

**Title: Multicellular Modeling of Ciliopathy by Combining iPS cells and  
Microfluidic Airway-on-a-chip Technology**

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**One Sentence Summary:** Combined induced pluripotent stem cells and airway-on-a-chip establish the global axis of planar cell polarity in patient-derived airway cell sheet.

**Abstract:** Muco-ciliary clearance is an essential lung function that facilitates the removal of inhaled pathogens and foreign matter unidirectionally from the airway tract and is innately achieved by coordinated ciliary beating of multiciliated cells. Should ciliary function become disturbed, mucus can accumulate in the airway causing subsequent obstruction and potentially recurrent pneumonia. However, it has been difficult to recapitulate unidirectional muco-ciliary flow using human-derived induced pluripotent stem cells (iPSCs) in vitro and the mechanism governing the flow has not yet been elucidated, hampering the proper humanized airway disease modeling. Herein, we combine human iPSCs and airway-on-a-chip technology, to demonstrate the effectiveness of fluid shear stress (FSS) for regulating the global axis of multicellular planar cell polarity (PCP), as well as inducing ciliogenesis, thereby contributing to quantifiable unidirectional muco-ciliary flow. Furthermore, we applied the findings to disease modeling of primary ciliary dyskinesia (PCD), a genetic disease characterized by impaired muco-ciliary clearance. The application of an airway cell sheet derived from patient-derived iPSCs and their gene-edited counterparts, as well as genetic knockout iPSCs of PCD causative genes, made it possible to recapitulate the abnormal ciliary functions in organized PCP using the airway-on-a-chip. These findings suggest that the disease model of PCD developed here is a potential platform for making diagnoses and identifying therapeutic targets, and that airway reconstruction therapy using mechanical stress to regulate PCP might have therapeutic value.

## Introduction

Multiciliated airway cells (MCACs) intercellularly cooperate to produce unidirectional fluid flow along luminal surfaces in several organ systems, including lung airways, nasal cavity, cerebral ventricles and fallopian tubes(1). In the lung, the muco-ciliary clearance by airway cells eliminates inhaled pathogens and foreign matter. This process requires coordinated ciliary beating of multiciliated cells to move small particles in one direction toward the hypopharynx. Failure to properly orient ciliary beating leads to chronic bronchitis and recurrent pneumonia and is implicated in various human diseases, including primary ciliary dyskinesia (PCD)(2). The planar orientation of multiciliated cells are determined by planar cell polarity (PCP) pathways including PCP core proteins, such as FZD receptors, *VANGL1* and *VANGL2* in MCACs which likely arise prior to ciliogenesis(3-5). Meanwhile, ensuring proper cilia orientation on MCACs induced from their progenitor cells, including human induced pluripotent stem cell (iPSC) derived MCACs(6), has proven challenging when using conventional air-liquid interface (ALI) culture in vitro(4, 7). Although mechanical stress, such as tension(8) or fluid shear stress (FSS)(5) reportedly regulate the PCP pathway of xenopus epidermal or mouse ependymal multiciliated cells, the effects of mechanical stress on human MCACs and their PCP, have not yet been completely elucidated. Recently, organ-on-a-chip technology has been employed to recapitulate the unidirectional muco-ciliary flow in vitro by culturing primary airway epithelial cells under a medium flow(9), however, the quantitative analysis of the coordinated muco-ciliary flow and PCP have not been shown. We hypothesized that combining human iPSCs with organ-on-a-chip technology will provide a more effective method for multicellular modeling of airway disease than conventional ALI culture, to elucidate the role played by mechanical stress on PCP. This study also leveraged the advantages of human iPSCs for investigating rare refractory lung diseases. Disease modeling based on human-

derived cells is attractive from the perspective of basic research and clinical medicine for achieving precision medicine for various refractory lung diseases(10-12). Although long-term culture of primary human bronchial basal cells was reported(13-16) and expected to be applicable for assessing the in vitro efficacy of readthrough therapeutic agents for treating PCD(17), development of a stable disease model using organ-specific primary cells, such as MCACs in the lung, remains difficult since they cannot proliferate on their own and are often damaged by environmental factors, such as smoking, pathogens and air pollution. Moreover, although patient-derived samples can be obtained from nasal brushing or bronchial biopsy, repeat procedures and subsequent cell analyses are often difficult for patients and clinicians. Alternatively, iPSCs can be derived from peripheral blood cells of patients and are expected to function as a source of various human cells(18), particularly lung cells as iPSCs can differentiate into airway and alveolar cells in organoids(6, 19-25). The primary advantages of iPSCs are their robustness for generating patient-derived functional somatic cells and isogenic control cells for disease modeling using gene-editing technologies(26). Indeed, disease modeling using patient-derived and genetic knockout iPSCs has elucidated the pathological mechanisms of various refractory lung diseases(20, 21, 24, 25, 27, 28). PCD is a predominantly autosomal recessive disorder with a reduced muco-ciliary clearance(29, 30), and an estimated prevalence of 1:10,000–20,000, which typically leads to recurrent respiratory infections, bronchiectasis, and progressive loss of lung function(29, 30). Although over 40 etiologic genes have been reported(31), they remain underestimated due to the uncertain causative mutations, phenotypic heterogeneity and difficulty associated with diagnosis(32). Making an appropriate diagnosis requires a combinatorial approach, including genetic analyses, nasal nitric oxide measurement (nNO), high-speed video microscopy (HSVM) analysis, and transmission electron microscopy (TEM) analysis. Hence, the complexity of diagnosis together with a lack of

available cell culture system for repeating analyses with appropriate isogenic controls, has made establishment of a definitive diagnostic method difficult(29, 30). We hypothesized that iPSCs might be a useful tool for definitive PCD diagnosis by enabling the determination of whether gene mutations are truly pathogenic. Although a few disease models have been reported for PCD using iPSCs, definitive methods for making a diagnosis based on genetic analyses have not been shown(25, 33, 34). Previously, we established a stepwise method of inducing functional MCACs in proximal airway epithelial cell (PAEC) spheroids from healthy donor-derived iPSCs(6). Herein, we generated PCD patient-derived iPSCs with gene-corrected counterparts, and iPSC lines with PCD causative gene knocked out. We then analyzed iMCACs combined with airway-on-a-chip technology, exploring whether or not ciliary beating and PCP coordinate intercellularly in PCD patient-derived iMCACs.

## **Results**

### **Unidirectional muco-ciliary transport by iMCACs cultured under fluid shear stress**

MCACs derived from healthy donor-derived iPSCs or primary airway epithelial cells reportedly lack intercellular synchronicity of ciliary beating in vitro(4, 6, 7). However, since FSS has recently been shown to regulate coordination of ciliary beating (5, 9), we hypothesized that unidirectional FSS over iMCAC sheets may facilitate coordination of ciliary beating and unidirectional muco-ciliary flow. We therefore explored whether ciliary beating could be coordinated intercellularly using the microfluidic device, “airway-on-a-chip,” where FSS could be loaded onto iMCACs (Fig. S1A). Human iPSCs were differentiated into MCACs via CPM-based sorting of *NKX2.1*+ lung progenitor cells, as described previously(6, 19). Proximal airway epithelial progenitor cell (PAEPC) spheroids were then dissociated from three dimensional (3D) Matrigel blocks on Day

28, reseeded on the laminin-coated membrane in the upper channel of the airway chips (Fig. 1A and Fig S1B). FSS flow was loaded at approximately 1500–3000  $\mu\text{l/h}$  over 14 days of differentiation, beginning 12 h after reseeding. VANGL1 was expressed in mature iMCACs (Fig. 1B and Fig. S1C). Immunofluorescence (IF) staining showed that the localization of VANGL1 was asymmetric, appearing in a crescent shape, in each iMCAC. In the airway chip culture with FSS flow, the crescents were aligned in the direction of the flow, whereas those in ALI-cultured iMCACs and airway chip culture without FSS were not aligned (Fig. 1B-C). Additionally, Ac-TUB was detected on the apical surface in iMCACs (Fig. S1D). Moreover, after culturing the iMCACs for 14 days, the membrane was removed from each device and placed on a microscope slide to analyze the fluorescent microbead flow on a horizontal stage (Fig. S1E). Fluorescent microbeads placed on iMCACs in ALI and airway chip culture without FSS flowed in almost random directions, whereas they flowed in the direction of the preloaded FSS on iMCACs in the airway chips with FSS, consistent with the angle histograms of the fluorescent microbead flows under each condition (Fig. 1D-E, Movie S1). We also quantified the degree of coordination among the VANGL1 crescents and fluorescent microbead flow for each condition using the indexes of orientation based on IF staining of samples under each condition (Fig. 1B-C) and tracking of the fluorescent microbead flows (Fig. 1D-E). Both indexes of the iMCACs on the airway chips with FSS were polarized in the direction of the FSS compared with cells of ALI or airway chip culture without FSS (Fig. 1F-G and Fig. S1F-G). Furthermore, we examined the effect of duration and timing of FSS on the muco-ciliary transport in the airway-on-a-chip. Specifically, the cells were perfused under FSS in the early or later 7-day period, and were compared with those for the whole 14-day period until Day 42 (Fig. S2A). Fluorescent microbeads flowed in almost random directions in both the early and later period groups in the airway chip culture with FSS, whereas

those in the whole period group flowed in the direction of the preloaded FSS (Fig. S2B–C). Moreover, the index of orientation for the direction of fluorescent microbeads indicated that 7 days of culture under FSS is not sufficient to establish the global PCP axis (Fig. S2D).

### **Ciliogenesis-driven by fluid shear stress in airway-on-a-chip**

Previous reports showed that ALI culturing had the advantage of driving ciliogenesis of primary airway epithelial cells compared to submerged culture techniques(35, 36). As expected, submerged culture on the airway chip did not induce efficient ciliogenesis of airway epithelial cells. However, FSS in submerged culture seemed to drive differentiation of airway epithelial cells to multiciliated cells. We then quantified the cells expressing FOXJ1, a representative MCAC marker, in ALI culture, and airway chip culture with or without FSS (Fig. S3A-B). The ratio of FOXJ1<sup>+</sup> cells significantly ( $P < 0.001$ ) increased by FSS. RT-qPCR analysis of multiciliated cell marker (*FOXJ1*, *DNAH5*, and *SNTN*) expression under each condition suggested that airway chip culture with FSS induced ciliogenesis more strongly than ALI or airway chip culture without FSS (Fig. 2A and Fig. S3C), which was supported by RNA-seq analysis of transcriptomes under each condition. Principal component analysis of the transcriptomes showed a clear separation of samples in each condition (Fig. 2B). A volcano plot of expressed genes in airway chip culture with FSS relative to that without FSS revealed that the expression of various multiciliated cell marker genes was upregulated (Fig. 2C and Table S7). Moreover, a heat map of gene expression in each condition showed that the representative genes related to motile cilia were promoted by FSS (Fig. 2D). Gene Ontology (GO) analysis of gene expression in the airway chip culture with FSS relative to that without FSS revealed upregulated genes enriched for cilia (Fig. 2E and Fig. S3D). Conclusively, FSS could promote ciliogenesis even in submerged culture condition.

## **VANGL1 deficiency impairs PCP formation and muco-ciliary transport in humans**

Although *VANGL1* mutation has not been reported to cause ciliopathy in humans, we generated *VANGL1* gene knockout iPSCs using a CRISPR-Cas9 system to investigate its potential function for PCP formation in human MCACs (Fig. 3A). Bi-allelic *VANGL1* gene knockout iPSCs with a normal karyotype were obtained (Fig. 3B and Fig. S4A). *VANGL1*(-/-) iPSCs were differentiated into iMCACs until Day 56(6) and the expression of core PCP protein markers (*VANGL1* and *VANGL2*) were evaluated. *VANGL1* and *VANGL2* expression decreased in *VANGL1*(-/-) iMCACs (Fig. 3C), which was confirmed by immunofluorescent staining, whereas both proteins were localized to the apical junctions in control iMCACs (Fig. 3D). *VANGL2* expression was only slightly detected in *VANGL1*(-/-) iMCACs in airway chip culture with or without FSS as well as ALI culture (Fig. 3D and Fig S4B-C). Next, the ciliary beat frequency (CBF) was measured using an HSVM, as described previously(6). Although *VANGL1*(-/-) iMCACs showed an apparently normal ciliary ultrastructure (Fig. S4D), the CBF of *VANGL1*(-/-) iMCACs under ALI condition was slightly decreased compared to parental iPSC-derived MCACs (Fig. 3E). In ciliary beating pattern (CBP) analyses, synchronicity of the beating in *VANGL1*(-/-) iMCACs seemed to be impaired (Movie S2, first half). Regarding muco-ciliary transport testing, the fluorescent microbead flow were interrupted in *VANGL1*(-/-) iMCACs (Fig. S4E). The muco-ciliary transport indexes (MTIs) of the fluorescent bead flow on the sheet of *VANGL1*(-/-) iMCACs were quantified as described previously(6), showing that the MTIs of *VANGL1*(-/-) iMCACs were significantly ( $P < 0.05$ ) decreased compared to the parental iPSC-derived MCACs (Fig. 3F). In airway chip analyses including quantification of the orientation of fluorescent microbead flow indexes, fluorescent microbeads placed on *VANGL1*(-/-) iMCACs in airway chip culture with FSS did not flow fluently (Fig. S4F), but their directions seemed to match the direction of preloaded medium



flow only slightly (Fig. 3G-H, Fig. S4F and Movie S2 later half). Hence, *VANGL1* deficiency in human iMCACs caused abnormal patterns of CBP and misoriented cilium, in agreement with the previous murine study(4).

### **Generation of PCD patient-derived iPSCs, gene-corrected counterparts, and PCD gene knockout iPSCs**

We generated iPSCs from three PCD patients: a 43 year-old female (PCD#1), 13 year-old female (PCD#2), and 6 year-old male (PCD#3)(37), with no family history of PCD who suffered repeated episodes of pneumonia and rhinitis. PCD#1 patient developed respiratory failure until she received a lung transplantation. Chest computed tomography and radiographs of each patient revealed bronchitis, atelectasis, bronchiectasis (Fig. 4A and Fig. S5A). MCACs of lung tissue of PCD#1 showed absent inner dynein arms (IDA) and outer dynein arms (ODA), while that of PCD#2 showed a normal ultrastructure (Fig. 4B). In PCD#1, *HEATR2* was suspected as a candidate causative gene by genetic testing(38), demonstrating mutations of the 5'-untranslated region (5'UTR) and exon 1 on the same allele (Allele 1) as well as in exon 12 on the other *HEATR2* allele (Allele 2) (Fig. 4C, left). In PCD#2, *DNAH11* was suspected as a candidate causative gene by genetic testing(38), demonstrating mutations of exon 59 on Allele 1 and in exon 75 on the *DNAH11* allele 2 (Fig. 4C, middle). In PCD#3, *PIH1D3* was suspected as a candidate causative gene by genetic testing(38), demonstrating mutations of the large deletions in *PIH1D3* (Fig. 4C right). These mutations had not been reported previously. IF staining further revealed that *HEATR2* was detected in the cytoplasm of epithelial cells of control lung tissue derived from a healthy donor but not in lung tissue of PCD#1 (Fig. S5B). We then established iPSCs with normal karyotypes (Fig.S5C), capacity to differentiate into all three germ layers in SCID mice (Fig. S5D), and

expression of undifferentiation markers (Fig. S5E) from each patient's peripheral blood mononuclear cells (PBMCs) and generated gene-edited iPSCs targeting candidate pathogenic gene mutations of PCD#1 and PCD#2 iPSCs (Fig. 4D). Since PCD caused by *HEATR2* and *DNAH11* mutations has been reported as autosomal recessive diseases(39, 40), we expected that single-allele correction would be sufficient to rescue gene function. To correct each mutation of iPSCs derived from PCD#1 and PCD#2, sgRNA was designed to target the exon 1 and exon 12 in *HEATR2* mutation sites and exon 59 and exon 75 in *DNAH11* mutation sites, respectively. Then, c*HEATR2* Ex1-iPSCs, c*HEATR2* Ex12-iPSCs, c*DNAH11* Ex59-iPSCs, and c*DNAH11* Ex75-iPSCs were generated from each patient iPSC line using CRISPR/Cas9-mediated homologous recombination (Fig. S6A) with each targeted gene mutation corrected in each clone (Fig. 4E and Fig. S6B), confirming that gene-corrected iPSCs have normal karyotypes (Fig. S6C). Furthermore, various ciliary abnormalities have been reported in MCACs of PCD patients. We, therefore, generated representative PCD gene knockout iPSCs using a CRISPR-Cas9 system to recapitulate the ciliary dysfunction patterns (Fig. 3A). Bi-allelic gene knockout iPSCs with normal karyotypes (Fig. S6C) defective of representative PCD causative genes [*RSPH4A*(41), *DNAH11*(42), and *DNAAF2*(43)] were obtained (Fig. 4F).

### **PCD iPSC lines recapitulates morphological and functional abnormalities**

Each iPSC clone was differentiated to iMCACs until Day 56 as described previously(6). IF staining detected *HEATR2* in the cytoplasm of c*HEATR2* Ex1-iMCACs and c*HEATR2* Ex12-iMCACs, but not in PCD#1-iMCACs (Fig. 5A upper row and Fig. S7A upper row). *DNAH11* stained in the proximal region of c*DNAH11* Ex59-iMCACs and c*DNAH11* Ex75-iMCACs, but not in PCD#2-iMCACs or *DNAH11*(-/-) iMCACs (Fig. 5A middle row and Fig. S7A-B upper row).

PIH1D3 was stained in the cytoplasm of control iMCACs, but not in PCD#3-iMCACs (Fig. 5A lower row and Fig. S7A lower row). RSPH4A was detected at the ciliary structure in control iMCACs, but not in *RSPH4A*(-/-) iMCACs (Fig. 5A lower row and Fig. S7A-B lower row). DNAAF2 was detected in the cytoplasm and nuclei of control iMCACs, but not in *DNAAF2*(-/-) iMCACs (Fig. 5A lower row and Fig. S7A-B lower row). On TEM analyses, PCD#1-iMCACs revealed the absence of both IDA and ODA, although cHEATR2 Ex1-iMCACs and cHEATR2 Ex12-iMCACs showed a normal ultrastructure. PCD#2-iMCACs, cDNAH11 Ex59-iMCACs, cDNAH11 Ex75-iMCACs, and *DNAH11*(-/-) iMCACs showed apparently normal ciliary ultrastructure, while PCD#3-iMCACs and *DNAAF2*(-/-) iMCACs lacked IDA and ODA. Alternatively, *RSPH4A*(-/-) iMCACs showed central microtubular abnormalities in the cilia (Fig. 5B and Fig. S8A). Although ciliary immotility was demonstrated in PCD#1-iMCACs, CBF and CBP were restored in cHEATR2 Ex1-iMCACs and cHEATR2 Ex12-iMCACs (Fig. 5C and Movie S3 first half). Moreover, PCD#2-iMCACs and *DNAH11*(-/-) iMCACs showed a median increase with a scattered pattern of CBF and hyperkinetic movement with shorter strokes, however, CBF and CBP were restored in cDNAH11 Ex59-iMCACs and cDNAH11 Ex75-iMCACs (Fig. 5C and Movie S4 first half). Complete ciliary immotility was noted in PCD#3-iMCACs and *DNAAF2*(-/-) iMCACs, and a decrease in the CBF and abnormal clockwise rotation of *RSPH4A*(-/-) iMCACs (Fig. 5C and Movie S5 first half). The fluorescent microbeads were placed on the sheet of iMCACs and traced to analyze muco-ciliary transport. Consistently, the microbeads flowed normally on gene-corrected iMCACs, but not on PCD patient-derived iMCACs or PCD gene knockout iMCACs (Fig. S8B and Movie S3-5 latter half). The microbead flow was then quantified as the MTI, which were shown to recover for cHEATR2 Ex1-iMCACs, cHEATR2 Ex12-iMCACs, cDNAH11 Ex59-iMCACs, and cDNAH11 Ex75-iMCACs compared to PCD#1- and PCD#2-

iMCACs, respectively. The MTIs of both PCD#3-iMCACs and *RSPH4A*(-/-) iMCACs were significantly lower ( $P < 0.05$ ) than those of healthy donor-iPSCs (Fig. 5D). Overall, *HEATR2* and *DNAH11* mutations and *RSPH4A*, *DNAH11*, or *DNAAF2* deficiency proved to be pathogenic, resulting in iMCACs recapitulating various ciliary dysfunction of PCD, consistent with the findings of previous reports of PCD patients with genetic abnormalities of *HEATR2*(39), *RSPH4A*(44), *DNAH11*(45) or *DNAAF2*(43).

### **Global PCP axis formed on both PCD patient-derived iMCACs and gene-corrected PCD iMCACs in airway-on-a-chip**

We explored whether or not ciliary beating and PCP were coordinated intercellularly in PCD patient-derived iMCACs and gene-corrected PCD iMCACs. Unidirectional microbead flow was detected on each gene-corrected iPSC-derived airway cell sheet in the direction of the preloaded FSS under the airway chip culture with FSS, whereas the fluorescent microbeads flowed in random directions in ALI and airway chip culture conditions without FSS, consistent with the angle histograms for fluorescent microbead flow under each condition (Fig. 6A, Fig. S9A, and Movie S6-7). The fluorescent microbeads on gene-corrected PCD iMCACs in airway chip culture with FSS moved not only in a field of view, but over the entire length of the airway chips in the direction of the preloaded medium flow (Movie S8). Alternatively, the fluorescent microbeads did not flow directionally on PCD#1 and PCD#2 iMCACs in all conditions (Fig. 6A, Fig. S9A and Movie S6-7). Index of orientation for microbead flow on each gene-corrected iMCAC indicated that the unidirectional flow was established in airway chip culture with FSS (Fig. 6B). IF staining showed that the *VANGL1* crescents in PCD#1-iMCACs and *cHEATR2* Ex1-iMCACs were randomly distributed in ALI and airway chip culture without FSS. Meanwhile, the *VANGL1* crescents in

PCD#1-iMCACs and cHEATR2 Ex1-iMCACs were located in the direction of the FSS (Fig. S9B). Angle histograms of VANGL1 crescent localization calculated based on IF staining of PCD#1-iMCACs and cHEATR2 Ex1-iMCACs under each condition indicated that the global axis of PCP was established in the cHEATR2 Ex1-iMCACs and PCD#1-iMCACs (Fig. S9C). The index of orientation calculated based on the VANGL1 crescent localization of both PCD#1-iMCACs and cHEATR2 Ex1-iMCACs revealed that the PCP was significantly ( $P < 0.001$ ) stronger in airway chip culture with FSS than in ALI or airway chip culture without FSS (Fig. S9D). We concluded that the PCP global axis was established in PCD iMCACs by preloading FSS during multiciliogenesis, irrespective of ciliary motility.

