with specific primer pairs for PC1-encoding Pkd1 (annealing 58°C, forward CGCCGCTTCACTAGCTTCGAC; reverse ACGCTCCAGAGGGAGTCCAC) and PC2-encoding Pkd2 (annealing 53°C, forward GCGAGGTCTCTGGGGAAC; reverse TACACATGGAGCTCATCATGC) genes (35 cycles) as well as with actin gene primer pairs (28 cycles) using KAPA2G Fast Multiplex PCR Kit (KK5801, Kapa Biosystems). PCR-amplified fragments were analyzed after their separation in agarose gels using image analysis software (Image J; La Jolla, CA) and normalized to actin gene levels.

Quantitative real-time PCR was performed using an iCycler real-time instrument (Bio-Rad Laboratories, Hercules, CA) and RT-PCR product was amplified using the iQ SYBR Green Supermix (Bio-Rad).25 Primer pairs were used for the Pkd1 (annealing 61°C, forward CAAGACACCCACATGGAAACG; reverse CGCCAGCGTCTCTGTCTTCT) gene (40 cycles) normalized to actin gene levels (annealing 62°C).

2.5 Western Blot Analysis

Proteins were resolved by electrophoresis in SDS-polyacrylamide gels with varying densities (6% for PC1; 8% for mTOR and p-mTOR; 10% for PC2, Jak2 and p-Jak2; 12% for p70S6K, p-p70S6K, Akt, p-Akt and PTEN; 15% for 4EBP1 and p-4EBP1) and transferred to a nitrocellulose membrane (Porablot NCP, Macherey- Nagel, Duren, Germany). Membranes were incubated overnight at 4°C with the primary antibodies (dilutions were 1:250 for antibodies against PC1, PC2, mTOR, 4EBP1, p70S6K, p-p70S6K; 1:500 for Jak2 and p-Jak2; 1:1000 for p-

mTOR, Akt, p-Akt, PTEN, p-4EBP1, actin in PBST containing 1% non-fat milk). Detection of the immunoreactive bands was performed with the LumiSensor Chemiluminescent HRP Substrate kit (GenScript, NJ). Relative protein amounts were evaluated by densitometric analysis using Image J software and normalized to the corresponding actin levels.

2.6 Cell Proliferation Assay

Cells were seeded in a 96-well plate at a density of 103-105 cells/well in 100µl of culture medium with the IgPKD1 antibody (1:50 and 1:100 dilutions) or non-immune rabbit serum. Cells were cultured in a CO2 incubator at 37°C for 24 and 48 hours. 10µl of the prepared XTT Mixture (XTT Cell Proliferation Assay Kit, 10010200; Cayman Chemical, USA) were added to each well and mixed gently. The cells were incubated for 4 hours at 37°C in a CO2 incubator. The absorbance of each sample was measured using a microplate reader at a wavelength of 450nm.

2.7 Cell Migration Assay

HT29, MCF7, PC3, A549 and GOS3 cells were cultured in 12 well culture plates. After coating the cells, the cell monolayer was etched with a 200µL sterile pipette tip. Culture medium was supplemented with the IgPKD1 antibody. Each location was photographed in a computer-connected microscope at the x10 magnification at the start (0h) and after 24h (24h) incubation with the inhibitory antibody. Images were analyzed using TScratch software. The results were expressed as percentages of the incised and the cell-coated region.

2.8 Statistical Analysis

All experiments were performed at least three times. Data are presented as mean ± SD and were analyzed by one-way ANOVA. GraphPad Prism 6 software was employed for these statistical analyses. All statistical tests were two-sided. $p < 0.05$ was considered statistically significant.

CHAPTER 2

RESULTS

2.1 Endogenous mRNA and protein expression of PC1 and PC2 in cell lines

PC1 and PC2 proteins have only been detected in SW480 colorectal cancer cells [126], therefore we firstly sought to determine the endogenous mRNA and protein expression levels of the two polycystins in MCF7, PC3, A549, HT29 and GOS3 cancer cell lines. We detected both mRNA **(Figure 2.1A)** and protein **(Figure 2.1B)** levels of PC1 and PC2 in all cell lines apart from PC2 protein in MCF7 cells. There were discrepancies between Pkd1 mRNA levels and PC1 protein levels in some cancer cell lines, as well as discrepancies between Pkd2 mRNA levels and PC2 protein levels. For example, in MCF7 cells the Pkd2 gene expression is increased but the PC2 protein expression is negligible. These differences may be due to post-transcriptional and post-translational regulatory mechanisms. In addition, we compared the mRNA and protein levels in the cancer cell lines to the levels in normal cell lines from the same embryonic origin. Our results show that PC1 protein levels were higher in prostate cancer cells (PC3) compared to normal cells (HPrEc) and lower in glioblastoma cells (GOS3) compared to normal brain cells (CHLA-259). PC2 protein levels were found to be higher in colorectal cancer (HT29) and prostate cancer (PC3) cells compared to normal cells (CACO2 and HPrEc respectively), while they were lower in breast cancer cells (MCF7) compared to normal breast cells (MCF10A) **(Figure 2.1B)**. There were no differences observed in the mRNA levels of PC1 and PC2 between cancer and normal cell lines.

