Loss of Adam10 Disrupts Ion Transport in Immortalized Kidney Collecting Duct Cells

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Abstract

The kidney cortical collecting duct (CCD) comprises principal cells (PCs), intercalated cells (IC), and the recently discovered intermediate cell type. Kidney pathology in a mouse model of the syndrome of apparent aldosterone excess revealed plasticity of the CCD, with altered PC:intermediate cell:IC ratio. The self-immortalized mouse CCD cell line, mCCDcl1, shows functional characteristics of PCs, but displays a range of cell types, including intermediate cells, making it ideal to study plasticity. We knocked out Adam10, a key component of the Notch pathway, in mCCDcl1 cells, using CRISPR-Cas9 technology, and isolated independent clones, which exhibited severely affected sodium transport capacity and loss of aldosterone response. Single-cell RNA sequencing revealed significantly reduced expression of major PC-specific markers, such as Scnn1g (γ-ENaC) and Hsd11b2 (11βHSD2), but no significant changes in transcription of components of the Notch pathway were observed. Immunostaining in the knockout clone confirmed the decrease in expression of γ-ENaC and importantly, showed an altered, diffuse distribution of PC and IC markers, suggesting altered trafficking in the Adam10 knockout clone as an explanation for the loss of polarization.

Key words: cell plasticity; Adam10 knockout; mCCDcl1 cell line, kidney cortical collecting duct; mouse SAME model, CRISPR-Cas9; polyploidy.

Introduction

Plasticity has been observed in kidney tubules as an adaptation to mechanical and chemical stimuli. This is observed not only during renal injury but also in healthy adult cells, indicating a capacity for plasticity and regeneration under both pathological and normal physiological conditions. That capacity is particularly evident in the collecting duct, with apparent switching between its two distinct cell types, principal cells (PCs)

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and intercalated cells (ICs), the latter being subdivided into α- and β-subtypes. Multiple studies have shown that these collecting duct cells are capable of interconversion.1,2

Chemical cues may cause collecting duct remodeling: ICs change from β- to α-subtype when subjected to acidic conditions,3 treatment with acetazolamide (IC marker CAII inhibitor) leads to an increase in α-ICs at the expense of β-ICs and PCs,4 and lithium treatment leads to an increased ratio of ICs to PCs.5 These characteristics suggest that plasticity in the collecting duct may be a mechanism for maintaining homeostasis in the kidney, as this segment plays a critical role in the regulation of extracellular volume, pH, and osmolarity. Single-cell sequencing of collecting ducts has identified the presence of an additional “intermediate” cell type between PCs and ICs.6

The mechanisms of cell plasticity in the collecting duct have been linked to the Notch pathway, in general associated with the sequential emergence of cell lineage from progenitor cells,7 and in particular to nephrogenesis.8 The knockout of several factors of the Notch pathway such as Delt19 or Mib110 suggests that blocking or downregulating Notch in the collecting ducts generally leads to a decreased number of principal cells and an increased number of intercalated cells, or in the case of Fox1 knockout to the appearance of the intermediate cell types.11 This indicates that the Notch pathway affects cell type determination. For example, the ratio of principal to intercalated cells, typically 70:30 in mice, was adversely influenced by cell-specific knockout of Adam10 (via deletion of floxed alleles through genetic crosses with Aqp2-cre mice).12 Adam10 controls the proteolytic processing of Notch and mediates lateral inhibition mechanisms during development.13 The apparent disruption of the Notch pathway resulted in a reduced number of principal cells and a corresponding increase in the number of intercalated cells. This was not accompanied by cell death or a significant shift in cell number, strongly suggesting a switch between cell types. Neither the role of cell plasticity nor the underlying mechanism has been completely elucidated in the collecting duct. It is not clear how diseases that disrupt collecting duct function affect cell determination and plasticity, either under normal or pathological conditions such as the syndrome of apparent mineralocorticoid excess (SAME), which is caused by the loss of Hsd11b2.14 Absence of the enzyme results in increased sodium recovery by the principal cell, mediated through the epithelial sodium channel, ENaC, leading to hypertension, hypokalemia, and pronounced changes to the distal nephron of the kidney.15,16

An important in vitro model for collecting duct studies is the mouse cortical collecting duct cell line (mCCD), which expresses principal cell factors and proteins enabling physiologically relevant aldosterone-stimulated sodium transport.17,18 However, mCCD cells have also been shown to express IC markers, with some intermediate cells expressing both PC and IC markers. The intermediate cells are phenotypically similar to those found in vivo, indicating a capacity for plasticity.19 This makes them an ideal system for gene targeting, using CRISPR/Cas9 gene targeting.

We initially studied the composition of collecting ducts from a mouse model of SAME and show important effects of Hsd11b2 knockout on cell type determination. We then knocked out Adam10 in mCCD cells, using CRISPR/Cas9 gene targeting. In-depth characterization of the resulting cell lines includes electrophysiological analysis, single-cell RNA sequencing, and immunocytochemical analyses. Here, we report that loss of Adam10 in mCCD cells led to dramatic effects on sodium trafficking, cell polarization, and PC:intermediate cell:IC ratios, in the absence of major transcriptional changes in the Notch pathway.

