Jagged2a-Notch Signaling Mediates Cell Fate Choice in the Zebrafish Pronephric Duct

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Introduction

In vertebrates, development of the excretory system is characterized by the successive formation of three distinct kidneys with increased complexity: pronephros, mesonephros, and metanephros. The pronephros is a functional filtration organ that develops very similarly to metanephros, and has been used as a model for kidney development. Nephrons, the fundamental functional units of the kidney, possess several segments, which regulate fluid balance, osmolarity, and the disposal of metabolic waste products [1]. While pronephros in amphibians and fish contain two functional nephrons, the metanephros of mammals have millions of nephrons [1–3]. The zebrafish pronephros consists of paired glomeruli coalescing at the midline ventral to the dorsal aorta, and two pronephric tubules that project bilaterally from the glomeruli to the pronephric (Wolfian) ducts that run caudally and fuse just before their contact with the exterior at the cloaca [2].

Morphogenesis and cell fate determination of different nephric segments have attracted much attention recently. Multiple transcription factors and signaling pathways have been shown to be involved in these processes in different model organisms. Wnt4 is essential for tubulogenesis in mouse metanephroi and Xenopus pronephroi [8,9]. Brn1 is required for the development of Henle’s loop, the distal convoluted tubule, and the macula densa in mice at the primitive loop stage [10], and so is pax2a for the differentiation of proximal tubule and duct epithelial cells and cloaca morphogenesis in zebrafish [11]. Some segments of the nephron comprise only one cell type, while others include two or more cell types. The mammalian collecting duct contains two major cell types: principal cells (for salt and water absorption) and intercalated cells (for acid/base transport) [12]. It was reported that Foxi1 plays a crucial role in the specification of intercalated cells [13].

Notch signaling is an evolutionarily conserved pathway that multicellular animals use in regulating pattern formation and cell fate determination through local cell interactions [14,15]. One of the well-known mechanisms of Notch signaling is lateral inhibition during neurogenesis: initially equivalent cells differentiate into a “salt and pepper” pattern of cells with different fates via a regulatory loop [14]. Notch is a
transmembrane receptor that interacts with Delta and Serrate/Jagged ligands. Ligand-activated intramembrane proteolysis, which is partly through the γ-secretase activity of Presenilin, is required to release the Notch intracellular domain (NotchICD), which is then translocated to the nucleus, where NotchICD and CSL (CBF1/RBPJK, Su(H), and Lag-1) proteins bind and activate downstream target genes, such as Hairy/Enhancer of split related (Hes) homologs [16]. Ubiquitylation is a multistep process that results in the conjugation of Ubiquitin to a substrate protein. Recent studies have identified the roles of Neuralized and Mind bomb (Mib) in ligand ubiquitylation and endocytosis, which is essential for activating Notch [17–20]. Similarly, Jagged2 is ubiquitylated by a Mib paralog, Skeletrophin [21].

Notch signaling is required for the development of different kidney segments. By manipulating Notch activity, Notch signaling in the tubule was shown to inhibit duct fate in the kidney segments. Two types of epithelial cells were found in the pronephric duct: multi-cilia cells and principal cells, which could be distinguished based on morphology and expression of different marker genes. In the pronephric duct, the multi-cilia cells and principal cells form a “salt and pepper,” or mosaic, pattern. Using existing zebrafish mutants and a knockdown technique, we demonstrated that the mosaic pattern and differentiation of these two cell types are controlled through a Notch-dependent lateral inhibition mechanism. Notch signaling has been shown to be essential for other aspects of kidney development, such as formation of the glomerulus and the tubule. Here, to our knowledge for the first time, we show that the same signaling pathway is required for the differentiation of two different epithelial cells in a kidney segment known as the distal pronephric duct. The same mechanism is very likely to be employed by other similar developmental processes in the same context to generate distinct cell types in a tissue.

