3D spheroid defects in NPHP knockdown cells are rescued by the somatostatin receptor agonist octreotide

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Ghosh AK, Hurd T, Hildebrandt F. 3D spheroid defects in NPHP knockdown cells are rescued by the somatostatin receptor agonist octreotide. Am J Physiol Renal Physiol 303: F1225–F1229, 2012.—Ciliopathies are a heterogeneous group of diseases that exhibit broad clinical phenotypes, including renal cysts, retinal degeneration, and cerebellar vermis aplasia. Nephronophthisis (NPHP) is a renal ciliopathy that causes chronic kidney disease and is characterized by kidney cysts at the corticomedullary border. Among the 10 different disease-causing genes (NPHP1-NPHP10), mutations in NPHP3, NPHP6, or NPHP8 cause the most severe ciliopathy variants of NPHP, Joubert syndrome, and Meckel Syndrome. In this study, we tested the hypothesis that loss of function of these three most severe disease-associated genes leads to developmental defects in a three-dimensional (3D) renal cell culture [murine (m) inner medullary collecting duct (mIMCD) 3] model by either lack of cilia formation and/or cell polarity defects. Stable knockdown cell lines were examined in 3D spheroid culture followed by rhodamine-phalloidin staining to assess spheroid architecture. We observed significantly higher percentages of abnormal spheroids for all three stable cell lines compared with control short-hairpin RNA cells. In addition, stable knockdown of Nphp3, Nphp6, and Nphp8 results in reduced cilia numbers and elevated cAMP levels in mIMCD3 cells. We demonstrate that, following gene knockdown of Nphp3, Nphp6, or Nphp8, treatment with the somatostatin agonist octreotide (2 μM) reduces the percentage of abnormal spheroids compared with control. This study reveals that the loss of Nphp3, Nphp6, or Nphp8 leads to cilia abnormalities and cell polarity defects, resulting in spheroid abnormalities, which can be rescued by inhibiting cAMP levels with octreotide treatment.

nephronophthisis; three-dimensional; polycystic kidney disease; spheroids

NPHRONOPHTHISIS-RELATED CILIOPATHIES (NPHP-RC) are a clinically and genetically heterogeneous group of diseases that exhibit shared clinical phenotypes, including renal cysts, retinal degeneration, and cerebellar vermis aplasia (6). Among the 10 different NPHP disease-causing genes (NPHP1-NPHP10), mutations in NPHP3, NPHP6, or NPHP8 cause the most severe NPHP-RC, Joubert syndrome, and Meckel Syndrome (7). Studies into these diseases have revealed that virtually all patients with polycystic kidney disease (PKD) (16). Elevated cAMP levels are found in the kidney, liver, vascular smooth muscle, and choroid plexus, in various animal models of PKD and NPHP (4, 9, 14, 16). A recent study from our group has demonstrated that Nphp10 (Sdccag8) disappears from cell junctions upon treatment of cells with the cAMP analog 8-bromo-cAMP (11). These studies prompted us to examine whether loss of function of NPHP-RC genes causes cellular abnormalities in the renal cystic disease model of three-dimensional (3D) spheroids (10) and whether the effect can be rescued by modulation of cAMP levels.

METHODS

Generation of stable knockdown murine inner medullary collecting duct cells: shRNA duplexes were designed and cloned into RNAi-Ready pSIREN-RetroQ vector (Clonetech). Short-hairpin RNA (shRNA) target sequences for Nphp genes were as follows: Nphp3 (5′-GGATTAATC-TACCAGGTGTTT-3′, 5′-GGATTCCACATCGAAGAAAAG-3′, and 5′-GGACATACACGAGCAGA-3′), Nphp6 (5′-GGAGAAGTTGGTTCAGGA-3′, 5′-GGAGAACCTTCATGTTTCA-3′, and 5′-GGACGAGACAGTAGCGACA-3′), and Nphp8 (5′-GGATCA-AGCATTGAGAATTTTA-3′, 5′-GGATGGGATGCTACTCAAA-3′, and 5′-GGGAAATCGGAAAGAGAG-3′).