**Materials and Methods**

**Animal Model and Tissue Preparation**

Mice with targeted knockout of the 11β-hydroxysteroid dehydrogenase type 2 (Hsd11b2) gene have previously been described20 as a model of the SAME. Kidneys were taken from wild-type (WT) and knockout (KO) mice at 4 weeks (n = 3) and 17 weeks (n = 3), respectively. Kidneys were perfusion fixed, removed, and fixed for further 24 h in 4% paraformaldehyde (PFA), as previously described.21 Fixed tissue was embedded in paraffin and sectioned at 5 μm thickness, followed by dewaxing, rehydration, and antigen retrieval steps.

**Immunohistochemistry**

Primary antibodies and dilutions were as follows: goat anti-Aqp2 (NB1-70378; Novus Biologicals) at 1:1000 and rabbit anti-V-ATPase B1 (PA535052; Life Technologies) at 1:200. Secondary antibodies used at a 1:500 dilution were donkey anti-goat Alexa Fluor 488 and donkey anti-rabbit Alexa Fluor 568 (A-11055 and A-10042, respectively; Life Technologies).

**Cell Culture**

The mCCDcl1, mouse cortical collecting duct cell line, was previously established and kindly provided by Bernard Rossier (University of Lausanne, Lausanne, Switzerland). For optimal culture conditions, the cells were cultured at 37 °C and 5% CO2 in Phenol red-free DMEM/F-12 media (Invitrogen, Life Technologies), with the following supplements: 1 mmol/L triiodothyronine, 5 μg/mL insulin, 50 mmol/L dexamethasone, 60 mmol/L sodium selenite, 5 μg/mL apotransferrin, 10 ng/mL epidermal growth factor (EGF), 2% fetal bovine serum (FBS), and 100 U/mL to 100 μg/mL penicillin–streptomycin (Pen–Strep) as previously described.17 During incubation with CRISPR-Cas9 reagents (as per IDT protocol, see later) medium without Pen/Strep was used. Cells were used from passage 28.

**Karyotyping and Fluorescence In Situ Hybridization (FISH) Analysis**

Fosmid clones for detection of Adam10 (W11-600D17) were selected using UCSC Genome Browser online tool (University of California, Santa Cruz, USA), and obtained from BACPAC Resources (Children’s Hospital Research Institute, Oakland, USA). mCCD cells were cultured until confluency, treated for 10 min at 37 °C with colcemid (KaryoMAX Colcemid, Gibco), trypsinized and burst (10 min at 37 °C) using a hypotonic solution (KCl and sodium citrate). The cell nuclei were then fixed using a 3:1 methanol/acetic acid solution and dropped on microscope slides. FISH analysis was conducted as previously described.22

**Immunocytochemistry**

On cells fixed in 4% PFA, Adam10 antibody (PA112500, Thermo Fisher Scientific) was used at 1:100 dilution. Rabbit ENaC-γ antibody was provided by the Loffing lab and used at 1:1000 dilution. Goat anti-Aqp2 (NB1-70378; Novus Biologicals) was used...
CRISPR-Cas9 Targeting

A detailed visual protocol, adapted from the IDT Altr-CRISPR-Cas9 user guide, can be found in Figure S1. The following reagents were used: CRISPRMAX Cas9 Transfection reagent (CMAX00001, Invitrogen), containing CRISPRMAX and Cas9Plus reagents; Cas9 nuclease (1081058, IDT); Opti-MEM reduced serum medium (11058021, Gibco); tracrRNA (transactivated crRNA) (20 nmol, 1072533, IDT); and nuclease-free duplex buffer (11-01-03-01, IDT). Two different sgRNAs (G1 and G2, 2 nmol, IDT) were designed for Adam10 using the following online resources: UCSC Genome Browser (USA), CRISPR design tool of the Broad Institute ( crispr.mit.edu, USA), Benchling ( benchling.com, USA), and CRISPR RGEN Tool ( genome.net/cas-offinder, Hanyang University, Korea) to inform our choice of optimal guides. We recommend choosing two guides in an exon (or neighboring exons) critical to the function of the target protein. If possible, the predicted deletion between the two target sites should not be divisible by 3, so any resulting protein product will be out of frame.

Four guide combinations were tested: negative control (no Cas9 enzyme), G1 (TM containing sgRNA1), G2 (TM containing sgRNA2), and G1+G2 (TM containing a mix of both sgRNA1 and sgRNA2). Technical triplicates were performed for each combination. In brief, guide duplexes were formed by incubating a mix of sgRNA, tracrRNA (transactivated crRNA), and duplex buffer at 95°C for 5 min in a thermal cycler (Veriti 96-well, Applied Biosystems, Foster City, CA). RNP (ribonucleoprotein) complexes were formed by mixing Cas9 enzyme, Cas9 PLUS reagent, Opti-MEM media, and the freshly prepared duplexes, and incubating at room temperature (RT) for 5 min. CRISPRMAX reagent and Opti-MEM media were then added to the RNP complexes, and the solution incubated at RT for 20 min to form the transfection mix. The transfection mix was then added to 200 μL of cell suspension at a concentration of 200 000 cells/mL in growth media lacking Pen/Strep and left to incubate overnight in usual culture conditions described earlier. mCCDcl1 cells were trypsinized after 5 days in culture.