Results

Multi-Cilia Cells Interpolate Principal Cells in the Zebrafish Pronephric Duct

Acetylated tubulin staining revealed that stumpy single primary cilia are present in the pronephric duct as early as the 20 somite stage (ss) (unpublished data). Cilia tufts or multi-cilia appeared later and were fully formed by 36 h post-fertilization (hpf). These cilia tufts were located along the distal pronephric duct between the proximal pronephric duct and cloaca, corresponding to somites 8–14 (Figures 1A, S1, and S3) [11,27,28]. Percenctriolar material 1 (Pcm1) staining of 36-hpf embryos revealed that multiple basal bodies are associated with each cilia tuft (Figure 1B–1D) [29,30] and Pcm1 is colocalized with γ-tubulin at the apical site of epithelial cells (Figure S2). To determine if each cilia tuft is generated from a single cell, we used antibodies against a membrane marker, wheat germ agglutinin (WGA), and a tight junction marker, Zonula occludens-1 (Zo-1) [11,31]. Triple labeling of Pcm1, acetylated tubulin, and WGA demonstrated that cilia tufts are in the lumens of the duct and that multiple basal bodies are within one cell. Individual Pcm1 staining was also found in the neighboring cells corresponding to individual basal bodies of the primary cilia (Figure 1E). Immunostaining of Zo-1 and Pcm1 confirmed that multiple Pcm1-staining basal bodies are localized to the apical side of one cell in the pronephric duct (Figure 1F).

Cilia identity was further confirmed by transmission electron microscopy imaging of a transverse section of the distal duct of 36-hpf embryos. We found two types of cells: cells with a cilia tuft of at least 15 cilia, and neighboring cells with a single primary cilium. All cilia tufts and primary cilia projected along the axis of the duct lumen and were of the typical 9 + 2 structure (Figure 1B and 1G), suggesting that they are motile [32]. Indeed, it was demonstrated that cilia in the zebrafish duct are motile, generating a corkscrew-like wave pattern in the duct lumen directed toward the cloaca [28].

In mammals, collecting ducts are composed of two major cell types: principal cells and intercalated cells [12]. Na⁺, K⁺ ATPases transport numerous solutes and water across epithelia [33] and are only expressed in the principal cells [34,35]. The zebrafish counterparts are expressed in the pronephric duct [36]. However, a meticulous examination of Na⁺, K⁺ ATPase α1a2 and Na⁺, K⁺ ATPase β1a expression using in situ hybridization revealed that these genes are not expressed in all the duct cells (Figure 1H–1K). Some sodium pump-negative cells intercalated principal cells. To investigate their identity, we cloned the zebrafish homologs and examined the expression patterns of pendrin1, pendrin2 [37,38], rhcg [39], and vacuolar-type ATPase B [40], all of which are marker genes in mammalian intercalated cells (see Materials and Methods). Although they were expressed in other tissues, none of these genes were expressed in the duct up to 72 hpf, suggesting that the sodium pump-negative cells are not intercalated cells (unpublished data). To determine whether they are multi-cilia cells, we cloned ciliogenic genes and analyzed their expression in the duct. Zebrafish rfx2 is the homolog of Caenorhabditis elegans daf-19, which controls cilium formation in sensory neurons [41]. Zebrafish centrin2 is the homolog of mouse Centrin2, which associates with centrosome-related structures of the basal bodies of the ciliated cells [42,43]. In addition to the ciliated tissues, including

Author Summary

The kidney is a complex organ that regulates blood homeostasis through the maintenance of fluid and ion balance and disposal of metabolic waste. We used zebrafish pronephros, a primordial vertebrate kidney, to address how a kidney tissue acquires its cell types and pattern. Two types of epithelial cells were found in the pronephric duct: multi-cilia cells and principal cells, which could be distinguished based on morphology and expression of different marker genes. In the pronephric duct, the multi-cilia cells and principal cells form a “salt and pepper,” or mosaic, pattern. Using existing zebrafish mutants and a knockdown technique, we demonstrated that the mosaic pattern and differentiation of these two cell types are controlled through a Notch-dependent lateral inhibition mechanism. Notch signaling has been shown to be essential for other aspects of kidney development, such as formation of the glomerulus and the tubule. Here, to our knowledge for the first time, we show that the same signaling pathway is required for the differentiation of two different epithelial cells in a kidney segment known as the distal pronephric duct. The same mechanism is very likely to be employed by other similar developmental processes in the same context to generate distinct cell types in a tissue.
Jag2a/N-Mediated Determination in Kidney