## Discussion

The present study demonstrated that FSS drove the global PCP axis formation of iMCACs in airway chip culture and induced multiciliogenesis. Moreover iPSCs were useful for PCD modeling even in cases with unreported mutations and for investigating candidate gene pathogenesis. Additionally, the combination of iPSC-based PCD modeling and airway-on-a-chip delineated that the global PCP axis was established by FSS, irrespective of ciliary motility.

VANGL1 is a PCP marker expressed opposite the basal feet of MCACs; its localization indicates the direction of MCAC ciliary beating(46). The directional flow of the muco-ciliary transport can be determined by coordinated alinement of VANGL1 localization in each MCAC of an airway epithelial cell sheet(46, 47). We, therefore, evaluated the coordination of ciliary beating by the muco-ciliary flow visualized by fluorescent microbeads placed on iMCACs as well as by the localization of VANGL1. FSS was then generated at medium flow rates of 10–3000  $\mu\text{L}/\text{h}$ (9). However, PCP appeared to show more alignment when the flow rate was increased to 1500–3000

$\mu\text{L/hr}$ . Although the mechanism by which the global PCP axis forms in airways and epithelial cell sheets under FSS is unclear, successful ciliary coordination directed the global PCP axis, which is consistent with the finding that the direction of ciliary beating of multiciliated ependymal cells was oriented in the direction of the flow only when FSS was initiated during growth(5). However, the global PCP axis driven by FSS appears to be imperfect compared with that of normal airway epithelial cells in vivo, suggesting that other factors, such as mechanical strain, might be involved in driving the global PCP axis in airway tissue. In addition, although hydrodynamic interaction of synchronously beating cilia has been reported to facilitate cilia movement of neighboring cells in a simulation study (48), the global PCP axis in an airway epithelial cell sheet was established in PCD patient-derived MCACs with only FSS and no beating cilia in the present study, indicating that driving the global axis of PCP in MCACs does not require ciliary motility(49). Hence, FSS may function as a substitute for ciliary beating in PCP formation.

We also observed that FSS strongly induced ciliogenesis and efficient iMCAC differentiation in the submerged condition in airway-on-a-chip. Although it has been generally noted that submerged culture conditions are not suitable for inducing MCACs(35) and ALI culture is conventionally adopted for MCAC differentiation, submerged culture under appropriate FSS could be equivalent to ALI culture, suggesting direct roles of FSS, such as oxygen supply, or the presence of mechanoreceptors for FSS in ciliogenesis. These findings suggest that fetal breathing movements filled with amniotic fluid instead of air might promote ciliogenesis as well as the global axis formation of PCP in airway epithelial cells(50). A previous study reported that *Vangl1*-inactivated mutant (*Vangl1<sup>gt/gt</sup>*) mice had tissue-level cilium orientation defects compared to wild-type littermates(4). Although *Vangl1* is a core PCP protein and its mutations lead to neural tube defects(51), its role in human ciliated cells was unclear. Therefore, we investigated the phenotypes

of *VANGL1* knockout iMCACs. Not only the expression of *VANGL1* but also *VANGL2* were decreased in *VANGL1*(-/-) iMCACs, consistent with a previous report that indicated the requirement of *Vangl1* for the recruitment and stable maintenance of *Pk2* and *Vangl2*(4). As expected, the global PCP axis of *VANGL1*(-/-) iMCACs in airway chip culture with FSS was insufficient relative to that of parental healthy donor-derived iMCACs (Fig. 3H). Muco-ciliary transport of *VANGL1*(-/-) iMCACs was also decreased in ALI culture compared with that of the parental iMCACs, in spite of a slight decrease in the CBF of *VANGL1*(-/-) iMCACs. We speculate that the *VANGL1*(-/-) iMCAC phenotype includes not only impaired CBF, but also CBP, a loss of synchronicity (Movie S2, first half). In *VANGL1*(-/-) iMCACs, the global PCP axis was not modulated by FSS, indicating that *VANGL1* could be downstream of FSS signaling. These findings were consistent with a previous study that reported PCP protein distribution as misoriented in *Vangl1*CKO<sup>Δ/Δ</sup> mice(4). Hence, human *VANGL1* mutations may serve as a causative gene of PCD, although PCD caused by *VANGL1* mutation has not been reported possibly due to poor viability during the embryonic period(4).

Gene-editing is a strong method for generating iPSCs harboring artificially introduced point mutations on the target genomic locus. Although *HEATR2*, *DNAH11* and *PIH1D3* were reported as causative genes of PCD(39, 42, 52-54), the mutations detected in the present study, in particular, single point mutations in *HEATR2* and *DNAH11*, have never been proven as such to our knowledge. It is difficult to ascertain whether these mutations are causative of PCD without identifying other patients with the same mutations. Therefore, we established patient-derived iPSCs and their gene-corrected counterparts to examine the effect of *HEATR2* and *DNAH11* mutations. All results were consistent with the previous reports of PCD patients with the genetic abnormality of *HEATR2* and *DNAH11*(39, 40). Considering that the genetic mutations in 30% of

PCD patients are undetermined(55), the method described in the present study may be useful for proving the causal relationship between candidate gene mutations and PCD, even if the mutations are unique to the patient. Additionally, we generated representative PCD gene knockout iPSCs and recapitulated the abnormal CBF, CBP and muco-ciliary transport of each PCD gene deficiency. These findings were consistent with those in previous reports of PCD patients(41, 43, 44). These panels of knockout iPSCs may be used to elucidate the pathological mechanism underlying each phenotype of PCD in the future.

Conclusively, coordinated ciliary beating—or at least the global axis of PCP—was established in the airway chip to where human iPSC-derived MCACs had been reseeded. *VANGL1* was demonstrated to be a key core PCP protein in iMCACs and to contribute to global PCP axis formation and ciliary beating synchronicity. Although air shear stress was not examined in the place of FSS in the present study, combined PCD modeling using iPSCs and airway-on-a-chip technology could provide a powerful tool for realizing multicellular, in-vivo-like functional airway epithelial cell sheets with well-organized planar polarity in vitro with potential use for investigating the mechanism of airway diseases and identifying or testing candidate therapeutic agents, ultimately contributing to precision medicine. Furthermore, characterization of the underlying mechanism regulating planar polarity will aid regenerative medicine techniques, such as repairing airways from severe lung injury and reconstructing functional artificial tracheas.

Our study has some limitations. First, the optimal conditions for recapitulating in vivo-like unidirectional muco-ciliary flow should be determined in future studies. The period, timing, and flow velocity of FSS and mechanical stretch are parameters that could be explored. Second, although FSS promoted ciliogenesis, the molecular mechanism is unclear. Third, *VANGL1* (-/-) iMCACs showed decreased expression of *VANGL2* and *VANGL1* and appeared to have



misoriented ciliary beating, but the PCP global axis seemed to still be slightly driven by FSS. Further research is needed to elucidate the roles of other PCP core proteins other than VANGL1 and VANGL2 in the regulation of iMCAC PCP.

## **Materials and Methods**

### **Study design**

This study was designed to recapitulate the unidirectional muco-ciliary flow of iPSC-derived airway epithelial cells via global axis formation of PCP in an airway cell sheet and therein to elaborate PCD modeling using iPSCs. We investigated the effects of FSS on regulating the global axis of multicellular PCP and ciliogenesis. Time-lapse imaging of fluorescent microbead flow on the iPSC-derived airway cell sheets and IF staining for VANGL1 as a PCP core protein were used to evaluate the PCP with or without FSS. RT-qPCR, RNA-Seq, and IF staining were used to evaluate ciliogenesis driven by FSS. We conducted a loss-of-function study of VANGL1 by generating *VANGL1*-knockout iPSCs using a CRISPR-Cas9 system. Next, those findings were applied to PCD modeling using disease-specific iPSCs. First, we established iPSCs from three PCD patients with different gene mutations (*HEATR2*, *DNAH11*, *PIH1D3*) and gene-corrected iPSCs (*HEATR2* and *DNAH11*). We also established representative PCD gene-knockout iPSCs (*DNAAF2*, *RSPH4A*, and *DNAH11*). The CBF and CBP of those PCD-specific iMCACs were evaluated using HSVM. The muco-ciliary transport was quantified using fluorescent microbead flow and the ciliary ultrastructure was examined using TEM. We evaluated whether PCD modeling using iPSCs was consistent with previous studies of PCD clinical specimens. Finally, we evaluated the planar polarity of PCD iMCACs using airway-on-a-chip technology. All experiments were

performed in three or more biological replicates and were unblinded. No samples were excluded from the study. For detailed information on PCD#3, see the clinical case report by Xu et al(37).

### **Statistical analyses**

All error bars indicate the standard error of the mean (SEM). Quantification data were collected from three or more independent experiments. All statistical analyses were performed with Prism 7 software program (GraphPad) or GeneSpring GX 13.0 (Agilent Technologies). Two groups were compared using Mann-Whitney test. Multiple group comparison used analysis of variance (ANOVA) with Tukey's multiple comparison test or Kruskal-Wallis test with Dunn's multiple comparison test.

### **Supplementary Materials**

Fig. S1. Established coordinated ciliary beating and unidirectional muco-ciliary flow using fluid shear stress in airway-on-a-chip.

Fig. S2. Analysis of duration and timing of fluid shear stress required for achieving coordinated ciliary movement.

Fig. S3. Multiciliated cell differentiation driven by fluid shear stress in airway-on-a-chip.

Fig. S4. Phenotypic analyses of MCACs derived from PCP gene knockout iPSCs.

Fig. S5. Validation of PCD#1-, PCD#2-, and PCD#3-iPSC lines.

Fig. S6. Generation of PCD patient-derived iPSCs, their gene-corrected ones and PCD causative gene knockout iPSCs.

Fig. S7. Immunofluorescence analysis of each cell line.

Fig. S8. Morphological and functional analyses of each cell line.

Fig. S9. Analysis of the global axis of planar cell polarity and coordinated ciliary beating of iMCACs derived from PCD cell line in airway-on-a-chip.

Table S1. CRISPR-sgRNA used for generation of gene-corrected iPSCs and knockout iPSCs.

Table S2. Predicted off-target sites of CRISPR-sgRNA used for generating cHEATR2 Ex1- and Ex12-iPSCs and PCD gene knockout iPSCs.

Table S3. Primers used to confirm off-target sites.

Table S4. Content of each medium for differentiation of iPSCs into iMCACs.

Table S5. Antibodies used in the present study.

Table S6. Primers for qRT-PCR used in the present study.

Table S7. List of genes that were significantly ( $P < 0.05$ , Fisher's exact test) upregulated in airway chip culture with fluid shear stress comparing to those without fluid shear stress or those in air-liquid interface culture by RNA-Seq.

Movie S1. Muco-ciliary transport of healthy donor-MCACs in airway-on-a-chip.

Movie S2. Ciliary beating and stacked images of fluorescent microbead flow on the airway epithelial cells derived from *VANGL1*(-/-) iPSCs.

Movie S3-5. Ciliary beating and stacked images of fluorescent microbead flow on iMCACs derived from each PCD cell line.

Movie S6-7. Muco-ciliary transport of MCACs derived from PCD#1-iMCACs, PCD#2-iMCACs and their gene-corrected counterparts.

Movie S8. Muco-ciliary transport over the entire length of the airway epithelial sheet in airway chip culture with FSS

Data file S1. Raw data for figures.

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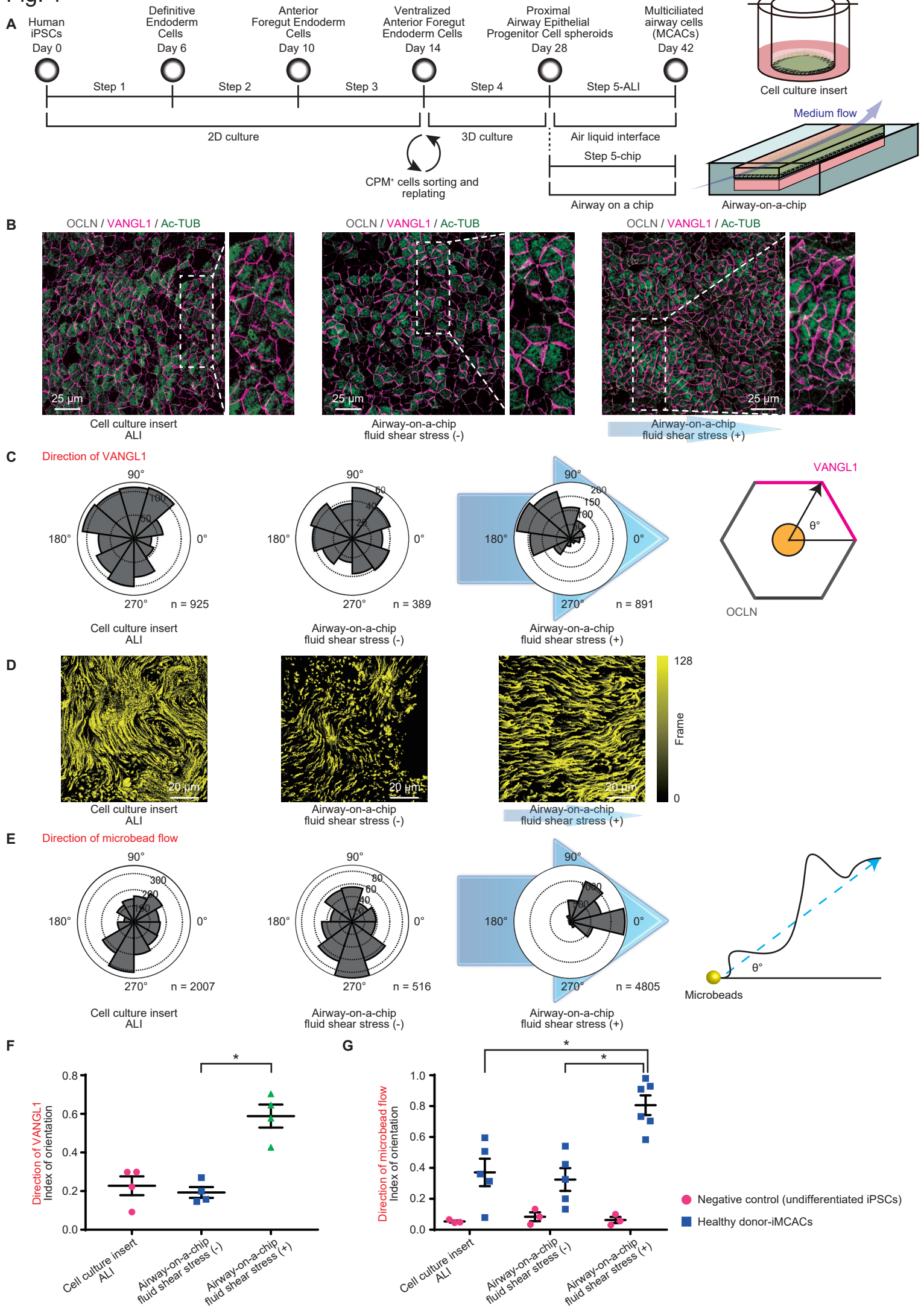
**Author contributions:** N.S., Sa.K., Y.T. and S.G. conceived and designed the study. N.S., Sa.K., K.I., Ko.T., S.I., Y.K., Sh.K., Y.Y. and S.G. performed the experiments. Y.T. generated airway-on-a-chip devices. N.S., Sa.K., K.I., T.N., Y.X. and S.G. analyzed the data. N.S., Sa.K., Y.T. and S.G. wrote the manuscript through fruitful discussions with and supervision by Ka.T. Y.A., M.H., T.F.C-Y., H.D., T.H., and S.T.

**Competing interests:** Sa.K., Y.Y., and S.G. are co-inventors of patents JP6730709, US10,377,991, EP3272859, and PCT/JP2016/059786, “Method for inducing differentiation of airway epithelial cells”. N.S., Sa. K., T.N., Y.Y., S.T., Y.T., and S.G. are co-inventors of a patent application, JP2020-081522 “Multiciliated airway cells, method of deriving the same, and method of manufacturing the same”. Y.Y., M.H., and S.G. are founders and shareholders of HiLung Inc. Sh. K. is an employee and shareholder of Kyorin Pharmaceutical. Co. Ltd.

**Data and materials availability:** All data associated with the present study can be found in the main text or the Supplementary Materials. RNA-seq raw data reported in this paper are deposited in the Gene Expression Omnibus with accession numbers GSE162012. The 201B7 iPSC line can be obtained from Cell Bank at Riken BioResource Research Center (Tsukuba, Japan). The other iPSC lines are available under a material transfer agreement upon request to the corresponding author.

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**Fig. 1**





## Figures:

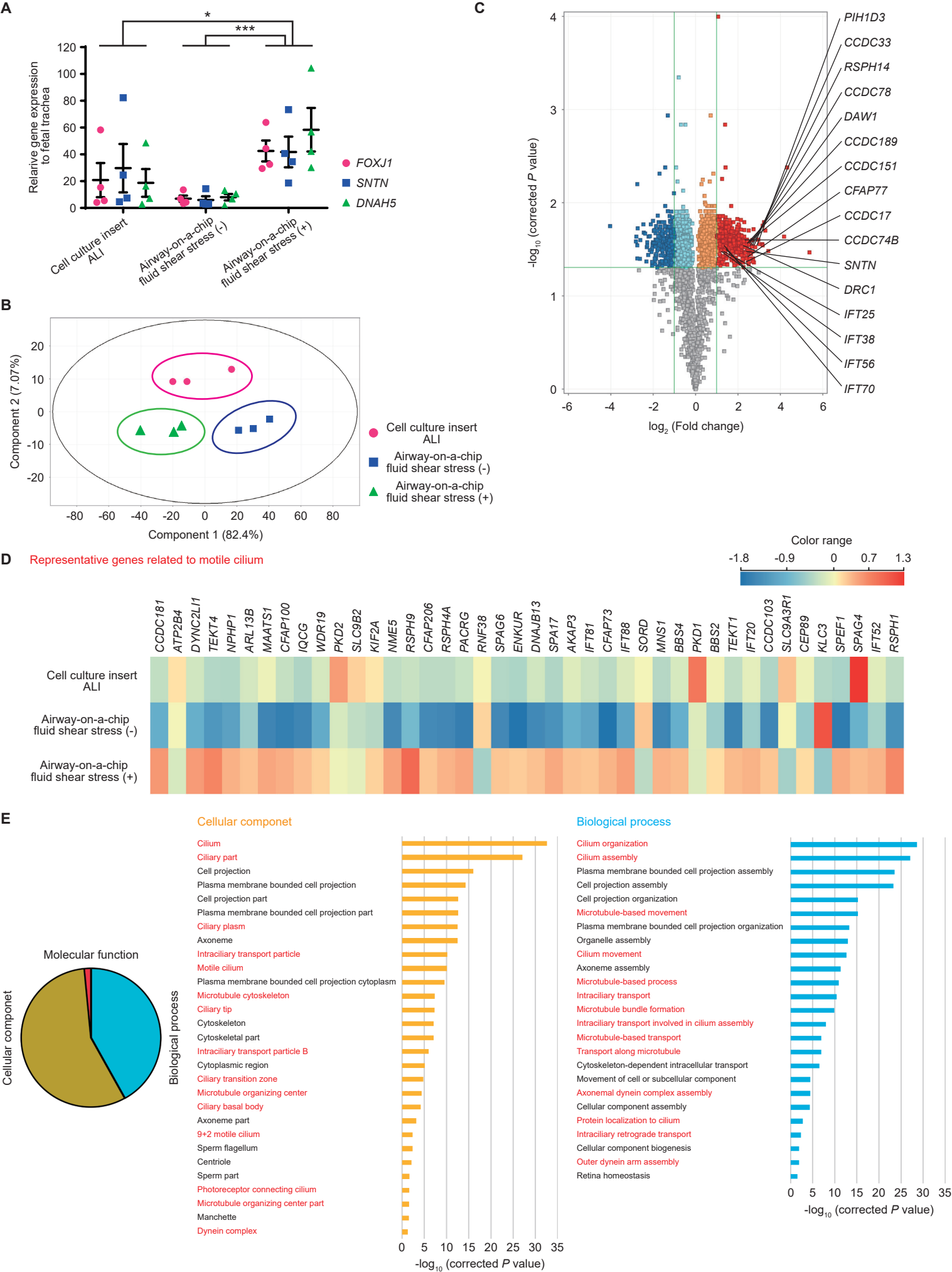
### **Fig. 1. Coordinated ciliary beating and unidirectional muco-ciliary flow in iPSC-derived multiciliated airway cells driven by fluid shear stress in airway-on-a-chip.**

**(A)** Schematic diagram of the stepwise method for inducing multiciliated airway cells (MCACs) from human induced pluripotent stem cells (iPSCs) in the airway-on-a-chip or cell culture insert. **(B)** Immunofluorescence (IF) staining of *VANGL1*, Ac-TUB and *OCLN* under each condition of air-liquid interface (ALI) and airway chip culture with and without fluid shear stress (FSS). **(C)** Angle histogram of *VANGL1* localization in iPSC-derived airway cell sheet calculated based on the IF staining of samples under each condition ( $n = 925, 389$ , and  $891$  cells from four independent experiments in ALI and airway chip culture with and without FSS, respectively). *OCLN* was used as a marker of the cell boundary.  $P_i$  was defined as  $(\cos\theta_i, \sin\theta_i)$ , and the Index of orientation ( $I_o$ ) was calculated by  $1/N\sum P_i$  (right panel). **(D)** Stacked images of the fluorescent microbeads placed on iMCACs under each condition. Color spectrum reflects time course. **(E)** Angle histograms of the fluorescent microbead flow calculated based on sample trajectories under each condition ( $n = 2007, 516$ , and  $4805$  trajectories from 5–6 independent experiments in ALI and airway chip culture with and without FSS, respectively).  $P_i$  was defined as  $(\cos\theta_i, \sin\theta_i)$ , and then the  $I_o$  was calculated by  $1/N\sum P_i$  (right panel). **(F)** The  $I_o$  of *VANGL1* localization was calculated based on the IF staining of samples under each condition (mean  $\pm$  SEM  $n = 925, 389$ , and  $891$  cells from four

independent experiments in ALI and airway chip culture with and without FSS, respectively).

Kruskal-Wallis with Dunn's multiple comparisons test was used.  $*P < 0.05$ . **(G)** The Io of the fluorescent microbead flow was calculated based on the trajectories of samples under each condition (mean  $\pm$  SEM n = 2007, 516, and 4805 trajectories from 5–6 independent experiments in ALI and airway chip culture with and without FSS, respectively). The left side of the data was obtained from undifferentiated iPSCs under each condition as a negative control. Kruskal-Wallis with Dunn's multiple comparisons test was used.  $*P < 0.05$ .