Sequence alignment between WT DNA sequence, sgRNAs, and selected primers for polymerase chain reaction (PCR) allowed us to predict sizing of products for each combination (Figure S2).

Indel Detection and Sequencing

Indels were detected in PCR products using EnGen® Mutation Detection Kit (New England Biolabs). Briefly, heteroduplexes are formed between PCR products with and without indels. The duplexes were then digested using the T7 enzyme and the products analyzed by running the fragments on agarose gel. PCR products were extracted from agarose gels and purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) and sent for sequencing at the MRC PPU DNA Sequencing and Services (University of Dundee, UK). Sequencing results were analyzed with the SnapGene® software (GSL Biotech LLC).

Cloning

Clonal cell lines were established by serial dilution as previously described (https://www.corning.com/catalog/cls/documents/protocols/Single_cell_cloning_protocol.pdf). Briefly, confluent mCCDcl1 cells in growth medium were trypsinized, suspended in complete culture medium, and serially diluted in a 96-well plate. Following appropriate dilution, the presence of single cells was independently verified and confirmed by observing the growth of the resulting single colonies in the wells over 3 days of culture. The colonies were then trypsinized and transferred to a 6-well plate for culture and screening.

DNA and RNA Extraction and PCR

DNA Total for each cell line was extracted using DirectPCR® DNA Extraction System (Viagen Biotech). Cells from a well of a 6-well plate were trypsinized, washed twice in phosphate buffered saline (PBS), and pelleted. The pellet was lysed by adding 140 μL of DirectPCR® reagent and 3 μL of proteinase K, mixed thoroughly, and incubated for 4 h at 55°C on a rocking platform, followed by a 45 min incubation at 85°C. The primer sequences for Adam10 were obtained using PrimerBank, with a WT product size for primer pair 1 of 766 and 458 bp, respectively.

Total RNA was extracted from mCCDcl1 cells and KO cell lines using Qiazol (Ambion, Life technologies). cDNA was obtained using 500 ng of RNA with a High-Capacity RNA-to-cDNA kit (Applied Biosystems). The primer sequences for Adam10 can be found in Table 1. Primers were designed on different exons of the Adam10 gene and used in different combinations of forward and reverse primer.

The reactions were carried out in a thermal cycler (Veriti 96-well, Applied Biosystems, Foster City, CA), and the amplified PCR products were separated by electrophoresis in a 1.5% agarose gel.

Electrophysiology

WT mCCDcl1 cells and clonal Adam10 KO mCCDcl1 lines were polarized by growing cells on Corning Costar Snapwell Perme-

Table 1. Guide RNAs and Primers for Adam10 Gene

| Guide | Sequence | Additional Information |
|-------|----------|------------------------|
| Adam10 sgRNA 1 (G1) | 5′-gaaggtgccctcttcctcattcgt-3′ | Exon 3 |
| Adam10 sgRNA 2 (G2) | 5′-gatactctctcatattcacc-3′ | Exon 3 |
| Adam10 F1 | 5′-gcctattcttacatctctgac-3′ | Exon3 |
| Adam10 R1 | 5′-cagcagctcagcagcagcagcagcag-3′ | Product size: 766 bp |
| Adam10 R2 | 5′-gggaagatggttgccgac-3′ | Exon 1 |
| Adam10 R3 | 5′-gggagatggttgccgac-3′ | Exon 4 |
| Adam10 F2 | 5′-gggagatggttgccgac-3′ | Exon 2 |
| Adam10 F3 | 5′-gcctattcttacatattcacc-3′ | Exon 5 |

* F for forward, R for reverse
able Support inserts (12 mm, 0.4 μm pore size). Cells were
seeded at a 1:1 split ratio and grown for 10 days. On day 8, the
cells were fed with basal medium containing charcoal-stripped
FBS and Pen−Strep supplements only and on day 9 with basal
media containing Pen−Strep only. Measurements for transep-
thelial voltage ($V_{te}$) and transepithelial resistance ($R_{te}$) were
made with a transepithelial volt-ohm-meter and a set of chop-
stick “STX” electrodes (EVO2; World Precision Instruments,
Sarasota, FL), and the equivalent short-circuit current ($I_{sc}$) was
calculated using Ohm’s law. By convention, a negative $I_{sc}$ reflects
either electrogenic secretion of cations, electrogenic absorption
of anions, or a combination of both. Aldosterone and amiloride
(Sigma Life Science) were used at 3 nm and 10 μM, respectively.

Preparation of Cells for 10X scRNA Sequencing

Cells were prepared as follows: mCCDcl1 and each clonal line
to be sequenced were seeded on four Corning Transwell filters
and cultured for 9 days with complete media. A separate control plate with the same mCCDcl1 cells was used for electrophysiological measurements to verify culture conditions through the development of a typical resistive monolayer of mCCDcl1 cells. On day 9, cells from 4 filters were trypsinized and resuspended gently, pooled, and the cell suspension diluted in chilled fluorescence-activated cell sorting (FACS) buffer (FBS with 2% fetal calf serum) to obtain approximately 1 million cells in 0.5 mL of buffer. Live−dead cell count was assessed by 4′,6-diamidino-2-phenylindole stain (DAPI-UV excitation 360 nm; emission filter 450/50), and singlets (FSC-A versus SSC-A) were gated to obtain 100 000 live cells using a flow cytometer (BD FACS Aria II SORP, Beckton Dickenson, Basel, CH).