Kupffer’s vesicle, olfactory pits, hair cells of the otic vesicle, and the neural tube (unpublished data) [44], rfx2 and centrin2 were expressed in a mosaic pattern in the duct at 36 hpf (Figure 1L–1N). Furthermore, fluorescent double in situ hybridization of rfx2 and Na\(^+\)\(\text{K}^+\) ATPase \(\beta 1a\) revealed a mutually exclusive pattern (Figure 1O–1Q). This indicates that multi-cilia cells and principal cells are two distinct cell populations in the zebrafish distal pronephric duct.

**notch1a, notch3, jagged2a, and her9** are expressed in the Duct

Notch signaling has been shown to be required for differentiation of ciliated cells in *Xenopus* skin [45] and in sensory patches of the zebrafish inner ear [46]. The mosaic pattern of multi-cilia cells and principal cells prompted us to explore whether Notch signaling is required for their differentiation in the pronephric duct.

Among four known Notch receptors, notch1a and notch3 were found to be expressed in the intermediate mesoderm (IM) in early stages and later in the duct. notch1a was expressed in the IM from 1 ss and subsequently in the distal duct region at 18 ss (Figure 2A and 2B). notch1a expression, however, was not detected in the duct after 20 hpf. notch3 was expressed in the IM from 1 ss onward and in the entire duct region, with a higher level of expression in the distal part, at 24 hpf (Figure 2C and 2D), where expression persisted until at least 48 hpf.

There are nine known zebrafish Notch ligands: deltaA [47], deltaB [48], deltaC [49], deltaD [50], dll4 (M. M. and Y.-J. J., unpublished data), jagged1a (also known as jagged1 or serrateC), jagged1b (also known as jagged3 or serrateA), jagged2a (also known as jagged2 or serrateB) [26,46,51], and jagged2b (M. M. and Y.-J. J., unpublished data). Of the ligands, deltaC is expressed in the anterior IM, presumably in the glomerulus, from 4 ss to 18 hpf [49], and jagged1b is expressed in the developing proximal tubule [51]. jagged2a expression in the IM appeared gradually from anterior to posterior, spanning from somite 3 to somite 13 at 15 ss (Figure 2E–2G). In the posterior IM, jagged2a expression displayed a salt-and-pepper-like pattern (a mixture of high- and low-expressing cells) from 17 ss to 20 ss (spanning from approximately somite 9 to somite 13) (Figure 2H and 2I). Beginning with 20 ss, jagged2a expression was limited to individual cells; this pattern persisted in the pronephric duct until at least 48 hpf (Figure...
Figure 2. Dynamic Expression of Notch Components in the IM and Pronephric Duct

(A–D) notch1a (A) and notch3 (C) are expressed in the IM at 10 ss. notch1a (B) is expressed in the distal duct region from somite 10 to 14 (see also Figure S3B) at 18 ss, and notch3 (D) is expressed in the whole duct from somite 3 to 20 at 24 hpf as indicated by the arrows.

(J–K) jagged2a expression is higher in some cells (arrows point to these cells in [J]) than in neighboring cells in the distal duct at 17 ss (H and I), and transcription is limited to individual cells from 20 ss (J), to 24 hpf (K), to at least 36 hpf (unpublished data) in the demarcated region from somite 8 to 14 (see also Figure S3D and S3F) as indicated by arrows.

(M) jagged2a expression is restricted to individual cells in the pronephric duct from 17 ss onwards to (C) 20 ss and (D) 24 hpf. Arrows demarcate the distal duct region that contains rfx2-expressing cells.

