Activation of Calcium-Sensing Receptor increases intracellular calcium and decreases cAMP and mTOR in PKD1 deficient cells

Annarita Di Mise, Grazia Tamma, Marianna Ranieri, Mariangela Centrone, Lambertus van den Heuvel, Djalila Mekahlí, Elena N. Levtchenko & Giovanna Valenti

Clinical and fundamental research suggest that altered calcium and cAMP signaling might be the most proximal events in ADPKD pathogenesis. Cells from ADPKD cysts have a reduced resting cytosolic calcium \([\text{Ca}^{2+}]_{i}\) and increased cAMP levels. CaSR plays an essential role in regulating calcium homeostasis. Its activation is associated with \([\text{Ca}^{2+}]_{i}\) increase and cAMP decrease, making CaSR a possible therapeutic target. Human conditionally immortalized Proximal Tubular Epithelial cells (ciPTEC) with stable knockdown of PKD1 (ciPTEC-PC1KD) and ciPTEC generated from an ADPKD1 patient (ciPTEC-PC1Pt) were used as experimental tools. CaSR functional expression was confirmed by studies showing that the calcimimetic NPS-R568 induced a significant increase in \([\text{Ca}^{2+}]_{i}\) in ciPTEC-PC1KD and ciPTEC-PC1Pt. Resting \([\text{Ca}^{2+}]_{i}\) were significantly lower in ciPTEC-PC1KD with respect to ciPTECwt, confirming calcium dysregulation. As in native cyst cells, significantly higher cAMP levels and mTOR activity were found in ciPTEC-PC1KD compared to ciPTECwt. Of note, NPS-R568 treatment significantly reduced intracellular cAMP and mTOR activity in ciPTEC-PC1KD and ciPTEC-PC1Pt. To conclude, we demonstrated that selective CaSR activation in human ciPTEC carrying PKD1 mutation increases \([\text{Ca}^{2+}]_{i}\), reduces intracellular cAMP and mTOR activity, reversing the principal dysregulations considered the most proximal events in ADPKD pathogenesis, making CaSR a possible candidate as therapeutic target.

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is the fourth leading cause of end stage renal disease (ESRD) in adults, characterized by the progressive, bilateral growth and enlargement of fluid-filled cysts in kidneys that leads to a decline in renal function. It has a frequency of 1:400–1:1000 and 50% of adult PKD patients will require dialysis or kidney transplantation by their 6th decade. ADPKD is a dominant inherited disease caused by loss-of-function mutations in the \(PKD1\) or \(PKD2\) gene, encoding polycystin-1 (PC1) or polycystin-2 (PC2), respectively. PKD1 is responsible for 85% of the cases in clinically-affected individuals (ADPKD1) and is associated with a more severe clinical course, while mutations in \(PKD2\) are present in the remaining 15% of the patients (ADPKD2), who generally show a milder renal functional decline and a lower renal complication rate.

During the past few years, understanding of ADPKD pathogenesis has been considerably deepened, nevertheless the function of the polycystins and the molecular mechanisms underlying cysts development are still poorly understood. Polycystins belong to a family of eight proteins containing transmembrane domains that form a heteromeric molecular complex in the plasma membrane and cilia. PC1 is localized to the primary cilium and to the cell junctions where it probably functions as a receptor and/or adhesion molecule. PC2 is a calcium-permeable nonselective cation channel, expressed on the primary cilium, endoplasmic reticulum, and the plasma membrane. PC1 and PC2 interact to form the PC complex, which localizes to the primary cilia and

1Department of Biosciences, Biotechnologies and Biopharmaceutics, University of Bari, Bari, 70125, Italy. 2Department of Pediatric Nephrology, Radboud University Nijmegen Medical Centre, Nijmegen, 6525 HP, The Netherlands. 3Department of Pediatric Nephrology, University Hospital Gasthuisberg, Leuven, 3000, Belgium. 4Department of Development & Regeneration, University of Leuven (KU Leuven), Leuven, 3000, Belgium. 5Istituto Nazionale di Biostrutture e Biosistemi, Roma, 00136, Italy. 6Center of Excellence in Comparative Genomics (CEGBA), University of Bari, Bari, 70125, Italy. Correspondence and requests for materials should be addressed to A.D.M. (email: annarita.dimise@uniba.it) or G.V. (email: giovanna.valenti@uniba.it)
acts as a mechanosensor that controls calcium influx through the plasma membrane, induced by mechanical stimuli. PC1 and PC2 are also known to regulate intracellular calcium release from the endoplasmic reticulum (ER) through their interaction with the inositol 1,4,5-trisphosphate receptor (IP3R). In conditionally immortalized, plasma membrane-permeabilized human proximal tubule epithelial cells, the simultaneous expression of both polycystins amplifies the IP3-induced calcium release, while PC1 alone or PC2 alone has no effect. Despite the diversity of conclusions reached in the numerous studies analyzing the mechanisms involved in intracellular calcium regulation operated by PC1 and PC2, most of them are consistent with the hypothesis that polycystins by themselves and through their interaction with other calcium channels in the endoplasmic reticulum prevent the depletion of intracellular stores, maintaining the amplitude of physiological calcium oscillations. The similar effect of both polycystins on the intracellular calcium homeostasis explains why loss-of-function mutations in the PKD1 or in the PKD2 genes both cause ADPKD.