10x Chromium Single Cell Library Workflow

Single cells were processed using the Chromium Single Cell 3’ Library and Gel Bead Kit v2 (10X Genomics, PN-120237) and the Chromium Single Cell A Chip Kit (10X Genomics, PN-120236) as per the manufacturer’s instructions. In brief, single cells were sorted into PBS + 2% FBS, and washed once. An estimated 7000–10 000 cells were added to each lane of a 10X chip and partitioned into Gel Beads in Emulsion, where cell lysis and barcoded sorting into PBS + 2% FBS, and wash were performed. Live−dead cell count was assessed using a QImaging camera (QImaging, Vancouver B.C., Canada) on a fluorescent microscope (Eclipse Ti, Nikon, Tokyo, JP), with DAPI, and TRITC filters applied, for detection of DAPI, Alexa Fluor 568, and Alexa Fluor 488 fluorophores, respectively. Immunostaining images were obtained with a QImaging camera (QImaging, Vancouver B.C., Canada) on a fluorescent microscope (Eclipse Ti, Nikon, Tokyo, JP), with DAPI, and TRITC filters applied, for detection of DAPI, Alexa Fluor 568, and Alexa Fluor 488 fluorophores, respectively. The 40 × 1.3 NA Plan Fluor oil objective was used. All images were processed and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). On mice kidney tissue, single channel and composite channel images were analyzed to ensure that all positively stained cells were counted. Secondary verification was applied to a number of images to ensure that the counting process was consistent and unbiased.

Sequencing images were obtained by using the “mapping” and “sequence” functions of the SnapGene® software.

Statistical Analysis

For mouse tissue data, ratios of cell type were analyzed using the R software. A $P < .05$ value was determined to be statistically significant. A minimum of 6 images were taken for each sample (prepared section microscope slide). Between 3 and 5 slides were prepared and imaged for each animal ($n = 3$). For electrophysiological measurements, statistical significance was assessed using a Student’s paired t-test. Data are expressed as means ± SD, and $n$ values refer to the number of repeats in an experiment.

Results

Effects of SAME on Collecting Duct Composition

Hsd11b2 was detected widely in the 4-week-old WT mouse kidney but was absent in 4-week-old Hsd11b2 KO mice, confirming the integrity of the knockout (Figure 1). Collecting ducts were identified by staining with antibodies specific for Aqp2 and/or V-ATPase B1 for PCs and ICs, respectively. Cells staining...
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Figure 1. Analysis of CCD Composition in SAME Mice. Top left panel: sample controls for WT and 11βHSD2 KO mice. Epifluorescence images of 5 μm thick kidney sections stained with anti-11βHSD2 antibody (green). DAPI staining of nuclei in blue. Scale bar 30 μm. Middle and bottom left panels: representative images of mouse kidney section stained for Aqp2 (green), and V-ATPase B1 (red) in 4 weeks old and 17 weeks old mice, both WT and KO for the HSD11B2 gene, with example of separate channels. Scale bars 15 μm. Top right panel: example of staining as used for calculating CD composition in 17 weeks KO mouse; ‘i’ indicates PCs, ‘∗’ indicates ICs, and full arrows indicate double-stained mixed cells. DAPI staining of cell nuclei in blue. Scale bars 10 μm. Bottom right panels: the proportions of PCs, ICs, and intermediate/mixed cells in collecting ducts were counted between groups of WT and Hsd11b2 KO mice at 4 weeks and 17 weeks (n = 3). The log-ratios of PC:IC and IC:intermediate cell are reported as mean ± SD. Significance between groups was assessed using MANOVA test with P < .05. ***P < .001, **P < .01.

Age-matched male mice at 4 and 17 weeks were used to investigate progression of the phenotype, since sodium handling changes from increased sodium retention to salt wasting at around 80 days. 28 The log-ratio of PC:IC cells in Hsd11b2 KO mice differed significantly from those observed in their WT counterparts at 4 weeks (0.36 ± 0.10 versus 0.57 ± 0.04) and 17 weeks (0.38 ± 0.05 versus 0.53 ± 0.03). Similarly, the IC:intermediate cell log-ratio was significantly lower in KO than WT mice at 4 weeks (−0.08 ± 0.05 versus 0.46 ± 0.05) and 17 weeks (0.083 ± 0.15 c.f. 0.54 ± 0.10). Since the relative proportion of IC cells in the CD did not change significantly between WT and KO mice of any age, these results show that loss of Hsd11b2 results in a shift from PC toward an intermediate cell type.

Ploidy of mCCDcl1 Cells and FISH Analysis

While mCCDcl1 cells are commonly used for the study of collecting duct physiology, their ploidy has not been reported previously. 18, 29–31 Since ploidy of cells has important implications for efficient gene targeting, we determined the ploidy of mCCDcl1 cells, by karyotyping. Chromosome counts from metaphase spreads revealed that the majority of cells contained 64 chromosomes (64 ± 1, n = 10) (Figure 2A), suggesting triploidy for the majority of chromosomes, but also some degree of aneuploidy.