Individual jagged2a-Positive Cells Are Multi-Cilia Cells

Since Jagged2a presumably starts signaling to the neighboring cells from 17 ss onwards, we investigated the onset of multi-cilia cell differentiation by examining the expression of rfx2 and centrin2 at earlier stages. Interestingly, rfx2 expression in the IM and duct was similar to that of jagged2a. rfx2 expression was uniform in all duct cells before 15 ss, which is consistent with the fact that all cells have cilia—either cilia tufts or a single cilium—in this kidney segment. Its expression was then limited to single cells in the distal duct from 17 ss until at least 36 hpf (Figure 3A–3D). Similarly, centrin2 expression was limited to single cells from 20 ss onwards (unpublished data). These observations suggest that multicilia cells are jagged2a-expressing cells. Indeed, jagged2a and rfx2 transcripts were colocalized in individual cells from 17 ss to at least 36 hpf (Figure 3E–3J). Furthermore, when we investigated whether her9 is expressed in the same distal duct.
is decreased in (H) jagged2a-atg morphants compared to (G) wt embryos as revealed by Na⁺, K⁺ ATPase β1a expression at 24 hpf.

I–L) Multi-cilia cell number is increased in (J) mib52b embryos compared to (I) wt embryos as shown by rfx2 expression at 24 hpf, but principal cell number is decreased in (L) jagged2a-sp morphants and (N) jagged2a-atg morphants at 24 hpf.

Panels M–R focus on the duct around somite 11 to 13. (M–O) Fluorescent double in situ hybridization of rfx2 (green) and Na⁺, K⁺ ATPase β1a (red) in 36-hpf (M) wt embryos, (N) jagged2a-sp morphants, and (O) mib52b mutants shows multi-cilia cell hyperplasia in jagged2a morphants and mib52b mutants. Arrows point to the rfx2-expressing cells in the duct of (M) wt embryos; arrowheads point to the Na⁺, K⁺ ATPase β1a-expressing cells in the pronephric duct of (N) jagged2a-sp morphants.

(P–R) Double immunohistochemistry of n6f (green) and Pcm1 (red) in 36-hpf (P) wt embryos, (Q) jagged2a-sp morphants, and (R) mib52b mutants shows multi-cilia cell hyperplasia in jagged2a morphants and mib52b mutants. Arrows point to the Pcm1 staining in the pronephric duct of (P) wt embryos; arrowheads point to n6f staining in the pronephric duct of (Q) jagged2a-sp morphants.

(S) Immunoprecipitation of Myc-Jagged2a and Myc-Jagged2aΔcd by Flag-Mib52b. IP, immunoprecipitation; IB, immunoblotting. (T–U) Expression of Myc-Jagged2a (T) and cotransfection of Myc-Jagged2a and Flag-Mib (U) in COS7 cells.

(V–Y) Compared to (V) wt embryos, mild cilia cell hyperplasia is observed in (W) notch1a (des935b) mutants and (X) notch3-utr mutants, while severe cilia cell hyperplasia is observed in (Y) notch3-utr MO-injected notch1a (des935b) mutants as shown by rfx2 expression at 24 hpf. All embryos, anterior to the left. Bar scale: 100 μm (B), 75 μm (C–L and V–Y), 50 μm (M–R), and 30 μm (T and U).

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domain as rfx2, we found that although her9 was expressed in the same domain, it was expressed primarily in non-rfx2-expressing cells (Figure 3K–3M).