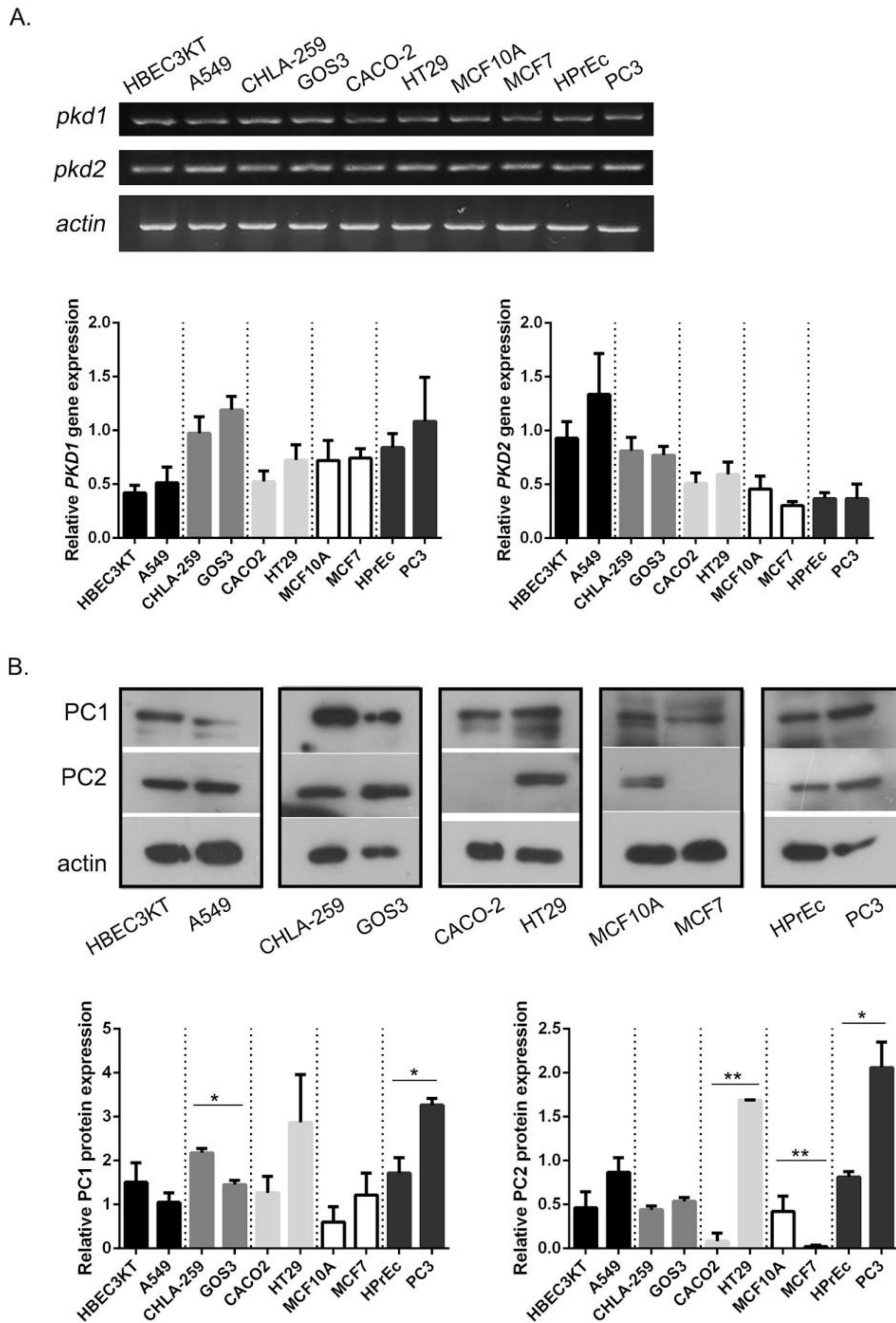


Figure 2.1 Endogenous Pkd1 and Pkd2 mRNA and PC1 and PC2 protein expression in cell lines. A, Semi-quantitative PCR analysis showing Pkd1 and Pkd2 mRNA levels in HBEC3KT, A549, CHLA-259, GOS3, CACO2, HT29, MCF10A, MCF7, HPrEc, PC3 cells. Actin was used as a mRNA loading control. Bars represent means ± SD. B, Western blot analysis of PC1 and PC2 protein levels in HBEC3KT, A549,

CHLA-259, GOS3, CACO2, HT29, MCF10A, MCF7, HPrEc, PC3 cells. Actin was used as a protein loading control. Bars represent means ± SD. *P < 0.05, **P < 0.01 versus respective normal cell line (CHLA-259 is the respective normal cell line of GOS3, HPrEc is the respective normal cell line of PC3, CACO2 is the respective normal cell line of HT29, MCF10A is the respective normal cell line of MCF7).

2.2 Effect of antibody-mediated PC1 inhibition on cell migration and proliferation in cancer cell lines

Next, we wanted to explore if PC1 affects cancer cell behavior. Thus, we decided to investigate whether PC1 affects cell migration and proliferation in cancer cell lines by incubating them with a blocking antibody, IgPKD1, raised against the Ig-like domains of extracellular PC1 [136]. Even though the function of PC1 remains obscure, and hence, there is still no specific assay to show that PC1 is inhibited, the IgPKD1 antibody is a valid method of inhibiting PC1. IgPKD1 has been used to block PC1 in murine, canine and human kidney epithelial cells [136-138], bone cells [30, 135], CRC cells and xenografts [126] and endothelial cells [139]. We found that in A549 cells, IgPKD1 treatment led to increased cell migration with the greatest effect observed at a 1:50 dilution of the IgPKD1 antibody **(Figure 2.2C)**. Conversely, in GOS3 cells, blocking PC1 resulted in decreased cell migration with the greatest effect observed at a 1:50 dilution of IgPKD1 **(Figure 2.2E)**. These results suggest that PC1 function in vitro is cancer cell type specific, promoting cell migration in GOS3 cells and suppressing cell migration in A549 cells. In terms of cell proliferation, our results show that MCF7, A549 and HT29 cells exhibited increased cell viability at both 24 and 48 hours after PC1 inhibition **(Figure 2.3A, C, D)**. However, in GOS3 cells, cell proliferation decreased at 48 hours after PC1 inhibition (1:50 dilution of the IgPKD1 antibody) **(Figure 2.3E)**. PC3 cells showed no significant effect on cell proliferation

(Figure 2.3B). These data indicate that PC1 enhances cell proliferation in GOS3 cells but hinders it in MCF7, A549, and HT29 cells.