Figure 2. Metaphase Spread and FISH Analysis of Adam10 in mCCDcl1 Cells. (A) Left panel: example of metaphase spread of mCCDcl1 cells. Scale bar 20 μm. Right panel: chromosome spread stained with DAPI for counting. Scale bar 5 μm. (B) Representative metaphase chromosome spread tagged with Adam10-specific probes (red channel, left panel). Three chromosomes were identified for the Adam10 gene (arrows). DNA was stained with DAPI.
In order to confirm the number of copies of the genes of interest, FISH analysis was performed. Gene-specific probes were created with fluorescent reporters and incubated on mCCDcl1 chromosome spreads. For the Adam10 gene, situated on chromosome 9, 3 gene-specific probe signals were detected in spreads (Figure 2B). Therefore, complete gene knockout required 3 copies to be targeted.

**Transfection and KO of Adam10 in mCCDcl1 Cells**

CRISPR targeting was achieved using sgRNAs, either singly or in combination. Targeting efficiency was assessed on the whole cell population by PCR (with F1 and R1; see Table 1), and T7 enzyme digestion of the PCR product was used to detect indels. Predicted product sizes for each targeting event for Adam10 were determined (Figure S2). PCR products lacking the expected WT product at 766 bp (Figure 3A) indicate the clone has a high probability of total KO. Two clones were identified from the G1+G2 combination (a 1:4 ratio for G1+G2 clones) and were named A1 and A5 for further analyses. No complete loss of parental PCR product was observed in clones targeted with G1 or G2 alone.

PCR products for each clone, both before and after T7 digestion, were sequenced and revealed a range of editing events (Figure 3C). More specifically, A1 showed a deletion between G1 and G2 loci as well as indels at one, or both target sites, leading to a shorter PCR product compared to truncated DNA products after T7 digestion. A5 showed a similar deletion event leading to a shorter PCR product compared with WT, as well as an insertion of random DNA sequence at G2 locus (a 1:4 ratio for G1+G2 clones) and were named A1 and A5 for further analyses. No complete loss of parental PCR product was observed in clones targeted with G1 or G2 alone.

Electrophysiological Characterization of Adam10 KO Cells

We next assessed the functional effects of Adam10 knockout. As seen in Figure 4, transepithelial electrophysiological measurements of WT mCCDcl1 cells revealed baseline Isc measurements of $-8.1 \pm 0.9 \mu A/cm^2$ ($n = 3$), consistent with previous reports. The application of amiloride (10 $\mu M$, 10 min) to the apical bath totally inhibited Isc, indicating that the basal current can be attributed to the transport of Na$^+$ via ENaC. The addition of aldosterone (3 nm, 3 h) increased Isc by a factor of 3.7 ± 0.3 fold, to reach values of $-29.6 \pm 2.9 \mu A/cm^2$ (see Table 2 for all values). In comparison, identical measurements performed on A1 and A5 show comparable Re to WT (values at day 10), but Vm failed to develop over the course of the experiment, particularly in clone A1. Both KO clones displayed negligible Vm at days 9 and 10, in media without additives (0.9 ± 0.2 and 0.5 ± 0.0 mV for A1 and A5, respectively). Baseline Isc was close to 0 for both KO clones, with a negligible effect of aldosterone and amiloride treatment. In summary, KO of Adam10 greatly impairs the transport of Na$^+$ via ENaC.

**scRNAseq Analysis of Adam10 KO Clone**

To uncover the mechanisms underlying the loss of sodium transport following knockout of Adam10, mCCD and A1 clone were grown on Corning Transwells for 9 days, to allow polarization, and were FAC sorted for single-cell RNA sequencing, following the 10X Genomics protocol.

The transcription profile of A1 was compared with parental mCCDcl1 cells by pooling the data while retaining source identity. We first performed linear dimensional reduction by PCA, and then clustered cells of closely related gene expression profile using a t-SNE plot. Figure 5A shows unsupervised clustering of pooled data from the two cell lines (resolution 0.1), resulting in the identification of 5 clusters. The accompanying table identifies the composition of these clusters (mCCDcl1, A1).

Average gene expression levels per cluster are provided (Dataset S1: PCA Cluster Average). Cluster 4 expresses high levels of cell cycle (cell division) related genes and is a consistent mix containing between 6.0% and 7.0% of cells of each line. The majority of WT mCCDcl1 cells are in cluster 0 (73.4%) and cluster 3 (16.9%), while the majority of A1 cells are in cluster 1 (53.7%) and cluster 2 (38.7%).

Figure 5B shows a heat map of the 10 most differentially expressed genes for each cluster. Notable genes, highly down-regulated in the knockout clone, include Tfc (transferrin receptor 1, regulation of intracellular iron levels) and Cripl (cysteine-rich protein 1, previously linked to blood pressure regulation). Parm1, previously identified as a specific intermediate cell marker in WT mice, is increased in A1 (data not shown).

Genes identified in the heat map allowed us to identify cluster 5 as dividing cells.