**Jagged2a and Mib Regulate Cell Fate Differentiation through Lateral Inhibition**

The above finding suggests that Jagged2a regulates differentiation of multi-cilia cells and principal cells. We designed morpholino antisense oligonucleotides (MOs) to knock down the function of Jagged2a, and used the mib52b mutant to study the function of the Jagged2a-Notch pathway in differentiation. In addition to two MOs, jagged2a-atg and jagged2a-utr, designed to be antisense to the jagged2a translation start site and the 5' UTR, respectively, one MO (jagged2a-sp) was designed to block RNA splicing between exon 1 and intron 1. The jagged2a-sp MO effectively blocked splicing until at least 48 hpf (Figure 4A) and the jagged2a-utr MO was specific in a sequence-dependent manner (Figure S4A–S4F). Jagged2a MOs did not affect duct development (Figure 4B). Jagged2a-atg morphants displayed uniform rfx2 (100%, n = 242) and centrin2 (89%, n = 224) expression in almost all of the duct cells, in contrast to a mosaic pattern found in wild-type (wt) embryos (Figure 4C–4F). Similar results were found in jagged2a-utr morphants (Figure 4H; Table 1). Furthermore, we observed that Pcm1 and acetylated tubulin were significantly increased in jagged2a-sp morphants (100%, n = 7; Figure S5A–S5F). In contrast, Na⁺, K⁺ ATPase β1a expression was highly reduced in the duct of jagged2a-atg morphants at 24 hpf (100%, n = 22; Figure 4G and 4H) and 36 hpf (100%, n = 40; unpublished data). Similarly, we observed multi-cilia cell hyperplasia in mib52b mutants as evidenced by rfx2 expression (Figure 4I and 4J) and immunostaining of acetylated tubulin and Pcm1 (Figure S5G–S5I). Statistically, mib52b mutants generated greater than 2-fold more multi-cilia cells than wt embryos (Table 1). Consistently, principal cells were

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**Figure 4.** Multi-Cilia Cell Hyperplasia Is Due to Mib-Mediated Jagged2a Signaling Pathway via Notch1a and Notch3 Receptors

(A) Effectiveness of splicing jagged2a-sp MO. RT-PCR of control embryos generates a 230-bp jagged2a fragment, bridging parts of exon 1 and exon 2 at 24 hpf (lane 1) and 48 hpf (lane 5), jagged2a-sp MO-injected embryos analyzed with the same primers at the same timepoints (lanes 3 and 7) show a larger amplicon of 708 bp caused by a nonsplicing intron 1, which encodes a premature stop codon. Lane 9 shows the amplicon from genomic DNA, and lane 10 shows the amplicon from jagged2a cDNA. No fragment can be amplified in the RT-PCR without reverse transcriptase in 24-hpf (lane 2) or 48-hpf (lane 6) wt embryos or in 24-hpf (lane 4) or 48-hpf (lane 8) jagged2a-sp MO-injected embryos. Lane L: 100-bp ladder.

(B) Pronephric duct (arrow) integrity is not affected in jagged2a morphants. Panels C–L focus on the duct between somite 10 and 13. (C–H) Multi-cilia cell number is increased in (D and F) jagged2a-atg morphants compared to (C and E) wt embryos as shown by (C and D) rfx2 and (E and F) centrin2 expression at 24 hpf, but principal cell number was decreased in (H) jagged2a-atg morphants compared to (G) wt embryos as revealed by Na⁺, K⁺ ATPase β1a expression at 24 hpf.

(I–L) Multi-cilia cell number is increased in (J) mib52b embryos compared to (I) wt embryos as shown by rfx2 expression at 24 hpf, but principal cell number is decreased in (L) jagged2a-sp morphants and (N) jagged2a-atg morphants at 24 hpf.
Table 1. Statistical Analysis of Multi-Cilia Cell Number in WT Embryos, des<sup>633b</sup> Mutants, mib<sup>652b</sup> Mutants, jagged2a Morphants, notch1a Morphants, and notch3 Morphants

| WT, Mutant, or Morphant | Penetrance | Markers (at 24 hpf) | Number of Embryos Examined | Number of Multi-Cilia Cells* |
|-------------------------|------------|---------------------|---------------------------|---------------------------|
| wt                      | —          | rf2                 | 10                        | 35.4 ± 6.8                |
| jagged2a-atg MO         | 100%, n = 242 | rf2               | 3                         | 145.7 ± 6.5               |
| jagged2a-atg MO         | 89%, n = 224   | centrin2           | n. c.                     | n. c.                     |
| jagged2a-utr MO         | 93%, n = 231   | rf2               | n. c.                     | n. c.                     |
| jagged2a-utr MO         | 92%, n = 165   | centrin2           | n. c.                     | n. c.                     |
| jagged2a-sp MO          | 64%, n = 53    | rf2               | n. c.                     | n. c.                     |
| jagged2a-sp MO          | 89%, n = 46    | rf2 (at 20 ss)     | n. c.                     | n. c.                     |
| notch1a-sp MO           | 100%, n = 44   | rf2               | 10                        | 41.5 ± 6.4                |
| notch3-utr MO           | 97%, n = 33    | rf2               | 10                        | 42.9 ± 4.7                |
| notch3-sp MO            | 87%, n = 105   | rf2               | 10                        | 44.3 ± 7.4                |
| notch3-sp MO into des<sup>635b</sup> | 97%, n = 33 | rf2               | 10                        | 55.7 ± 12.2               |
| her9-atg MO             | 92%, n = 105   | rf2               | 10                        | 91.1 ± 12.2               |
| her9-utr MO             | 96%, n = 75    | rf2               | 10                        | 60.1 ± 5.1                |
| mib<sup>652b</sup>      | —            | rf2               | 10                        | 84.4 ± 14.4               |