Calcium signaling dysregulation is strictly correlated to another ADPKD hallmark represented by elevated cAMP levels. Numerous animal models of PKD show increased content of cAMP in the kidney, an effect also observed in cholangiocytes, in vascular smooth muscle cells, and in choroid plexus. Several hypotheses have connected the increased levels of cAMP in PKD tissues to the dysregulation of intracellular calcium signaling, specifically correlating the reduced cytosolic calcium to both cAMP synthesis and hydrolysis. The decrement in cytosolic calcium is supposed to cause the activation of the calcium-inhibitable adenylyl cyclase 6 (AC6), to directly inhibit calcium/calmodulin-dependent phosphodiesterase 1 (PDE1) and to increase the levels of cyclic guanosine monophosphate, thus inhibiting indirectly the cyclic guanosine monophosphate-inhibitable PDE3. Increased cAMP levels are also attributable to the dysfunction occurred in the PC ciliary complex where the disruption of the PC2-mediated calcium entry, activates AC5/6 and inhibits phosphodiesterase 4C (PDE4C). Another study proposed the activation of the calcium/calmodulin-dependent phosphodiesterase 1 (PDE1) and the oligomerization and translocation of STIM1 to the plasma membrane, caused by the ER calcium stores depletion. cAMP increase causes protein Kinase A (PKA) activation, which leads to ERK-mediated phosphorylation of tuberin (the TSC2 gene product), inducing upregulation of the mammalian target of rapamycin, mTOR, implicated in the pro-proliferative pathway. This process has also been linked to the abnormal transcriptional activation of aerobic glycolysis and intracellular ATP accumulation, allowing liver kinase B1 inhibition which, together with AMP-activated protein kinase (AMPK) inhibition, may further enhance mTOR signaling.

The two crucial dysregulations in ADPKD, intracellular calcium and cAMP levels, are two pathways both regulated in the kidney by the activation of the extracellular calcium-sensing receptor (CaSR). CaSR is a G-protein-coupled receptor, originally cloned from the bovine parathyroid gland and successively identified in various organs. Besides the parathyroid gland, the key CaSR-expressing organs are intestine, bone, and kidney. The CaSR senses changes in extracellular calcium concentrations and regulates parathyroid hormone (PTH) secretion and renal tubular calcium reabsorption to maintain serum calcium levels within the normal range. Increased serum calcium concentrations activate CaSR expressed in the parathyroid gland eliciting a Gq-protein cascade that activates the phospholipase C (PLC) pathway and prevents exocytosis of PTH. In the proximal tubule, CaSR is expressed on the apical membrane and its activation evoked by an increment in luminal calcium, decreases PTH-induced intracellular calcium accumulation by inhibiting adenylyl cyclase.

In a recent work, we have shown that conditionally immortalized human proximal tubule epithelial cells (ciPTEC), isolated from urine of a healthy volunteer (ciPTECw) and in the wild type clone (ciPTEC-PKI), were used in this study. Both cell type expressed the monomeric form at 130 kDa, the glycosylated monomeric one at 160 kDa, and the dimeric receptor at 250 kDa. No difference in CaSR expression levels was observed in the two cell lines (data not shown). Immunofluorescence CaSR localization in monolayers of polarized ciPTEC revealed an apical plasma membrane expression of the receptor in both cell lines as it occurs in native renal proximal tubule epithelial cells.

Results
Expression and functional characterization of CaSR in ciPTECw and ciPTEC-PKI. The endogenous CaSR expression in ciPTECw stably knocked down for polycystin-1 (ciPTEC-PKI) and in the wild type clone (ciPTECw) was evaluated by Western blotting. Both cell type expressed the monomeric form at 130 kDa, the glycosylated monomeric one at 160 kDa, and the dimeric receptor at 250 kDa. Moreover, no difference in CaSR expression levels was observed in the two cell lines (data not shown).