Violin plots (Figure 5C) revealed the transcript distribution of key components of the Notch pathway and PC-specific genes across clusters. Despite the introduction of indels into the Adam10 gene, transcripts are still produced (in common with many knockout strategies), but no active protein is predicted from transcript sequences (Figure 3D). Neither Notch1 nor Adam10 (which showed a similar pattern of expression across clusters to Adam17) was highly expressed in any of the clusters. There was a significant decrease in transcription of the gamma and beta subunits of ENaC (Scnn1g and Scnn1b) in knockout cell-rich clusters (see violin plots in Figure 5C), while subunit alpha (Scnn1a) did not show significant change.

Velocyto analysis allowed us to investigate the distribution of spliced versus unspliced transcripts in the individual datasets (Figure 5D; note that circles represent average of 10 cells). This clearly demonstrated varying degrees of active transcription and splicing of Adam10 and Scnn1g transcripts across cells of each library but at 50% reduced level in clone A1 compared with mCCDcl1 cells.

To interrogate the response of clone A1 to Adam10 knockout, we performed CCA as a means of dimensional reduction. This identifies common sources of variation (such as cell types) in the pooled datasets, and then looks for variation within resolved clusters. The dataset for A1 was mapped closely onto the mCCD dataset using CCA, and five clusters were identified (Figure 6A; resolution 0.3). Feature plots revealed variations in transcript expression between the two libraries, across the clusters. Adam10 transcription was observed in a smaller percentage of A1-derived cells across the clusters (Figure 6B). Transcription of a number of genes was dramatically reduced in A1, including Crip1 and Tfc (Figure 6C), while Haldsb and Scnn1g transcription appeared to be restricted to cluster 1 in A1 (Figure 6D and E) as was Sgk1. Of note, a number of transcription factors, including Klf4 (Figure 6F), showed increased transcription in the knockout clone, while the ligand Apel, which is known to regulate fluid balance, was downregulated (Figure 6G). (The full
Figure 3. Identification of 2 Clones Resulting From Targeting of the Adam10 Gene in mCCDcl1. (A) Transfection results on mCCDcl1 cell population for the Adam10 gene. “Del” for deletion; WT represents the predicted size of the WT product. T7 product of G2 is a doublet. (B) T7 screening for Adam10 transfected mCCDcl1 cells populations, following single-cell cloning. Red arrows indicate the clones selected after screening, based on the disappearance of a WT-sized band. “Ins” for insertion; “Del” for deletion; WT represents the predicted size of the WT product. The numbers above each gel track are the initial identification numbers of each clone. (C) Sequencing of PCR products from both clonal cell lines for Adam10. (D) Immunostaining of mCCDcl1 cells using anti-Adam10 antibody in wild-type mCCDcl1 cells (WT) and KO clonal lines A1 and A5. Composite image with DAPI staining of cell nuclei (blue). All scale bars 30 μm.
Figure 4. Electrophysiological Analysis of Adam10 KO Clones. Top left: transepithelial resistance (Rte) measured across monolayers of mCCDcl1, A1, and A5 cells grown on Snapwell filters, between days 3 and 10 after seeding. Top right: transepithelial voltage (Vte) measured across monolayers of mCCDcl1, A1, and A5 cells. Bottom left: short-circuit current (Isc) was calculated using Ohm’s law. Bottom right: effects on baseline Isc of aldosterone (3 nm) and amiloride (10 μM, apical bath) added at t = 0 and t = 180 min, respectively. Values are shown as means ± SE (n = 3).

Table 2. Electrophysiological Measurements for mCCDcl1 Cell Line and Clonal KO Lines A1 and A5, and Calculated Isc Fold Change After Aldosterone Treatment

|                  | Baseline ± SD | Aldosterone 3 h ± SD | Amiloride 10 min ± SD | Isc Fold Change, Aldosterone Treatment ± SD |
|------------------|---------------|----------------------|-----------------------|---------------------------------------------|
| mCCDcl1          |               |                      |                       |                                             |
| Isc, μA/cm²      | –8.1 ± 0.9    | –29.6 ± 2.9          | 0.5 ± 0.3             |                                             |
| Rte, kΩ·cm²      | 1.0 ± 0.1     | 0.8 ± 0.1            | 1.5 ± 0.2             | 3.69 ± 0.03                                 |
| Vte, mV          | –7.9 ± 0.7    | –23.3 ± 1.8          | 0.7 ± 0.3             |                                             |
| A1               |               |                      |                       |                                             |
| Isc, μA/cm²      | 1.1 ± 0.3     | 0.1 ± 0.3            | 0.9 ± 0.4             |                                             |
| Rte, kΩ·cm²      | 0.9 ± 0.1     | 1.0 ± 0.1            | 1.2 ± 0.2             | N/A                                         |
| Vte, mV          | 0.9 ± 0.3     | 0.1 ± 0.4            | 1.0 ± 0.3             |                                             |
| A5               |               |                      |                       |                                             |
| Isc, μA/cm²      | 0.7 ± 0.1     | 0.1 ± 0.4            | 0.5 ± 0.1             |                                             |
| Rte, kΩ·cm²      | 0.9 ± 0.2     | 0.8 ± 0.2            | 1.0 ± 0.2             | N/A                                         |
| Vte, mV          | 0.5 ± 0.1     | 0.2 ± 0.3            | 0.5 ± 0.2             |                                             |

Abbreviation: N/A, not applicable.
lists of upregulated and downregulated transcripts in clusters 0 to 4 are given in Dataset S2: CCA A1 Response.)