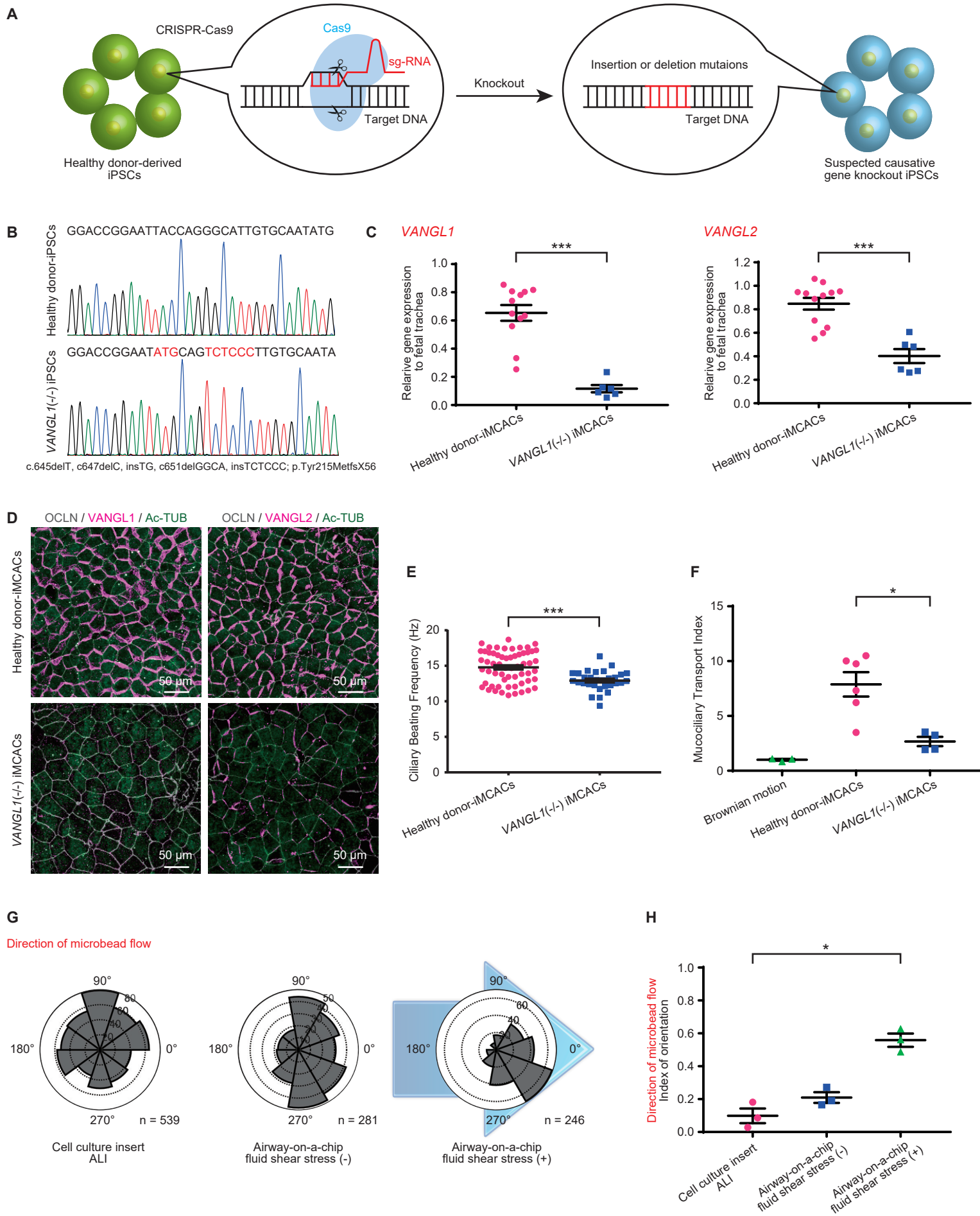
Fig. 2



**Fig. 2. Ciliogenesis driven by fluid shear stress in airway-on-a-chip.**

**(A)** RT-qPCR of multiciliated cell markers (*FOXJ1*, *SNTN*, and *DNAH5*) under each condition (mean  $\pm$  S.E.M.,  $n = 4$  independent experiments). Two-way ANOVA with Tukey's multiple comparisons test was used to culture method ( $P = 0.0004$ ), multiciliated cell markers ( $P = 0.8581$ ) and their interaction ( $P = 0.7786$ ).  $*P < 0.05$ ,  $***P < 0.001$ . **(B)** Principal component analysis (PCA) of the RNA-seq data of iMCACs under each condition ( $n = 3$  independent experiments). **(C)** Volcano plot of gene expression from airway chip culture with or without FSS, identifying differentially expressed genes. Representative genes related to motile cilia. **(D)** Heat map of gene expression related to motile cilia under each condition ( $n = 3$  independent experiments). **(E)** Pie chart showing Gene Ontology (GO) term distribution for upregulated genes in airway chip culture with FSS relative to that without FSS. GO terms analysis ("cellular component", [29 terms]; "biological process", [25 terms]) revealed enrichment of cilia-related genes by Fisher's exact test ( $P < 0.05$ ).

**Fig. 3**

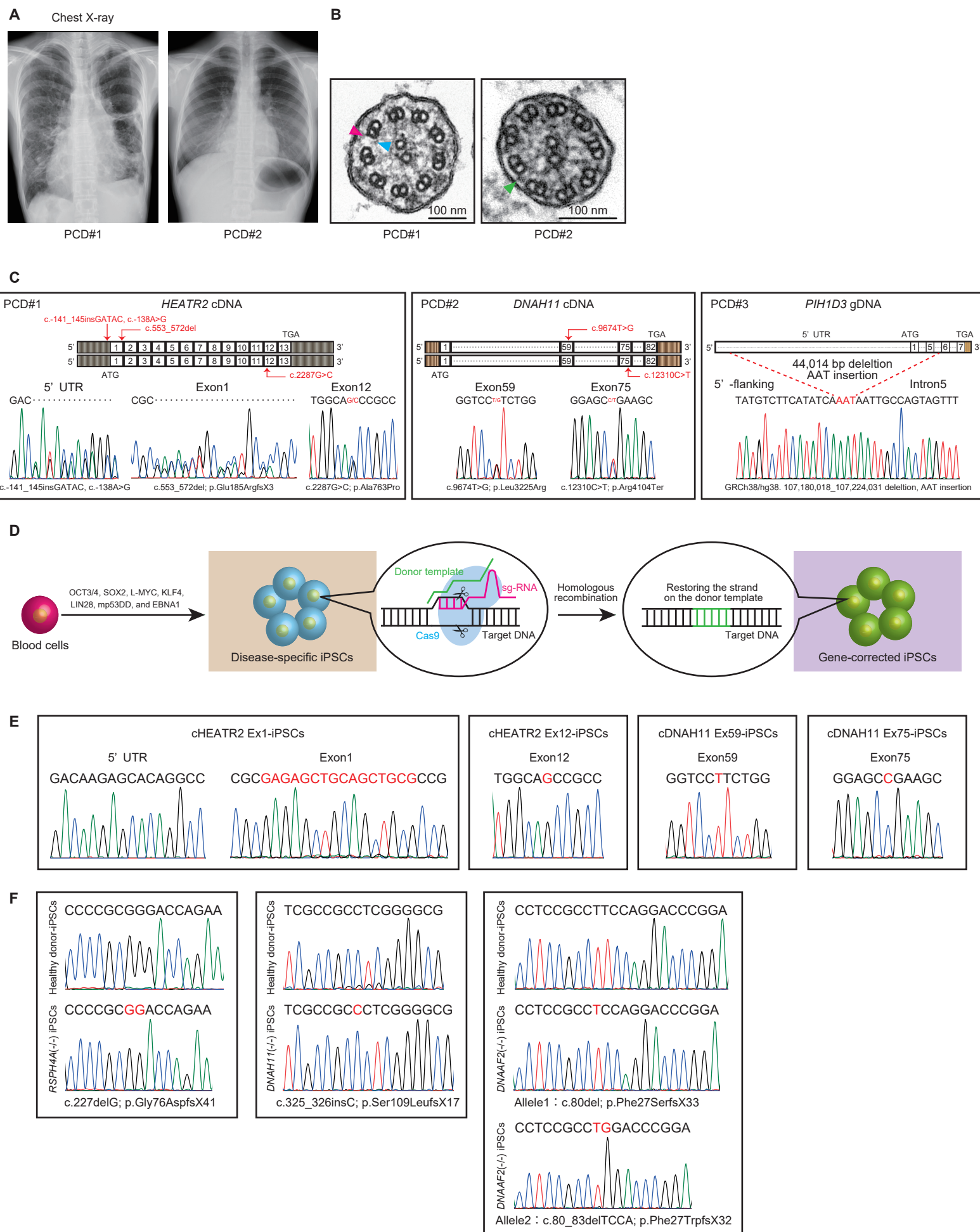


**Fig. 3. Generation and phenotypic analyses of PCP gene knockout human iPSCs.**

**(A)** Schematic overview of the generation of gene knockout iPSCs using a CRISPR-Cas9 system. **(B)** Sequence data for *VANGL1*(-/-) iPSCs compared to parental healthy donor-iPSCs. **(C)** RT-qPCR of PCP markers (*VANGL1* and *VANGL2*; mean  $\pm$  S.E.M.,  $n = 6$ –12 independent experiments). Mann-Whitney test was used.  $***P < 0.001$ . **(D)** IF staining of *VANGL1*, and *VANGL2* in *VANGL1*(-/-) iMCACs. The healthy donor-iPSC line was used for a control. **(E)** Ciliary beat frequency (CBF) of *VANGL1*(-/-) iMCACs analyzed by high-speed video microscopy (HSVM). Mean  $\pm$  S.E.M.,  $n = 3$ –6 independent experiments with each comprising ten fields of view. Mann-Whitney test was used.  $***P < 0.001$ . **(F)** Muco-ciliary transport index (MTI) of *VANGL1*(-/-) iMCACs calculated from trajectories of the fluorescent microbeads per sample. Mean  $\pm$  S.E.M.  $n = 3218$  and  $734$  trajectories from  $6$  and  $4$  independent experiments in healthy donor-iMCACs, and *VANGL1*(-/-) iMCACs, respectively. Mann-Whitney test was used.  $*P < 0.05$ . **(G)** Direction of the fluorescent microbead flow on a *VANGL1*(-/-) iPSC-derived airway cell sheet. Angle histograms of the fluorescent microbead flow were calculated based on sample trajectories under each condition ( $n = 539$ ,  $281$ , and  $246$  trajectories from three independent experiments in ALI and airway chip culture with and without FSS, respectively). **(H)** The Io of the fluorescent microbead flow was calculated based on sample trajectories under each condition (mean  $\pm$  SEM,  $n = 539$ ,  $281$ , and  $246$  trajectories from  $3$ – $6$  independent experiments in ALI and airway chip culture with and without FSS

respectively. Kruskal-Wallis with Dunn's multiple comparisons test was used.  $*P < 0.05$ . See Fig. 1G for control data obtained from the parental healthy donor-iMCACs in airway chip culture with FSS.

**Fig. 4**

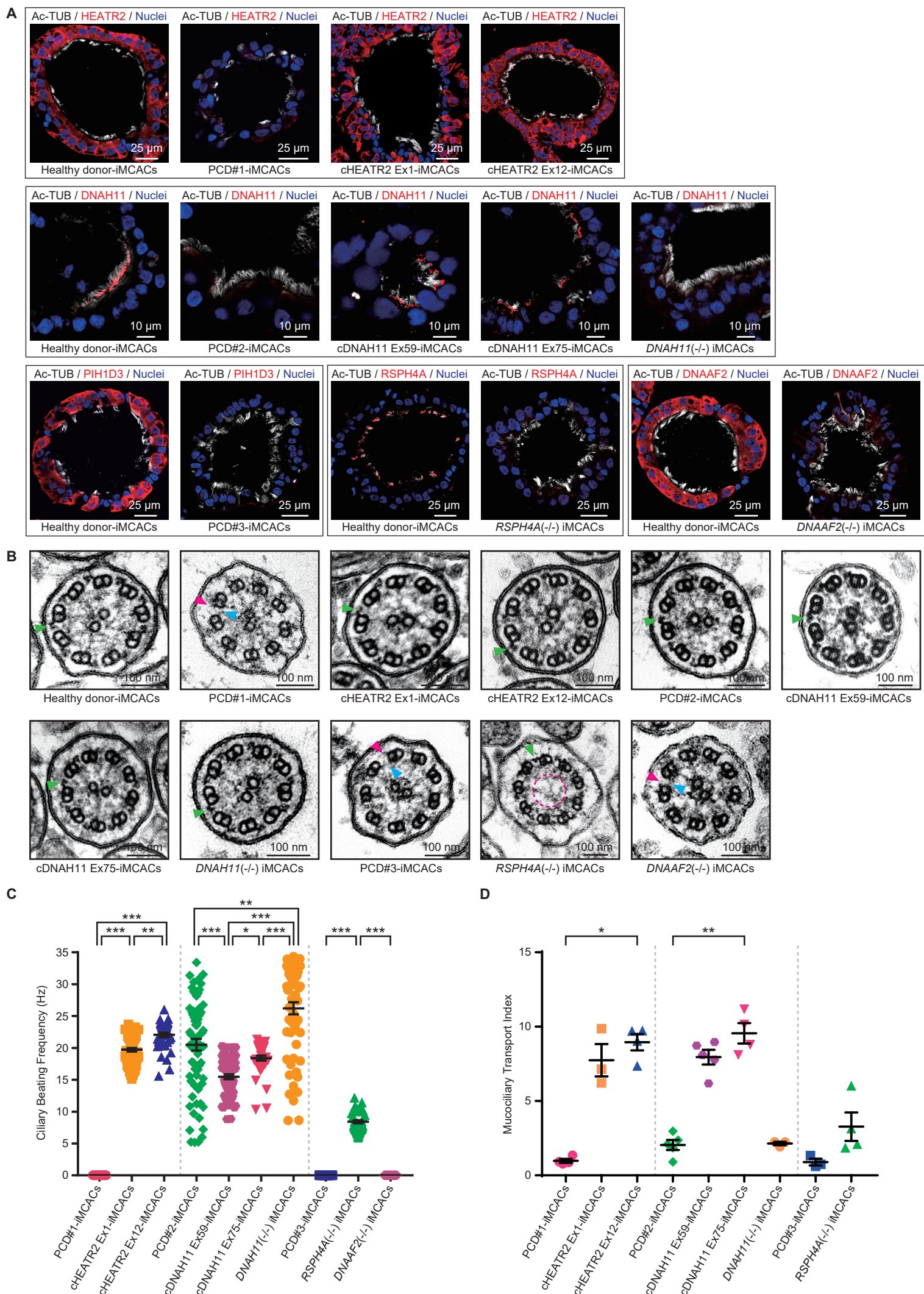




**Fig. 4. Generation of patient-derived iPSCs, gene-corrected counterparts and knockout iPSCs of PCD causative genes.**

**(A)** Chest X ray for PCD#1 and PCD#2. **(B)** Transmission electron microscopy images of the transverse section of cilia in each patient's tissue. Lack of outer dynein arms (ODA) and inner dynein arms (IDA) indicated by magenta and blue arrowheads, respectively. Green arrowhead indicates the ODA. **(C)** Map of suspected causative gene and mutations of each patient. In PCD#1, the 5'UTR and exon 1 in *HEATR2* gene mutations were on the same-side allele, and the exon 12 gene mutation in *HEATR2* was on the opposite-side allele. In PCD#2, the exons 59 and 75 gene mutations in *DNAH11* were on different alleles. In PCD#3, large deletions in *PIH1D3* were identified on the X chromosome. **(D)** Schematic overview of PCD#1 and PCD#2 iPSC gene corrections. **(E)** Sequence data for each mutation region in cHEATR2 Ex1-iPSCs, cHEATR2 Ex12-iPSCs, cDNAH11 Ex59-iPSCs and cDNAH11 Ex75-iPSCs. Each mutation was corrected in each cell line. **(F)** Sequence data of *RSPH4A*(-/-) iPSCs, *DNAH11*(-/-) iPSCs, and *DNAAF2*(-/-) iPSCs compared to parental healthy donor-iPSCs.

Fig. 5



**Fig. 5. Morphological and functional analyses of each PCD iPSC line.**

**(A)** IF staining of HEATR2, DNAH11, PIH1D3, RSPH4A and DNAAF2 in an airway organoid derived from each iPSC line. Healthy donor-derived 201B7 iPSC line used as control.

**(B)** TEM images of the transverse section of cilia in iMCACs derived from each cell line. Lack of ODA and IDA indicated by magenta and blue arrowheads, respectively. Green arrowheads indicate ODA. Magenta circle represents to lack of the central pair. **(C)** CBF of each iPSC

line-derived MCACs analyzed by HSVM (mean  $\pm$  S.E.M.,  $n = 4-7$  independent experiments with each consisting of ten fields of view). Kruskal-Wallis with Dunn's multiple comparisons test was used.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ . See Fig. 3E for control data obtained from

the healthy donor-iMCACs. **(D)** The MTI of iMCACs calculated from the trajectories of the fluorescent microbeads per cell line (mean  $\pm$  S.E.M.,  $n = 3218, 1091, 2599, 4972, 985, 3492,$

4846, 466, and 506 trajectories from 3-5 independent experiments in PCD#1-iMCACs, cHEATR2 Ex1-iMCACs and cHEATR2 Ex12-iMCACs, PCD#2-iMCACs, cDNAH11 Ex59-iMCACs, cDNAH11 Ex75-iMCACs, *DNAH11*(-/-) iMCACs, PCD#3-iMCACs, *RSPH4A*(-/-)

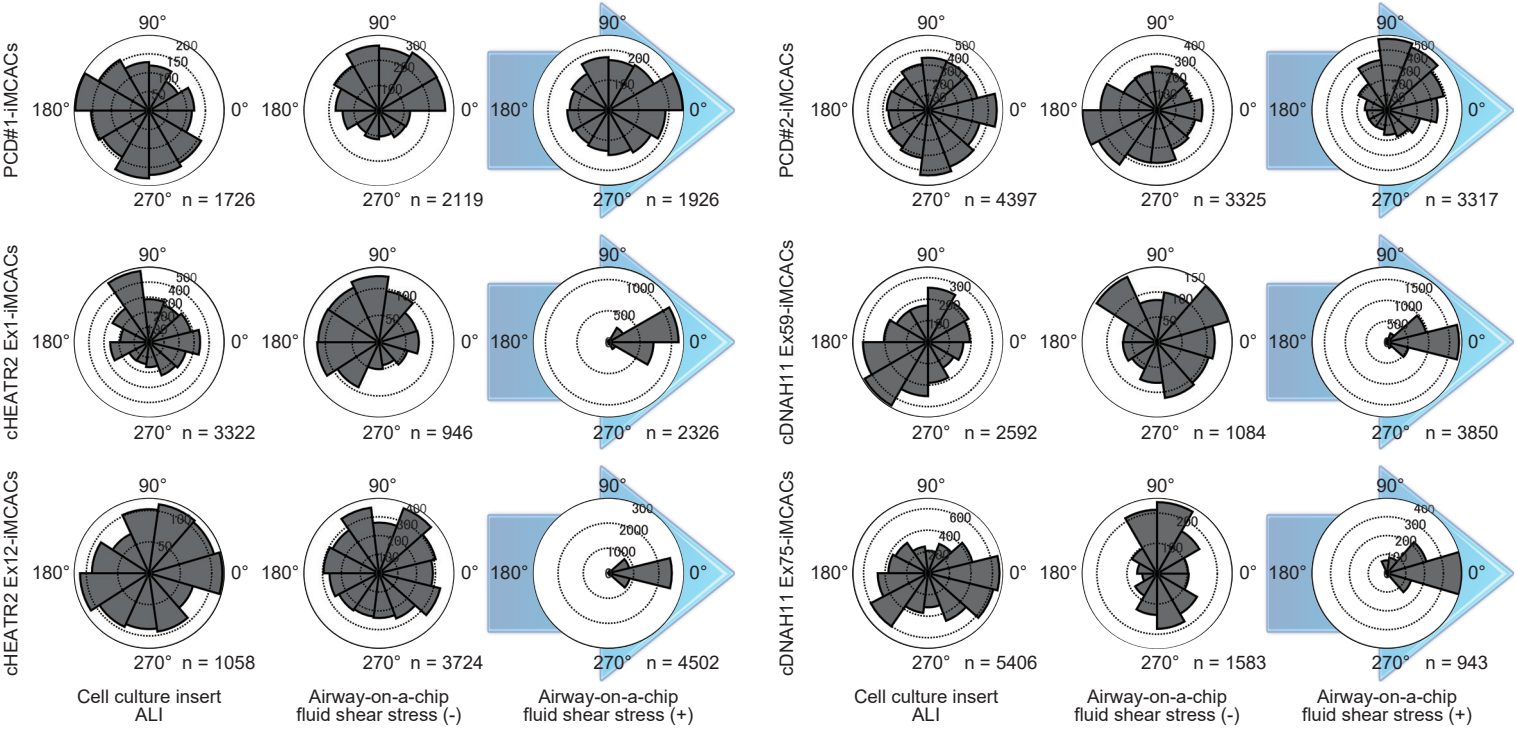
iMCACs respectively). Kruskal-Wallis with Dunn's multiple comparisons test was used or

Mann-Whitney test was used.  $*P < 0.05$ ,  $**P < 0.01$ . See Fig. 3F for Brownian motion and control data obtained from the healthy donor-iMCACs.

