# Deletion of hensin/DMBT1 blocks conversion of β- to α-intercalated cells and induces distal renal tubular acidosis

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Acid–base transport in the renal collecting tubule is mediated by two canonical cell types: the β-intercalated cell secretes $HCO_3$ by an apical Cl:$HCO_3$ named pendrin and a basolateral vacuolar (V)-ATPase. Acid secretion is mediated by the α-intercalated cell, which has an apical V-ATPase and a basolateral Cl:$HCO_3$ exchanger (kAE1). We previously suggested that the β-cell converts to the α-cell in response to acid feeding, a process that depended on the secretion and deposition of an extracellular matrix protein termed hensin (DMBT1). Here, we show that deletion of hensin from intercalated cells results in the absence of typical α-intercalated cells and the consequent development of complete distal renal tubular acidosis (dRTA). Essentially all of the intercalated cells in the cortex of the mutant mice are canonical β-type cells, with apical pendrin and basolateral or diffuse/bipolar V-ATPase. In the medulla, however, a previously undescribed cell type has been uncovered, which resembles the cortical β-intercalated cell in ultrastructure, but does not express pendrin. Polymerization and deposition of hensin (in response to acidosis) requires the activation of β1 integrin, and deletion of this gene from the intercalated cell caused a phenotype that was identical to the deletion of hensin itself, supporting its critical role in hensin function. Because previous studies suggested that the conversion of β- to α-intercalated cells is a manifestation of terminal differentiation, the present results demonstrate that this differentiation proceeds from $HCO_3$ secreting to acid secreting phenotypes, a process that requires deposition of hensin in the ECM.

The intercalated cells (ICs) of the kidney mediate acid–base transport and exist in two functionally distinct subtypes (1): the β-type secretes $HCO_3^-$, whereas the α-form secretes $H^+$. An apical Cl:$HCO_3$ exchanger and a basolateral vacuolar $H^+$-ATPase (V-ATPase) mediate secretion of base by the β-cells, whereas α-cells secrete acid by an apical V-ATPase and a basolateral Cl:$HCO_3$ exchanger. In both cell types, the same or a very similar V-ATPase is located in the apical membrane of the α-form and in the basolateral membrane of the β-type (2, 3). The apical Cl:$HCO_3$ exchanger of the β-intercalated cell is pendrin (Slc26a4) (4), whereas the basolateral exchanger of the α-cell is an alternately spliced form of the red cell anion exchanger, AE1 (Slc4a1).

Metabolic acidosis converts the collecting tubule from a state of $HCO_3$ secretion to $HCO_3$ absorption (i.e., $H^+$ secretion). We found that the number of β-intercalated cells was reduced by metabolic acidosis, whereas the number of α-intercalated cells increased. However, the total number of intercalated cell remained the same (1). We interpreted these results as indicating that the β-intercalated cell converts to an α-phenotype. Although the nomenclature is somewhat contentious, there is no doubt about the presence of an acid secreting "canonical" α-cell type with an apical V-ATPase and a basolateral AE1 and β-cell type with an apical pendrin and a basolateral V-ATPase. The presence of intermediate cells raises many questions about the origin and diversity of these cell types, and some AE1-negative intercalated cells display bipolar and/or a diffuse cytoplasmic distribution of the V-ATPase (2, 3)

and some cortical intercalated cell types express pendrin and the V-ATPase on the apical surface (the so-called non-A non-B type) (4). Induction of metabolic acidosis or alkalosis produces a profound change in the population distribution of these different cell types with acidosis shifting the distribution to the type with apical ATPase, whereas alkalosis increases the number of canonical β-cells at the expense of α-cells (5, 6). That an individually identified β-intercalated cell actually converts to an α-intercalated cell was provided more recently when we found that its exposure to a basolateral low pH medium caused a significant fraction of such cells, which had an apical Cl:$HCO_3$ exchanger to convert to ones with basolateral Cl:$HCO_3$ exchangers (7). However the molecular identity of these exchangers was not identified.

To identify the molecular basis of the conversion, we generated a clonal immortalized cell line of a rabbit β-intercalated cell and found that when these cells were seeded at subconfluent density and allowed to form confluent monolayers they developed into $HCO_3$ secreting cells (8). We discovered that the α-resembling intercalated cells deposited an extracellular matrix protein which, when purified, was able by itself to induce conversion of intercalated cells seeded at low density to a cell type that resembled the α-intercalated cell. We termed this protein hensin (9) (also termed DMBT1 by the Mouse Genome Project). We proposed that the conversion of intercalated cells is an example of terminal differentiation (10). Hensin/DMBT1 is expressed in most epithelia, often in alternately spliced forms, suggesting that hensin might be involved in the differentiation of other epithelia as well. That global deletion of hensin resulted in early embryonic lethality at the time of appearance of the first columnar epithelium, visceral endoderm, supported this proposal. Hensin/DMBT1 hensin has been found to be deleted in a large number of epithelial malignancies, again supporting its role in epithelial differentiation (see ref. 10 for review).