Intracellular and ER calcium content in ciPTECw and ciPTEC-PKI. Polycystin dysfunctions have been proven to cause a reduction in steady state calcium levels which contributes to cyst formation. It has been shown that cultured epithelial cells derived from human ADPKD cysts have a basal intracellular...
calcium content approximately 20 nM lower than normal human kidney (NHK) cells. Therefore, we first measured the cytosolic calcium levels at rest in our cell models. Calcium calibration experiments revealed a significant lower \([\text{Ca}^{2+}]\)_i in ciPTEC-PC1KD compared to ciPTECwt (ciPTEC-PC1KD = 93.44 ± 4.13 nM, n = 107; ciPTECwt = 113.4 ± 3.7 nM, n = 95; \(P = 0.0004\)), confirming calcium dysregulation in ADPKD cells and in animal models (Fig. 3A).

Since it is known that PC complex disruption has implications in abnormal ER calcium depletion, FRET experiments were performed to measure calcium levels in the ER. ciPTEC-PC1KD had significant lower ER

---

**Figure 1.** Endogenous CaSR expression and localization in ciPTECwt and ciPTEC-PC1KD. (A) Immunodetection of CaSR in homogenates of ciPTECwt and ciPTEC-PC1KD, after 11 days of maturation at 37 °C. Specific anti-CaSR antibodies revealed both CaSR forms at 130 and 250 kDa, corresponding to the monomeric and mature receptor. The figure shows a representative blot. (B) Immunofluorescence localization of CaSR in polarized ciPTEC, showing its predominant apical plasma membrane localization.

**Figure 2.** Effects of CaSR positive allosteric modulator, NPS-R568, on (\(\text{Ca}^{2+}\))_i levels. ciPTECwt (A) and ciPTEC-PC1KD (B) were grown for 11 days at 37 °C and stimulated with NPS-R568 10 \(\mu\)M and ATP 100 \(\mu\)M. Fluorescence ratio 340/380 nm was recorded. Each trace is representative of 4 different experiments with similar results. (C) Fluorescence ratio 340/380 nm was recorded and responses to NPS-R568 were calculated as the percentage of changes in fluorescence (Delta Ratio Fura-2%), normalized to the fluorescence ratio observed in the presence of the ATP stimulus (100%). Histogram shows a significant lower intracellular calcium increase in ciPTEC-PC1KD compared to ciPTECwt. Data were analyzed with One-way ANOVA followed by Newman-Keuls multiple comparisons test and are expressed as means ± SEM (**\(P < 0.01\) vs. ATP; ****\(P < 0.0001\) vs. ATP or ciPTECwt).
calcium levels compared with ciPTECwt (Fig. 3B), consistent with the lower increase in intracellular calcium after CaSR stimulation showed in Fig. 2C (ciPTEC-PC1KD = 90.46 ± 2.09%, n = 140, vs. ciPTECwt = 100%, n = 152; P = 0.0008).

CaSR activation decreases cAMP levels in ciPTEC-PC1KD. As in native cells from cysts 41, significantly higher cAMP levels were found under basal conditions in ciPTEC-PC1KD with respect to ciPTECwt (ciPTEC-PC1KD = 104.6 ± 0.81%, n = 96, vs. ciPTECwt = 100%, n = 114; P < 0.0001) (Fig. 4). Interestingly, treatment of cells with the CaSR positive allosteric modulator NPS-R568 (10 μM for 30 min) induced a significant decrease in ciPTEC-PC1KD intracellular cAMP content (ciPTEC-PC1KD + NPS-R568 = 99.76 ± 0.68%, n = 115), approaching the levels observed in ciPTECwt at basal conditions. This result represents the first evidence that cells knocked down for PC1 expressing an endogenous functional CaSR, respond to the calcimimetic NPS-R568 with a significant reduction in cAMP intracellular levels, thus attenuating one of the pivotal dysregulations characterizing ADPKD.