Expression and Localization of Key Markers

Considering the reported effect of Adam10 in vivo on the composition of the CCD, further immunostaining and analysis were conducted in clone A1 compared with parental mCCD$_{cl1}$ on markers specific to PCs ($\gamma$-ENaC and Aqp2) and ICs (V-ATPase B1).

Immunostaining of markers specific to PCs and ICs, Aqp2 and V-ATPase B1, respectively, shows altered expression levels and localization of proteins. Parental mCCD$_{cl1}$ cells showed varied expression levels in individual cells, and a staining distribution in line with previous report$^{19}$: $\sim$44% of cells with no staining; $\sim$41% with dual staining of Aqp2 and V-ATPase B1; and
Figure 6. Canonical Correlation Analysis (CCA) of mCCD and A1. (A) Visualization of CCA combined libraries projected into maximally correlated subspace before and after cluster analysis (resolution 0.3). Typical FeaturePlots of genes, split according to source library, including (B) Adam10 and (C) genes Crip1 and Tfrc, downregulated in A1; (D) FC-specific genes Hsd11b2 and (E) Scom1; (F) transcription factor Klf4, upregulated in A1 compared with mCCD, and (G) ligand Apela, which was downregulated in A1. Positive expression, purple; no expression, gray.
a small proportion of cells staining for only Aqp2 (8.7%) or V-ATPase B1 (5.3%). In contrast, A1 showed an overall decrease in Aqp2 expression (−41.4% ± 3.7% in A1) but also a more uniform expression of both Aqp2 and V-ATPase B1 across the whole cell population (Figure 7A, C, and D). Dual staining cells represent 86% ± 2% of A1 cells, mainly due to the decrease in nonstaining cells compared with mCCDcl1. Confocal imaging shows that A1 cells lose polarization, with diffuse staining of Aqp2 throughout the cytoplasm, compared with the localization of Aqp2 at the apical membrane in mCCDcl1 (Figure 7B).

Equally, γ-ENaC is expressed at low levels but more uniformly throughout the A1 cell population compared with mCCDcl1 cells (Figure 7E). High magnification imaging shows typical “punctate” staining of ENaC channels on the apical membrane of mCCDcl1 cells, absent in A1 (Figure 7F). These results are consistent with the loss of sodium transport function confirming the electrophysiological assays, and with the lower expression mirrored in the sequencing results.

Discussion

Knockout of Hsd11b2 in PCs led to a reduction in PCs and increase in intermediate cells. The functionality of intermediate cells displaying both PC and IC markers in SAME mice is unknown. The reduction in PCs may be a homeostatic mechanism to limit the increase in sodium retention, due to loss of Hsd11b2 function. The intermediate cell phenotype suggests an undifferentiated state, or dedifferentiation after duct development. The phenotype appears similar to the one described in the work of Blomqvist et al.,11 where Foxi1 gene knockout led to a collecting duct composed entirely of intermediate cells. It has to be noted that the Foxi1 KO led to renal tubular acidosis, which in turn could lead to a shift of some b-IC to a-IC as shown before in acidic conditions3 to manage that acidosis. The intermediate cell phenotype19 (expressing both PC and IC markers), absent in A1 (Figure 7F). These results are consistent with these findings. While the role of Notch signaling is well established, the role of α-secretase (Hsd11b2) remains unknown. The reduction in PCs may be a homeostatic mechanism to limit the increase in sodium retention, due to loss of Hsd11b2 function. The intermediate cell phenotype suggests an undifferentiated state, or dedifferentiation after duct development. The phenotype appears similar to the one described in the work of Blomqvist et al.,11 where Foxi1 gene knockout led to a collecting duct composed entirely of intermediate cells. It has to be noted that the Foxi1 KO led to renal tubular acidosis, which in turn could lead to a shift of some b-IC to a-IC as shown before in acidic conditions3 to manage that acidosis. The Hsd11b2 knockout mice have previously been reported to display a reduced number of collecting ducts, and additionally, hyperplasia of the distal convoluted tubule21 suggesting that potential changes in genomic, environmental, or chemical cues at the collecting duct can affect other parts of the kidney tubules. Our study on SAME mice supports the view that dynamic regulation of the PC/IC ratio is complex and extends beyond genes in the Notch pathway. Though a decreased number of PCs was previously observed in Adam10 KO mice,12 leading to polyuria and hydrenephrosis and suggesting a role for Aqp2 in mediating the KO phenotype, very different mechanisms affecting cell plasticity may be at play. Equally, plasticity may be a key response of the collecting duct to various stresses.