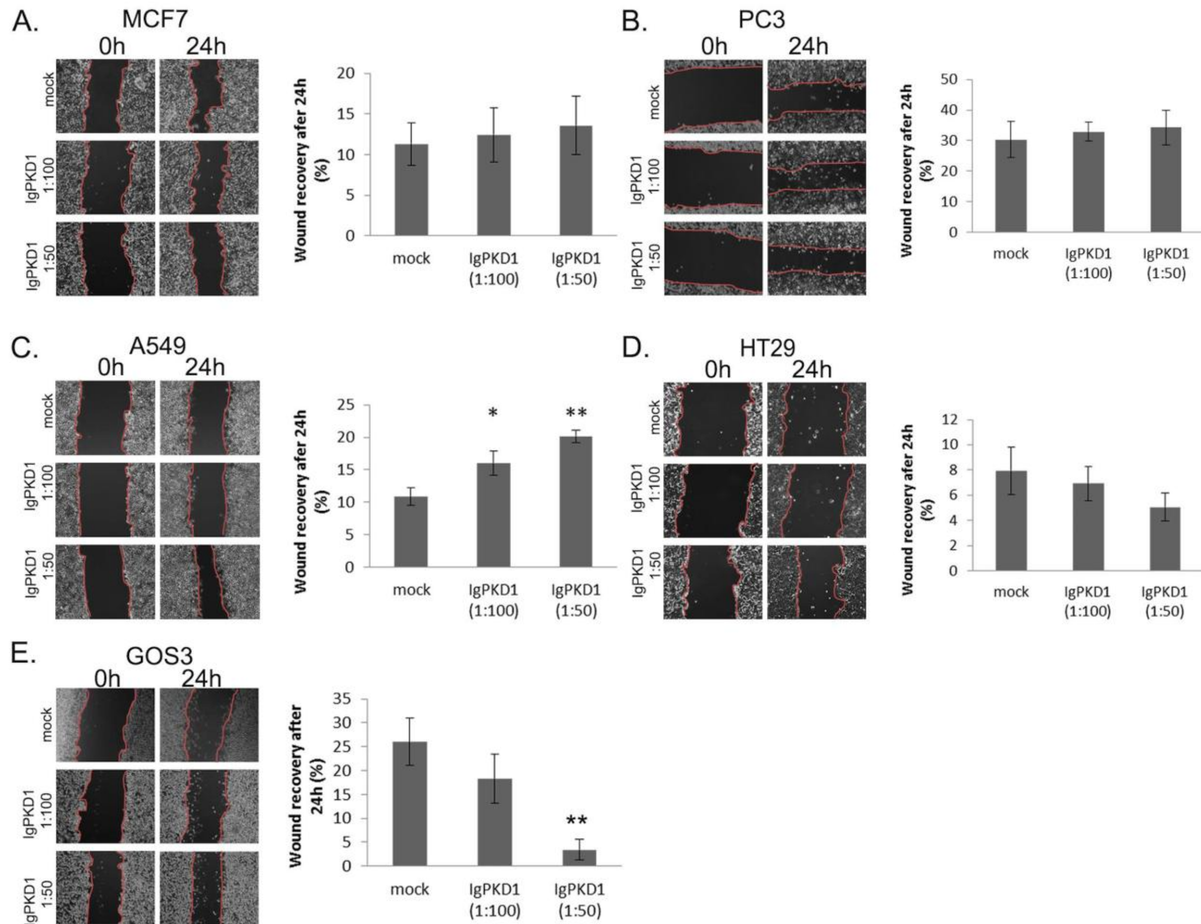


Figure 2.2 Effect of PC1 inhibition on cancer cell migration. A-E, Cell migration assay in MCF7, PC3, A549, HT29 and GOS3 cells. IgPKD1 is the inhibitory antibody against PC1. Mock represents cells that have been incubated with non-immune rabbit serum (without the IgPKD1 antibody). The images were analyzed using Tscratch software. Bars represent mean areas ± SD. $*P < 0.05$, $**P < 0.01$ versus mock.

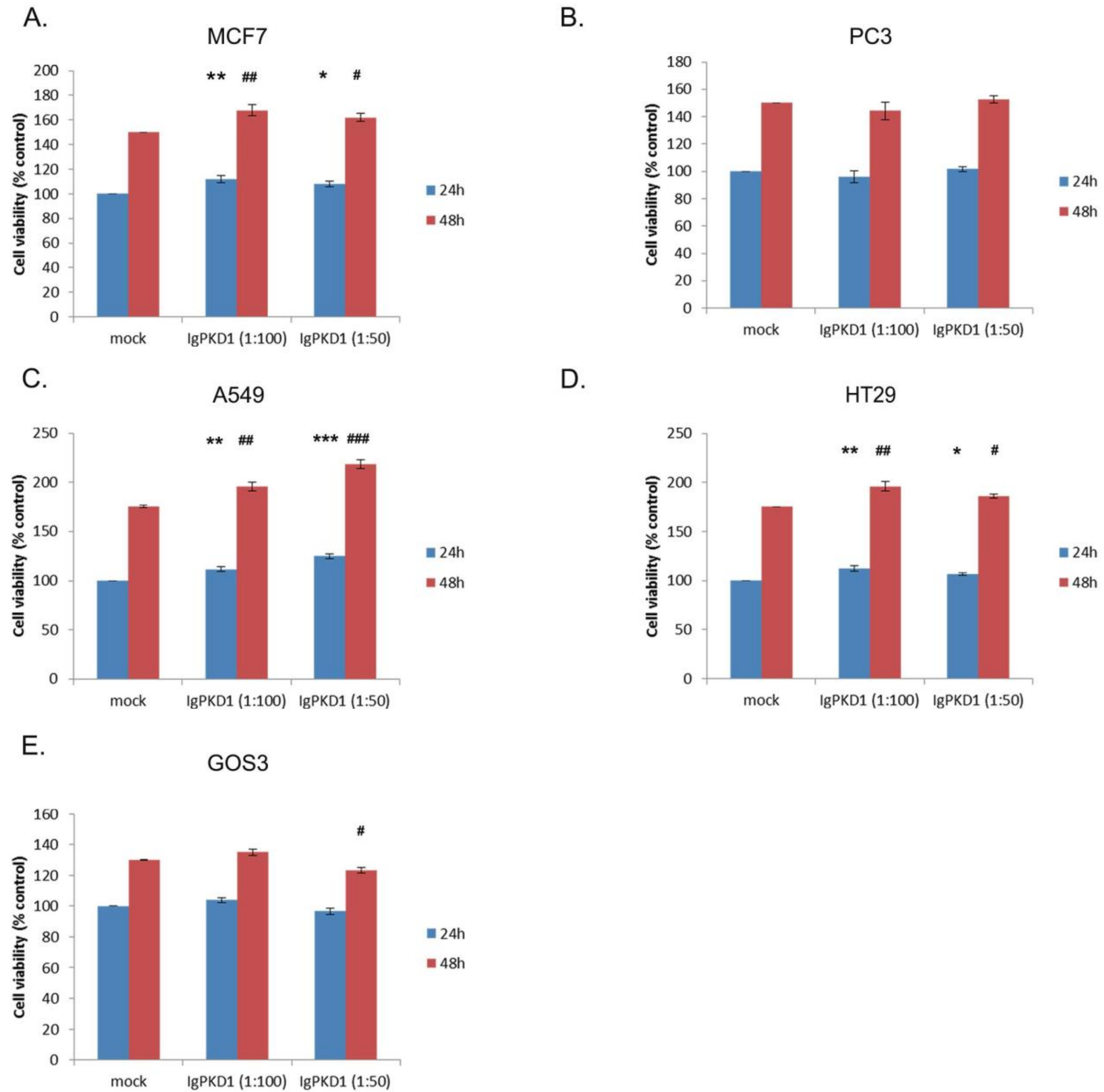


Figure 2.3 Effect of PC1 inhibition on cancer cell proliferation. A-E, Cell proliferation assay in MCF7, PC3, A549, HT29 and GOS3 cells. IgPKD1 is the inhibitory antibody against PC1. Mock represents cells that have been incubated with non-immune rabbit serum (without the IgPKD1 antibody). Each bar represents mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus mock at 24 hours. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ versus mock at 48 hours.

2.3 Effect of Pkd1 silencing on mTOR pathway in cancer cell lines

Subsequently, we sought to explore the effect of PC1 on mTOR signaling in our cancer cell lines when PC1 protein expression is knocked down by siRNA. The knockdown efficiency of the Pkd1 siRNA was confirmed by qRT-PCR **(Figure 2.4)**. Our results revealed that p70S6K phosphorylation was increased in PC3 cells treated with siRNA targeting the Pkd1 mRNA (siPKD1) **(Figure 2.5B,G)**, while it was decreased in GOS3 cells treated with siRNA targeting the Pkd1 mRNA (siPKD1) **(Figure 2.5E,J)**. mTOR phosphorylation was increased in HT29 cells treated with siRNA targeting the Pkd1 mRNA (siPKD1) **(Figure 2.5D,I)**. Akt phosphorylation was decreased in A549 cells treated with siRNA targeting the Pkd1 mRNA (siPKD1) **(Figure 2.5C,H)**. It should be mentioned that PC3 cells are PTEN-deficient. PC1 knockdown resulted in significantly affecting the phosphorylation of only one mTOR pathway component in most cancer cell lines; therefore, based on our data, PC1 appears to downregulate mTOR signaling in PC3 and HT29 cells, while it upregulates mTOR signaling in GOS3 and A549 cells. All these results suggest that PC1 interacts in vitro with the mTOR pathway in cancer cells.