Retrovirus was generated by transfecting 293T cells with the shRNA constructs along with pRD-G and p-gag-pol constructs. Murine (m) inner medullary collecting duct (mIMCD) 3 cells were infected with the supernatant. IMCD3 cells were then selected with puromycin resistance for 10 days at 10 μg/ml.

Quantitative real-time PCR. RNA was isolated using the RNeasy Mini Kit (Qiagen). First-strand cDNA was performed using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Accumulation of PCR products was measured in real-time by using iTaq SYBR green supermix with the MyiQ Single Color Real-time PCR Detection System (Bio-Rad).

Spheroid assay. 3D spheroid cell culture was performed using Geltrex Reduced Growth Factor Basement Membrane Matrix (Invitrogen) in chamber slides. Spheroids were stained with rhodamine-phalloidin and DAPI. The spheroid architecture was examined under an inverted confocal microscope (Leica).
**RESULTS AND DISCUSSION**

In the present study, we generated mIMCD3 cell lines of stable knockdown of Nphp3, Nphp6, or Nphp8 by retroviral delivery of three shRNA duplexes for each gene. Quantitative RT-PCR analysis revealed that all three shRNA duplexes for each gene reduced mRNA expression of each gene. However, Nphp3.1, Nphp6.1, and Nphp8.1 were more efficient than the other two shRNA duplexes for the same gene (Fig. 1, A–C). To test whether knockdown of Nphp subtype affects other Nphp mRNA expression, we used the cDNAs from these cells and tested in the context of Nphp3, Nphp6, and Nphp8 primer sets in a reciprocal real-time PCR analysis. These results confirm the specificity of the knockdown by shRNA duplexes (Fig. 1D). To test the effects of Nphp knockdown on the cell proliferation, growth assay was performed by plating an equal number of cells and counting them by an automatic counter (TC10; Bio-Rad) at different time intervals. Our data suggested retarded growth of Nphp knockdown cells compared with the sh-neg control cell line (Fig. 2A). To investigate the roles of Nphp3, Nphp6, and Nphp8 gene products in tissue architecture, we examined Nphp knockdown cell lines in a 3D spheroid culture system that reflects the cell biology of kidney collecting duct. Nphp3, Nphp6, and Nphp8 knockdown mIMCD3 cell lines were grown in the 3D culture system for 3–4 days to develop spheroid structure. Spheroids were then stained with rhodamine-phalloidin for F-actin and DAPI (for nuclear staining) to visualize spheroid architecture under an inverted confocal microscopy. Spheroid architecture was examined by focusing at the equator of the spheroids to visualize lumen formation. Spheroids with no lumen and/or misaligned nuclei were considered as abnormal (Fig. 3A).

We found significantly higher percentages of abnormal spheroids for all three stable knockdown cell lines (Nphp3, 73%; Nphp6, 84%; and Nphp8, 56%) compared with control shRNA cells (21%) (Fig. 3B). Spheroid abnormalities from cells with knockdown of Nphp genes by small-interfering RNA have also been reported in a recent study (13). We investigated the cilia defects by growing sh-neg, Nphp3, Nphp6, and Nphp8 cell lines on membrane filters under conditions of ciliogenesis (see METHODS). Cilia quantification data (Fig. 2, B and C) suggested that lack of all three Nphp genes...
under this study resulted in significant reduction in cilia numbers in the stable knockdown cells. Because cilia are sensory organelles for the kidney cells, lack of cilia might perturb signaling events critical for normal polarity maintenance.

Previous studies have established that cAMP plays a central role in the progression of cystic disease in patients with autosomal dominant PKD by stimulating epithelial cell proliferation and secretion of fluid into cysts (12). This has provided a strong rationale for therapies targeting cAMP signaling. Somatostatin analogs acting on Gi protein-coupled receptors provided an alternative path to inhibit cAMP signaling in tubular epithelial cells in patients with PKD. Research studies showed that somatostatin receptor inhibitor octreotide significantly reduces cAMP levels and rates of cysts expansion and secretion of fluid into cysts (12).
Fig. 4. Protective effects of octreotide on spheroid abnormalities caused by knockdown of Nphp3, Nphp6, or Nphp8 genes in mIMCD3 cells. IMCD3 cells with stable knockdown of Nphp3, Nphp6, and Nphp8 were treated with either vehicle (DMSO) or 2 μM octreotide during spheroid growth for 96 h. Spheroids were stained with rhodamine-conjugated phalloidin and DAPI. The morphology was examined by a confocal microscope. The percentages of abnormal spheroids were plotted in the bar diagram. Scale bars = 10 μm (B). Representative images for different cell lines are shown in A. A total of 180 (n = 180) spheroids from three different experiments were evaluated. Error bars represent the SD from the mean values.