Here we have conditionally deleted hensin from intercalated cells and found that this resulted in an almost complete absence of typical α-intercalated cells as defined by existing standard markers of this cell type. Activation of integrin β1 was necessary for polymerization and deposition of hensin in the ECM (11) and here we found that deletion of β1 integrin in the intercalated cells also prevented the conversion of β- to α-intercalated cells. Furthermore, the presence of only $HCO_3$-secreting β-cells, as expected produced complete distal renal tubular acidosis (dRTA).

## Results

**Conditional Deletion of Hensin.** We found that hensin is secreted as a monomer by the low-density phenotype of immortalized intercalated cells (the β-intercalated cell equivalent) and that acidosis (or high-density seeding) led to its polymerization and deposition in the ECM. To delete hensin from the intercalated cells we constructed a loxPhensin mouse line as shown in Fig. S1*A*. We crossed this line withone expressing Cre under the control of the HoxB7 promoter, resulting in deletion of hensin in the entire collecting duct (Fig. S1*B*). We also bred loxPhensin mice to those expressing Cre under the control of the promoter for the B1 subunit of the V-ATPase, which is expressed at high levels in the intercalated cells (we refer to this line of mice as the VB1Cre line) (Fig. S1*C*) (12). A critical initial step was that activation of hensin polymerization requires inside out signaling of β1 integrin (11). Hence, we crossed the VB1Cre mice with mice expressing a LoxP integrin β1 transgene (Fig. S1*D*). This cross resulted in deletion of this integrin from the intercalated cell lineage (Fig. S2). All resulting progeny of the crosses showed Mendelian ratios of mutant and wild-type pups. The animals were fertile and appeared in good health.

We examined the expression of hensin by Western blot analysis. Comparison of the intensity of the bands from wild-type mice to those of the three mutant lines is shown in Fig. S1*E*. We used two loading markers, an ECM protein laminin and an anonymous band in the Ponceau Red stained blots. There was no significant difference in the use of these two markers. Fig. S1*E* shows a representative gel taken from four independent studies where all of the lanes of wild-type and the three mutant lines provided interpretable results and were present in the same gel. The collecting duct-specific (HoxB7) mutants had reduced expression of hensin protein on average to 34% of the wild-type levels. That it did not reduce it to 0% is likely due to the recently reported lack of expression of the Cre recombinase in some parts of the connecting tubule (ref. 13 and see below). The intercalated cell-specific mutant (VB1Cre) mice reduced their hensin protein expression to 69% of wild type, a value compatible with the finding that intercalated cells represent 39% of the mouse collecting tubule cells (see below). The levels of hensin in the β1 integrin Cre mice were no different from the wild type, as expected. These results demonstrate that hensin was indeed deleted from the intercalated cells in these mice. All primers are presented in Table S1 and the PCR screening strategy is described in Fig. S3.

**Hensin Knockout Causes Distal Renal Tubular Acidosis.** All three lines of mutant mice had significant metabolic acidosis with plasma HCO3 concentration and pH both lower than their wild-type littermates (Fig. 1). The urine pHs were higher in the mutant mice than in the wild type animals. These studies were performed using the iSTAT analyzer, which uses a variety of electrodes to estimate pH and pCO2. We also measured $pCO_2$ and pH by traditional clinical blood gas analyzer in retroorbital mixed venous blood taken from awake and spontaneously breathing mice and found that those results showed that mutant mice had metabolic acidosis. These studies demonstrate that deletion of hensin results in spontaneous complete distal renal tubular acidosis. That deletion of β1 integrin from the intercalated cells also led to distal RTA indicates that polymerized hensin is the critical intermediate in the signaling pathway.