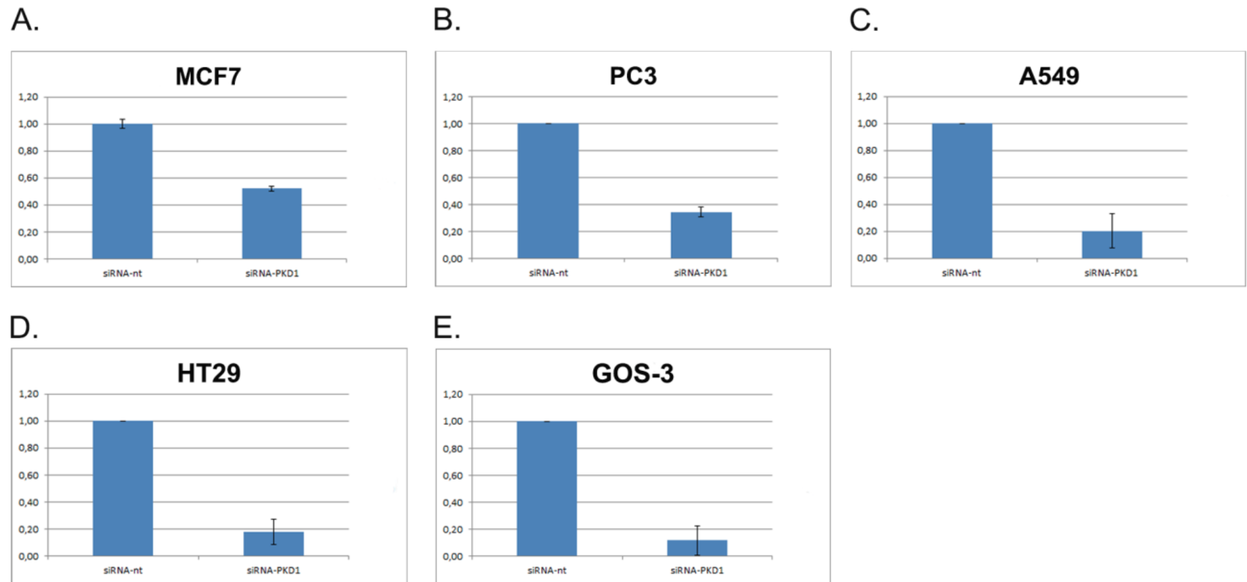
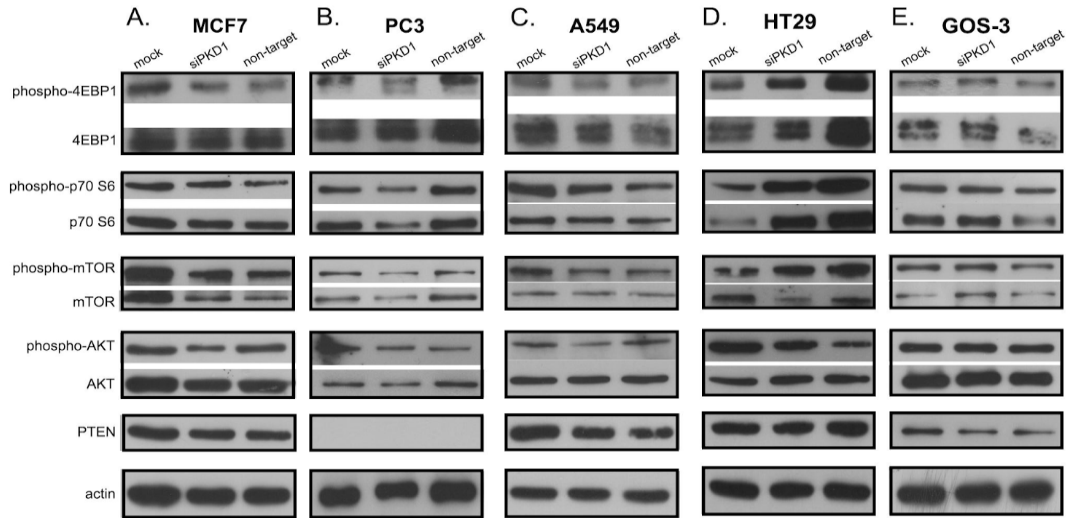
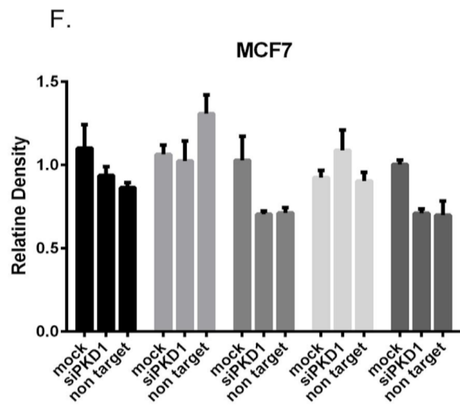
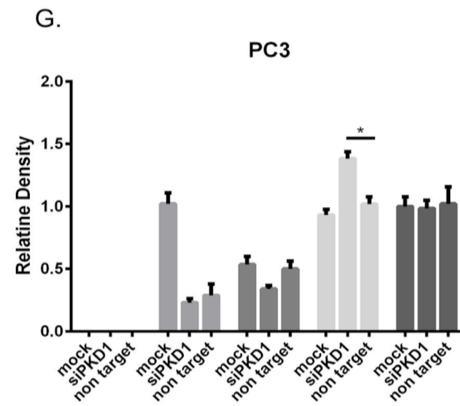
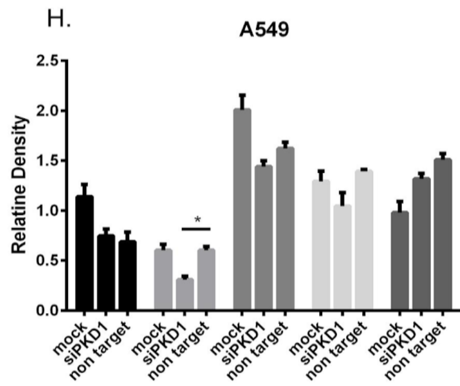


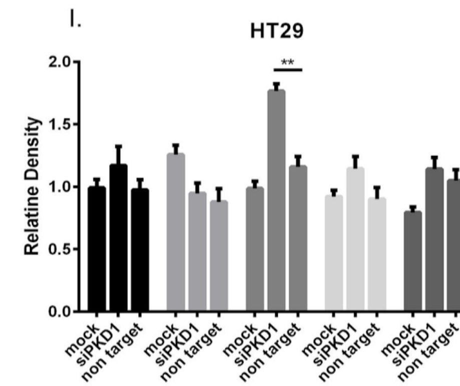
Figure 2.4 Knockdown efficiency of the *Pkd1* siRNA in cancer cell lines confirmed by quantitative Real-Time PCR (qRT-PCR). A, MCF7 cancer cell line. B, PC3 cancer cell line. C, A549 cancer cell line. D, HT29 cancer cell line. E, GOS3 cancer cell line. siRNA-PKD1 represents cells transfected with siRNA targeting the mRNA of Pkd1; siRNA-nt represents cells transfected with non-targeting siRNA.