We treated Nphp3, Nphp6, and Nphp8 knockdown spheroids with the somatostatin agonist octreotide (2 μM) and observed that treatment of knockdown spheroids with octreotide (2 μM) reduced the percentage of abnormal spheroids to 29% (Nphp3), 35% (Nphp6), and 35% (Nphp8), whereas vehicle control abnormal spheroids were 68% (Nphp3), 87% (Nphp6), and 65% (Nphp8) (Fig. 4). Because octreotide was a known ligand for somatostatin receptors (Sstr), we evaluated the mRNA expression level of somatostatin receptors (Sstr1–5) using cDNA from the mIMCD3 cell line by RT-PCR analysis. Our expression data suggested that, except for Sstr4, all other Sstr mRNAs were abundant in mIMCD3 cells (Fig. 5A). In addition, imaging analysis revealed that only Sstr3 was localized to the primary cilium (Fig. 5B). Our data are in agreement with

Fig. 5. Octreotide treatment inhibits elevated cAMP levels upon Nphp knockdown in IMCD3 cells. A: IMCD3 cells express somatostatin receptors. Designed primers for five somatostatin receptors (Sstr1, Sstr2, Sstr3, Sstr4, and Sstr5) and cDNAs from IMCD3 cells were used to analyze mRNA expression by the RT-PCR method. Agarose gel electrophoresis data revealed that all five somatostatin receptors except Sstr4 were abundant in IMCD3 cells. M, molecular weight marker. B: somatostatin receptor 3 (Sstr3) is localized to the cilia. Ciliated IMCD3 cells were stained with acetylated α-tubulin (Ac-tub) and Sstr3 antibodies. Confocal immunofluorescence images were taken for cilia (red) and Sstr3 (green) localization. Merged image revealed Sstr3 localization at the cilia. Magnified image was shown in the inset. C: bar diagram represents elevated intracellular cAMP levels upon Nphp3, Nphp6, and Nphp8 knockdown compared with the control sh-neg cells. Treatment of octreotide (2 μM) significantly reduced cAMP levels. Enzyme-linked immunosorbent assay was performed on triplicate samples using the Direct Cyclic AMP Enzyme Immunoassay kit (Arbor Assays). Statistical significance level was determined by t-test using means and SDs of triplicate samples. Two-tailed t-test was performed to test the significance of difference using means and SDs. *Significance level (P < 0.05).
the studies by others where it has been clearly demonstrated that a consensus sequence of third intracellular loop3 of Sstr3 was critical for cilia localization (1). We then analyzed the intracellular cAMP concentrations in octreotide-treated or untreated control, Nphp3-sh, Nphp6-sh, and Nphp8-sh cell lines. Our data suggested that elevated intracellular cAMP levels in Nphp knockdown cells compared with the control sh-neg cells. Octreotide treatment significantly reduces cAMP levels in the Nphp knockdown cells or control cells (Fig. 5C).

This study reveals that lack of Nphp3, Nphp6, or Nphp8 leads to cell polarity defects, resulting in spheroid abnormalities that can be rescued by inhibiting intracellular cAMP levels with octreotide treatment. Recently, in a randomized clinical trial, long-acting octreotide has been reported to slow down disease progression in patients with polycystic liver disease (8). Our study confirms that manipulation of the cAMP pathways could be one of the therapeutic approaches in treating patients with NPHP-RC.

REFERENCES


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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: A.K.G., T.W.H., and F.H. conception and design of research; A.K.G. performed experiments; A.K.G. analyzed data; A.K.G. interpreted results of experiments; A.K.G. prepared figures; A.K.G. drafted manuscript; T.W.H. and F.H. edited and revised manuscript; F.H. approved final version of manuscript.

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