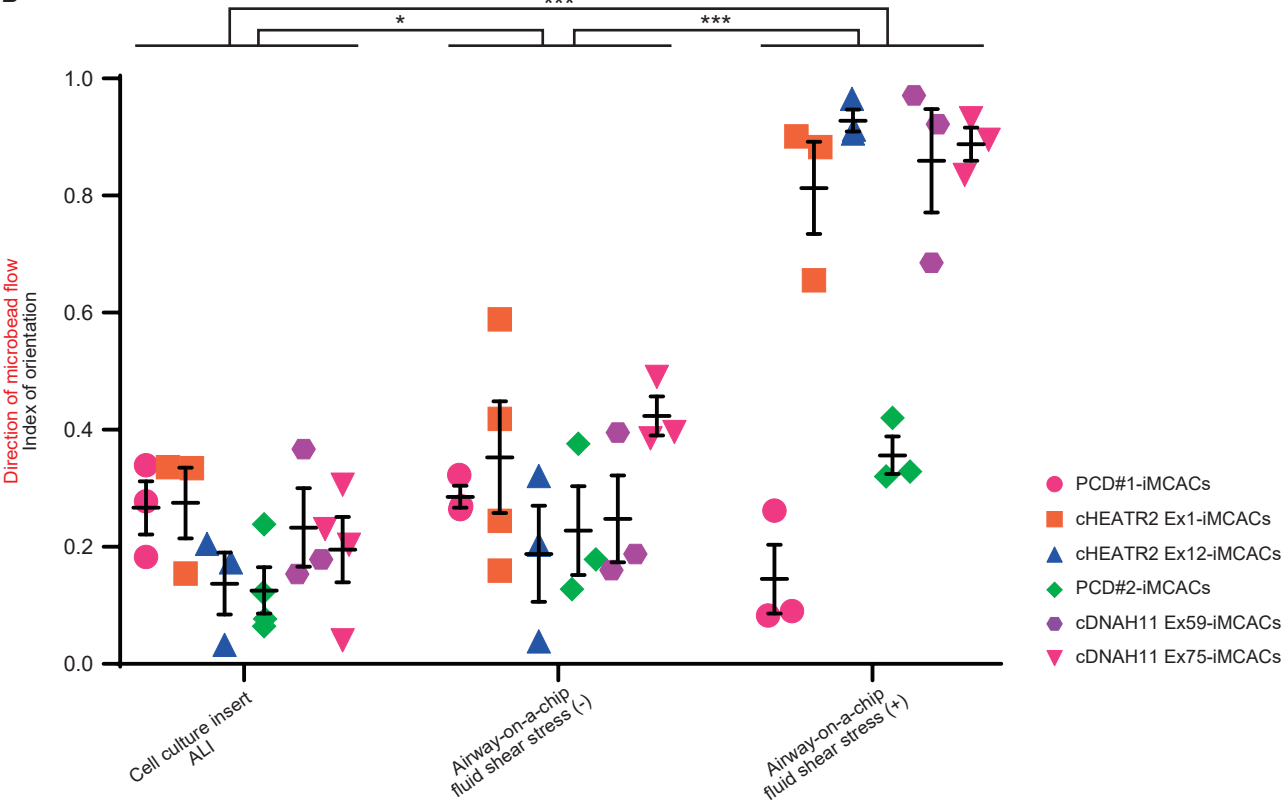
Fig. 6

A

Direction of microbead flow



B



**Fig. 6. Coordinated ciliary beating of iMCACs derived from gene-corrected PCD patient-derived iPSC lines in airway-on-a-chip.**

**(A)** Direction of the fluorescent microbead flow in each iPSC-derived airway cell sheet.

Angle histograms of the fluorescent microbead flow calculated based on sample trajectories under each condition (n = 3–4 independent experiments in ALI and airway chip culture with and without FSS, respectively). **(B)** The Io of the fluorescent microbead flow was calculated based on the trajectories of samples under each condition (mean  $\pm$  SEM 3–4 independent experiments in ALI and airway chip culture with and without FSS, respectively). A two-way ANOVA with Tukey's multiple comparisons test was used to examine the effect in culture method ( $P < 0.0001$ ), cell line ( $P < 0.0001$ ), and their interaction ( $P < 0.0001$ ). \* $P < 0.05$ , \*\*\* $P < 0.001$ .

## **Materials and Methods**

### **Ethics**

Animal experiments were approved by the Animal Research Committee of Kyoto University (MedKyo20259-2). The generation and use of patient-derived iPSCs for research as well as patient-derived blood and tissue samples were approved by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine (R91/G259). Written informed consent was obtained from each patient.

### **Generation of patient-derived iPSCs**

PBMCs were collected using BD Vacutainer cell preparation tubes with sodium citrate (BD Biosciences) and cryopreserved. A total of  $3 \times 10^6$  PBMCs were thawed and seeded with 1.5 ml of StemSpan- Animal Component Free (ACF) (STEMCELL Technologies) supplemented with 100 ng/mL Stem Cell Factor (SCF) (R&D Systems), 100 ng/mL Thrombopoietin (TPO) (R&D Systems), 100 ng/mL Flt3L (R&D Systems), 50 ng/mL IL-6 (R&D Systems), and 20 ng/mL IL-3 (R&D Systems) in 1 well of a 6-well plate (Corning) and cultured for 5 days. To induce putative iPSC colonies, cells were transfected with human iPSC generation episomal vector mix containing cDNA of OCT3/4, SOX2, KLF4, L-MYC, LIN28, mp53-DD and EBNA1 (Takara Bio), and cultured until the putative iPSC colonies were manually selected, according to manufacturer's instruction. The individual iPSC colonies were maintained in StemFit AK03N/AK02N on iMatrix-511-coated plates and cryopreserved in Stem Cell-Banker (Takara Bio). PCD#1-iPSCs (Clone: PCD1-1), PCD#2-iPSCs (Clone: PCD9-1), PCD#3-iPSCs (Clone: PCD6-2) were then established. The teratoma formation assay was performed, as described previously(27).

### **Generation of gene-corrected PCD#1-iPSCs and PCD#2-iPSCs**

Prior to gene correction, PCD#1-iPSCs were maintained on mitomycin C-treated STO feeder cells in Primate ES medium (ReproCELL) supplemented with human FGF-2 (4 ng/ml) (DS Pharma Biomedical) and PCD#2-iPSCs were maintained on iMatrix-511-coated plates in StemFit AK02N. In both cases, the media was supplemented with Y-27632 at 10  $\mu$ M at least 1 h before electroporation. When maintained with feeder cells, the cells were washed with PBS and incubated in CTK (Collagenase type IV, Trypsin and Knockout serum replacement) solution at room temperature (RT) for 1–3 min to remove feeder cells. The cells were dissociated into single cells with Accutase (Innovative Cell Technologies) at 37 °C for 20 min and neutralized with DMEM/F12 (Nacalai Tesque) supplemented with 2% fetal bovine serum (Sigma-Aldrich). Three plasmid vectors comprising 5  $\mu$ g of Cas9, 5  $\mu$ g of sgRNA and 5  $\mu$ g of donor template with a G418 resistant gene cassette were simultaneously electroporated into  $1.0 \times 10^6$  cells using a NEPA 21 electroporator (poring pulse: pulse voltage, 125 V; pulse width, 5 ms; pulse number, 2; Nepagene) as previously reported(27, 56). The cells were then reseeded on STO feeder cells in Primate ES medium supplemented with 10  $\mu$ M Y-27632 or iMatrix-511-coated plates in StemFit AK02N medium supplemented with 10  $\mu$ M Y-27632 for 1–2 days. Two days after electroporation, G418 (Gibco) selection was initiated at 100  $\mu$ g/ml. After selection for 7–10 days, the cells were dissociated and replated at limiting dilutions of 200, 500, and 1500 cells per 10-cm dish with STO feeder cells or iMatrix-511-coated 10-cm dish. Each subclone was selected and screened by genomic PCR. When homologous recombination was suspected, Sanger sequencing was



performed. Ultimately, 10/98 clones (11.36%) in cHEATR2 Ex1-iPSCs, 42/781 (5.37%) in cHEATR2 Ex12-iPSCs and 9/96 (9.38%) in cDNAH11 Ex59-iPSCs had a neomycin-resistant gene cassette knocked in at the target locus. Although 1/240 (0.42%) in cDNAH11 Ex75-iPSCs were gene-corrected, the cells did not contain a neomycin-resistant gene cassette. In cHEATR2 Ex1-iPSCs, cHEATR2 Ex12-iPSCs and cDNAH11 Ex59-iPSCs, 10  $\mu$ g of Cre expression vector was delivered to knockin clones by electroporation and cells were reseeded at limiting dilutions. Excision of the G418-selection cassette was confirmed by genomic PCR and sequencing. cHEATR2 Ex1-iPSCs (Clone: res100-14), cHEATR2 Ex12-iPSCs (Clone: res296-1), cDNAH11 Ex59-iPSCs (Clone: res9-6) and cDNAH11 Ex75-iPSCs (Clone: res103-13) were established for subsequent experiments. All gene-corrected iPSCs were verified as derived from their parental iPSCs using short tandem repeat analysis (Promega). The predicted off-target sites with up to 3-bp mismatches were obtained from the database (GGGenome; <https://gggenome.dbcls.jp/en/>) and examined by Sanger sequencing (Table S1–3).

### Generation of knockout iPSCs

201B7 iPSCs were maintained on mitomycin C-treated STO feeder cells in Primate ES medium (ReproCELL) or iMatrix-511-coated plates in StemFit AK02N (Ajinomoto), as described previously(19, 57). The medium was supplemented with Y-27632 at 10  $\mu$ M for at least 1 h before electroporation. iPSCs were dissociated into single cells as described for gene correction. Two plasmid vectors comprising 5  $\mu$ g of Cas9 and 5  $\mu$ g of sgRNA (*VANGL1*-sgRNA, *RSPH4A*-sgRNA, *DNAH11*-sgRNA, and *DNAAF2*-sgRNA, respectively) were incorporated into  $1.0 \times 10^6$  cells using a NEPA 21 electroporator (poring pulse: pulse voltage, 125 V; pulse width, 5 ms; pulse number, 2). The cells were placed on STO feeder cells or iMatrix-511-coated plates for recovery in the medium supplemented with 10  $\mu$ M Y-27632 for 2 days. At 7–10 days after electroporation, the cells were replated at limiting dilutions of 200, 500, and 1500 cells per 10-cm dish with feeder cells or iMatrix-511-coated 10-cm dishes. Each subclone was then screened by genomic PCR and Sanger sequencing to obtain homozygous knockout iPSC clones. *VANGL1*(-/-), *RSPH4A*(-/-), *DNAH11*(-/-) and *DNAAF2*(-/-) iPSCs were ultimately established in 1/82 clones (1.22%), 20/96 clones (20.83%), 54/96 clones (56.25%), and 2/96 clones (2.08%), respectively. We chose *VANGL1*KO-12, *RSPH4A*KO-87, *DANH11*KO-54, and *DNAAF2*KO-29 clones for subsequent experiments after confirming that no base changes were present in the potential off-target sites with up to 3-bp mismatches (<https://gggenome.dbcls.jp/en/>) (Table S1–3).

### Differentiation of iPSCs into airway epithelial cells

Each cell line was cultured in Essential 8 medium (Thermo Fisher Scientific) before differentiation studies and tested for mycoplasma using the MycoAlert mycoplasma detection kit (Lonza). No contamination was detected. Cells were used for differentiation studies within 30 passages after generation. The 201B7-iPSCs were used as healthy donor-iPSCs. Human iPSCs were stepwise differentiated into the functional airway epithelial cells using each medium (Table S4), as previously described(6). The optimized concentrations of ATRA in the ventralization medium (Step 3) for PCD#1-iPSCs (PCD1-1), cHEATR2 Ex1-iPSCs (res100-14) and cHEATR2 Ex12-iPSCs (res296-1) were 1.0  $\mu$ M, PCD#2-iPSCs (PCD9-1), cDNAH11 Ex59-iPSCs (res9-6) and cDNAH11 Ex75-iPSCs (res103-13) were 0.1  $\mu$ M, and PCD#3-iPSCs (PCD6-2) were 0.5  $\mu$ M,

while that for all 201B7 derivative cell lines was 0.05  $\mu\text{M}$ . The concentration of CHIR99021 was set at 3.0  $\mu\text{M}$ . On Day 14, Y-27632 was supplemented in the medium, and cells were dissociated with Accutase. *NKX2-1*<sup>+</sup> ventralized anterior foregut endodermal cells (VAFECs) were immunostained with mouse anti-human CPM (Abcam; ab49278/1C2 or Wako; 014-27501) at 0.2  $\mu\text{l}$  in 20  $\mu\text{l}$  of 1% BSA/PBS per million cells. CPM<sup>+</sup> VAFECs were then isolated with anti-mouse IgG or IgG1-microbeads (Miltenyi Biotec) as the secondary antibody using an LS column (Miltenyi Biotec) or auto-MACS Pro Separator (Miltenyi Biotec) and the airway epithelial cells were induced in organoids until Day 42, reseeded onto ALI culture, and harvested until Day 56 for analyses of CBF and MTI as well as their immunostaining, as described previously(6). For the airway-on-a-chip studies, the cells in Matrigel were dissociated on Day 28 and replated on the laminin 511-coated membrane of the top channel of an airway chip and the Step 5 medium was replaced depending on the conditions.

### **Fabrication of microfluidic devices**

The microfluidic device consists of two layers of microchannels separated by a semipermeable membrane. The microchannel layers were fabricated from polydimethylsiloxane (PDMS) using a standard soft lithographic method. PDMS prepolymer (Sylgard 184; Dow Corning) at a ratio of 10:1 base to curing agent was cast against a mold composed of SU-8 2150 (MicroChem) patterns formed on a silicon wafer. The cross-sectional size of the microchannels was 1 mm  $\times$  300  $\mu\text{m}$  (w  $\times$  h). Two PDMS layers were bonded to a semipermeable PET membrane containing 0.4  $\mu\text{m}$  pores (#353090; Falcon) using a thin layer of liquid PDMS prepolymer as mortar(58). PDMS prepolymer was spin-coated (4000 rpm for 60 s) onto a glass slide. Subsequently, both the top and bottom channel layers were placed on the glass slide to transfer the thin layer of PDMS prepolymer onto the embossed PDMS surfaces. The membrane was then placed onto the bottom layer and sandwiched with the top layer. The combined layers were left at RT for 1 day to remove air bubbles and then placed in an oven at 60  $^{\circ}\text{C}$  overnight to cure the PDMS glue.

### **Microfluidic cell culture in airway-on-a-chip**

The microfluidic devices were oxidized in an oxygen plasma (Covance MP, Femto Science) for 5 min to generate a hydrophilic surface. Shortly thereafter, PBS was introduced into the channels and the devices were treated with ultraviolet light in biological safety cabinet (MHE-131AJ; SANYO) for 30 min. Before cell culture, the semipermeable membrane of the device was coated with recombinant laminin-511 (5.0  $\mu\text{g}/\text{cm}^2$ ; Matrixome) at 37  $^{\circ}\text{C}$  for 24 h. PAEPC spheroids in 3D Matrigel blocks were dissociated on Day 28 and placed on the laminin-coated membrane in the upper channel of the device at a density of  $1.0 \times 10^7$  cells/ml (50–100  $\mu\text{l}$ ) and 100  $\mu\text{l}$  of Step 5 medium was added to the lower channel. Four hours later, 200  $\mu\text{l}$  of Step 5 medium was added to the cell monolayer in the upper channel. After 8 h, the attached cells were perfused with culture medium using a micro peristaltic pump (RP-TXP5F or RP-HXP5F; Aquatech Co Ltd) at a flow rate of 1500–3000  $\mu\text{l}/\text{h}$  (0.22–0.44 dyne/ $\text{cm}^2$ ). The microfluidic device was placed in an incubator set at 5%  $\text{CO}_2$  and 37  $^{\circ}\text{C}$  and the Step 5 medium of both channels was changed every other day until Day 42. To examine the effect of FSS duration and timing on the muco-ciliary transport in the airway-on-a-chip, the cells were perfused with medium flow at 1500–3000  $\mu\text{l}/\text{h}$  (0.22–0.44 dyne/ $\text{cm}^2$ ) during the first half 7-day (early period; Day 1 to Day 7) or the second half 7-day (later



period; Day 8 to Day 14) periods compared with that for consecutive 14-day (whole; Day 1 to Day 14) period, beginning on Day 28 until Day42 (Fig. S2A-D).

### Quantitative analysis of the muco-ciliary transport of cell sheet of iMCACs

Each sample and membrane was placed on a glass slide and immersed in 100  $\mu$ l of Step 5 medium. The flow of fluorescent microbeads (a 500-fold dilution of Fluoresbrite, 0.5  $\mu$ m; Polysciences) placed on the iMCAC sheet was recorded at 36-42 fps and a total of 64 frames per take were acquired using an Orca-ER CCD camera (Hamamatsu) connected to an upright fluorescent microscope (BX53; Olympus) equipped with a 60 $\times$  water immersion objective. The samples were analyzed immediately after they were placed on a Thermo plate (37  $^{\circ}$ C; Tokai Hit) and fluorescent microbeads were traced with TrackMate (ImageJ/Fiji software program plugin). To improve microbead detection, we applied Gaussian blur filtering to the original sequential images of the fluorescent microbeads and subtracted the processed images from the original sequential images to remove the diffraction rings of the fluorescent microbeads. After extracting the time-series data for the fluorescent microbead positions, we evaluated the MTI, as described previously(6). Brownian motion was set as a negative control when analyzing the muco-ciliary transport(59). To calculate the MTI, more than 150 trajectories in each experiment of each iPSC line were analyzed. We also evaluated the direction of each trajectory of the fluorescent microbeads of the cell sheet cultured on an airway chip using the time-series data on the position of the fluorescent microbeads. The angle histogram for the trajectories of the microbeads on the cell sheets cultured under each condition were analyzed using the Matlab software program (Mathworks), and the degree of trajectory unidirectionality was assessed for microbeads using the index of orientation. The index of orientation was calculated by extracting vector sets connecting the start and end points of each trajectory of the fluorescent microbeads. We excluded short trajectories with a duration less than four frames to avoid overestimation of the muco-ciliary transport effect. When we analyze the muco-ciliary transport of the negative control (undifferentiated iPSCs), PCD#1-iMCACs and PCD#2-iMCACs with weak muco-ciliary transport (MTI < 2.0), the trajectories with duration less than four frames were included for analysis to avoid bias by excluding short trajectories. (Fig. 1G, Fig. 6 and Fig. S1F-G). We defined the angle between each vector and horizontal axis by  $\theta_i$  ( $i = 1, \dots, N$ ), where  $N$  represents the total number of trajectories detected by TrackMate (ImageJ/Fiji software program plugin). The unit vectors in two-dimensional  $p_i$ s were defined according to Equation 1:

$$p_i = (\cos \theta_i, \sin \theta_i) \quad (1)$$

Based on this definition, a single mean vector  $p$  was calculated according to Equation 2:

$$p = \frac{1}{N} \sum_{i=1}^N p_i \quad (2)$$

The index of orientation is defined as  $|p|$  and is in the range of [0,1]. This value is an indicator of the unidirectionality of the trajectories of fluorescent microbeads. If all trajectories of the fluorescent microbeads are in the same direction, the index of orientation will be 1. If the orientations of the trajectories of the fluorescent microbeads are random, the index of orientation will be 0. More than 150 trajectories in each experiment of each iPSC line were analyzed.

### IF staining

To compare ALI and airway chip culture, airway epithelial cells were induced as described in Fig. 1A. Meanwhile, other airway epithelial cells were induced in organoids until Day 42, converted to ALI culture, and harvested at Day 56 for immunostaining, as described previously(6). The ALI and airway chip culture samples and cytospin slides, were fixed with 4% paraformaldehyde/PBS for 15 min. However, for double IF staining using anti-*DNAH11*, anti-*OCN* and anti-*VANGL1* antibodies, samples were fixed with 100% methanol at -20 °C for 7 min. The samples were then immunostained with primary and secondary antibodies, as previously described(19). Matrigel-embedded 3D organoids were fixed with 4% paraformaldehyde for 30 min at RT, washed with PBS thrice, and immersed in 30% sucrose overnight at 4 °C. Samples were then embedded in OCT compound (Sakura Finetek) and frozen in liquid nitrogen. The frozen organoids were sliced into 10 µm thick sections on slides, permeabilized and immersed in blocking solution as described previously(6). They were immunostained with primary antibodies overnight at RT and with secondary antibodies for 1 h at RT. Nuclei were stained with Hoechst-33342 (Dojindo) supplemented in the secondary antibody solution. All antibodies used are listed in the Table S5. IF images were acquired using a BZ-X710 (Keyence) and a TCS SP8 confocal microscope (Leica Microsystems).

### **Quantification of fluorescence area of target proteins**

The fluorescence area of each target protein expression was measured using ImageJ/Fiji (National Institutes of Health). The area was normalized with FOXJ1 or Ac-TUB to correct for differences of iMCAC differentiation efficiency. Each expression ratio was compared among the corresponding isogenic counterparts of each cell line.

### **Electron microscopy**

Airway epithelial cells were induced in organoids until Day 56, as described previously(6). Matrigel-embedded organoids were immersed in fixative containing 2.5% glutaraldehyde, 4% paraformaldehyde, 1.0% tannic acid (Koso Chemical), and 0.1 M phosphate buffer (pH 7.4) at 4 °C overnight. The fixative solution was then changed to the fixative solution without tannic acid. After washing three times in 0.1 M phosphate buffer (pH 7.4) for 20 min, the samples were fixed in 1.0% osmium tetroxide (Nacalai Tesque) for 2 h and gradually dehydrated and embedded in pure epon, as described previously(6). The samples were then sliced using an ultramicrotome (EM UC6; Leica) and stained with uranyl acetate and lead citrate. Finally, the sections were examined with a Hitachi H-7650 or JEM-1400Flash transmission electron microscope.

### **Measurement of the CBF**

PAEC spheroids in 3D Matrigel blocks were dissociated on Day 42 and replated on a Geltrex-coated or iMatrix-511-coated 12-well cell culture insert in a total volume of 0.5 ml at a density of  $9.33 \times 10^5$  cells/cm<sup>2</sup>. Step 5 medium (1 ml) was added to the lower chamber and changed every other day for 14 days unless otherwise indicated. The medium was reduced to 0.125 ml in the upper chamber, and ALI culture began on Day 43. Each sample was placed on a slide glass and immersed in 100 µl of Step 5 medium. For analysis of the airway epithelial cell sheet in an airway chip, the microfluidic device was dismantled with a disposable scalpel (Feather No.11) and the cell culture membrane was removed. The membrane was placed on a slide glass and immersed in

100  $\mu$ l Step 5 medium. The CBF of MCACs was measured by acquiring images at 250 frames per second (fps) using a high-speed video camera (FASTCAM MC2.1; Photron) equipped with the Photron FASTCAM Viewer Ver. 3.0 software program connected to an upright microscope (Zeiss Axioplan; Carl Zeiss) with 63 $\times$  water immersion objective. Prior to measurement, the samples were placed in an incubator (INUG2-UK; Tokai Hit) maintained at 5% CO<sub>2</sub> and 37 °C for 30 min on the microscope stage, as the CBF increased over time and reached a plateau after 30 min. The CBF was then calculated using the ImageJ/Fiji (National Institutes of Health) and Scilab software programs (Scilab Enterprises), as described previously(6). To minimize the effect of noise, regions of interest that included beating cilia were selected by setting a threshold range for the CBF between 3 and 35 Hz. When the cilia were completely immotile under the direct view through the high-speed camera, the CBF was set to 0.