*rf2-expressing cells were counted on both sides of the pronephric duct.

n. c., not checked

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decreased dramatically in mib<sup>652b</sup> mutants, as shown by Na<sup>+</sup>, K<sup>+</sup> ATPase α1a2 expression at 24 hpf (Figure 4K and 4L).

While the number of rfx2-expressing cells was dramatically increased in jagged2a-sp morphants and mib<sup>652b</sup> mutants (Table 1), only three to five Na<sup>+</sup>, K<sup>+</sup> ATPase β1a-expressing cells were found in the jagged2a-sp morphants (89%, n = 19), and a dramatically decreased number of Na<sup>+</sup>, K<sup>+</sup> ATPase β1a-expressing cells were found in mib<sup>652b</sup> mutants (Figure 4M–4O). Similarly, double immunostaining with α6F (raised against the chick α1 subunit of the Na<sup>+</sup>/K<sup>+</sup> ATPase [61]) and Pcm1 showed that most of the duct cells adopted a multi-cilia cell fate and expressed Pcm1; only two to three cells were positive for the principal cell maker α6F in the distal duct of jagged2a-sp morphants (93%, n = 15), and principal cells were dramatically decreased in mib<sup>652b</sup> mutants (Figure 4P–4R). This observation suggests that the multi-cilia cell hyperplasia in jagged2a morphants and mib<sup>652b</sup> mutants is at the expense of the principal cells through lateral inhibition (see below). It is unlikely to be due to an inhibitory activity of Jagged2a on proliferation of multi-cilia cells, since there is no difference in cell proliferation between wt embryos and jagged2a morphants (unpublished data). The phenotypic severity of mib<sup>652b</sup> was not as strong as that of jagged2a morphants (Table 1), indicating that some residual Notch activity remains in mib<sup>652b</sup> mutants, as reported previously [20].

Mib Binds and Internalizes Jagged2a in Cells

mib<sup>652b</sup> mutants display a global compromise in Notch activation, and mib was identified to encode an E3 ligase that activates Notch signaling by ubiquitylating and endocytosing Delta [20].

The phenotypic analysis of multi-cilia cells and principal cells in the duct suggests that mib genetically interacts with jagged2a. Since Delta has been shown to be a substrate of Mib and endocytosed after ubiquitylation [20], we asked if Mib physically interacts with Jagged2a, as shown for a human Mib paralog, Skeltothphin [21]. We checked the in vivo interaction of Jagged2a and Mib by immunoprecipitation and cotransfection experiments. Indeed, Mib bound to full-length Jagged2a and Jagged2aicd (Figure 4S). Moreover, Myc-Jagged2a was localized to the cell surface (membrane) and cytoplasm when transfected alone (Figure 4T) and to the perinuclear granules when cotransfected with Flag-Mib (Figure 4U). The fact that Mib binds Jagged2a and facilitates its internalization suggests that Mib regulates Jagged2a in a way similar to Delta.

Notch1a and Notch3 Receptors Function Redundantly in Jagged2a-Mediated Lateral Inhibition

In the duct, we observed slight multi-cilia cell hyperplasia in notch1a (deadly seven) mutants and notch1a morphants (Figure 4W; Table 1). notch3-utr MO and notch3-sp splicing MO against the exon 1-intron 1 boundary were designed; the former was specific in a sequence-dependent manner (Figure S4G–S4L) and the latter was able to induce splicing defects until at least 48 hpf (Figure S4M). More multi-cilia cells were found in notch3 morphants (Figure 4X; Table 1). The stronger cilia phenotype seen in notch3 morphants compared with that of notch1a mutants or morphants was consistent with the persistent notch3 expression in the pronephric duct and also demonstrated that Notch3 plays a more important role than Notch1a.