We also measured acid excretion and found that, whereas NH4 and titratable acid and net acid excretion were not different between the mutant mice and their wild-type littermates, the urine pH of mutant mice was significantly higher than the wild type. Studies in patients with distal RTA (14) usually show that the NH4 excretion is lower in these patients; however, where documented, these patients had significant renal failure largely attributed to nephrocalcinosis (figures 6 and 7 in ref. 14). When studies were conducted in patients with renal function close to normal there was no difference in these parameters similar to what we found here (figure 11 in ref. 14). Mutant mice had normal glomerular filtration rates.
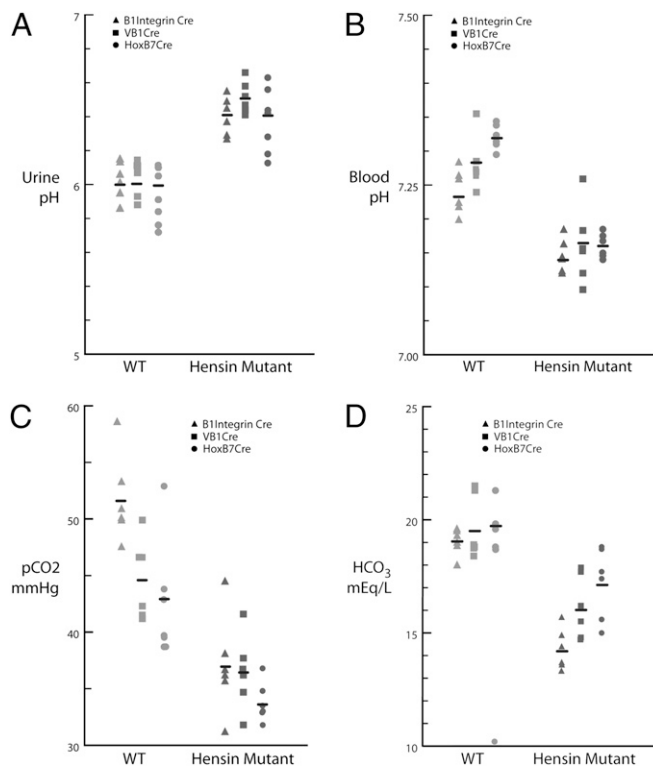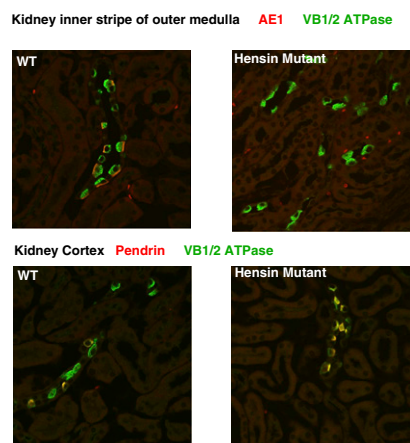


**Fig. 1.** Acid–base parameters in all three lines of hensin-deleted mice compared with their wild-type littermates. Each time point represents data from a single mouse. Mice were placed in a metabolic cage for 24 h and given water and food ad libitum. Urine was collected under oil and blood was drawn using intracardiac puncture and measured. Wild-type mice were littermates of the mutants. Bar represents average value. The panels show the results of studies of (*A*) urine pH; (*B*) plasma pH; (*C*) blood $pCO_2$; and (*D*) plasma $HCO_3$ concentration in the three lines of mutant mice, each of which is identified with a different symbol and compared to their nonmutant littermates.

Because hensin is expressed by both principal and intercalated cells, it is possible that the "acid signal" that leads to the increased abundance of the α-intercalated cell phenotype is received by the principal cells and transmitted through hensin to the intercalated cell in a "juxtacrine" manner. To test this possibility, we bred the hensin loxP mice to those expressing Cre under the control of the Hoxb7 promoter, which should result in hensin deletion in all cells derived from the ureteric bud (15). The results were quite similar to those in the mice lacking hensin only in the intercalated cells (Fig. 1, HoxB7Cre). These studies demonstrate that the mechanism of action of hensin is "cell autonomous" i.e., acidosis does not stimulate the principal cells to secrete functional hensin molecules, which then alters intercalated cell response to acidosis in a hensin-dependent manner or that the main site of hensin production is in intercalated cells, as suggested by the PCR data on the different mice strains.
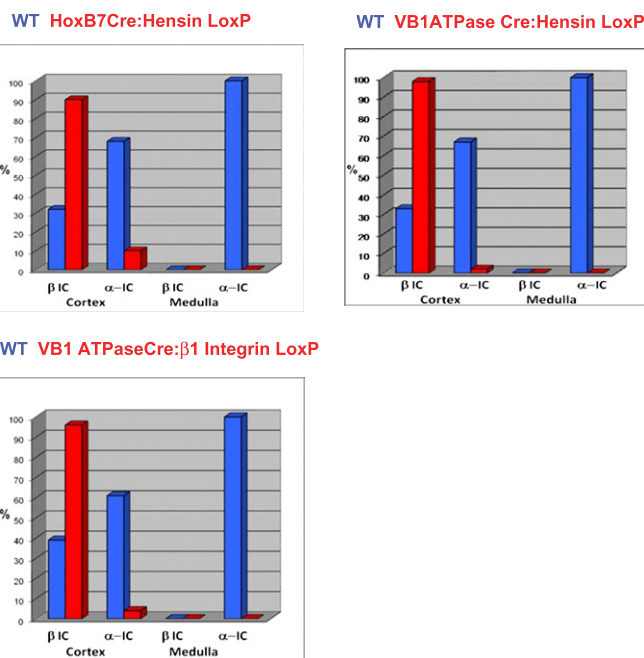
Many syndromes of distal RTA are associated with hearing loss, in part because both intercalated cells and cochlea cells express the same transport proteins, such as the B1 subunit of the V-ATPase (16) and a critical transcription factor foxi1 (17). We studied wild-type mice and the VB1 hensin mutant mice by measuring the whole auditory nerve response (known as the compound action potential, CAP) to tones over a range of frequency from 0.2 to 40 kHz. By monitoring the sound level required to elicit a criterion "threshold" response, a CAP threshold tuning curve was constructed, with tone frequency on the horizontal axis, and the threshold sound level on the vertical axis (*SI Text, Hearing Tests*, and Figs. S4 and S5). There was no difference in these curves between wild-type and mutant mice.