mTOR dysregulation is improved by CaSR activation in ciPTEC-PC1KD. mTOR activity was investigated by the evaluation of the phosphorylated forms of its downstream effector, the S6 ribosomal protein, and its upstream effector, AMP-activated protein kinase (AMPK). Specifically, S6 is used as a marker of mTORC1 pathway activation 42. mTOR signaling activates p70S6 kinase which in turn phosphorylates Ser235, Ser236, Ser240 and Ser244 of S6 43. Anti-pS6 (Ser235/236) antibodies were used for pS6 immunodetection (Fig. 5). pS6 expression at rest was significantly higher in ciPTEC-PC1KD compared with ciPTECwt (ciPTECwt = 1 ± 0.06, n = 10; ciPTEC-PC1KD = 2.88 ± 0.6, n = 8; P < 0.001). Importantly, CaSR activation by NPS-R568 (10 μM for 30 min), reduced S6 protein levels in ciPTEC-PC1KD to those observed in ciPTECwt at basal conditions (ciPTEC-PC1KD + NPS-R568 = 1.07 ± 0.3, n = 10). CaSR stimulation did not affect pS6 levels in ciPTECwt with respect to untreated cells.

Conversely, mTOR is known to be inhibited by AMPK 44, and AMPK activity is decreased in PC1 knocked down cells compared with control. We evaluated AMPK in ciPTEC by Western blotting experiments (Fig. 6) examining its phosphorylation at Thr172 in the α subunit (pAMPK). The phosphorylated levels of AMPK were significantly lower in ciPTEC-PC1KD compared with ciPTECwt (ciPTECwt = 1 ± 0.05, n = 15; ciPTEC-PC1KD = 0.58 ± 0.09, n = 15; P < 0.0001). After treatment with NPS-R568 (10 μM for 30 min), pAMPK levels in ciPTEC-PC1KD were reversed to levels comparable to wt cells (ciPTEC-PC1KD + NPS-R568 = 1.32 ± 0.2, n = 15). No changes were observed in pAMPK expression in ciPTECwt after NPS-R568 treatment with respect to untreated cells.

AMPK regulates mTOR also through the direct phosphorylation of the tumor suppressor TSC2 44, resulting in mTOR inhibition. We therefore evaluated the serine/threonine kinase Akt, an upstream negative regulator of TSC2, whose activation mediates cell growth, proliferation, and survival. In ciPTEC-PC1KD cells, the levels of phosphorylated Akt (pAkt, Ser-473) were significantly reduced with respect to wt (ciPTECwt CTR = 1 ± 0.05,
ciPTEC-PC1KD CTR = 0.57 ± 0.11, n = 8; P < 0.01) (Fig. 7). Interestingly, CaSR stimulation with NPS-R568, by increasing cytosolic calcium concentration, elicited an increase in pAkt in ciPTEC-PC1KD with respect to ciPTECwt, restoring the basal content (ciPTEC-PC1KD + NPS-R568 = 1.16 ± 0.13, n = 8). Since increased Akt activity inhibits cAMP-dependent B-Raf/ERK, ERK/pERK were next evaluated. pERK 1/2 levels were found increased in ciPTEC-PC1KD compared to ciPTECwt (ciPTECwt = 1 ± 0.02, ciPTEC-PC1KD = 1.89 ± 0.34, n = 7; P < 0.05) (Fig. 8). Interestingly, CaSR activation decreased pERK 1/2 expression in ciPTEC-PC1KD to the levels observed in wt cells (ciPTEC-PC1KD + NPS-R568 = 0.82 ± 0.3, n = 7).
CaSR activation reverses PKD1 dysregulations in ciPTEC isolated from an ADPKD1 patient.

The effect of CaSR activation in ciPTEC-PC1Pt was next evaluated. The absence of immunoreactive PC1 band in the ADPKD patient (10.032) was previously reported. CaSR expression was confirmed by Western blotting experiments, showing the presence of both the forms of the receptor, the monomeric and mature protein (Fig. 9A), and by confocal analysis in monolayers of polarized ciPTEC-PC1Pt proving the apical localization of CaSR (Fig. 9B).

The functional expression of the receptor present in ciPTEC-PC1Pt was next evaluated by single-cell epifluorescence imaging. Cells were loaded with 6 μM Fura-2 AM and treated with 10 μM NPS-R568 (Fig. 10A). As ciPTEC-PC1KD, ciPTEC-PC1Pt responded to CaSR activation with a significant increase in cytosolic calcium (29.51 ± 3.93% vs. ATP 100%, n = 38, P < 0.0001) (Fig. 10B). Interestingly, the calcium levels observed were comparable with those reported in ciPTEC-PC1KD, suggesting a similar dysregulation in calcium homeostasis in cells isolated from ADPKD1 patient urine as well.