Polyplody, a well-known phenomenon in immortalized cell lines,34 has not been reported previously for mCCDcl1 cells. FISH analysis of mCCDcl1 indicated triploidy for the genes of interest. However, the total chromosome count returned an abnormal number (64 instead of the expected 60 for a triploid cell line), suggesting aneuploidy for some of the chromosomes. Our results show the value of preliminary cell line characterization before undertaking genetic modification work. CRISPR-Cas9-targeted deletion between the two guides, G1 and G2, was the most frequent event observed in knockout clones. Ideally, G1 and G2 should be chosen in an exon (or neighboring exons) critical to the function of the protein. Alternatively, the guides should be chosen so that the resultant deletion produces an out-of-frame product. No total knockout was achieved using a single guide, which may be due in part to the number of clones screened in this study. Using G1+G2, 25% of targeted Adam10 clones were complete knockouts. Since mCCDcl1 is polyploid, and each allele is knocked out independently, it is critical to carefully analyze the resultant targeted clones in order to verify complete knockout. Our results indicate total knockout is achieved efficiently using 2 guides and should require only a single round of single-cell cloning and screening. We have used the G1+G2 combination strategy for the knockout of another gene of interest (Tfcp2l1, unpublished), where approximately 14% of the clones were completely targeted. This is the strategy that we recommend for total knockout in polyploid cell lines.

The transcription profile of components of the Notch pathway was remarkably consistent between mCCD and clone A1, except for Tfcp2l1, which was downregulated, and Jag1, which was upregulated in A1. It is possible that knockout of Adam10 was complemented by Adam17, which is expressed at equivalent levels in each cell line and across clusters. Adam17 is known to overlap with Adam10 in its alpha-secretase activity.35 RNA sequencing may indicate similar levels of expression of key markers; however, effects on protein trafficking and localization will lead to drastically different phenotypes.

Despite including a significant portion of cells with an intermediate phenotype39 (expressing both PC and IC markers), mCCDcl1 cells exhibit the expected functions of PCs such as amiloride-sensitive sodium transport. It is not clear whether intermediate cells in vitro (or in vivo) are capable of some physiological function. In this study, the knockout of Adam10 had dramatic consequences on cell functionality, in particular the capacity to transport sodium. No difference in either Dot1l or Mib1 transcription was observed, suggesting that neither epigenetic effects on ENaC subunit transcription nor protein ubiquitination are likely to explain this observation.

Our results suggest loss of Adam10 increases the intermediate phenotype ratio, and that the loss of function results from a lack of cell polarization. Immunocytochemistry of A1 suggests that lack of polarization may occur in both intermediate and principal cells, given the diffuse antibody staining observed for Aqp2, V-ATPase B1, and γ-ENaC. Clues to explain the loss of polarization in A1 cells may come from the scRNAseq CCA analysis. Cldn7 is drastically reduced in A1 compared with mCCD cells. Since Cldn7 is involved in tight junctions, this may have a detrimental effect on cell polarity. Gene lists were analyzed by Gene Ontology (http://geneontology.org/), which revealed enrichment in downregulated genes, encoding proteins involved in plasma membrane rafts, caveola, ER membranes, and focal adhesion (Dataset S2), suggesting a significant change in cell trafficking. This may also be relevant to the Adam10 knockout mouse.12

The importance of cell polarization in the maturation process has previously been reported,36 and our data are consistent with these findings. While the role of Notch signaling is now well recognized for the modulation of the PC:IC ratio in the collecting duct, our study shows particular effects of disturbing Adam10 on cell polarization and cellular function that suggest a cellular dedifferentiation phenomenon. In reference to earlier reports on cellular plasticity terminology,37 this would suggest that CD cells can “transdifferentiate” through a process of dedifferentiation into an intermediate cell type. The intermediate cell type has indeed previously been observed in adult collecting ducts.6,38 Recently, Uchimura et al.39 demonstrated the roles played by aldosterone and vasopressin on PC and IC differentiation in kidney organoids, suggesting their importance in collecting duct maturation.
Figure 7. Characterization of A1 by immunohistochemistry. (A) Immunostaining of mCCD cl1 cells (mCCDs) and A1 cells (A1) with anti-Aqp2 and V-ATPase B1 antibodies. In the composite images, DAPI staining of cell nuclei is in gray. Scale bars 50 μm. (B) Confocal imaging of Aqp2 immunostaining in mCCD cl1 and A1 cells. On the right, z-projection of the images z-stack, localized on the left image by the red arrows. DAPI staining of cell nuclei is in gray. Scale bars 20 μm. (C) Gray value per cell line for Aqp2 staining. ****P < .0001. (D) Quantification of the proportion of cells (%) staining for Aqp2 only, V-ATPase B1 only, both, and neither in mCCD cl1 and A1 cells. ***P < .001; ****P < .0001. (E) Representative images of mCCD cl1 and A1 cells stained with anti-γ-ENaC antibody. Scale bars 20 μm. (F) Representative images of mCCD cl1 and A1 cells stained with anti-γ-ENaC antibody (red), and DAPI in blue. Scale bars 10 μm.

In summary, our study shows that PC:intermediate cell:IC cell ratio is altered in SAME, offers new insights into the functional effects of Adam10 knockout, and highlights the potential of mCCD cells for understanding collecting duct cell biology. Recognition of the potential cell dedifferentiation occurring under pathological conditions could lead to new targets for treatment of kidney diseases affecting the collecting duct.

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Supplementary Material
Supplementary material is available at the APS Function online.

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Conflict of Interest Statement
None declared.

Data Availability
The data underlying this article are available in the article and in its online supplementary material. The raw scRNA data are deposited in Edinburgh Datashare, at https://doi.org/10.7488/ds/3022, subject to an embargo until October 12, 2021 during which data can be accessed using the “request a copy” function.
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