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**Intercalated Cells in Hensin-Deleted Mice.** We quantified the number and subtypes of intercalated cells in the cortical- and medullary collecting tubules using double immunofluorescence staining for aquaporin 2, a marker for principal cells and the B1 subunit of the V-ATPase for the intercalated cells. More than 5,000 cells were counted in the cortical collecting tubule and an equal number in the medulla in each line of mice. In wild-type mice, intercalated cells represented 39% of collecting duct cells in the cortex and 38% in the medulla. In mice deleted for hensin in the entire collecting duct, the fraction of intercalated cells was identical to the wild type, 38% in the cortex and 39% in the medulla. Hence, deletion of hensin did not reduce the number of intercalated cells in the kidney.

Intercalated cells exist in many forms and for the sake of simplicity, we will assume here that there are two "canonical" versions: α-intercalated cells with apical V-ATPase and a basolateral anion exchanger, which is an alternately spliced product of the erythrocyte AE1 gene, and β-intercalated cells with apical pendrin and basolateral (or bipolar) V-ATPase (Fig. 2, wild type). We estimated the expression of AE1 and pendrin in mutant kidney cortex using quantitative PCR in the Hoxb7Cre:Hensin LoxP mice and found that AE1 expression in the kidney cortex was reduced to below 4% of WT levels, whereas that of pendrin was increased to 1.37-fold of the WT levels ($n = 2$). Similarly a quantitative Western blot of pendrin showed that mutant mice had 1.34-fold ($n = 2$) the amount of pendrin protein using laminin as a loading control (Fig. S6). Because the number of β-intercalated cells was 2.5-fold that of the WT, pendrin expression per cell must have been reduced. The most likely explanation is acidosis, which all mutant mice exhibited and is well known to reduce pendrin activity presumably due to reduced expression in identified β-intercalated cells (18). We next evaluated the number of α- and β-intercalated cells in wild-type and knockout mice. Intercalated cells were defined as those cells that stained for the B1 subunit of the V-ATPase; α-intercalated cells were defined as those that also had AE1 staining, whereas β-intercalated cells had pendrin staining. In the cortex of wild-type mice, we counted 6,293 cells from four different animals and the results are displayed as blue bars in Fig. 3. Thirty-two percent of intercalated cells stained for pendrin, whereas 68% of cells had AE1 staining. In the medulla we counted 2,017 cells and found no pendrin-positive intercalated cells. In the medulla of wild-type mice almost all of the intercalated cells (99.6%) were AE1 positive, as expected from previous data. In the collecting duct-specific (HoxB7 Cre:HensinLoxP) mutant mice the results were dramatically different: we counted 8,662 cells (red



**Fig. 3.** Quantitative analysis of the number of α- and β-intercalated cells in the cortex and medulla of kidneys taken from wild-type and the three lines of hensin mutant mice. Each panel was derived from three wild-type and three mutant mice. Wild-type mice were littermates of the mutants hence they were actually HoxB7Cre or VB1Cre rather than true wild-type mice.