Of note, as observed in ciPTEC-PC1KD, CaSR stimulation with NPS-R568 increased pAkt to levels detected in ciPTECwt at rest. One-way ANOVA followed by Newman-Keuls multiple comparisons test was used to analyze data.
The expression and activity of mTOR and AMPK were also evaluated in ciPTEC-PC1Pt. CaSR activation with NPS-R568 caused a significant decrease in pS6 levels compared to unstimulated cells which reflects a lower mTOR activity in response to CaSR activation in this cell line (ciPTEC-PC1Pt+NPS-R568 = 0.52 ± 0.08, n = 10, vs. ciPTEC-PC1Pt CTR = 1 ± 0.09, n = 10; P = 0.0004; Fig. 12). Conversely, CaSR stimulation resulted in an increased AMPK activity as demonstrated by the significantly higher levels of the phosphorylated form in NPS-R568 treated cells (ciPTEC-PC1Pt+NPS-R568 = 1.51 ± 0.22, n = 8, vs. ciPTEC-PC1Pt CTR = 1 ± 0.07, n = 11; P = 0.02; Fig. 13).

Discussion
The major results obtained in this work can be summarized as follows: a. ciPTEC with stable knockdown of PKD1 or isolated from the urine of an ADPKD1 patient endogenously express functional CaSR; b. both cell lines showed the same dysregulations in intracellular calcium, cAMP and mTOR pathways reported in animal models of ADPKD112,14,20,46–50 and human polycystic kidneys38,51; c. these dysregulations were reversed by activation of CaSR with the allosteric modulator NPS-R568. These results indicate that CaSR may represent a therapeutic target in Autosomal Dominant Polycystic Kidney Disease 1. The first evidence that ciPTEC isolated from urine of a healthy volunteer endogenously express a functional CaSR was recently provided by our group35. We showed that CaSR activation causes a decrease in cytosolic cAMP...
content and an increase in intracellular calcium attributable to CaSR coupling to Gq, resulting in PLC activation and IP3-dependent release of calcium from intracellular stores35. In the aim of exploring, at cellular level, the potentiality of CaSR as therapeutic target in the treatment of ADPKD, we considered, as valuable model system, ciPTEC obtained by immortalizing cells exfoliated from urine sediments of an ADPKD1 patient and ciPTEC obtained from healthy subject, knocked down for PKD1.

The complexity of the renal cyst formation still makes ADPKD a poorly understood disease, nevertheless it is known to involve cell clonal proliferation, increased apoptosis, abnormal epithelial cell phenotype, extracellular matrix alterations and inflammation52,53. Recent evidence suggests that for cystogenesis to occur, there is

Figure 10. Functional characterization of endogenous CaSR in ciPTEC-PC1Pt. (A) Cells were grown for 11 days at 37°C and stimulated with NPS-R568 10μM and ATP 100μM. Fluorescence ratio 340/380 nm was recorded. Each trace is representative of 3–4 different experiments with similar results. (B) Histogram shows that NPS-R568 stimulation in ciPTEC-PC1Pt elicited an intracellular calcium increase comparable to the one obtained in ciPTEC-PC1KD. Fluorescence ratio 340/380 nm was recorded and responses to NPS-R568 were calculated as the percentage of changes in fluorescence (Delta Ratio Fura-2%), normalized to the fluorescence ratio observed in the presence of the ATP stimulus (100%). Data were analyzed with One-way ANOVA followed by Newman-Keuls multiple comparisons test and are expressed as means ± SEM (****P < 0.0001 vs. ATP).

Figure 11. Effect of CaSR activation on cAMP content in ciPTEC-PC1Pt. FRET experiments revealed that CaSR stimulation with NPS-R568 10μM significantly reduced cAMP levels in ciPTEC-PC1Pt with respect to untreated cells. Data are expressed as means ± SEM (***P = 0.0006).
no requirement of a complete loss of PC1 or PC2 function, but their functionality must be reduced to a certain threshold level, which marks the correspondence between PC1 dosage and rate of disease severity. In the renal collecting duct, mutations in PKD1 or PKD2 are associated with a decrease in intracellular calcium and increase in cAMP with consequent activation of PKA exposing collecting duct principal cells to the constant tonic effect of vasopressin, activating downstream signaling pathways responsible for impaired tubulogenesis, cell proliferation, increased fluid secretion, and interstitial inflammation.