Loss of function of a single Notch receptor resulted in a phenotype that was less severe than that of jagged2a morphants. This suggests that Notch1a and Notch3 act redundantly. In fact, 91.1 ± 12.2 multi-cilia cells were generated in notch3-sp MO-injected des<sup>635b</sup> mutants in contrast to 42.9 ± 4.7 and 55.7 ± 12.2 multi-cilia cells in notch1a-sp and notch3-sp morphants, respectively (Figure 4V–4Y; Table 1). However, the multi-cilia cell phenotype in notch3-sp MO-injected des<sup>635b</sup> mutants was not as severe as that of jagged2a morphants (Table 1). Consistently, Na<sup>+</sup>, K<sup>+</sup> ATPase β1a down-regulation in notch3-sp MO-injected des<sup>635b</sup> mutants was not as severe as that in jagged2a morphants (unpublished data). These data suggest that there may be a yet-unidentified Notch involved in this differentiation process.
Her9 Acts Downstream in Jagged2a-Notch1a/Notch3-Mediated Lateral Inhibition

The temporal and spatial expression of her9 in the distal pronephric duct suggests that it is one of the downstream target genes of Jagged2a-Notch1a/Notch3 signaling. We asked whether activation of her9 in the duct requires Jagged2a, Notch1a, and Notch3. her9 expression in the pronephric duct was reduced in jagged2a-sp morphants (77%, n = 54; Figure 5A and 5B). While her9 expression was slightly reduced in desAβ5β6/ notch1a mutants (Figure 5C and 5D) and in notch3-sp morphants (100%, n = 45; Figure 5E), its expression was almost completely lost in notch3-sp MO-injected desAβ5β6 mutants (94%, n = 36; Figure 5F). Similarly, its expression was almost completely lost in mibMo52b mutants (Figure 5G and 5H). We next examined whether her9 is activated by Notch1a and Notch3. The constitutively active form, the intracellular domain (icd) of both Notch1a and Notch3, were used. her9 expression was activated by both Notch1aicd (22%, n = 98; Figure 5I–5K) and Notch3icd (17%, n = 70; unpublished data). These experiments demonstrate that the activation of her9 expression in the pronephric duct requires Notch1a and Notch3, in addition to Jagged2a and Mib.

We further studied her9 function in the duct with her9-atg (effectiveness verified in [62]) and her9-utr MOs. her9 morphants exhibited multi-cilia cell hyperplasia, as demonstrated by rfx2 expression (Figure 5L and 5M; Table 1). The requirement of Jagged2a, Notch1a, and Notch3 for activation of her9 expression in the duct, and the multi-cilia cell hyperplasia in her9 morphants demonstrate that Her9 acts downstream of the Jagged2a-Notch1a/Notch3 pathway. However, multi-cilia cell hyperplasia in her9 morphants was not as severe as that in jagged2a morphants or notch3-sp MO-injected desAβ5β6 mutants (Table 1). One possibility is that Her9 is not completely knocked down by her9 MOs, because of the potential negative autoregulatory feedback on the transcription by its protein, similar to Hes7 [63]. Alternatively, there may be more effector(s) working in parallel with Her9. The latter explanation is particularly likely, since the her9 expression domain only partially overlaps with that of jagged2a (Figure S3).