bars in Fig. 3). Most (88%) of the intercalated cells in the cortex were pendrin positive. As in the wild-type mice there were no pendrin-positive intercalated cells in the medulla. However, there were few AE1 positive cells (9%) in the cortex (Fig. S7 also shows images taken from cortical labyrinth containing connecting tubules) and none in the medulla (0%). We do not know at present whether the 9% presence of AE1 in the cortex is due to variable penetrance of Cre expression in the cortex as compared with the medulla. We performed similar studies in mice that were deleted in hensin only in the intercalated cells using the ATPase B1Cre mice. As in the total collecting duct knockout animals, these mice had very few AE1-expressing cells in the cortex; wild-type mice had 1,656 AE1-positive cells out of 2,471 total intercalated cells (67%), whereas the hensin KO had only 49 AE1-positive cells out of 2,404 intercalated cells counted (2%). Note that in the knockout mice the antibody to AE1 was functional as seen in staining of the few red blood cells that remain after perfusion of the kidney before fixation (Fig. 2, *Upper Right*). In the medulla the effect was also dramatic, reducing the α-intercalated cell count (identified by AE1 staining) from 1,209/1,210; 100%) to 0/1,209; 0%). Pendrin-expressing cells represented 794/2,406 or 33% of the intercalated cells of the cortex of wild-type mice, whereas in the knockout animals they were 2,371/2,419; 98%. There were no medullary pendrin positive cells in either group of mice. Fig. 2 presents representative images from the kidneys from wild-type or mutant mice stained for pendrin or AE1 and the B1 subunit of the V-ATPase. In summary, these results show that hensin is required for the generation of the α-intercalated cell phenotype in cortical collecting ducts, providing strong support of our hypothesis that during acidosis, β-intercalated cells can convert to α-intercalated cells in a hensin-dependent manner.

**β1 Integrin Is Critical for Hensin Function.** Hensin is secreted as a monomer but under the influence of acidosis (in vivo, ref. 7) or high-density seeding in vitro (11), it polymerizes and deposits in the ECM. It is only the ECM form of hensin that functions in conversion of polarity. Polymerization and deposition of hensin is a complex process that requires the secretion of two additional



**Fig. 2.** Immunofluorescence confocal images from the kidney of wild-type and hensin-deleted mice. Merged images are shown where antibodies to AE1 or pendrin (both in red) were simultaneously used with those against the VB1 ATPase. Kidneys were perfused and fixed and then subjected to immunofluorescence and confocal microscopy. Sections represent those from outer medulla and inner cortex. Note that in the mutant mice the antibody to AE1 stained the red blood cells remaining after perfusion.

proteins, cyclophilin A through its *cis trans* prolyl isomerase activity (19, 20) and galectin 3 (21, 22). We had recently shown that the initial event in the polymerization of hensin is the "activation" of β1 integrin (11). Blocking antibodies to β1 integrin prevented the conversion of cell types in the intercalated cell line and an activating antibody to β1 integrin reproduced the phenotype of the α-intercalated cell without any additional requirement. To examine whether deletion of β1 integrin in the intercalated cell in vivo produces the same syndrome as that of deletion of hensin described above, we crossed the VB1Cre mice with transgenic mice that had LoxP surrounding the β1 integrin locus. Remarkably these mice have the same physiology as the conditional hensin knockout mice. They developed distal renal tubular acidosis and their urine pH was higher than their wild-type littermates (Fig. 1). When we examined the distribution of intercalated cells in cortex and medulla, we again found that the cortical collecting tubule contains almost no α-intercalated cells and most of the cells had apical pendrin (Fig. 3). Similarly, the medullary collecting tubule had intercalated cells with diffuse distribution of the V-ATPase but no expression of kAE1 or pendrin.

**Ultrastructure of the Intercalated Cells.** The medullary collecting ducts of wild-type mice, VB1-, and Hox B7-hensin–deleted mice were examined by electron microscopy to determine the ultrastructural appearance of intercalated cells in this region. In wild-type mice, most intercalated cells had an appearance typical of α-intercalated cells. They contained variable numbers of cytoplasmic vesicles that were restricted to the subapical pole of the cell, as well as well-defined apical microvillar extensions that also varied in number among individual cells. Very few vesicles were seen in the basolateral pole of these cells in wild-type mice (Fig. 4A). In striking contrast, most of the medullary intercalated cells in VB1-hensin knockout mice had the appearance of β-intercalated cells. They had very few if any apical microvillar projections and the entire cytoplasm from the subapical to the basolateral pole was filled with large numbers of vesicles (Fig. 4B). In the outer stripe, toward the cortex, a minority of intercalated cells had the appearance of α-intercalated cells, but these were rare. The intercalated cells in the medullary collecting ducts of HoxB7 mice also looked mostly "nonspecialized" with few apical villi and numerous cytoplasmic vesicles (Fig. 4C). However, whereas the intercalated cells in these mice also had vesicles distributed in the cytoplasm from apex to base, the number of vesicles was lower than in cells from the VB1-hensin deleted mice. Again, a few cells with the typical appearance of α-intercalated cells were seen, but these were in the minority. However, stud-like projections previously identified as proton pumps (23) were found on vesicles and regions of the plasma membrane in cells from both wild-type and knockout mice (Fig. 4B, small *Inset*), supporting the fluorescence studies that the V-ATPase is still expressed in these cells.