The pivotal role of cAMP in the pathogenesis of ADPKD represents a key point for treatment strategies rational to lower its levels in cystic tissues. Clinical trials of vasopressin receptor 2 (V2R) antagonists have shown encouraging results. In fact, several studies demonstrated that V2 receptor antagonists (mozavaptan and/or tolvaptan) attenuate the progression of PKD in 

Figure 12. Effect of CaSR stimulation on mTOR expression and activity in ciPTEC-PC1Pt, evaluated by pS235/236-S6 (pS6) levels analysis. (A) Equal amount of proteins from cells (30 μg) were immunoblotted for total S6 and pS6. The figure shows representative blots. (B) Densitometric and statistical analysis performed with unpaired t-test (means ± SEM, ***P = 0.0004) revealed that pS6 expression was significantly decreased by the treatment with NPS-R568 compared to untreated cells.

Figure 13. NPS-R568 stimulation effect on AMPK phosphorylation levels in ciPTEC-PC1Pt. (A) Equal amount of proteins (30 μg) were immunoblotted with antibodies specific for total AMPK or for pT172-AMPK (pAMPK). The figure shows representative blots. (B) Signals were semiquantified by densitometry. Statistical analysis (means ± SEM, *P = 0.02) demonstrated that CaSR activation elicited a significant increase in pAMPK levels with respect to untreated cells. Data were analyzed by unpaired t-test.
suboptimal efficiency of tolvaptan therapy. Interestingly, a recent study showed that treatment of pcy mice (a NPHP orthologous animal model), with the calcimimetic NPS-R568, a positive allosteric CaSR modulator, inhibited progression of cysts growth and renal fibrosis. NPS-R568 was less effective in later-stage NPHP but did significantly reduce kidney weight. In an interesting work, Gattone and coworkers showed that NPS-R568, administrated to male Cy/+- rats from 20 to 38 weeks of age, an age when CKD is well established, reduced the development of renal cysts and ameliorated the kidney cystic disease. In contrast, another study evaluating the effect of CaSR activation with NPS-R568 in animal models orthologous to human ADPKD and/or ARPKD (Autosomal Recessive Polycystic Kidney Disease), reported no detectable effect on cystogenesis but possible beneficial effect on interstitial fibrosis. A possible explanation for the lack of calcimimetic effect is that the administration of NPS-R568 resulted in hypocalcemia and therefore it is likely that the effects of NPS-R568 on intracellular calcium and cAMP are negated by the reduction in extracellular calcium.

In the present study, we show the cellular effect of CaSR activation with NPS-R568 on the most proximal events in ADPKD pathogenesis, calcium and cAMP, in ciPTEC of human proximal tubule origin stably knocked down for PKD1 or isolated from the urine of an ADPKD1 patient. Both the cell lines expressed the monomeric and the mature glycosylated forms of CaSR that localized at the apical plasma membrane as in native proximal tubule cells. At basal conditions, ciPTEC-PC1KD showed lower cytosolic calcium concentrations with respect to ciPTECwt, reporting one of the two major dysregulations observed in ADPKD1 animal models. Of note, functional experiments showed that CaSR activation, elicited by NPS-R568 stimulation, caused an increase in cytosolic calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt both having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solicits calcium both in ciPTEC-PC1KD and ciPTEC-PC1Pt having calcium values significantly lower than ciPTECwt. In a previous work, we already showed that CaSR expressed in ciPTEC couples to Gq and its activation solici...
Antibodies. Monoclonal CaSR antibody recognizing amino-acid 15–29 at the extracellular N-terminus was from Sigma-Aldrich, Milan, Italy. Rabbit anti-Phospho-S6RP (Ser235/236), anti-Tot S6RP, anti-Phospho-AMPK (Thr172 of its α subunit) and anti-Tot AMPK antibodies were purchased from Cell Signaling Technology (Beverly, Massachusetts, USA). Mouse anti-ERK 1/2 and rabbit anti-Phospho-ERK 1/2 (Thr185/Tyr187) were from Merck Millipore (Darmstadt, Germany). Secondary goat anti-rabbit, goat anti-mouse and goat anti-mouse IgG biotin antibodies were purchased from Sigma-Aldrich, Milan, Italy. Streptavidin-488 conjugate was from Alexa Fluor (Molecular Probes, Eugene, Oregon, USA).

Generation of ciPTEC knocked down for polycystin-1 or from ADPKD1 patient. ciPTEC were generated as described by Wilmer and colleagues. Primary cells were cultured by collecting mid-stream urine within 5h after collection. Urine sediment was resuspended in DMEM Ham’s F12 medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 mg/ml streptomycin, ITS (5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml selenium), 36 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor (EGF) and 40 pg/ml triiodothyronine. The suspension was placed at 37°C in a 5% CO2 incubator.