A. MCF7
B. PC3
C. A549
D. HT29
E. GOS-3
mock
siPKD1
non-target
phospho-4EBP1
4EBP1
phospho-p70 S6
p70 S6
phospho-mTOR
mTOR
phospho-AKT
AKT
PTEN
actin


F.
MCF7
Relatine Density
mock
siPKD1
non target


G.
PC3
Relatine Density
mock
siPKD1
non target


H.
A549
Relatine Density
mock
siPKD1
non target


I.
HT29
Relatine Density
mock
siPKD1
non target

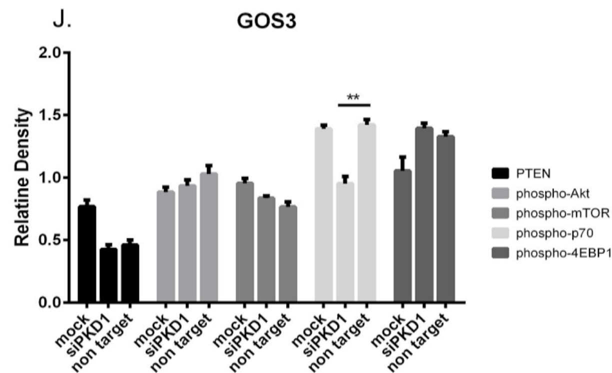

J.
GOS3
Relatine Density
mock
siPKD1
non target
PTEN
phospho-Akt
phospho-mTOR
phospho-p70
phospho-4EBP1

Figure 2.5 Effect of Pkd1 silencing on the mTOR pathway in cancer cell lines. A-E, Western blot analysis showing the effect of Pkd1 silencing on the phosphorylation of mTOR pathway components in MCF7, PC3, A549, HT29 and GOS3 cells. F-J, Quantitative data showing the effect of Pkd1 silencing on the phosphorylation of mTOR pathway components in MCF7, PC3, A549, HT29 and GOS3 cells. Bars represent means ± SD. *P < 0.05, **P < 0.01 versus non target. siPKD1 represents cancer cells transfected with siRNA targeting the Pkd1 mRNA; non target represents cancer cells transfected with a non-targeting siRNA; mock represents cancer cells transfected with only transfection reagents (without siRNA).

2.4 Effect of antibody-mediated PC1 inhibition on mTOR pathway in cancer cell lines

In a similar fashion to the above experiments we investigated the effect of PC1 on mTOR signaling in our cancer cell lines, but this time we inhibited PC1 with the blocking antibody IgPKD1. This was done by incubating all cancer cells with IgPKD1 for 3 hours, followed by collection and analysis of protein extracts at different time points (1, 3, and 6 hours) so as to investigate if the effect of PC1 on mTOR signaling is time-dependent as well. We show that phosphorylation of mTOR and 4EBP1 increased in MCF7 cells treated with the IgPKD1 antibody. On the other hand, phosphorylation of p70S6K decreased in MCF7 cells treated with the IgPKD1 **(Figure 2.6A,F)**. In A549 cells, IgPKD1 treatment decreased phosphorylation of mTOR and p70S6K **(Figure 2.6C,G)**. In PC3 cells, the phosphorylation of mTOR decreased after IgPKD1 treatment, while the phosphorylation of Akt and p70S6K increased in IgPKD1 treated cells **(Figure 2.6B,H)**. In HT29 cells, treatment with IgPKD1 increased mTOR phosphorylation, while it reduced 4EBP1 phosphorylation **(Figure 2.6D,I)**. Finally, in GOS3 cells, mTOR and Akt phosphorylation increased in IgPKD1 treated cells, while the

phosphorylation of 4EBP1 and p70S6K decreased in IgPKD1 treated cells. Total PTEN in GOS3 cells increased in IgPKD1 treated cells **(Figure 2.6E,J)**. According to these results, we were not able to clearly identify whether mTOR signaling is up- or downregulated in each cancer cell line; of the mTOR pathway-related proteins that we analysed in individual cell lines, some demonstrated increased phosphorylation while others showed decreased phosphorylation. We could probably state that mTOR signaling is upregulated in A549 cells, as there is an increase in both mTOR and p70S6K phosphorylation; however, these two proteins were the only ones to show a significant change in their phosphorylation after PC1 inhibition. Our difficulty in determining mTOR pathway activity after PC1 inhibition may be due to the complexity of its regulation which includes activating or inhibitory inputs from several other pathways. Furthermore, our results display a time-dependent in vitro effect of PC1 on mTOR signaling in cancer cells. For example, in PC3 cells, the phosphorylation of mTOR is gradually decreased over time (1, 3 and 6 hours) in IgPKD1 treated cells **(Figure 2.6B,H)**. Taken together, our results further support that PC1 interacts with the mTOR pathway in cancer cells.