**Expression of Other Anion Exchangers.** A number of other anion exchangers are expressed in the kidney and we examined their expression in the kidney to determine whether any of them was upregulated in the mice deleted for hensin. As seen in Fig. S8 the expression of AE2 (Slc4a2), AE3 (Slc4a3), AE4 (Slc4a9), Slc26a7, and Slc26a11 did not differ between wild-type and mutant mice. Furthermore, the expression of the transcription factor Foxi1, a gene critical for intercalated cell development, was also not affected by the mutation.

## Discussion

Previous studies have shown that substantial conversion of β- to α-intercalated cells can occur after only 3 h of exposure to an acid basolateral medium, a process that was blocked by protein, DNA, and RNA synthesis inhibitors and hence is likely due to induction of new genes (7). However both cell types can respond to a variety of stimuli within a few minutes of exposure to the stimulus. For instance, the α-intercalated cell responds to an increase in the $pCO_2$ by exocytosis of $H^+$ ATPase containing vesicles (24). Similarly, cAMP causes redistribution of the ATPase to the apical surface and increases the length of the apical microvilli (25). Fur-
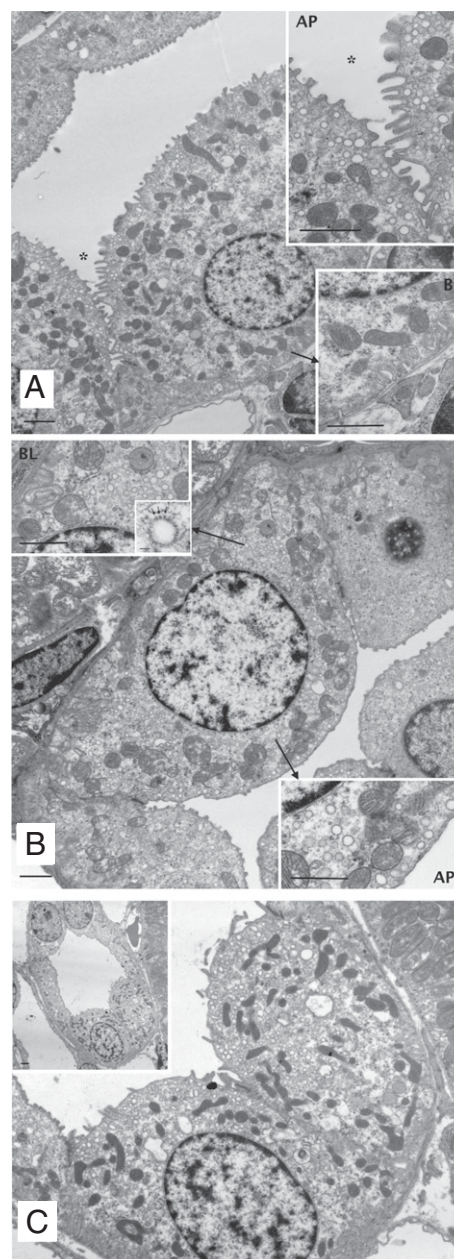


**Fig. 4.** Ultrastructural studies of Intercalated cells in mouse kidneys. (A) EM of collecting duct from inner stripe of outer medulla of a wild-type mouse kidney. Intercalated cells show typical, well-developed apical microvilli with numerous subapical vesicles. Higher magnification of the apical region (AP) indicated by an asterisk shows these apical vesicles lying beneath the apical pole of this A-type intercalated cell. In contrast, very few vesicles are seen in the basolateral region (BL) of the cell indicated by an arrow, as shown in the *Inset, Bottom Right*. (Scale bar, all panels, 1 μm.) (B) EM of collecting duct from the inner stripe of the outer medulla of a VB1 hensin-deficient transgenic mouse. Many intercalated cells in the medullary region of these animals showed features more typically associated with β-intercalated cells. The detailed *Insets* show that these ICs had few apical microvilli and numerous cytoplasmic vesicles that were located at both the apical (AP) and basolateral (BL) poles. The cytoplasmic vesicles did retain the typical stud-like coat of projections characteristic of the V-ATPase, however (small *Inset, Upper Left*). (Scale bar, 1 μm.) (Small *Inset*, scale bar, 50 nm.) (C) EM of collecting duct from the inner stripe of the outer medulla of a Hox B7 hensin-deficient transgenic mouse. The two intercalated cells shown here have fewer apical microvilli than wild-type cells, and the vesicles typical of ICs are more widely scattered throughout the cytoplasm, some coming close to the basolateral plasma membrane. The *Inset* shows a lower magnification image of the entire tubule containing these cells. (Scale bar, 1 μm.)