Primary cells were immortalized as previously described. Briefly, cells were infected with SV40T and hTERT vectors, containing respectively genetin (G418) and hygromycin resistance. Subconfluent cell layers were transferred to 33°C and selected by using G418 (400 μg/ml) and hygromycin B (25 μg/ml) for 10 days. Stable knocked down ciPTEC for polycystin-1 (ciPTEC-PC1KD) were obtained transducing a cloned ciPTEC line (ciPTECwot) of a healthy individual (34.8) by adding lentiviral vectors encoding miR-shRNA directed against polycystin-1, cloned in tandem (pCHMWS Bsd 2xmiRNA PKD1), to the culture medium. Transduced cells were selected using 10 μg/ml blasticidin. Alternatively, ciPTEC derived from an ADPKD1 patient (10.032) with known germline PKD1 mutation (ciPTEC-PC1P1) were isolated as described. Experiments were performed prior cellular maturation for 11 days at 37°C. The reduced expression of PC1 was showed by Mekahli and coworkers which biochemically characterized these cell lines.

Immunofluorescence Microscopy. Immunofluorescence localization of CaSR in polarized ciPTEC was performed as previously described. Cells were incubated with antibodies diluted in block solution containing 2% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) Tween-20 in HBSS against the calcium-sensing receptor (CaSR, 1:800 dilution) at 4°C overnight. Following treatment with secondary rabbit-anti-mouse-biotin antibodies followed by Streptavidin-488, samples were mounted on glass slides with Mowiol. Images were obtained with a confocal microscope Leica TCS SP2 (Leica Microsystems, Heerbrugg, Switzerland).

Cell Preparations. ciPTEC were seeded onto 100-mm dishes and grown at 37°C for 11 days, then were lysed in Cell Fractionation Buffer (20 mM NaCl, 130 mM KCl, 1 mM MgCl2, 10 mM Heps, pH 7.5) in the presence of proteases (1 mM PMSE, 2 mg/ml leupeptin and 2 mg/ml pepstatin A) and phosphatases (10 mM NaF and 1 mM sodium orthovanadate) inhibitors. Cellular debris was removed by centrifugation at 12,000 x g for 20 min at 4°C. The supernatants were collected and used for immunoblotting studies.

Gel Electrophoresis and Immunoblotting. ciPTEC lysates were separated on 10% bis-tris acrylamide gels under reducing conditions. Protein bands were electrophoretically transferred onto Immobilon-P membranes (Millipore Corporate Headquarters, Billerica, USA) for Western blot analysis, blocked in TBS-Tween-20 containing 5% BSA and incubated with primary antibodies O/N. Anti-CaSR was used at 1:800 dilution, anti-Phospho-S6RP and anti-Tot S6RP were used at 1:1000 dilution, anti-Phospho-AMPK and anti-Tot AMPK at 1:500 dilution. Immunoreactive bands were detected with secondary antibodies conjugated to horseradish peroxidase (HRP) obtained from SantaCruz Biotechnologies (Tebu-Bio, Milan, Italy). Membranes were developed using Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, USA) with Chemidoc System (Bio-Rad Laboratories, Milan, Italy). Representative figures are shown. Densitometry analysis was performed with Scion Image. Data were summarized in histograms with GraphPad Prism (Graphpad Software Inc. La Jolla, CA, USA).

Video-Imaging Experiments. ciPTEC were grown on 25-mm glass coverslips at 37°C for 11 days, then were loaded with 6 μM Fura-2 AM for 15 min at 37°C in DMEM. Ringer’s Solution was used to perfuse cells during the experiment containing 120 mM NaCl, 4 mM KCl, 15 mM NaHCO3, 1 mM MgCl2, 15 mM Heps, 0.5 mM NaH2PO4, 10 mM Glucose, 1 mM CaCl2, 0.5 mM Na2HPO4, 0.4 mM MgSO4, pH 7.4 (modified by 9,36). Measurements were performed using an inverted microscope (Nikon Eclipse TE2000-S microscope) equipped for single cell fluorescence measurements and imaging analysis. The sample was illuminated through a 40 × oil immersion objective (NA = 1.30). The Fura-2 AM loaded sample was excited at 340 and 380 nm. Emitted fluorescence was passed through a dichroic mirror, filtered at 510 nm (Omega Optical, Brattleboro, VT, USA) and captured by a cooled CCD camera (Cool SNAP HQ, Photometrics). The ratio of fluorescence intensities at 340 and 380 nm was plotted using Metafluor software (Molecular Devices, MDS Analytical Technologies, Toronto, Canada).