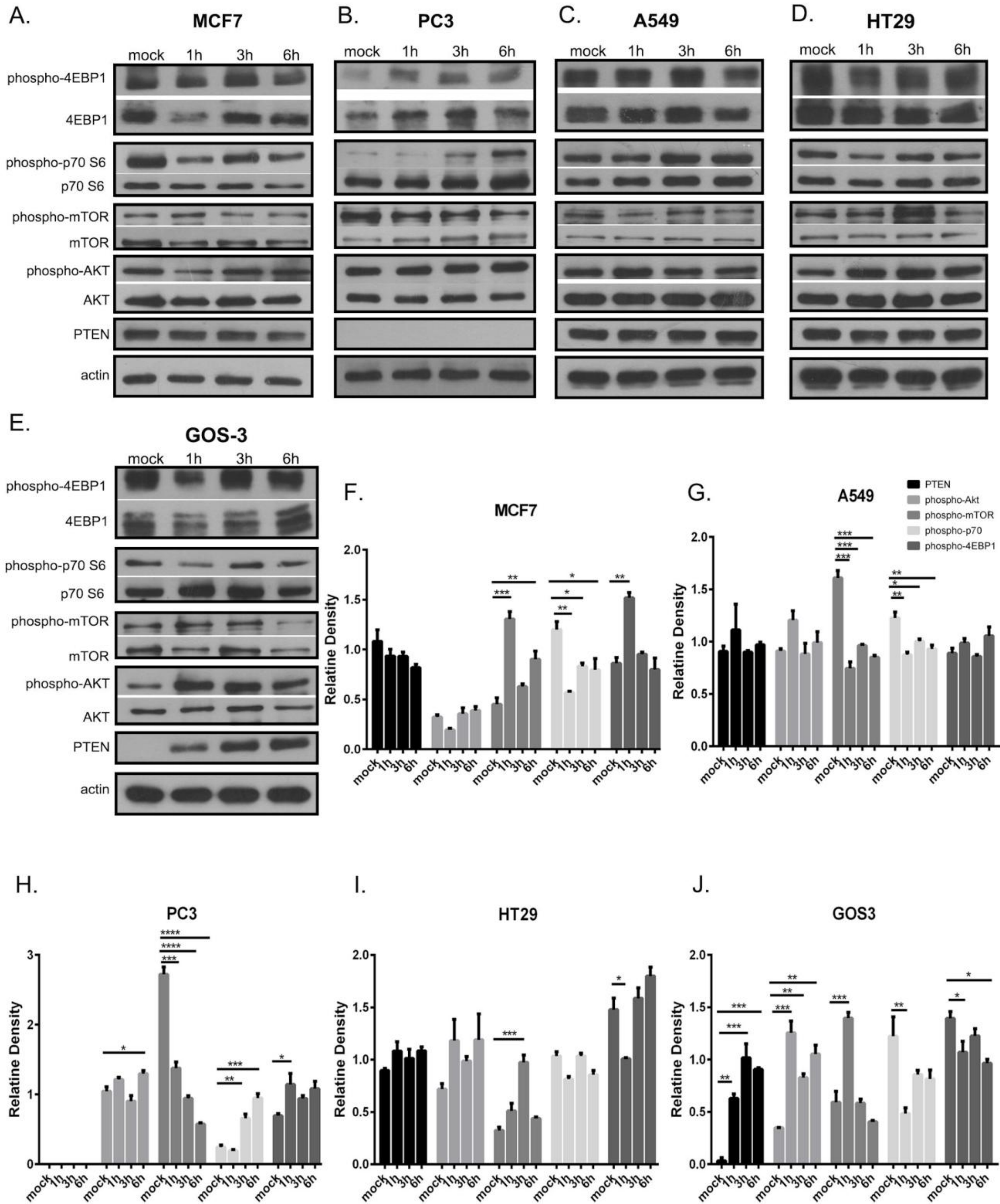


Figure 2.6 Effect of PC1 inhibition on the mTOR pathway in cancer cell lines. A-E, Western blot analysis showing the effect of IgPKD1 on the phosphorylation of mTOR pathway components in MCF7,

PC3, A549, HT29 and GOS3 cells. F-J, Quantitative data showing the effect of IgPKD on the phosphorylation of mTOR pathway components in MCF7, PC3, A549, HT29 and GOS3 cells. Bars represent means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 versus mock. Mock represents cells that have been incubated for 3 hours with non-immune rabbit serum; 1h, 3h, and 6h represent time points of cell harvesting after 3 hour incubation of cancer cells with the IgPKD1 antibody.

2.5 Effect of Pkd1 silencing on Jak pathway in cancer cell lines

To determine the effect of PC1 on the Jak pathway in our cancer cells, we silenced PC1 protein expression through siRNA. The knockdown efficiency of the Pkd1 siRNA was confirmed by qRT-PCR **(Figure 2.4)**. Our data demonstrate that the phosphorylation of Jak2 is increased in MCF7 **(Figure 2.7A,F)** and GOS3 **(Figure 2.7E,J)** cells treated with siRNA targeting the Pkd1 mRNA (siPKD1) compared to MCF7 and GOS3 cells treated with non-targeting siRNA (non-target), while it is decreased in PC3 **(Figure 2.7B,G)** and A549 **(Figure 2.7C,H)** cells treated with siRNA targeting the Pkd1 mRNA (siPKD1) compared to PC3 and A549 cells treated with non-targeting siRNA (non-target). According to this evidence, PC1 downregulates Jak signaling in MCF7 and GOS3 cells, whereas it upregulates Jak signaling in PC3 and A549 cells. These results suggest that PC1 interacts with the Jak pathway in cancer cells.

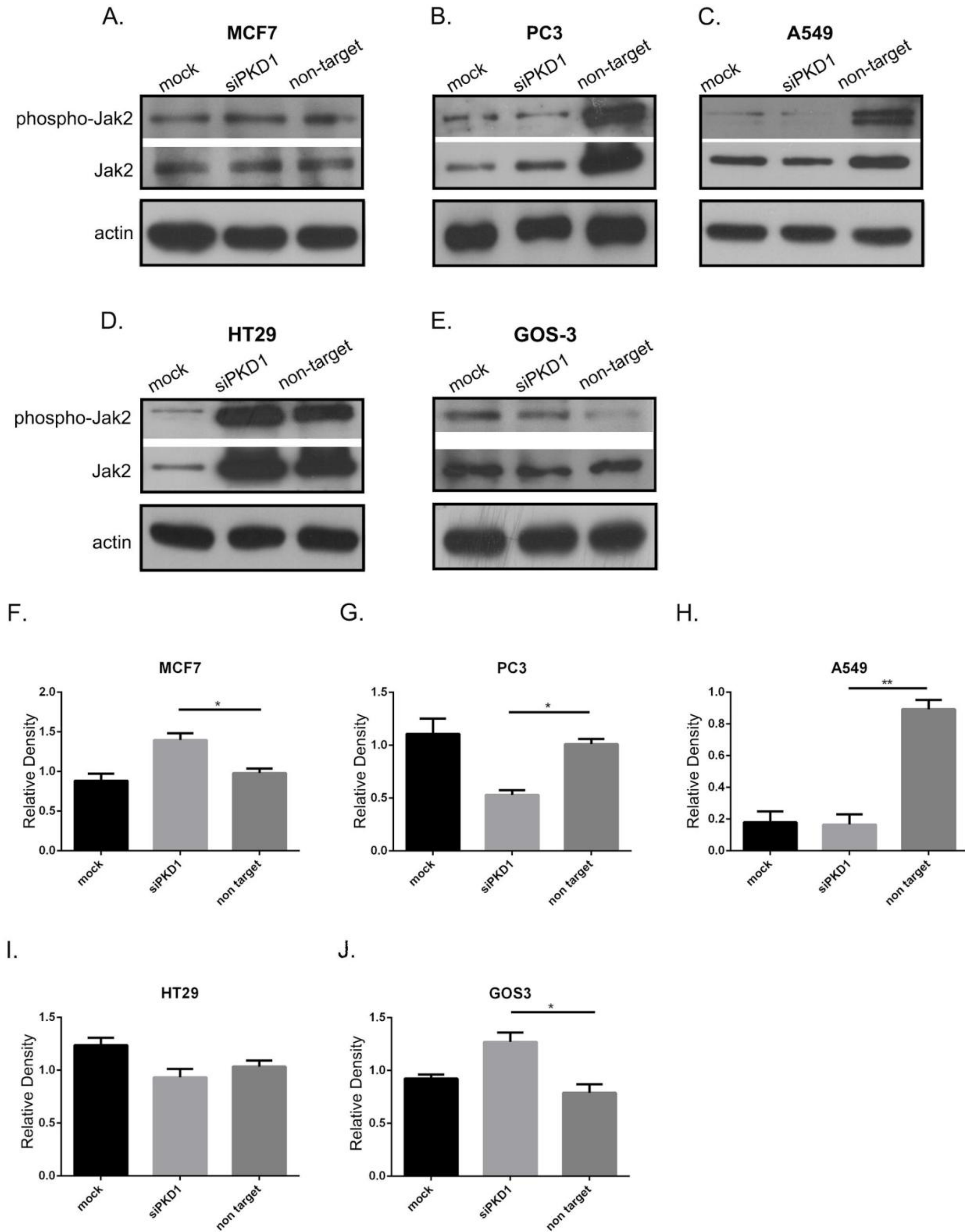


Figure 2.7 Effect of Pkd1 silencing on the Jak pathway in cancer cell lines. A-E, Western blot analysis showing the effect of Pkd1 silencing on the phosphorylation of Jak2 in MCF7, PC3, A549, HT29

and GOS3 cells. F-J, Quantitative data showing the effect of Pkd1 silencing on the phosphorylation of Jak2 in MCF7, PC3, A549, HT29 and GOS3 cells. Bars represent means ± SD. *$P < 0.05$, **$P < 0.01$ versus non target. siPKD1 represents cancer cells transfected with siRNA targeting the Pkd1 mRNA; non target represents cancer cells transfected with a non-targeting siRNA; mock represents cancer cells transfected with only transfection reagents (without siRNA).