### **Quantitative analysis of the degree of coordination for VANGL1 crescents of iMCACs**

To analyze the degree of coordination for VANGL1 crescents of iMCSCs in the cell sheet, we simultaneously immunostained the cell sheet for VANGL1, OCLN, and Ac-TUB and recorded the immunostaining using a confocal microscope (TCS SP8; Leica Microsystems) equipped with a 63 $\times$  oil-immersion objective lens. We converted the recorded 3D images into 2D images by maximum intensity projection in the z-direction. We then detected iMCACs using Ac-TUB-stained multiple cilia and extracted the position of the center of mass and the tip of the VANGL1 crescents of all iMCACs in the cell sheets under each condition using the ImageJ/Fiji software program. The angle between the line from the tip of the crescent to the center of the mass and the horizontal axis was then calculated, followed by calculation of the index of orientation, as described for the quantitative analysis of the muco-ciliary transport of the cell sheet of iMCACs. More than 35 cells per experiment in each iPSC line were analyzed.

### **Quantitative reverse transcription-PCR (qRT-PCR) analyses**

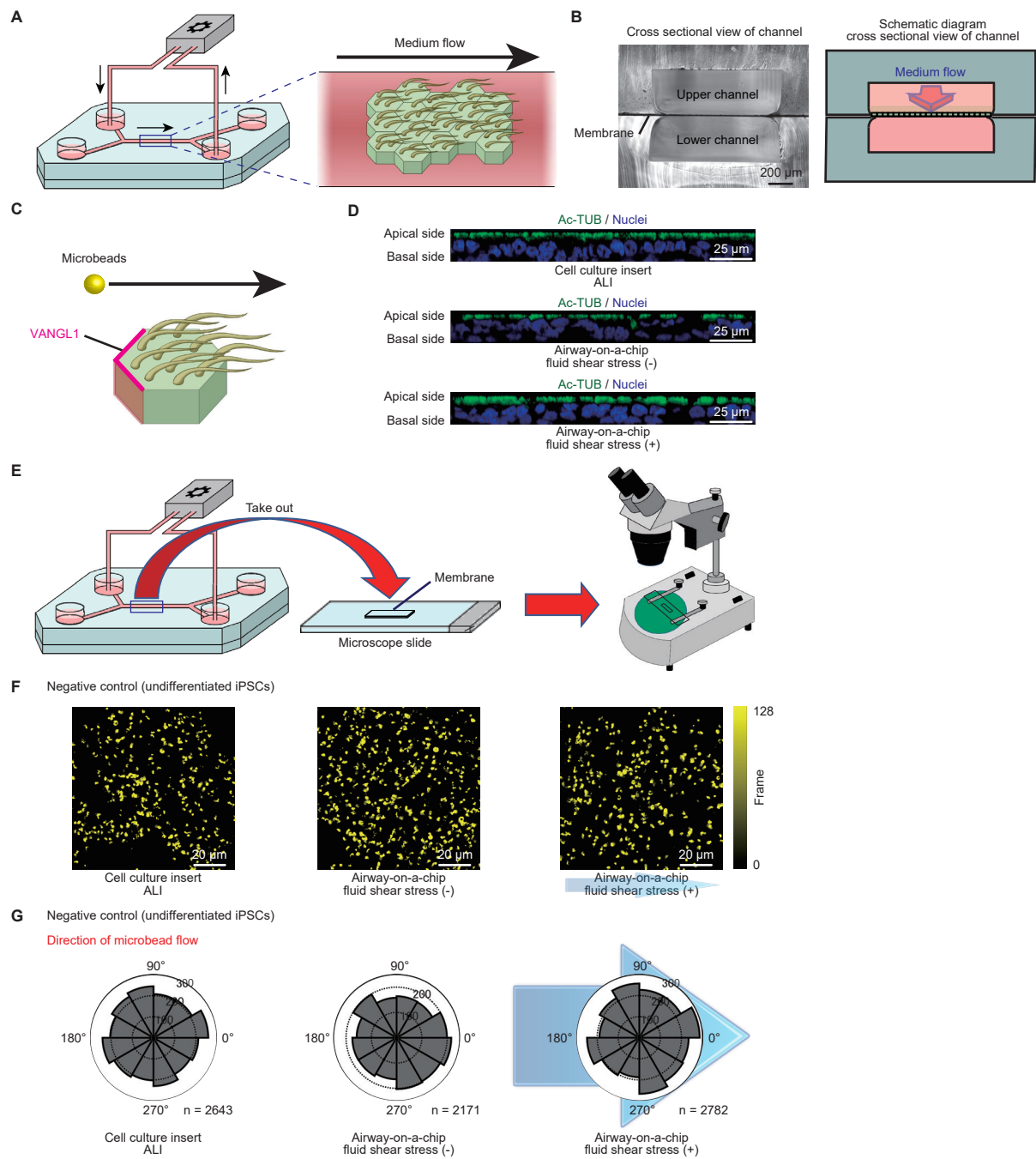
Total RNA was isolated with a PureLink RNA mini kit (Invitrogen). For the microfluidic devices, total RNA was isolated with a NucleoSpin RNA Plus XS (Takara Bio). cDNA was synthesized, amplified and quantified by QuantStudio 3 (Applied Biosystems), as described previously(27). PCR was performed in duplicate for each sample. The expression of each gene was normalized to that of *ACTB* and compared with to control RNA of fetal trachea samples (29 weeks' gestation; #R1244160-10, Lot.B402231; BioChain) as the relative gene expression. The primers used in qRT-PCR are listed in the Table S6.

### **RNA-seq**

The iPSC-derived airway cell sheet cultured in ALI and airway chip culture with and without FSS (n = 3) were subjected to RNA-seq analysis. All cells were harvested on Day 42 and total RNA was isolated with a PureLink RNA mini kit (Invitrogen) or NucleoSpin RNA Plus XS (Takara Bio). The libraries were then prepared using a SMART-Seq v4 Ultra Low Input RNA Kit (TAKARA) and Nextera XT DNA Library Prep Kit (Illumina), according to the manufacturer's instructions. Each sample was sequenced on the NovaSeq 6000 (Illumina) with 150-bp paired-end reads. Sequenced reads from fastq files were mapped to GRCh37/hg19 whole genome using the STAR 2.6.0c aligner. Fragments per kilobase of exon per million mapped reads (FPKM) values

for each gene was calculated using GeneData Profiler Genome (version 13.0.11). All expression analyses were performed with GeneSpring GX 13.0 (Agilent Technologies; Fig. 2B-E) or Metascape (<http://metascape.org/gp/index.html#/main/step1>) (Fig. S3D). Fold change in gene expression between groups was determined by ANOVA using a linear threshold of 2 and  $P < 0.05$  with Benjamini-Hochberg FDR. GO enrichment analysis was performed on the 770 upregulated genes in airway chip culture with FSS relative to that without FSS ( $P < 0.05$ , Fisher's exact test).

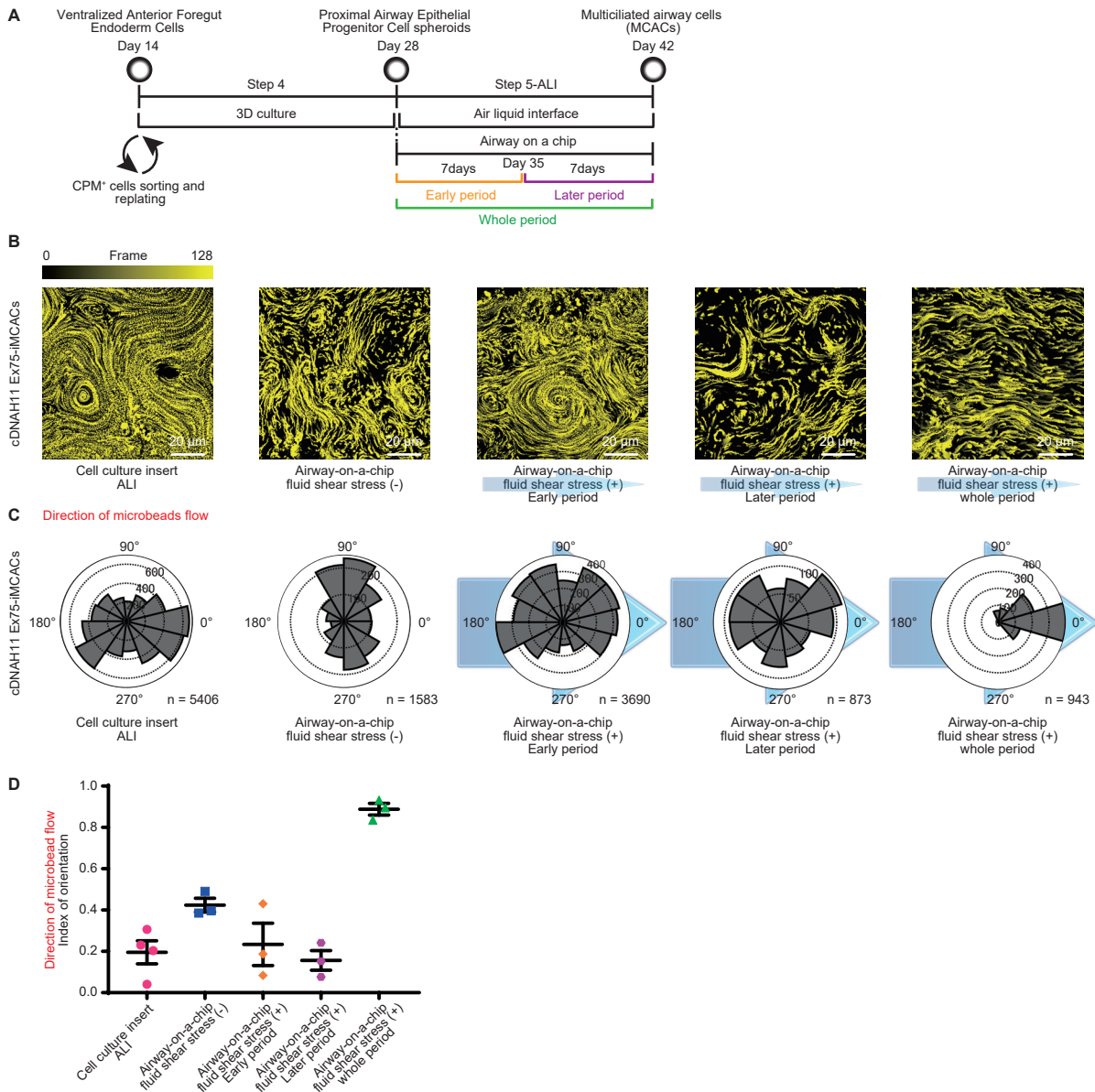
Fig. S1



**Fig. S1. Established coordinated ciliary beating and unidirectional muco-ciliary flow using fluid shear stress in airway-on-a-chip.**

**(A)** Schematic diagram of the airway-on-a-chip. The iMCACs were cultured under FSS induced by medium flow using a micro peristaltic pump. **(B)** Cross sectional view of upper and bottom channels in airway-on-a-chip. **(C)** Scheme of VANGL1 localization at the cell boundary on the opposite side of the beating cilia. Microbeads are depicted to move along the muco-ciliary flow. **(D)** A cross sectional view of IF staining of Ac-TUB in healthy donor-iMCACs in each condition. Ac-TUB was detected on the apical surface. **(E)** Schematic illustration of the pipeline for analyzing muco-ciliary transport. The membrane was removed from the airway-on-a-chip and placed on a horizontal stage of a microscope for examination. **(F)** Stacked images of the fluorescent microbeads placed on undifferentiated iPSCs under each condition. Color spectrum reflects the time course. **(G)** Direction of the fluorescent microbead flow on the undifferentiated iPSCs in each condition. Angle histograms of the fluorescent microbead flow were calculated based on the trajectories of samples under each period (n = 2643, 2171, and 2782 trajectories from 3 independent experiments in each condition, respectively).

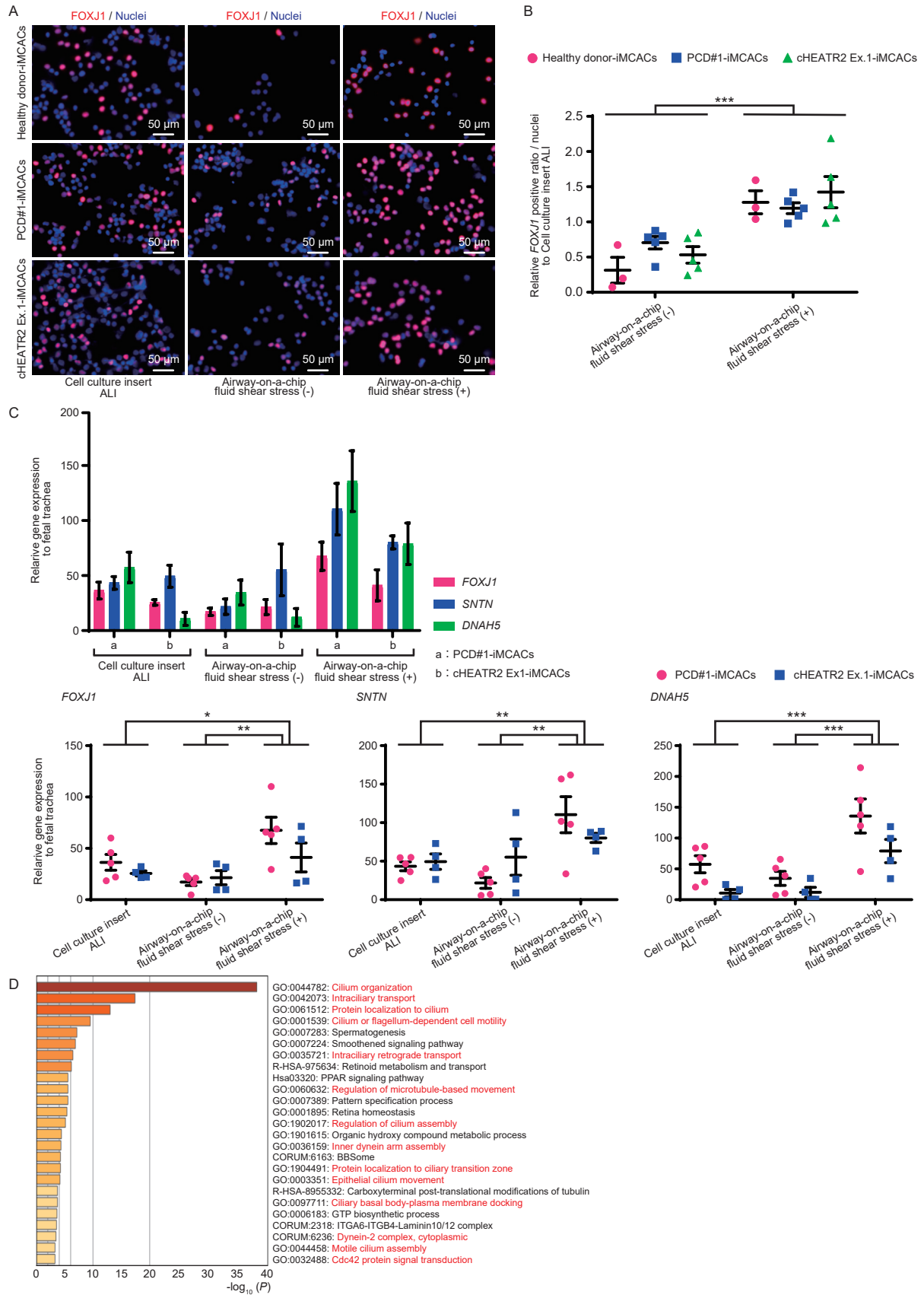
Fig. S2



**Fig. S2. Analysis of duration and timing of fluid shear stress required for achieving coordinated ciliary movement.**

(A) Schematic diagram to examine the effect of duration and timing of fluid shear stress on the muco-ciliary transport in the airway-on-a-chip. (B) Stacked images of the fluorescent microbeads placed on an iPSC-derived airway cell sheet under each condition. Color spectrum reflects time course. Experiment was conducted at the same time as Fig. 6. (C) Direction of the fluorescent microbead flow on the iPSC-derived airway cell sheets in each condition. Angle histograms of the fluorescent microbead flow were calculated based on the trajectories of samples under each period ( $n = 5406, 1583, 3690, 873$ , and  $943$  trajectories from 3–4 independent experiments in each condition, respectively). (D) The Io of the fluorescent microbead flow was calculated based on the sample trajectories under each condition (mean  $\pm$  S.E.M.  $n = 5406, 1583, 3690, 873$ , and  $943$  trajectories from 3–4 independent experiments, respectively). Kruskal-Wallis with Dunn's multiple comparisons test was used. Early period vs whole period  $P=0.3205$ . Later period vs whole period  $P=0.0786$ .

Fig. S3

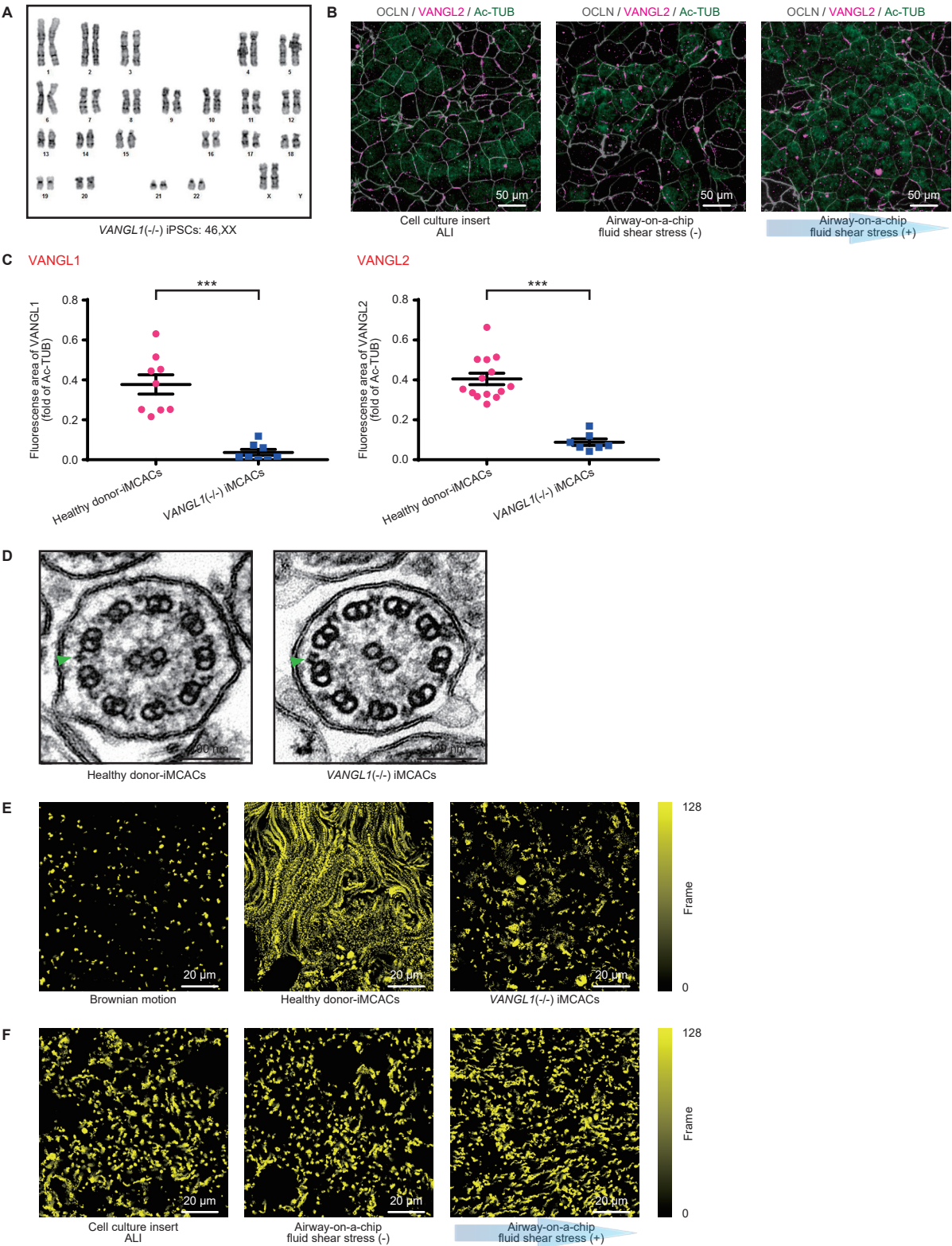




**Fig. S3. Multiciliated cell differentiation driven by fluid shear stress in airway-on-a-chip.**

**(A)** Representative images of FOXJ1+ cells of iMCACs under each condition with counterstaining of nuclei. **(B)** Induction efficiency of iMCACs calculated from the number of FOXJ1+ cells in the total number of nuclei under each condition (mean  $\pm$  S.E.M.,  $n = 3\text{--}5$  independent experiments). The score of each experiment was an average of ten randomly selected images. A two-way ANOVA with Tukey's multiple comparisons was used to examine the differences in culture method ( $P < 0.0001$ ), multiciliated cell markers ( $P = 0.5103$ ) and their interaction ( $P = 0.2441$ ). \*\*\* $P < 0.001$ . **(C)** RT-qPCR of multiciliated cell markers (*FOXJ1*, *SNTN* and *DNAH5*) under each condition (mean  $\pm$  S.E.M.,  $n = 4\text{--}5$  independent experiments). Two-way ANOVA with Tukey's multiple comparisons was used to examine the differences among each condition for *FOXJ1*, *DNAH5* and *SNTN*, respectively: culture method ( $P = 0.0031$ ,  $0.0020$  and  $0.0001$ ), cell lines ( $P = 0.1548$ ,  $0.8010$  and  $0.0064$ ), and their interaction ( $P = 0.2619$ ,  $0.1257$  and  $0.5929$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  **(D)** Pathway enrichment analysis of iMCACs under airway chip culture with and without FSS using Metascape. Significantly upregulated genes ( $P < 0.05$ ) from each condition were used for analysis. The top 25 differential pathways with  $P < 0.05$  are shown.