For the experiments at steady state, intracellular calcium level was calibrated and then calculated as described by Grynkiewicz. Briefly, intracellular calcium concentration (Ca2+) was determined from the emission fluorescence ratio of the two excitation wavelengths accordingly to the formula (Ca2+) = Kd · (Q(R - Rmin)/(Rmax - R)), where Kd (224 nM) indicates the dissociation constant of Fura-2 AM for (Ca2+), and Q indicates the ratio of the fluorescence intensities (F) at the minimum and the maximum calcium concentration at 380 nm. Each sample was calibrated by the addition of 5 μM ionomycin in the presence of 1 mM EGTA (Rmin) followed by 5 μM ionomycin in 5 mM CaCl2 (Rmax).
Fluorescence Resonance Energy Transfer (FRET) Measurements. To evaluate intracellular cAMP levels and endoplasmic reticulum (ER) calcium content, fluorescence resonance energy transfer (FRET) experiments were performed as described\(^\text{5,6}\). Briefly, cPiTEC were seeded onto 20-mm glass coverslips at 37 °C for 11 days. For cytosolic cAMP evaluation, cells were transiently transfectected with a plasmid encoding the H96 probe containing cAMP binding sequence of Epac1 between CFP and cp173Venus-Venus\(^\text{5,6}\). For ER calcium levels measurements, cells were transiently transfectected with a plasmid encoding the D1ER Cameleon\(^\text{5,6}\). Experiments were performed 48 hours post-transfection. Cells were left under basal condition or, for intracellular cAMP evaluation, also stimulated with NPS-R568 (10 μM for 30 min at 37 °C) in Ringer’s solution described above, containing 2 mM CaCl\(_2\).

FRET measurements were carried out using MetaMorph software (Molecular Devices, MDS Analytical Technologies, Toronto, Canada). CFP and YFP were excited at 436 and 500 nm, respectively; fluorescence emitted was measured at 480/40 nm for CFP and 535/30 nm for YFP and FRET. Corrected normalized FRET values were determined as already described\(^\text{7,8}\). Each image was corrected for CFP cross-talk and YFP cross- excitation. Therefore, netFRET = |IFRETbg − ICFPbg K\(_{1}\) − IYFPbg (K\(_{2}\) − αK\(_{1}\)))/(1 − δK\(_{1}\)) where IFRETbg, ICFPbg, and IYFPbg are the background-corrected pixel gray values measured in the FRET, CFP and YFP windows, respectively; K\(_{1}\), K\(_{2}\), α and δ are calculated to evaluate the crosstalk between donor and acceptor. The integrated fluorescence density values of the images from each cell were analyzed using MetaMorph and Microsoft Excel software.

Statistical analysis. One-way ANOVA followed by Newman–Keuls multiple comparisons test or t-test were used for the statistical analysis. All values are expressed as means ± SEM. A difference of P < 0.05 was considered statistically significant.

Data availability statement. All data generated or analysed during this study are included in this published article.

Ethical approval and informed consent. The current study is in accordance with the institutional ethical guidelines for obtaining human cell lines for research and was approved by the corresponding ethical committee at University Hospitals Leuven. An informed consent was obtained from all participants and/or their legal guardian/s.

References

45. Mekahli, D. et al. Polycystin-1 but not polycystin-2 deficiency causes upregulation of the mTOR pathway and can be synergistically targeted with rapamycin and metformin. *Pflugers Arch* **466**, 1591–1604 (2014).

Acknowledgements
The authors thank Amgen (Amgen Dompé S.p.a., Milan, Italy) for providing NPS-R568. We thank Diede Booltink for the assistance during her internship in our laboratory. This study was supported in part by Telethon funding (grant number GGP13227) and by ASI (Italian Space Agency, grant number 2013-091-R.0). EL is supported by the Fund for Scientific Research, Flanders, (grant numbers ZKC5782 and G0B1313N).

Author Contributions
A.D.M. designed, performed research and wrote the paper; G.T. designed research; M.C. performed research; M.R. performed research and analysed data; B.V.H., D.M. and E.L. contributed cells and analytic tools; E.L. designed research; G.V. designed research and wrote the paper.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-23732-5.

Competing Interests: The authors declare no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2018