2.6 Effect of antibody-mediated PC1 inhibition on Jak pathway in cancer cell lines

Finally, we explored the effect of PC1 on the Jak pathway by treating cancer cells with IgPKD1 for 3 hours, followed by analysis of protein extracts at different time points (1, 3, and 6 hours). According to our results, the phosphorylation of Jak2 in PC3 **(Figure 2.8B,G)**, A549 **(Figure 2.8C,H)**, HT29 **(Figure 2.8D,I)**, and GOS3 **(Figure 2.8E,J)** cells increased in IgPKD1 treated cells compared to mock cells where Jak2 phosphorylation was negligible. On the other hand, the phosphorylation of Jak2 in MCF7 cells decreased in IgPKD1 treated cells compared to mock cells **(Figure 2.8A,F)**. All the above indicate that PC1 upregulates Jak signaling in MCF7 cells, while it downregulates Jak signaling in PC3, A549, HT29 and GOS3 cells. We also observed that this in vitro effect of PC1 on Jak2 phosphorylation status was time-dependent. For example, in PC3 cells, the phosphorylation of Jak2 gradually decreased over time (1, 3 and 6 hours) in IgPKD treated cells compared to mock cells **(Figure 2.8B,G)**. These data further support that PC1 is linked in vitro to the Jak pathway in cancer cells.

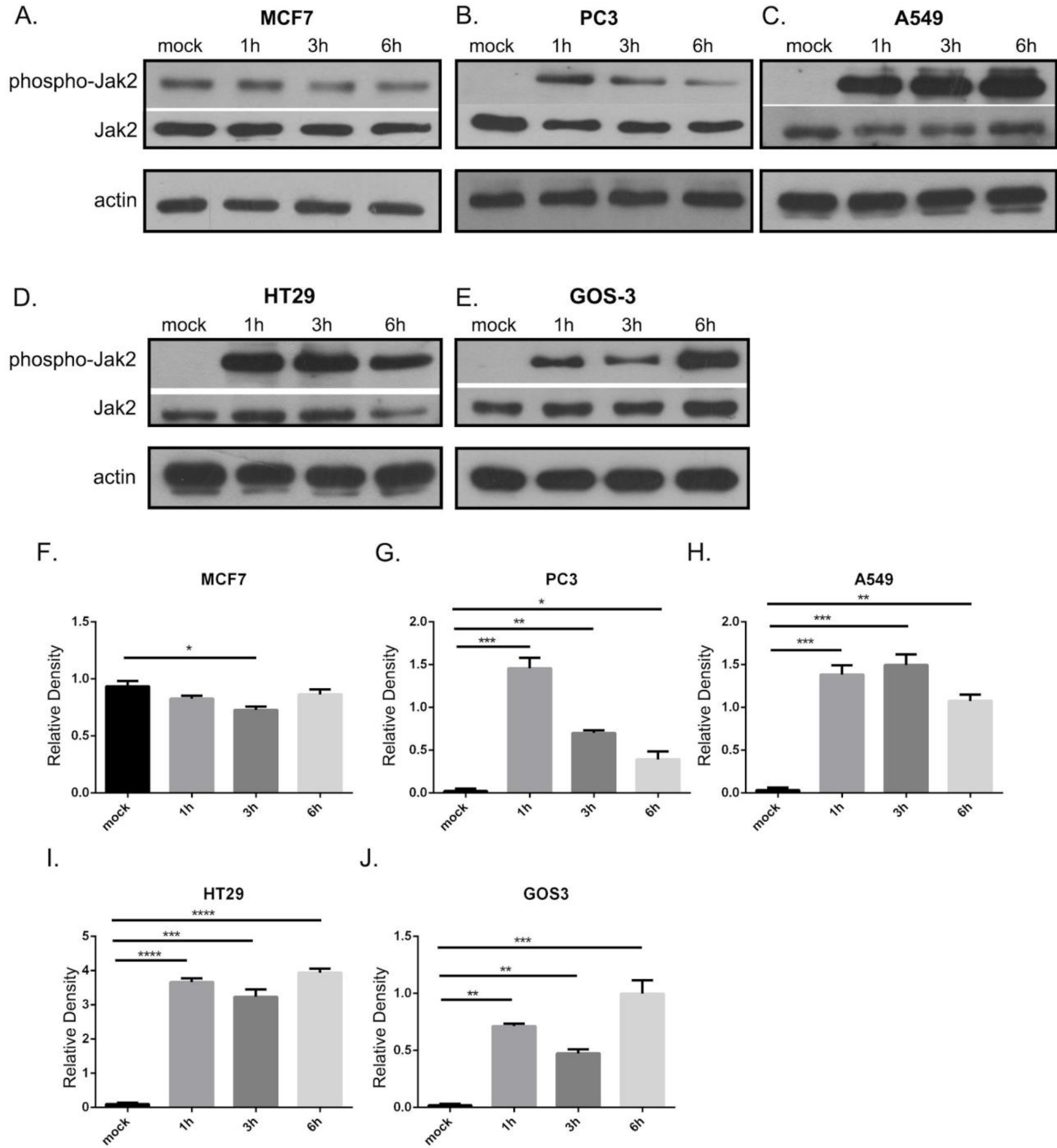


Figure 2.8 Effect of PC1 inhibition on the Jak pathway in cancer cell lines. A-E, Western blot analysis showing the effect of IgPKD1 on the phosphorylation of Jak2 in MCF7, PC3, A549, HT29 and

GOS3 cells. F-J, Quantitative data showing the effect of IgPKD1 on the phosphorylation of Jak2 in MCF7, PC3, A549, HT29 and GOS3 cells. Bars represent means ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ versus mock. Mock represents cells that have been incubated for 3 hours with non-immune rabbit serum; 1h, 3h, and 6h represent time points of cell harvesting after 3 hour incubation of cancer cells with the IgPKD1 antibody.