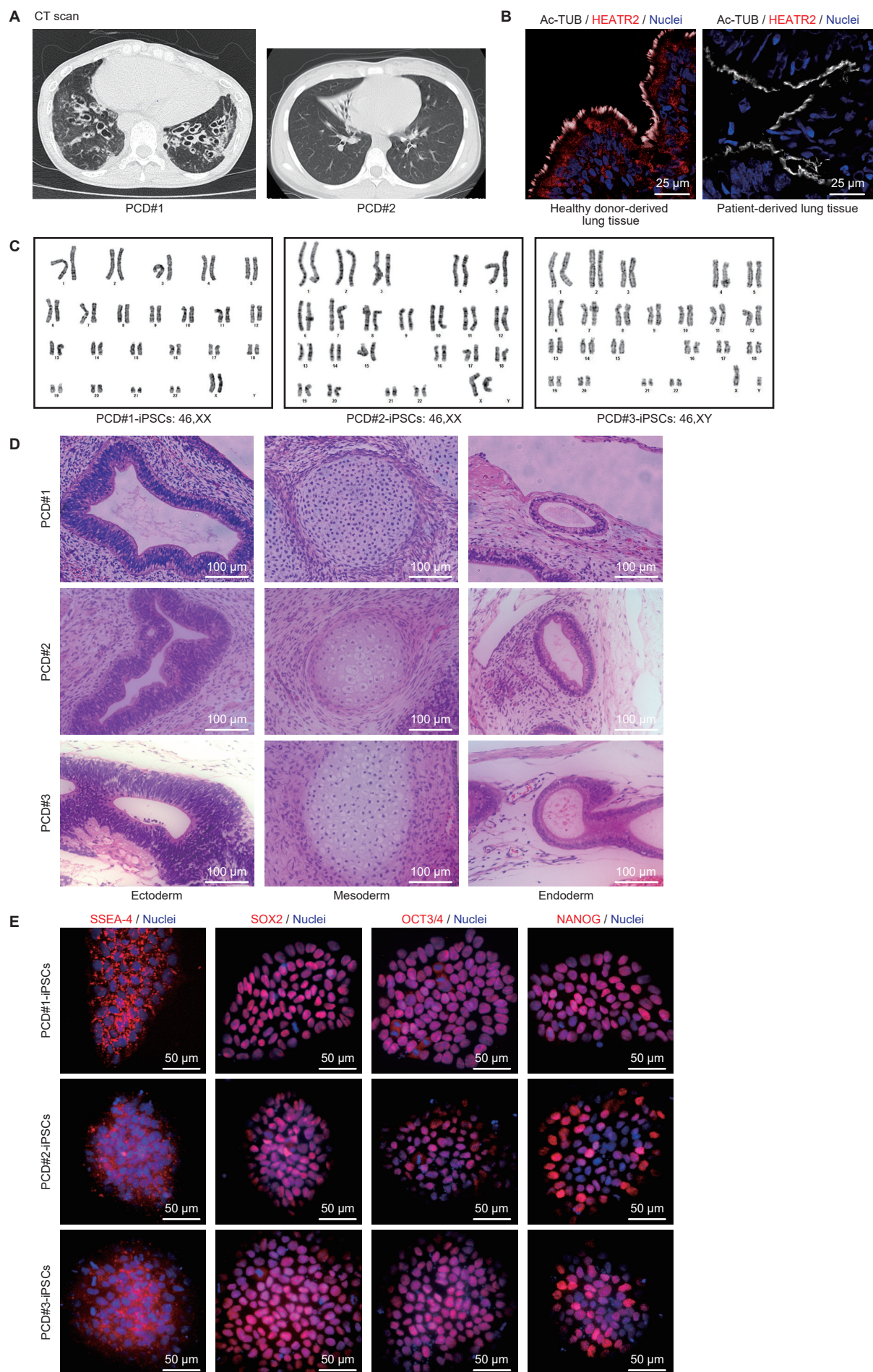
Fig. S4



**Fig. S4. Phenotypic analyses of MCACs derived from PCP gene knockout iPSCs.**

**(A)** G-banding analysis of the *VANGL1*(-/-) iPSC karyotypes. **(B)** IF staining of VANGL2, Ac-TUB and OCLN in *VANGL1*(-/-) iMCACs under each condition. **(C)** Quantification of fluorescence area of immunostained VANGL1 and VANGL2 (normalized by Ac-TUB) (VANGL1 and VANGL2; mean  $\pm$  S.E.M., n = 7–14 different views). Mann-Whitney test was used. \*\*\* $P < 0.001$ . **(D)** TEM images of the transverse section of cilia in *VANGL1*(-/-) iMCACs. Green arrowheads indicate ODA. **(E)** Stacked images of the fluorescent microbeads placed on *VANGL1*(-/-) iMCACs. **(F)** Stacked images of the fluorescent microbeads placed on *VANGL1*(-/-) iMCACs in ALI and airway chip culture with and without FSS, respectively. Color spectrum reflects the time course.

Fig. S5

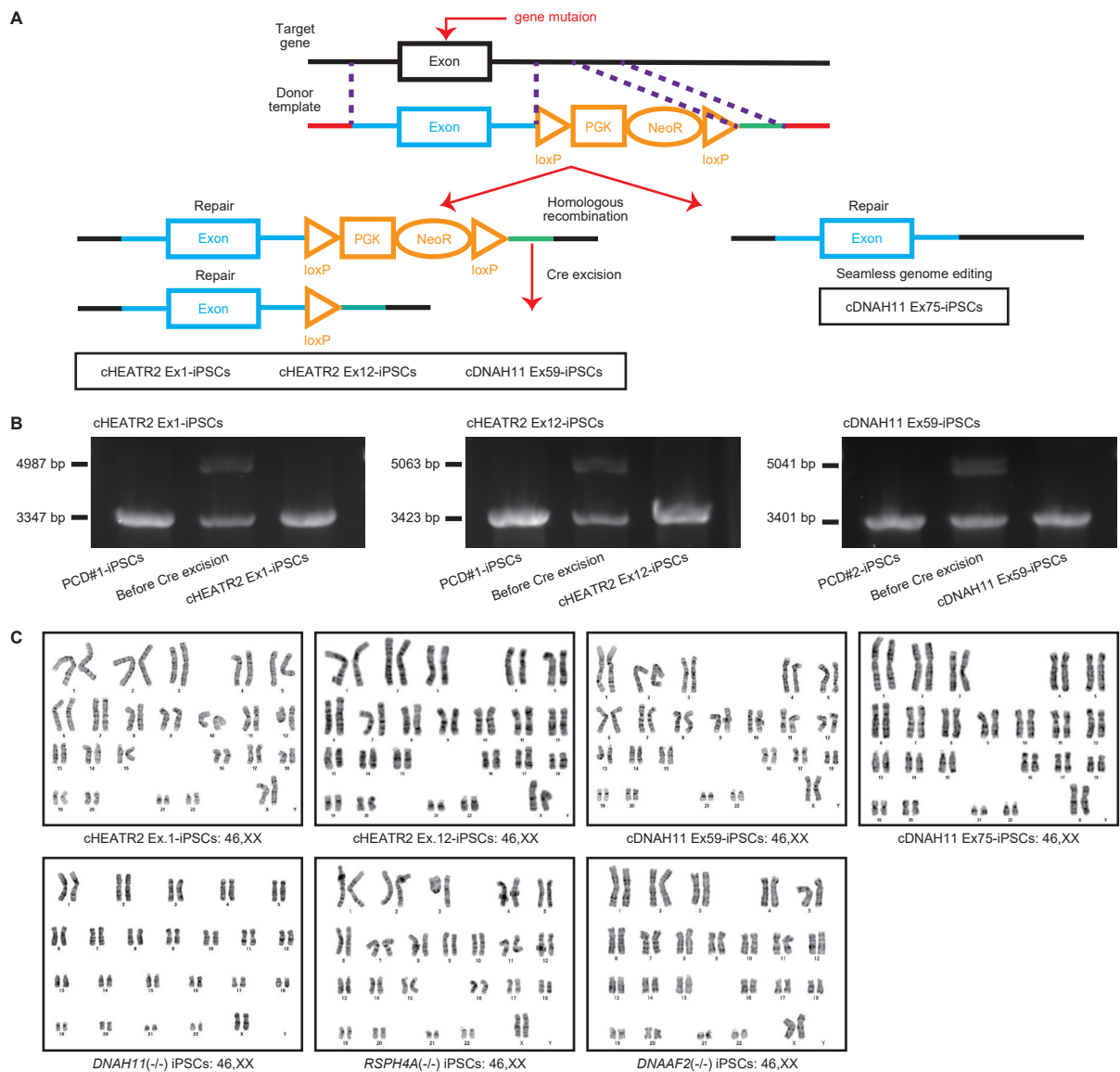


**Fig. S5. Validation of PCD#1-, PCD#2- and PCD#3-iPSC lines.**

**(A)** Chest CT scan images of PCD#1 and PCD#2, respectively. **(B)** IF staining of HEATR2 in PCD#1 lung tissue. A healthy donor-derived lung tissue was used as a control. **(C)** G-banding analysis for the PCD#1-iPSCs (PCD#1-1), PCD#2-iPSCs (PCD#9-1) and PCD#3-iPSCs (PCD6-2) karyotypes. **(D)** Hematoxylin-eosin staining of the teratomas derived from PCD#1 (PCD#1-1), PCD#2 (PCD#9-1) and PCD#3 (PCD#6-2) in SCID mice. Each panel shows ectoderm (neuronal cells), mesoderm (cartilage), and endoderm (gland) tissues, respectively. **(E)** IF staining of undifferentiated markers SSEA4, SOX2, OCT3/4 and NANOG in each cell line.



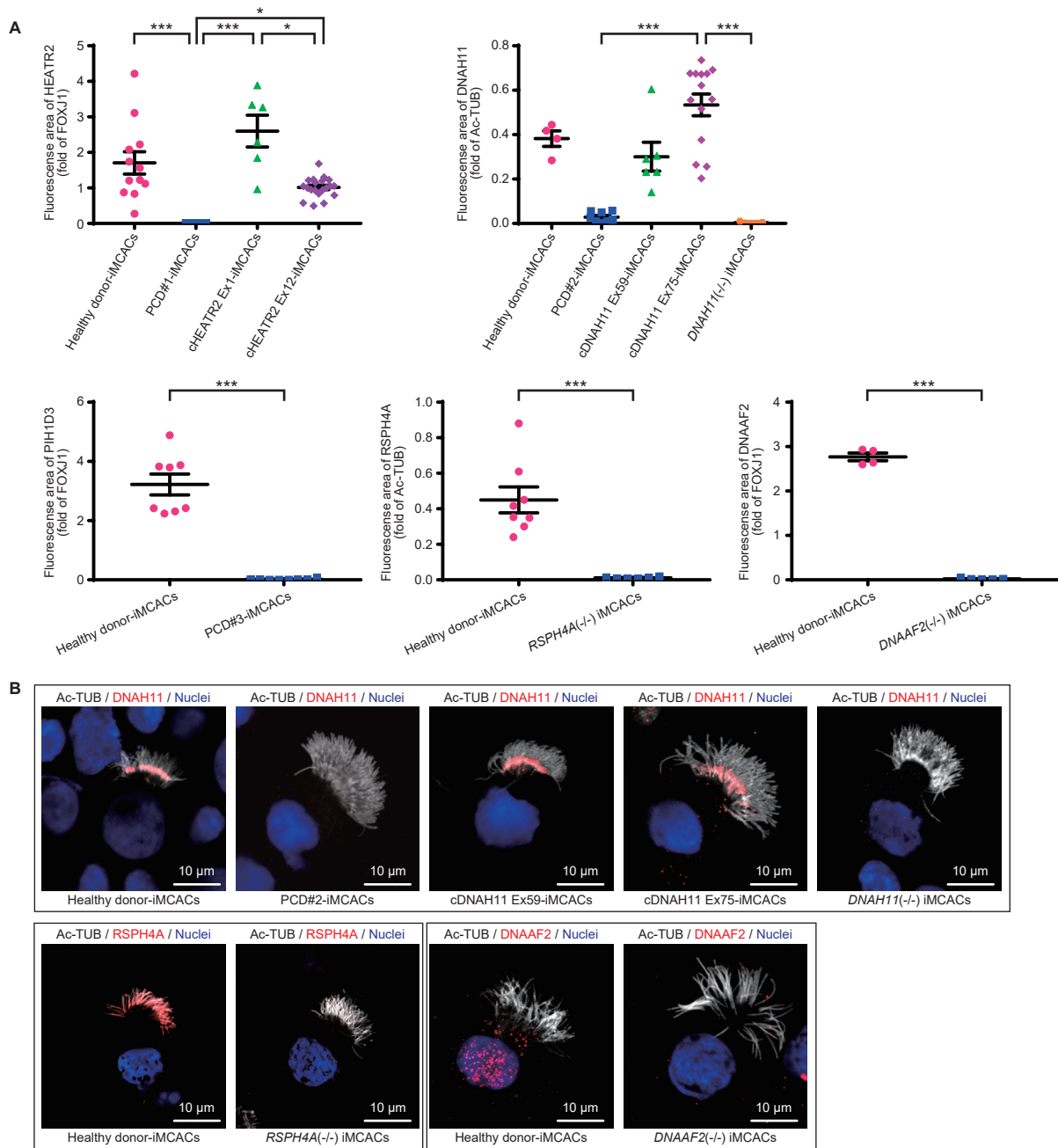
Fig. S6



**Fig. S6. Generation of PCD patient-derived iPSCs, their gene-corrected ones and PCD causative gene knockout iPSCs.**

(A) Strategy for correcting each gene mutation using homologous recombination. (B) Detection of PCR products suggesting homologous recombination in electrophoresis. (C) G-banding analysis for the cHEATR2 Ex1-iPSCs (res100-14), cHEATR2 Ex12-iPSCs (res296-1), cDNAH11 Ex59-iPSCs (res9-6), cDNAH11 Ex75-iPSCs (res103-13), DNAH11 (-/-) iPSCs, RSPH4A(-/-) iPSCs and DNAAF2(-/-) iPSCs karyotypes.

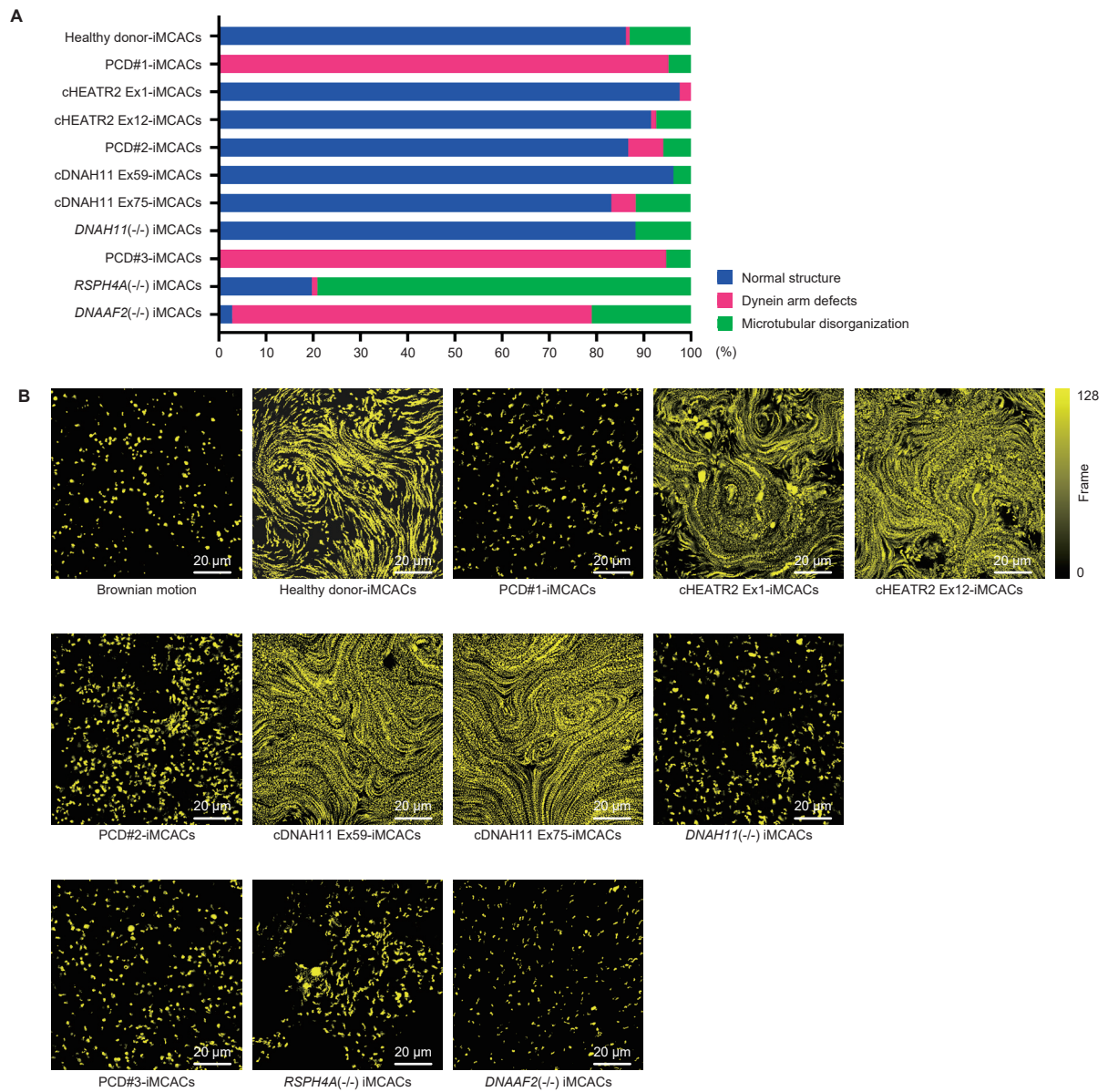
Fig. S7



**Fig. S7. Immunofluorescence analysis of each cell line.**

(A) Quantification of fluorescence area of immunostained target proteins in each cell line (normalized by FOXJ1 or Ac-TUB) (mean  $\pm$  SEM  $n = 4-20$  different views). Kruskal-Wallis with Dunn's multiple comparisons test was used. \* $P < 0.05$ . \*\*\* $P < 0.001$ . (B) IF staining of, DNAH11, RSPH4A and DNAAF2 in MCACs derived from each iPSC line. The Healthy donor-iMCACs were used for a control.

Fig. S8

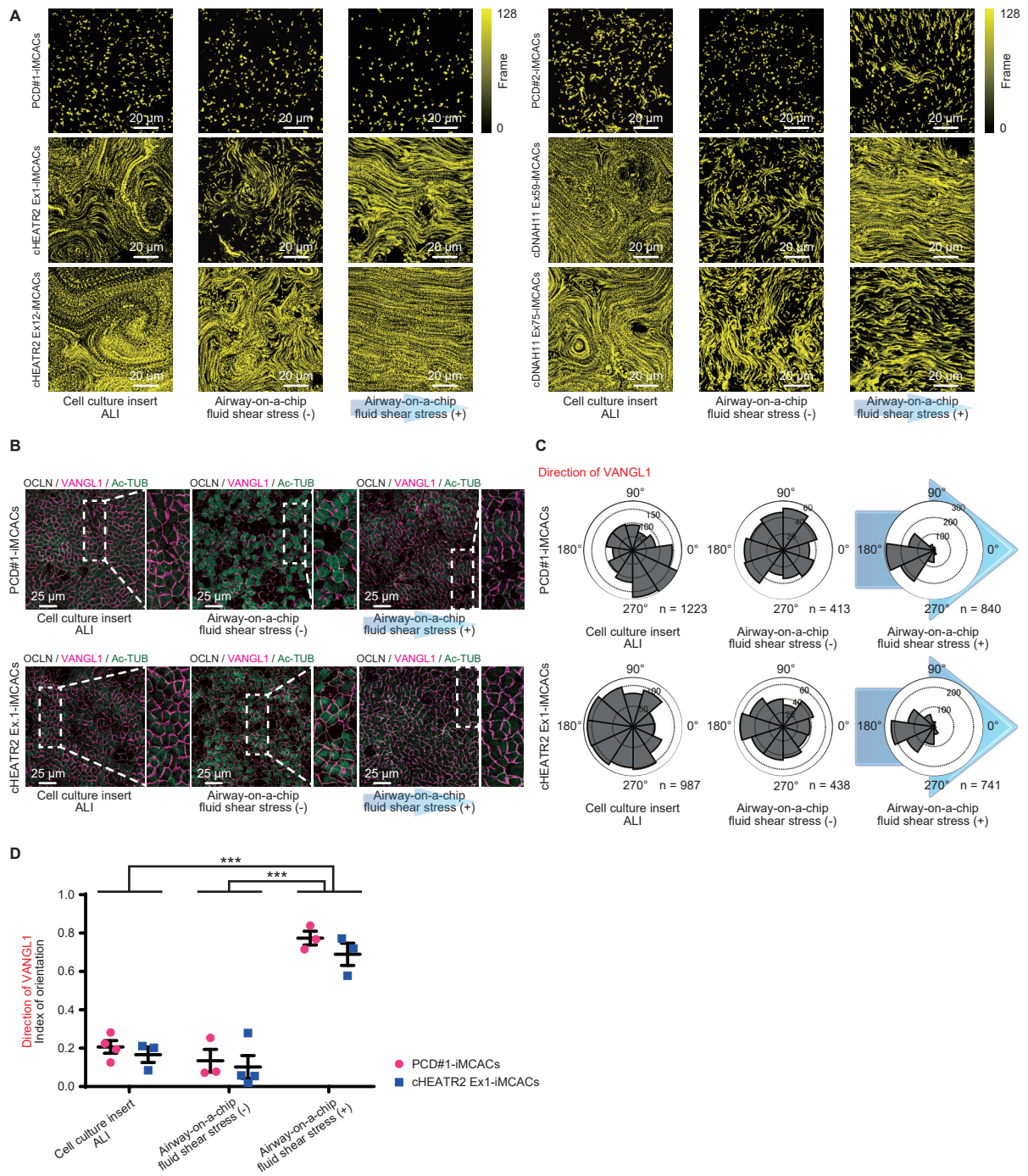


**Fig. S8. Morphological and functional analyses of each cell line.**

(A) Classification of ciliary structure images of each cell line using transmission electron microscopy. More than 30 cilia in each iPSC line were analyzed. (B) Stacked images of the fluorescent microbeads placed on an airway epithelial cell sheet derived from each cell line. Color spectrum reflects time course.