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ther, isoproterenol by increasing cAMP in the β-intercalated cell stimulated apical Cl:HCO$_3$ exchange (26). The complexity of regulation of acid/base transport is highlighted by these short-term and the hensin-mediated long-term changes described in this paper.

The data presented here demonstrate that deletion of hensin leads to accumulation of β-intercalated and lack of α-intercalated cells indicating that there is a path of differentiation in which the β-cell is less differentiated than the α-type. However this scheme seems to apply only to the cortex, suggesting that medullary α-intercalated cells appear to be derived from a different type of cell, which, unlike ß-intercalated cells, does not express pendrin. These cells remaining in the medulla in hensin-deleted mice represent a previously unidentified type of renal cell whose ultrastructure resembles that of β-intercalated cells in that they do not protrude into the lumen and do not have large numbers of cytoplasmic vesicles in their apical pole. However, these cells, as illustrated in Fig. 4 *B* and *C*, also differ notably from β-intercalated cell because in addition to not expressing pendrin, they lack some of their morphological features such as dense and clustered mitochondria, an eccentric nucleus, darkly stained cytoplasm, and a vesicle-free apical band. Rather we propose that they are progenitors of the medullary α-intercalated cell. It is known that cortical α-intercalated cells differ from those in the medulla at least with respect to their lectin binding (27). However, the function of those "new" cell types in the mutant mice in acid–base transport is unknown, except that they cannot secrete protons because they lack basolateral AE1, although they express the V-ATPase in an albeit diffuse distribution.

Development of medullary intercalated cells begins early during nephrogenesis where cells expressing V-ATPase and AE1 begin to appear by E15.5 (28). Similarly, pendrin-expressing intercalated cells appear in the connecting tubule as early as E14, but appear in the cortical collecting tubule only after birth (29). In medullary collecting tubules, pendrin-positive cells appear during embryonic development but are eliminated after birth by apoptosis. However, we note that the cortex unlike the medulla develops slowly during embryonic life and its rapid growth is only achieved after birth (30). Hence the differentiation and development of the cells of the cortical and medullary segments occurs in widely differing environments. Because both cortical and medullary intercalated cells are absent from Foxi1 knockout mice (17) a likely first differentiation step would be to specify an intercalated cell fate different from the adjacent principal cell. This is followed by a bifurcation to form two (or perhaps more) lineages, one that would lead to the formation of the β-lineage in the cortex and the other to the "new" cell type uncovered by our study in the medulla. Both cortical and medullary lineages ultimately result in formation of α-intercalated cells, which is the more differentiated and proposed terminally differentiated phenotype in the intercalated cell lineage.

The concept of terminal differentiation implies that once a cell reaches that phenotype its fate is fixed as it becomes "postmitotic." However, there is now an extensive literature especially during embryonic development where a terminally differentiated cell could readily convert to a different phenotype, often another differentiated phenotype even one from a completely different lineage including epithelial-to-mesenchymal and mesenchymal-to-epithelial transitions. These transitions are now known to occur even in mature tissues. Hence an alternative view would be that both α- and β-intercalated cells are in different states of differentiation with their acidosis-dependent conversion being a shift from one differentiated state to another. Recent studies show that HCO$_3$ treatment leads to a beginning of conversion of α- to β-intercalated cells (31). Whether that process is also hensin dependent, i.e., requiring hensin degradation, is unknown at present.

The mechanism by which hensin mediates its effect on differentiation is unknown. In particular, we do not know that polymerized hensin by itself acts as a ligand for a receptor that transduces the signal from the outside to the nucleus. Recently we found that blockade of α6 integrin prevents the function of polymerized hensin. This might be interpreted as a result favoring the role of this integrin as a signaling receptor for hensin. However, it is possible that polymerized hensin binds to a small molecule and modulates its affinity

for its receptor and that the role of the α6 integrin is to bring it closer to the cognate receptor of the putative small molecule. Many growth factors need to bind to extracellular matrix molecules, which changes their affinity to their receptors (32). Future studies will have to distinguish between these possibilities.