CHAPTER 3

DISCUSSION

Considering the common cellular features and signaling pathways between PKD and cancer, we speculated whether polycystins PC1 and PC2 play a role in cancer biology. First, we sought to evaluate the mRNA and protein levels of polycystins in cell lines from different types of cancer, including glioblastoma, prostate, lung, breast, and colorectal cancer. We detected both mRNA and protein of PC1 and PC2 in the cancer cell lines. Our results also showed that there were differences in PC1 and PC2 protein levels between cancer cells (PC3, GOS3, HT29, MCF7) and their respective normal cells (HPrEc, CHLA-259, CACO2, MCF10A). The protein expression of polycystins and their subcellular localization have been studied primarily in renal tissues and cultured cell lines of renal epithelial origin. With respect to our study, PC1 and PC2 protein expression has been detected in normal developing brain, breast ductal epithelium, colonic epithelium, prostate epithelium, and lung epithelium [9, 43, 140]. Nevertheless, immunohistochemistry data on polycystin protein expression in human tissues have not been consistent. Although in the present study we evaluated the protein expression of PC1 and PC2 in cancer and normal cell lines, it is also important to determine the subcellular localization of both polycystins because it is essential to their function. The subcellular localization of polycystins is complex and still debated by researchers. Results based on renal epithelial cells demonstrate that PC1 and PC2 are found on the primary cilia and in other subcellular compartments and membrane domains. Their localization, particularly PC2, has been found to be regulated by chemical chaperones, proteasome inhibitors, protein-protein interactions, and phosphorylation. In addition, given that PC1 and PC2 physically interact via their cytoplasmic C-terminal tails, it is

possible that they modulate each other’s subcellular localization, but this remains controversial [1]. Similarly to renal epithelial cells, all cell lines used in our study, apart from glioblastoma cells (GOS3), are epithelial in origin. However, whether PC1 and PC2 localization in our cancer epithelial cell lines follows the same pattern as in normal renal epithelial cells needs to be investigated in future studies.

For our next experiments we focused on PC1 because of its large size, flexible nature, participation in cell-cell and cell-matrix contacts, and known communication with many downstream signaling pathways via its intracellular C-terminal tail [19, 95, 98, 132, 133, 141-144]. First, we evaluated the effect of PC1 on two important cellular features, cell proliferation and migration, which are commonly deregulated in cancer [122]. Increased cell proliferation is a major feature of a polycystic kidney; cysts have even been characterized as “neoplasia in disguise” [145]. Several studies have reported that PC1 also regulates cell migration [82, 146-149]. We found that blocking PC1 in vitro with IgPKD1 affected both cell proliferation and migration in cancer cells in a cell type dependent manner. According to our results, PC1 functions as a tumor-suppressor protein in A549 cells inhibiting cell migration. In contrast, PC1 probably acts as an oncogene protein in GOS3 cells enhancing cell migration. Since PC1 has been reported to promote cell migration, could it be that GOS3 glioblastoma cells hijack this function of PC1 and turn it into a malignant signal that enhances their migratory and invasive abilities? Concerning cell proliferation, our results show that PC1 might be a tumor-suppressor protein in MCF7, A549, and HT29 cells that impedes cell proliferation. Conversely, in GOS3 cells PC1 appears to be an oncogene that promotes cell proliferation. Because PC1 has been shown to inhibit cell proliferation in non-cancerous cells [1, 94, 98, 150], we wondered whether MCF7, A549 and HT29 cancer cells are deregulated in such a way that interferes with the

normal PC1-mediated inhibition of proliferation. Do cancer cells achieve this effect by abolishing the function of the PC1 protein to transmit inhibitory signals to the cell's interior or by making the targets of these signals insensitive to inhibition? More study is required to confirm the influence of PC1 on cancer cell proliferation and migration and to uncover the mechanisms of this effect.

Next, we wanted to determine if PC1 regulates signaling pathways that are constitutively activated in cancer. Cancer and PKD are frequently accompanied by aberrant activation of the mTOR pathway [151-156]. Previous data have demonstrated that PC1 overexpression in SW480 colon cancer cells leads to downregulation of mTOR signaling [126]. Jak signaling also becomes upregulated in cancer [157-159] and studies have shown that PC1 activates Jak signaling in PKD [94, 134, 160-162]. Therefore, we investigated if the mTOR and Jak pathways are affected by changes in the function of PC1 in cancer cells. Inhibiting PC1 via the use of IgPKD1 resulted in alterations in the phosphorylation level of upstream regulators and downstream effectors of mTOR and Jak2. Likewise, silencing PC1 gene expression via siRNA modified the phosphorylation level of mTOR pathway-associated molecules and Jak2. Specifically, p-p70 in PC3 cells, p-mTOR in HT29 cells and p-Jak2 in GOS3 cells were upregulated in both assays, and p-p70 in GOS3 cells was downregulated in both assays. These findings indicate that PC1 stimulates mTOR signaling and inhibits Jak signaling in GOS3 cells, while it suppresses mTOR signaling in PC3 and HT29 cells. The mTOR suppression observed in HT29 colon cancer cells is consistent with the previous finding that PC1 downregulates mTOR signaling in SW480 colon cancer cells [126]. These results also suggest that the effect of PC1 on mTOR signaling in cancer is cell type dependent. Moreover, these data contribute to our knowledge on the regulation of mTOR and Jak signaling in cancer. It should be noted that the conclusions on whether the mTOR

cascade is activated or inhibited in cancer cells after changes in PC1 function are based only on the phosphorylation of a single mTOR-related protein. Likewise, we focused only on Jak2 phosphorylation and did not evaluate any downstream effectors or target genes as surrogate markers of Jak pathway activity. Therefore, our conclusions in terms of Jak signaling activation are solely based on the phosphorylation status of Jak2. The inhibitory or activating effect of PC1 on the two cascades has to be validated through further studies.

A challenge that we encountered in the present study was the following: the two methods used to inhibit PC1 activity, PC1 knockdown with siRNA and PC1 inhibition with IgPKD1, did not generate the same results for most cancer cell lines as far as Jak pathway activity is concerned. The siRNA experiment data propose that PC3 and A549 cells use PC1 in order to activate Jak signaling, while MCF7 and GOS3 cells use PC1 to suppress Jak signaling. In contrast, data from the antibody-mediated PC1 inhibition experiment imply that PC3, A549, HT29 and GOS3 cells use PC1 to downregulate Jak signaling, whereas MCF7 cells use PC1 to upregulate Jak signaling. These discrepancies could be due to limitations inherent in the two methods used to perturb the function of PC1; in contrast to RNAi where the PC1 protein is absent, the IgPKD1-inhibited PC1 protein may lack certain activities but may still execute other activities and/or interact with other proteins. Moreover, both methods can have substantial off target effects.

In summary, our study demonstrates that PC1 regulates cell proliferation and migration and interacts with mTOR and Jak signaling in various cancer cell lines. Given that there is a lack of prior research on the subject of polycystins and cancer biology, this study represents the first steps towards understanding the function of polycystins in the pathophysiology of cancer. As we expected, our research prompted more questions than answers. Future research should focus on the mechanism through which PC1 promotes or inhibits cell proliferation and migration, and the

molecular details of the interaction between PC1 and mTOR and Jak signaling. Moreover, future studies on polycystins and cancer should explore whether polycystins are associated with any other signaling pathways in cancer cells. It would also be interesting to evaluate the clinical relevance of polycystins in cancer by studying human cancer tissues. All the above will reveal the significance of polycystins in cancer biology and may lead to the identification of new therapeutic targets or prognostic markers in cancer.

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