Fig. S9



**Fig. S9. Analysis of the global PCP axis and coordinated ciliary beating of PCD iMCACs in airway-on-a-chip.**

**(A)** Stacked images of the fluorescent microbeads placed on iMCACs derived from PCD cell line under each condition. Color spectrum reflects time course. **(B)** IF staining of VANGL1, Ac-TUB, and OCLN in PCD#1-iMCACs and cHEATR2 Ex1-iMCACs in ALI and airway chip culture with and without FSS. **(C)** Angle histogram of *VANGL1* localization calculated based on IF staining per sample in PCD#1-iPSCs and cHEATR2 Ex1-iPSCs under each condition (n = 1223, 413, and 840 cells from 3–4 independent experiments in PCD#1-iPSCs and n = 987, 438, and 741 cells from 3–4 independent experiments in cHEATR2 Ex1-iPSCs in ALI and airway chip culture with and without FSS, respectively). **(D)** The Io of *VANGL1* localization in PCD#1-iMCACs and cHEATR2 Ex1-iMCACs under each condition was calculated based on IF staining per sample (mean  $\pm$  S.E.M. n = 1223, 413, and 840 cells in PCD#1-iMCACs and n = 987, 438, and 741 cells in cHEATR2 Ex1-iMCACs in ALI and airway chip culture with and without FSS from 3–4 independent experiments, respectively). Two-way ANOVA with Tukey's multiple comparisons test was used to examine differences among culture method ( $P < 0.0001$ ), cell lines ( $P = 0.2189$ ), and their interaction ( $P = 0.8627$ ). \*\*\* $P < 0.001$ .

**Table S1. CRISPR-sgRNA used for generation of gene-corrected iPSCs and knockout iPSCs**

**cHEATR2 Ex1-iPSCs and cHEATR2 Ex12-iPSCs**

| Gene               | Chromosome | Strand | Start  | End    | sgRNA                   |
|--------------------|------------|--------|--------|--------|-------------------------|
| <i>HEATR2</i> Ex1  | chr7       | -      | 727262 | 727284 | AGCGCCAGGGCGGCGGCACGG   |
| <i>HEATR2</i> Ex12 | chr7       | -      | 779980 | 780002 | GGGTGCCATCCTCACATCGTTGG |

**cDNAH11 Ex59-iPSCs and cDNAH11 Ex75-iPSCs**

| Gene               | Chromosome | Strand | Start    | End      | sgRNA                   |
|--------------------|------------|--------|----------|----------|-------------------------|
| <i>DNAH11</i> Ex59 | chr7       | +      | 21786683 | 21786705 | GGCAGCCGTGATGGTCCGTCTGG |
| <i>DNAH11</i> Ex75 | chr7       | -      | 21880802 | 21880824 | ATAGCTTCAGCTCCAGCCCTGGG |

**Knockout iPSCs**

| Gene          | Chromosome | Strand | Start     | End       | sgRNA                   |
|---------------|------------|--------|-----------|-----------|-------------------------|
| <i>VANGL1</i> | chr1       | +      | 115664087 | 115664109 | ACTCGGGACCGGAATTACCAGGG |
| <i>RSPH4A</i> | chr6       | -      | 116616843 | 116616865 | GATGATGTTTCTGGTCCCGCGGG |
| <i>DNAH11</i> | chr7       | +      | 21543553  | 21543575  | GGTTTAGCTTCGCCGCCTCGGGG |
| <i>DNAAF2</i> | chr14      | +      | 49635054  | 49635076  | GAACTCCGGGTCCTGGAAGGCGG |

**Primers for genome sequencing**

| Gene               | Forward                 | Reverse                  | Size (bp) |
|--------------------|-------------------------|--------------------------|-----------|
| <i>HEATR2</i> Ex1  | AGCTCCAGTGTCTTTAGCC     | TGCGGTGGTTTCCAGGTATC     | 1839      |
| <i>HEATR2</i> Ex12 | ATGCCTTTGTGGACGTTTAGA   | TCACACGGCTCAGCTACAGAT    | 344       |
| <i>DNAH11</i> Ex59 | GTTCTACGGCACCCCAATAAG   | CACAACCTGTCTAAACTCTTGCC  | 3679      |
| <i>DNAH11</i> Ex75 | GCCTCACAGAACTGCCAAATC   | CGGTGGTCAACTGGTGTTTC     | 3263      |
| <i>PIH1D3</i>      | CAAAACTCCCCTCTCCCTTCT   | GACCAGGTCCAGGACACAAAT    | 50764     |
| <i>VANGL1</i> Ex3  | AGCCAAACAGACAGAAAGATCAC | CCTCCCCAAACCAGAAAAGAG    | 993       |
| <i>RSPH4A</i> Ex1  | CAACCAGGACCCAGAAATCGC   | TCAAGCCCGAGATTCCATAATTCC | 1097      |
| <i>DNAH11</i> Ex1  | GAGGCGCGAGACTTCAGAG     | TGTTAAACTGAGCATTGCGCTAC  | 1027      |
| <i>DNAAF2</i> Ex1  | CCCTCCTCCCGAATGTTAGTG   | CTTTCTCGTTACCTCCAGCG     | 1099      |

**Table S2. Predicted off-target sites of CRISPR-sgRNA used for generating cHEATR2 Ex1- and Ex12- iPSCs and PCD gene knockout iPSCs**

**cHEATR2 Ex1-iPSCs**

| Chromosome           | Strand | Start     | End       | Sequence                  |
|----------------------|--------|-----------|-----------|---------------------------|
| Chr1                 | +      | 53738065  | 53738086  | GGCGCCGGGGCGG-GGCGCACGG   |
| Chr1                 | +      | 243849517 | 243849540 | AGCGCCAGGGCGCGCGGCAG-ACGG |
| chr1_KI270763v1_alt  | +      | 865977    | 866000    | AGCGCCAGGGCGCGCGGCAG-ACGG |
| Chr2                 | +      | 70142778  | 70142799  | TGCGCCAGGGCGGCTGCGCA-GG   |
| Chr2                 | +      | 222319726 | 222319747 | AGAGCCAGGGCGGCGG-GCGCGG   |
| chr2_GL383521v1_alt  | +      | 6636      | 6657      | AGCGCCGAGGG-GGCGGCGCA-GG  |
| Chr3                 | +      | 50414826  | 50414848  | TGCGCCAGGCCGGCGGCGCAGGG   |
| Chr4                 | +      | 3765925   | 3765945   | AGCG-CAGGGCGGCGGCG-GCGG   |
| Chr4                 | +      | 7192429   | 7192449   | AGCGCCTGGGCGGCGG-GC-CGG   |
| Chr4                 | +      | 105146591 | 105146612 | GGCCCCAGGGCGGCGGCGCA-GG   |
| Chr5                 | +      | 169300657 | 169300678 | AGCGCCA-GGCGG-GCGCGCACGG  |
| Chr6                 | +      | 34792119  | 34792140  | AGCGCCATGGCGGCGGCG-GCGG   |
| Chr6                 | +      | 40134129  | 40134152  | AGCGCAAGGGACGGCGGCGCAAGG  |
| Chr6                 | +      | 150143229 | 150143252 | GGGGCCAGCGGCGGCGGCGCACGG  |
| Chr7                 | +      | 1530788   | 1530808   | AGCGCCA-GGCGGCGGCG-GCGG   |
| Chr8                 | +      | 17156748  | 17156768  | AGCGCCA-GGCGGCGGTGC-CGG   |
| Chr8                 | +      | 43140444  | 43140465  | AGCG-CAGGGCGG-GGCGCAGCGG  |
| Chr10                | +      | 75235882  | 75235903  | AGGGCCAGCGCGGCGG-GCACGG   |
| Chr11                | +      | 76860948  | 76860969  | AGCGCCTGGGCGGCGGCG-GCGG   |
| Chr12                | +      | 53995691  | 53995712  | AGCGTCAGGGCGGGGGCGC-CGG   |
| Chr15                | +      | 28094901  | 28094920  | AG-GCCAGGGG-GCGGCGC-CGG   |
| chr15_KI270905v1_alt | +      | 229200    | 229219    | AG-GCCAGGGG-GCGGCGC-CGG   |
| Chr16                | +      | 165952    | 165972    | AG-GCCAGGGCGGCGG-GCGCGG   |
| Chr16                | +      | 2179821   | 2179841   | AG-GCC-GGTCGGCGGCGCACGG   |
| Chr17                | +      | 10697648  | 10697668  | AGC-CCAGGG-GCCGGCGCACGG   |
| Chr17                | +      | 29566938  | 29566960  | AGCGCGAGGGCGGCGCCCCACGG   |
| Chr17                | +      | 63477494  | 63477514  | AGCGCC-GGGCTGC-GCGCACGG   |
| Chr19                | +      | 12790253  | 12790272  | A-C-CCAGGGCGG-GGCGCACGG   |
| Chr19                | +      | 55080833  | 55080854  | AGCG-CAGGGCGGCGGCGCTCAG   |
| chr19_GL949746v1_alt | +      | 984231    | 984252    | AGCG-CAGGGCGGCGGCGCTCAG   |
| chr19_GL949747v2_alt | +      | 726035    | 726056    | AGCG-CAGGGCGGCGGCGCTCAG   |
| chr19_GL949748v2_alt | +      | 1060819   | 1060840   | AGCG-CAGGGCGGCGGCGCTCAG   |
| chr19_GL949749v2_alt | +      | 1088356   | 1088377   | AGCG-CAGGGCGGCGGCGCTCAG   |
| chr19_GL949750v2_alt | +      | 1062905   | 1062926   | AGCG-CAGGGCGGCGGCGCTCAG   |
| chr19_GL949751v2_alt | +      | 999198    | 999219    | AGCG-CAGGGCGGCGGCGCTCAG   |
| chr19_GL949752v1_alt | +      | 983615    | 983636    | AGCG-CAGGGCGGCGGCGCTCAG   |
| chr19_GL949753v2_alt | +      | 792994    | 793015    | AGCG-CAGGGCGGCGGCGCTCAG   |
| chr19_KI270938v1_alt | +      | 1063315   | 1063336   | AGCG-CAGGGCGGCGGCGCTCAG   |
| Chr20                | +      | 37346410  | 37346429  | AGCGCC-GGGC-G-GGCGCACGG   |

|       |   |           |           |                           |
|-------|---|-----------|-----------|---------------------------|
| Chr20 | + | 50191606  | 50191626  | GGCGCC-GGGCGGCGGCGCA-GG   |
| Chr21 | + | 39313406  | 39313429  | AGCGCCAGGGCGGGGGTGGCACGG  |
| Chr22 | + | 41301612  | 41301634  | AGCTGCCGGGGCGGCGGCGC-CGG  |
| Chr1  | - | 10210647  | 10210666  | CCGT-CGCCG-CGCCCTGGC-CT   |
| Chr2  | - | 36356659  | 36356680  | CC-TGCGCCGCC-CCCTCGGCGCT  |
| Chr3  | - | 49470233  | 49470254  | CCGCGCGCC-CCGCCCTTGCGCT   |
| Chr3  | - | 99818092  | 99818115  | CCGTGCGCCTCAGCCCTGGTCGCT  |
| Chr3  | - | 141738144 | 141738164 | CC-TGC-CCGCCCCCTGGCGCT    |
| Chr3  | - | 184314757 | 184314779 | CCCTGCGCCGCGCCCCGCGGCC    |
| Chr4  | - | 26321206  | 26321227  | CCGTGCGCTGCCGCTCT-GCGCT   |
| Chr6  | - | 35776617  | 35776638  | CCATG-GCCGCAGCCCTGGCGCT   |
| Chr7  | - | 16421280  | 16421301  | CCG-GCTCCGCGGCGCTGGCGCT   |
| Chr9  | - | 75088374  | 75088393  | CCGTGCG-CGCCGCCC--GCGCT   |
| Chr9  | - | 129319051 | 129319072 | CCGT-CGCCCCCGCCCTGGCCCT   |
| Chr9  | - | 137218479 | 137218499 | CTGTGC-CCGCCG-CCTGGCGCT   |
| Chr10 | - | 91410400  | 91410420  | CCG-CCGCCGCGG-CCTGGCGCT   |
| Chr10 | - | 96043426  | 96043448  | CCGCCGCGCGCGCG-CCTGGCGCT  |
| Chr10 | - | 101579720 | 101579741 | CCG-GTGCCGCGCCCTGGCCCT    |
| Chr12 | - | 132687282 | 132687303 | CCGCGCGCGCGCGCCC-GCCGCT   |
| Chr14 | - | 81220309  | 81220329  | CCGCGC-CCGCCGCCC-GGCGCT   |
| Chr16 | - | 677072    | 677091    | CCGTGC-CCG-CG-CCTGGCGCT   |
| Chr16 | - | 23149489  | 23149510  | CCG-GCGCCGCCCCGCCC-GGCGCT |
| Chr16 | - | 33707269  | 33707290  | CC-TGCGCCGCGCCCTGG-GCCT   |
| Chr16 | - | 89949260  | 89949279  | CCGTGC-CC-CCGCCCTGG-GCT   |
| Chr17 | - | 7437051   | 7437073   | CCGTGCGCCTCCG-CCTGGCAGCT  |
| Chr17 | - | 81902296  | 81902318  | CTG-GCGGCCGCGCCCTGGCGCT   |
| Chr19 | - | 45496982  | 45497003  | CCG-CCGCCGCGCGCCCTGGTGCT  |
| Chr22 | - | 20950692  | 20950714  | CCGTGAGCCGCGCGCCTGGC-CT   |

### cHEATR2 Ex12-iPSCs

| Chromosome          | Strand | Start     | End       | Sequence                |
|---------------------|--------|-----------|-----------|-------------------------|
| Chr1                | +      | 23344963  | 23344985  | GGCTGCCGTCCACACATCGTTGG |
| Chr2                | +      | 198156345 | 198156364 | GGGTGCATCCTCAATGTTGG    |
| Chr10               | +      | 129742109 | 129742131 | GTGTGGCATCCTCCCATCGTTGG |
| Chr12               | +      | 62237900  | 62237919  | GGGTGCATCCTACATCTTGG    |
| Chr15               | +      | 89322833  | 89322853  | GGGTGCCATCCTCCATCTTGG   |
| ChrX                | +      | 81647401  | 81647421  | GGGTGCCATCCTCCATGTGGG   |
| ChrX                | +      | 106935038 | 106935057 | GGGTGCCATCCTCCTGTTGG    |
| Chr3                | -      | 150656292 | 150656311 | CCAAGATTGAGGATGCACCC    |
| Chr5                | -      | 13044935  | 13044956  | CCAAGATTGAGGATGCGCACCC  |
| Chr7                | -      | 923085    | 923106    | CCCACGATGTGAGGAAGCACCC  |
| Chr7                | -      | 37026178  | 37026200  | CCAACCATGTGAGATGGGCACCC |
| Chr7                | -      | 145045316 | 145045336 | CCACAATGTGGGATGGCACCC   |
| Chr8                | -      | 2221741   | 2221762   | CCAAGGATGTGAGGATGCACCT  |
| Chr8_KI270821v1_alt | -      | 404619    | 404640    | CCAAGGATGTGAGGATGCACCT  |

|       |   |          |          |                         |
|-------|---|----------|----------|-------------------------|
| Chr9  | - | 30000096 | 30000117 | CCAAGATTGAGGATGCGCACCC  |
| Chr10 | - | 20812799 | 20812819 | CCACGATGTGAGGGTGGACCC   |
| Chr15 | - | 53822912 | 53822933 | CCAACGTTGAGGATGTGCACCC  |
| Chr15 | - | 88219129 | 88219151 | CCTACATGTGCAGGATGGCACCC |
| Chr19 | - | 46579925 | 46579946 | CCAAGGATGGGAGGATGGCCCC  |
| Chr20 | - | 32084216 | 32084237 | CCAAGGATGGGAGGATGGCCCC  |
| ChrX  | - | 10692266 | 10692288 | CCTACTGATGTGAGATGGCACCC |
| ChrY  | - | 12387305 | 12387325 | CCAAGGATGGGGATGGCACCC   |

***DNAH11*(-/-) iPSCs**

| Chromosome | Strand | Start     | End       | Sequence                |
|------------|--------|-----------|-----------|-------------------------|
| Chr9       | -      | 128115604 | 128115624 | CCCCGAGG-GGC-AAGATAAACC |

***DNAAF2*(-/-) iPSCs**

| Chromosome | Strand | Start     | End       | Sequence                 |
|------------|--------|-----------|-----------|--------------------------|
| Chr1       | +      | 16167628  | 16167650  | GAACTCCGGGTCCCGTAGGGCGG  |
| Chr1       | +      | 27154585  | 27154607  | GAACTCCGGG-CCTGGGAAGGGGG |
| Chr5       | +      | 90910009  | 90910030  | GAACTCCGCGTCCTGG-AGGCGC  |
| Chr11      | +      | 64805157  | 64805179  | GCACTCAGGGTCCTGGAGGGCGG  |
| Chr15      | +      | 41861583  | 41861604  | GAAC-CAGGGTCCTGGAAGGCAG  |
| Chr19      | +      | 49125610  | 49125632  | GAACTCCTGGGTCCTGG-AGGAGG |
| Chr4       | -      | 93795956  | 93795979  | CCGCCTTTCTGGAGCCCGGAGTTC |
| Chr5       | -      | 473526    | 473547    | CCTCCTCCCAGGACCCGG-GTTC  |
| Chr6       | -      | 158758835 | 158758857 | CTC-CCTTCCAGGACCCGGAGCTC |
| Chr8       | -      | 90001461  | 90001480  | CCGCCTTCC-GG-CCC-GAGTTC  |
| Chr16      | -      | 49517361  | 49517381  | CC-CC-TCCAGGACCCAGAGTTC  |

In *VANGL1*(-/-) iPSCs and *RSPH4A*(-/-) iPSCs, there were no potential off-target sites with up to 3-bp mismatches allowed in the database.

**Table S3. Primers used to confirm off-target sites****cHEATR2 Ex1-iPSCs**

| Off target               | Forward                | Reverse                  | Size (bp) |
|--------------------------|------------------------|--------------------------|-----------|
| GGCGCCGGGGCGG-GGCGCACGG  | TGGACCGTAGTCTCGGGG     | GCTTTCCGCATTCTAACTGGC    | 318       |
| AGCGCCAGGGCGGCGGCAG-ACGG | AGCATCACTGGATCAGCCAAA  | CTGCTGTCTGCGCAACTCT      | 439       |
| AGCGCCAGGGCGGCGGCAG-ACGG | AGCATCACTGGATCAGCCAAA  | CTGCTGTCTGCGCAACTCT      | 439       |
| TGCGCCAGGGCGGCTGCGCA-GG  | CTAGACTAGGCATCGGCACC   | AGATGCTTGGGGATGCTAAGA    | 600       |
| AGAGCCAGGGCGGCGG-GCGCGG  | CTCGGCTAGCTTGGTGTGT    | ACTGCGGAGACGCAGGA        | 338       |
| AGCGCCGAGGG-GGCGGCGCA-GG | CAGAGCCCTCTTTCCTCCAGAA | GGGATTTACGGAACCTGCGAC    | 206       |
| TGCGCCAGGGCGGCGGCAGGG    | GCCTAGCCCTGTTGACAGAT   | CTCTGAAGCCATGCGTCACT     | 382       |
| AGCG-CAGGGCGGCGGCG-GCGG  | GAGAGGTTGCTCTGCCATCA   | CGTGTAAGTGACAGAGCAGGA    | 872       |
| AGCGCCTGGGCGGCGG-GC-CGG  | TTATAATCTGCGCCGGGAGC   | CGAGAGCGCAGAGAAAGGAG     | 411       |
| GGCCCCAGGGCGGCGGCAG-GG   | TTGCTCTGCTTCTTCTCCAGG  | TGACGTCTGTTCTCATCACTCAGC | 500       |
| AGCGCCA-GGCGG-GCGCGCACGG | CTGACCAAGCCTACAGACCC   | CGATGGAGCTGCTGTTGCT      | 577       |
| AGCGCCATGGCGGCGGCG-GCGG  | ACCCCTCAGAGGCGAAAAGAA  | AGGTGCGAGAGGGTTAGGAAG    | 481       |
| AGCGCAAGGGACGCGGCGCAAGG  | ATGCTACTACAGTAGGGCCGA  | GGGCAATAACAGCTTGAGTCC    | 292       |
| GGGGCCAGCGGCGGCGGCACGG   | GTCCTCGGCGGCTTCTTT     | TCACTGTCACTTTTCCCTGCT    | 460       |
| AGCGCCA-GGCGGCGGCG-GCGG  | CTCCCGGCCTCCAGAGAT     | TGACTCAGCGGCCAGGTA       | 470       |
| AGCGCCA-GGCGGCGGTGC-CGG  | CTGCGGGATGGGAGTTAG     | GCCACCTTAACCCGAGAGTT     | 509       |
| AGCG-CAGGGCGG-GGCGCAGCGG | AGACAAACACTCCCAACGCA   | CGAAGTCGCAGCAACAGC       | 520       |
| AGGGCCAGCGGCGG-GCACGG    | AGCGGCTCAGAAGATACTGC   | GCACAGGCGGAAAATTCAG      | 539       |
| AGCGCCTGGGCGGCGGCG-GCGG  | CTCCGAGTGGGCCTTACAC    | AGGCCACACTCACAGAACTC     | 348       |
| AGCGTCAGGGCGGGGCGC-CGG   | CTCAGTCTGGGCTCAAAGTC   | CTACACACCAGGAGTCAGGTC    | 330       |
| AG-GCCAGGGC-GCGGCGC-CGG  | ATCGTTCGTGTGGGCTACTG   | CTTGGCTGCTGCTCATTAC      | 436       |
| AG-GCCAGGGC-GCGGCGC-CGG  | ATCGTTCGTGTGGGCTACTG   | CTTGGCTGCTGCTCATTAC      | 436       |
| AG-GCCAGGGCGGCGG-GCGCGG  | AGTCTGCAGTAAAGGTGCC    | GAAGGACCTTGGATGGCGTT     | 417       |
| AG-GCC-GGTCGGCGGCGCACGG  | AGACCACCGAGTAAGGAGGT   | TTGGCCAAGGTGGAAGCC       | 498       |
| AGC-CCAGGG-GCCGCGCACGG   | GCGCTGTACATAGCCCGTA    | GTGGGTCTCTAGGCAGTGTG     | 299       |
| AGCGCGAGGGCGGCGCCACGG    | CGAAGTGGCAGAGGGTCTG    | TCTGCTCCCACTTGTGCAA      | 572       |
| AGCGCC-GGGCTGC-GCGCACGG  | ATCACCGCGGAGAATGCAAG   | TGCGGGAGACCTTAGTTTGC     | 484       |
| A-C-CCAGGGCGG-GGCGCACGG  | ACCATTGCTTGAGGGGAGAG   | CCCCTCGATGGTAACCTCTT     | 399       |
| AGCG-CAGGGCGGCGGCGCTCAG  | ACAGGTGTGAACGGTAGCC    | CACACAGGCTCAAAAGGCTC     | 500       |
| AGCG-CAGGGCGGCGGCGCTCAG  | ACAGGTGTGAACGGTAGCC    | CACACAGGCTCAAAAGGCTC     | 500       |
| AGCG-CAGGGCGGCGGCGCTCAG  | ACAGGTGTGAACGGTAGCC    | CACACAGGCTCAAAAGGCTC     | 500       |
| AGCG-CAGGGCGGCGGCGCTCAG  | ACAGGTGTGAACGGTAGCC    | CACACAGGCTCAAAAGGCTC     | 500       |
| AGCG-CAGGGCGGCGGCGCTCAG  | ACAGGTGTGAACGGTAGCC    | CACACAGGCTCAAAAGGCTC     | 500       |
| AGCG-CAGGGCGGCGGCGCTCAG  | ACAGGTGTGAACGGTAGCC    | CACACAGGCTCAAAAGGCTC     | 500       |
| AGCG-CAGGGCGGCGGCGCTCAG  | ACAGGTGTGAACGGTAGCC    | CACACAGGCTCAAAAGGCTC     | 500       |
| AGCG-CAGGGCGGCGGCGCTCAG  | ACAGGTGTGAACGGTAGCC    | CACACAGGCTCAAAAGGCTC     | 500       |
| AGCG-CAGGGCGGCGGCGCTCAG  | ACAGGTGTGAACGGTAGCC    | CACACAGGCTCAAAAGGCTC     | 500       |
| AGCGCC-GGGC-G-GGCGCACGG  | CTCCCGTGCGTCCGTCTG     | GGCTTCTCTGGGGGATCCTA     | 351       |
| GGCGCC-GGGCGGCGGCAG-GG   | ACCTCTTCTCCGACGACTACG  | ACTCGTCGCTGTGCTTGTG      | 462       |
| AGCGCCAGGGCGGGGTGGCACGG  | GATAGGTACCGGCGGATAAG   | AGCTCCGGTCCTCTCTTG       | 465       |