**Distal Renal Tubular Acidosis Likely Due to Continuous HCO$_3$ Secretion.** Similar to all other types of complete dRTA the hensin-deficient animals are acidotic and cannot maximally acidify the urine. Defects in net H$^+$ secretion are the defining characteristics of dRTA either due to defects in basolateral anion exchange, active proton pumping into the tubule, or increased back-leak of H$^+$, all reflecting deficiencies of α-intercalated cell function. In such animals or patients, their acidosis will result in conversion of most cortical β-intercalated cells into α-intercalated cells, albeit ones that are incapable of pumping protons. Hence, there will be no HCO$_3$ secretion in the cortical collecting tubule. The evidence for this is shown in the studies of Walsh et al. who found that furosemide did not change the urine pH significantly (33). Had there been functional β-intercalated cells, one would have expected that the excess delivery of Cl to the cortical collecting tubule would have resulted in increased HCO$_3$ secretion via pendrin. This scenario can be contrasted with that of the hensin knockout mice where almost all intercalated cells have the apical Cl:HCO$_3$ exchanger pendrin despite the presence of significant metabolic acidosis. The lack of hensin prevented these cells from converting to the α-phenotype. Continued HCO$_3$ secretion explains the appearance of significant spontaneous metabolic acidosis. In other forms of distal RTA, spontaneous acidosis is often absent or mild. This is likely due to the presence of other robust H$^+$ transporting activities in inner medullary collecting tubule or elsewhere in the nephron where there are no intercalated cells. Whether human syndromes of hensin deficiency exist will require direct tests in families with distal RTA.

## Materials and Methods

**Transgenic Mice.** *Hensin LoxP Mice.* We constructed the targeting vector using a C57Black6 BAC clone from which we subcloned an ~11.1-kb region. The region was designed such that the short homology arm (SA) extends 1.5 kb 3′ to exon 1. The long homology arm (LA) starting at the 5′ side of exon 1 is ~9.2 kb long. The single loxP site was inserted upstream of exon 1, and the loxP-flanked Neo cassette is inserted downstream of exon 1. The target region is about 1.9 kb including exon 1. The targeting vector was confirmed by restriction analysis and sequencing after each modification step. A pGKNeo cassette flanked by loxP sites were inserted into the gene. After linearization it was electroporated into C57Black6 embryonic stem cells (InGenious Targeting Laboratory). After selection in G418, surviving clones were expanded for PCR analysis to identify recombinant ES clones. Of these ES clones, 375 proved to be positive. The loxP-containing clone would give a positive PCR signal at 1.6 kb. Seven clones were expanded for microinjection into blastocysts. After the sixth blastocyst injection, new chimeras were born yielding a litter that had transmitted the transgene. These mice, which are C57Black/6J, were then bred with the same or other strains of mice as described below. All animal studies have been performed under protocols approved by the Columbia University Animal Care Facility.

*Hensin Deletion Mutants.* We generated two lines of mice that were deleted in hensin: one in the entire collecting duct and the other in the intercalated cells only. The loxPhensin mice were of the C57Black strain, which were then crossed with HoxB7–Cre to delete hensin from the ureteric bud lineage, which was on the FVB/NJ strain (15). Homozygous hensin$^{-/-}$ mice in the HoxB7 background were born in Mendelian ratios and were fertile and survived until adulthood without any apparent phenotype. The mice were backcrossed to the C57Black/6J strain and all studies were performed on the progeny after a minimum of four generations. To delete hensin in the intercalated cells we used mice expressing Cre under the control of the B1 subunit of the V-ATPase. These mice were in a C57Black6/CBA strain (12). The mice were also viable and fertile and appeared to have no obvious phenotype. All studies reported here were performed after a minimum of three backcrosses to the parental C57Black6 strain. A third line of mice was generated using a β1 integrin loxP line (C57Black6;129-*Itgb1tm1Efu*/J) obtained from The Jackson Laboratory). This strain was crossed with the B1 ATPase Cre described above and all studies were done after three backcrosses. The screening and genotyping strategy is shown in Fig. S1.

The rest of the methods used in this paper have been previously reported in detail from our combined laboratories and are presented in the *SI Materials and Methods*. These include analysis and collection of blood and urine, Western blot, kidney perfusion, tissue fixation, immunofluorescence confocal microscopy, and electron microscopy.

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