|                          |                         |                        |     |
|--------------------------|-------------------------|------------------------|-----|
| AGCTGCCGGGGCGGCGGCGC-CGG | AGGCATGAGCTTTCGACGG     | GCCGAGCCCGATAAATGGTA   | 370 |
| CCGT-CGCCG-CGCCCTGGC-CT  | CTCACCTACGATTGGCTGG     | GCATCGAGGTGGGAGGTG     | 319 |
| CC-TGCGCCGCC-CCCTCGGCGCT | GTGACGAGTCCAAGTGC GA    | AACAAGGAGAGGGAACGCAG   | 388 |
| CCGCGCGCC-CCGCCCTTGGCGCT | GGCTTCCATAATGGGGCGTG    | AGCCAGGCACAGCGGATAG    | 456 |
| CCGTGCGCCTCAGCCCTGGTCGCT | TGTGTGAAACTGCGACGA      | AATGTCACAGCTGTCTGTTTAC | 456 |
| CC-TGC-CCGCCCCCTGGCGCT   | ACATTTGGGAGGGAAGAGACAAA | GCAGGTTTCTCTCCGTCTTC   | 441 |
| CCCTGCGCCGCCGCCCGGCGCC   | TTGTGGACTACGTCTGTGCG    | GGCCAGTGCCAAAGAACAAC   | 462 |
| CCGTGCGCTGCCGCTCT-GCGCT  | GGGTAAGTCTGAGGGAATCGG   | GGGTAGCGGTGAAAGACAAAAG | 413 |
| CCATG-GCCGCAGCCCTGGCGCT  | TGGAAGTAACACGTATGGAGGC  | TTGAAGTCCGTCTGAGGATGC  | 462 |
| CCG-GCTCCGCCGGCCTGGCGCT  | CTTGAATAACTGAGCGCGGC    | CGTAGCTGTGGGATCGTC     | 574 |
| CCGTGCG-CGCGCCCC--GCGCT  | TCCTCTGGGTTAGAGGGATG    | GTCTGGCTTACAACCGTGC    | 471 |
| CCGT-CGCCCCCGCCCTGGCCCT  | AAACCCTTCTGCCCTTGGTAA   | GGCTCTGGAGAAACCAAAACAG | 500 |
| CTGTGC-CCGCCG-CCTGGCGCT  | CTGCATCTATCACCGCAAC     | GTTGTGGGTTAGCAATGGG    | 447 |
| CCG-CCGCCGCCG-CCTGGCGCT  | AGCAAGAAACCTGTGGGACCT   | GCGTGCCAGGGTAAACG      | 477 |
| CCGCCGCGCCGCCG-CCTGGCGCT | CGAATCCATGCCGTGAAGAC    | CCCATTGTTAAAGGACCGGC   | 448 |
| CCG-GTGCCGCCGCCCTGGCCCT  | GGAGGCCTAAGAGCCTGAAGA   | TAGACATCAGGAGAGACCTGGG | 415 |
| CCGCGCGCCGCCGCCG-GCCGCT  | CTCGTTTCCCCGAAAGAAG     | AAGCAGAGGTGGTAGCCAAC   | 306 |
| CCGCGC-CCGCCGCCG-GCGCT   | CAGCCGCCAAAAACAACCTAA   | AATGGCGAACTCGGCAAATAC  | 547 |
| CCGTGC-CCG-CG-CCTGGCGCT  | CGCTGGTACTTCTACCGTCA    | AAACGGGTACGAAGGTGG     | 432 |
| CCG-GCGCCGCCGCCG-GCGCT   | CGCCGTACCTTGACATGG      | CCCACACTAGACATGGCCTC   | 383 |
| CC-TGCGCCGCCGCCCTGG-GCCT | CTGGCTTACGCAAAGTGCTC    | TATTCACCTCGATGTGGCC    | 575 |
| CCGTGC-CC-CCGCCCTGG-GCT  | GTCCACACCAGTGCCTG       | GTGACCACCAGGAAGCGG     | 405 |
| CCGTGCGCCTCCG-CCTGGCAGCT | GTTTACCACCGTCTCCTG      | AATAGTGAGGCAACGTCCCG   | 468 |
| CTG-GCGGCCGCCGCCCTGGCGCT | CATGGGTGGGAGCTTGAG      | CCCTCTGTAGGGCTGGGAG    | 446 |
| CCG-CCGCCGCCGCCCTGGTGCT  | CCTTTGTCTCCTATGCCTCCTC  | CGTTCACCATCCTCTCCCAAG  | 641 |
| CCGTGAGCCGCCGCGCTGGC-CT  | AGTCTCCGCTCCTGAGTTC     | AGTACCCCCAACTCTGATGC   | 501 |

### cHEATR2 Ex12-iPSCs

| Off target              | Forward                 | Reverse                | Size (bp) |
|-------------------------|-------------------------|------------------------|-----------|
| GGGTGCCGTCCACACATCGTTGG | GCTGAACCAATGAGGGTGCT    | TTCCTAAGGGTGGGGCATTG   | 846       |
| GGGTGCATCTCAATGTTGG     | CCAGACCAAACCAATGTACTGC  | TCCCAAGAACAGCAGCATGG   | 401       |
| GTGTGGCATCCTCCATCGTTGG  | TTCGAGTGGGGTCACCATCA    | GCCCTACGCATGGTGTGAAG   | 690       |
| GGGTGCATCTACATCTTGG     | CCGGGTTTCTTGCTTTGTCC    | CACCATCTGGGTTCCCTTG    | 579       |
| GGGTGCCATCTCCATCTTGG    | TCTGTTGGAGCTACGAAGGG    | GCACGTCGTATGTTGGTACG   | 678       |
| GGGTGCCATCTCCATGTGGG    | GTTAGGAACCTGCTGTGCTG    | GGTGCAAACTAGGCATGAAGA  | 418       |
| GGGTGCCATCTCCTGTTGG     | TGCTCAGGAGAAAAGCCAGTTA  | AAATGCTAAAGCCAGCCAC    | 846       |
| CCAAGATTGAGGATGCACCC    | TTGAACACGGGAGGCAGAAG    | ATCTCAGCTCAGGTGCAGTG   | 519       |
| CCAAGATTGAGGATGCGACCC   | TGGAACACCTACCAGACTTACC  | GCCTTTCTGGATAGAACCAACG | 412       |
| CCCACGATGTGAGGAAGCACCC  | GGAAAATAGCCCGGGTGGTG    | TGACGTCGGGAAAGAAAGTC   | 699       |
| CCAACCATGTGAGATGGGCACCC | AGGAGGAGCAGAAGATGAAAAGG | CCCTAGACGTGGCTAATGCTC  | 370       |
| CCACAATGTGGGATGGCACCC   | GTGAAATTTGTGCTGCTGGTG   | AGGTGCAATGGAAGTACCCTC  | 660       |
| CCAAGGATGTGAGGATGCACCT  | CTCCGCCGTGATAGTGATGG    | CAGAAAGTCACCCAGGCAGG   | 674       |
| CCAAGGATGTGAGGATGCACCT  | CTCCGCCGTGATAGTGATGG    | CAGAAAGTCACCCAGGCAGG   | 674       |
| CCAAGATTGAGGATGCGACCC   | ACTGGGTGCTTGGCTAAGTG    | AAGTCCCTTCCCCGCTTCTA   | 504       |
| CCACGATGTGAGGGTGACCC    | CCAGGTGAATGCCAGAAATCCC  | GACAATCTGCACGCAGCCAG   | 466       |



|                         |                       |                         |     |
|-------------------------|-----------------------|-------------------------|-----|
| CCAACGTTGAGGATGTGCACCC  | GCTGTTTTGCCAGAACTCACC | CCTGCCTGCCATTCTATTCAAAG | 410 |
| CCTACATGTGCAGGATGGCACCC | CCGCATTAACCCCTGTGAGA  | GCTGGGACTTCTCCTCTTGG    | 587 |
| CCAAGGATGGGAGGATGGCCCC  | CAGAAGACGATCGTTGGCAC  | CTTTTGAGTCCCGCCACATTC   | 643 |
| CCAAGGATGGGAGGATGGCCCC  | AGCTACTCTTTGTCCGTGCG  | TCCAGACTTCCCCAAACTGC    | 645 |
| CCTACTGATGTGAGATGGCACCC | ACCCTCAGGCTTGCTAGGAT  | CTGGGATTATAGGCACCCGC    | 525 |

### ***DNAH11(-/-) iPSCs***

| Off target              | Forward              | Reverse              | Size (bp) |
|-------------------------|----------------------|----------------------|-----------|
| CCCCGAGG-GGC-AAGATAAACC | GAAGCCCTGTTCTCACCTGG | TAAGATGAGGGGGTGGGAGG | 539       |

### ***DNAF2(-/-) iPSCs***

| Off target               | Forward                  | Reverse                 | Size (bp) |
|--------------------------|--------------------------|-------------------------|-----------|
| GAACTCCGGGTCCCGTAGGGCGG  | CATCCAGTCTCCTCCCTCAAAG   | CAATGGTATGCAAAGTGCATGGG | 701       |
| GAACTCCGGG-CCTGGAAGGGGG  | GGTGACATCCCAATCGAGC      | CGCGATACTTCTTCCCTCG     | 1100      |
| GAACTCCGCGTCTGG-AGGCGC   | CTTGCCAGCTTCTTGTTCTG     | AAAGAAACCACCCCTCACTG    | 1245      |
| GCACTCAGGGTCCTGGAGGGCGG  | TTGTCCAGTGCTGGCTTCTTG    | CAGCTACAACTACTGCCGGG    | 1112      |
| GAAC-CAGGGTCCTGGAAGGCAG  | AGAAGCTCCTTGGTGTTCCG     | AGCCCGCGTATGTTAGAAG     | 970       |
| GAACTCCTGGGTCCTGG-AGGAGG | CTGGCTTGCCCTCTTAGAATCG   | GGACCATGCTTGTCATTCTTCAC | 803       |
| CCGCCTTTCTGGAGCCCGAGTTC  | AGAAAGGCAGTAGTGATATTTTGC | CCTCCTTCTTTGCGTGTAGGG   | 852       |
| CCTCTCCCAGGACCCGG-GTTC   | GATCTGGGGTTTCTCTGGGAC    | GCTTCGCTGTTGCTGCTT      | 293       |
| CTC-CCTTCCAGGACCCGGAGCTC | GCTCCGACCTGGACATAAGG     | TGGTCTTGACACATCCGGTC    | 984       |
| CCGCCTTCC-GG-CCC-GAGTTC  | AGAGTTTCGCCCTTGATAGGC    | CTCCTTAAGGGGTGGACGGG    | 1058      |
| CC-CC-TCCAGGACCCAGAGTTC  | GTTGAGCTACCTAGGGTGTCTG   | TTTTGGAGCAAAGCGGGAAC    | 901       |

**Table S4. Content of each medium for differentiation of iPSCs into iMCACs**

|                               | Endodermization medium<br>(Step1)                                                                                                                | Anteriorization medium<br>(Step2)                                                                                                                | Ventralization medium<br>(Step3)                                                                                                                                                                                                                                                        |
|-------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Basal<br>Medium               | RPMI<br>B27 supplement (2 %)<br>Penicillin/Streptomycin<br>(50 U/ml)                                                                             | DMEM / F12<br>Glutamax<br>B27 supplement (2 %)<br>L-ascorbic acid (0.05 mg/ml)<br>Monothioglycerol (0.4 mM)<br>Penicillin/Streptomycin (50 U/ml) |                                                                                                                                                                                                                                                                                         |
| Chemicals<br>and<br>Cytokines | Activin A (100 ng/ml)<br>CHIR99021 (1.0 $\mu$ M)<br>Y-27632 (Day0; 10 $\mu$ M)<br><i>Sodium butyrate (NaB)</i><br>(Day 1, 2 and 4)               | Noggin (100 ng/ml)<br>SB431542 (10 $\mu$ M)                                                                                                      | ATRA (depends on cell lines)<br>201B7 iPSCs and knockout<br>iPSCs: 0.05 $\mu$ M,<br>PCD#1-iPSCs and their<br>gene-corrected iPSCs: 1.0 $\mu$ M<br>PCD#2-iPSCs and their gene-<br>corrected iPSCs: 0.1 $\mu$ M<br>PCD#3-iPSCs: 0.5 $\mu$ M<br>BMP4 (20 ng/ml)<br>CHIR99021 (3.0 $\mu$ M) |
|                               | PAEPC medium (Step4)                                                                                                                             | PAEC and MCAC medium<br>(Step5-ALI, Step5-chip)                                                                                                  |                                                                                                                                                                                                                                                                                         |
| Basal<br>Medium               | DMEM / F12<br>Glutamax<br>B27 supplement (2 %)<br>L-ascorbic acid (0.05 mg/ml)<br>monothioglycerol (0.4 mM)<br>Penicillin/Streptomycin (50 U/ml) | PneumaCult-ALI Basal Medium<br>PneumaCult-ALI Supplement<br>PneumaCult-ALI Maintenance<br>Supplement                                             |                                                                                                                                                                                                                                                                                         |
| Chemicals<br>and<br>Cytokines | CHIR99021 (3.0 $\mu$ M)<br>FGF10 (100 ng/ml)<br>Y-27632 (10 $\mu$ M)                                                                             | Heparin (4 $\mu$ g/ml)<br>Hydrocortisone (1.0 $\mu$ M)<br>DAPT (10 $\mu$ M)<br>Y-27632 (10 $\mu$ M)                                              |                                                                                                                                                                                                                                                                                         |

**Table S5. Antibodies used in the present study**

| Species                                 | Antibodies                      | Company                                  | Catalog No./ Clone | Dilution | Fixation                    |
|-----------------------------------------|---------------------------------|------------------------------------------|--------------------|----------|-----------------------------|
| For isolating NKX2-1 <sup>+</sup> cells |                                 |                                          |                    |          |                             |
| Mouse                                   | Anti-CPM                        | Abcam                                    | ab49278/1C2        | 1:200    | -                           |
| Mouse                                   | Anti-CPM                        | Wako                                     | 014-27501          | 1:200    | -                           |
| Rat                                     | Anti-mouse IgG1-MicroBeads      | Miltenyi Biotec                          | 130-047-101        | 1:5      | -                           |
| Goat                                    | Anti-mouse IgG-MicroBeads       | Miltenyi Biotec                          | 130-048-401        | 1:5      | -                           |
| For immunofluorescence staining         |                                 |                                          |                    |          |                             |
| Mouse                                   | Anti-FOXJ1                      | eBioscience                              | 2A5/14-9965-82     | 1:500    | paraformaldehyde / methanol |
| Mouse                                   | Anti-Acetylated tubulin         | Sigma-Aldrich                            | T7451              | 1:4000   | paraformaldehyde / methanol |
| Rabbit                                  | Anti-RSPH4A                     | Atlas Antibodies                         | HPA031198          | 1:200    | paraformaldehyde            |
| Rabbit                                  | Anti-DNAH11                     | Atlas Antibodies                         | HPA045880          | 1:200    | methanol                    |
| Rabbit                                  | Anti-DNAAF2                     | Atlas Antibodies                         | HPA004113          | 1:200    | paraformaldehyde            |
| Rabbit                                  | Anti-HEATR2                     | proteintech                              | 24578-1-AP         | 1:200    | paraformaldehyde            |
| Rabbit                                  | Anti-PIH1D3                     | proteintech                              | 25309-1-AP         | 1:200    | paraformaldehyde            |
| Rabbit                                  | Anti-VANGL1                     | Atlas Antibodies                         | HPA025235          | 1:200    | methanol                    |
| Rat                                     | Anti-OCN                        | Kindly provided by Prof. Sachiko Tsukita | MOC37              | 1:1      | methanol                    |
| Mouse                                   | Anti-SSEA-4                     | BioLegend                                | 330401/MC-813-70   | 1:100    | paraformaldehyde            |
| Rat                                     | Anti-SOX2                       | Millipore                                | 14-9811-82/Btjce   | 1:500    | paraformaldehyde            |
| Mouse                                   | Anti-OCT3/4                     | Santa Cruz Biotechnology                 | sc-5279/C-10       | 1:100    | paraformaldehyde            |
| Rabbit                                  | Anti-NANOG                      | ReproCELL                                | RCAB004P-F         | 1:100    | paraformaldehyde            |
| Donkey                                  | Anti-mouse IgG-Alexa Fluor 488  | Thermo Fisher Scientific                 | A-21202            | 1:500    | -                           |
| Goat                                    | Anti-mouse IgG1-Alexa Fluor 647 | Thermo Fisher Scientific                 | A-21240            | 1:500    | -                           |

|        |                                  |                          |             |       |   |
|--------|----------------------------------|--------------------------|-------------|-------|---|
| Goat   | Anti-mouse IgG2b-Alexa Fluor 488 | Thermo Fisher Scientific | A21141      | 1:500 | - |
| Donkey | anti-rat IgG-Alexa Fluor 488     | Thermo Fisher Scientific | A21208      | 1:500 |   |
| Donkey | Anti-rabbit IgG-Cy3              | Jackson ImmunoResearch   | 711-165-152 | 1:500 | - |
| Goat   | Anti-rat-Alexa Fluor 647         | Jackson ImmunoResearch   | 712-605-153 | 1:500 | - |

**Table S6. Primers for qRT-PCR used in the present study**

| Gene           | Forward               | Reverse                | Size (bp) |
|----------------|-----------------------|------------------------|-----------|
| <i>β-ACTIN</i> | CAATGTGGCCGAGGACTTTG  | CATTCTCCTTAGAGAGAAGTGG | 126       |
| <i>FOXJ1</i>   | CCTGTCGGCCATCTACAAGT  | AGACAGGTTGTGGCGGATT    | 94        |
| <i>DNAH5</i>   | GCAATTGTGGCTTCCTGTTT  | GGAGACCTCCAACAGCAAAA   | 109       |
| <i>SNTN</i>    | GCTGCAAACCCAATTTAGGA  | TGCTCATCAAGTTCAGAAAGGA | 84        |
| <i>VANGL1</i>  | GAACGGCGAGTAAAGAAGCG  | GGTGATGCGCAGGTACTTCT   | 186       |
| <i>VANGL2</i>  | AAACAGCACCAACAACCTCCA | CAGCCGCTTAATGTGAGTGA   | 181       |

**Movie S1. Muco-ciliary transport of healthy donor-MCACs in airway-on-a-chip.**

Bright-field movie of healthy donor-iMCACs in ALI and airway chip culture with or without FSS on Day 42 (upper row) and stacked images of the fluorescent microbeads placed on the iMCACs in each corresponding field of view (lower row in the first part). Direction of the preloaded medium flow in airway chip culture with FSS is from left to right. The fluorescent microbeads were traced with TrackMate (ImageJ/Fiji software program plugin) (lower row in the second part).

**Movie S2. Ciliary beating and stacked images of fluorescent microbead flow on the airway epithelial cells derived from *VANGL1*(-/-) iPSCs.**

Ciliary beating pattern (CBP) of *VANGL1*(-/-) iMCACs in ALI culture was analyzed by HSVM on Day 56 (first portion). Bright-field movie of the *VANGL1*(-/-) iMCACs in ALI and airway chip culture with or without FSS on Day 42 (upper row in second portion) and stacked images of the fluorescent microbeads placed on the *VANGL1*(-/-) iMCACs in each corresponding field of view (lower row in second portion). Fluorescent microbeads were traced with TrackMate (ImageJ/Fiji software program plugin).

**Movie S3-5. Ciliary beating and stacked images of fluorescent microbead flow on iMCACs derived from each PCD cell line.**

Bright-field movie of iMCACs derived from each PCD cell line in ALI culture analyzed by HSVM on Day 56 in the first portion. CBP of iMCACs derived from each PCD cell line in ALI culture on Day 56 in the second portion. Stacked images of the fluorescent microbeads placed on iMCACs derived from each PCD cell line in the ALI culture on Day 56 as well as bright-field of each corresponding view shown in the third portion. Sequential images of the fluorescent beads traced with TrackMate (ImageJ/Fiji software program plugin) shown in the fourth section.

**Movie S6-7. Muco-ciliary transport of MCACs derived from PCD#1-iMCACs, PCD#2-iMCACs and their gene-corrected counterparts.**

Bright-field movie of iMCACs derived from each cell line in ALI and airway chip culture with or without FSS on Day 42 (upper row) and stacked images of the fluorescent microbeads placed on iMCACs derived from each cell line (lower row) shown in the first portion. Sequential images of the fluorescent beads traced with TrackMate (ImageJ/Fiji software program plugin) shown in the lower row in the second portion.

**Movie S8. Muco-ciliary transport over the entire length of the airway epithelial sheet in airway chip culture with FSS.**

The fluorescent microbeads were placed on the cHEATR2 Ex12-iMCACs in ALI and airway chip culture with and without FSS after each insert membrane was removed from the chip on Day 42. Microbead flow along the entire length of the airway epithelial sheet in airway chip culture was captured. Direction of the preloaded medium flow in airway chip culture with FSS is from left to right.

**Data file S1. Raw data for figures (Excel format).**