Multi-Cilia Cell Differentiation Requires Jagged2a from 17ss Onwards

rfx2 and jagged2a displayed mosaic patterns from 17 ss onwards (Figure 3E and 3F); her9 was expressed in the distal duct domain from 17 ss onwards (Figures 2L and 3L). Moreover, her9-expressing cells were not multi-cilia cells (Figure 3K–3M). The dynamic expression of these genes suggests that multi-cilia cells start to differentiate from 17 ss onwards. We next investigated whether Jagged2a-Notch signaling is required from as early as 17 ss. We found that rfx2 expression is uniform in the IM in wt embryos (Figure 6A), mibMo52b mutants (Figure 6B), and jagged2a-sp morphants (91%, n = 33; Figure 6C) at 13 ss, while a neurogenic phenotype was obvious in mibMo52b mutants, indicating that multi-cilia cells do not start to differentiate before 15 ss. However, when rfx2 expression was limited to individual cells at 18 ss in wt embryos (Figure 6D), rfx2-expressing cells were increased in the mibMo52b mutant (Figure 6E) and jagged2a-sp morphants (90%, n = 43; Figure 6F). Similarly, her9 and notch3 morphants exhibited multi-cilia cell hyperplasia from as early as 17 ss (unpublished data). These data indicate that Jagged2a-Mib-Notch3-Her9 is required for cell differentiation from as early as 17 ss.

Duct Cells Adopt a Principal Cell Character at the Expense of Multi-Cilia Cells When Notch Is Constitutively Activated

Multi-cilia cell hyperplasia is found in the mutants and morphants defective in the Jagged2a-Notch1a/Notch3-Her9 pathway. The increase of multi-cilia cells is most likely at the expense of principal cells, since no cell proliferation and apoptosis were detected in the duct of either wt or mibMo52b embryos (Figure 7A–7F; Videos S1 and S2). Thus, we demonstrated that Jagged2a-Notch1a/Notch3-Her9 is required for specification of multi-cilia cells and principal cells through a lateral inhibition mechanism. We next asked
Discussion

In this paper, we have shown that there are two major epithelial cell types found in the zebrafish distal pronephric duct. The mosaic pattern of multi-cilia cells and principal cells is controlled by Jagged2a/Notch-mediated lateral inhibition. Using available mutants and morphants deficient in genes functioning in Notch signaling, we demonstrated that one ligand, Jagged2a; two receptors, Notch1a and Notch3; and one downstream effector, Her9, are required for the differentiation and patterning of these two cell types. In addition, we showed that Mib is essential for this process, since it interacts with Jagged2a and facilitates Jagged2a internalization. In summary, our findings indicate a new function of Notch signaling in cell fate choice within a zebrafish kidney segment.

Interestingly, such a function of Jagged2-Notch signaling has not to our knowledge been found in mammals, although Jagged2 is expressed in the postnatal murine kidney [64,65].

This may be due to the early lethality of Jagged2 knockouts, which prevents the detection of such a function in mammalian kidneys. There are two zebrafish Jagged2 homologs, jagged2a and jagged2b. Most likely, the subfunctionalization of these two genes makes it possible for us to identify the function of jagged2a in zebrafish pronephros. Our findings warrant further study of the role of Jagged2 in mammalian kidneys by conditional knockouts.

The physiological functions of multi-cilia cells and principal cells are apparently different. While motile cilia on the apical side of the multi-cilia cells propel urea along the lumen of the pronephric duct [28], principal cells, which account for the majority of the cells in the kidney, reabsorb ions and other molecules according to fluid balance requirements. A plausible physiological significance of the intercalating pattern of multi-cilia cells and principal cells may be to coordinate the movement of the fluid and the process of reabsorption of the ions and other small molecules.
Expression of procilia genes could encode bHLH transcription factors, stimulating to signal to neighboring cells. Procilia genes are hypothetical and have interacting with Jagged2a and facilitating Jagged2a endocytosis in order of the principal cells. In this model, Mib affects Notch activity by receptors to prevent generating excessive multi-cilia cells at the expense Her9 is one of the effectors that works downstream of the Notch organism expresses jagged2a, thereby activating Notch in neighboring cells, it will not only inhibit these neighbors from adopting the primary fate, but it will also down-regulate their expression of jagged2a. This generates a feedback loop that, over time, tends to amplify differences between adjacent cells so as to create a mixture of different cell types (Figure 8; [72]).

A Similar Mechanism for Other Similar Systems?
Multi-cilia cells are largely absent in mammalian kidneys, even though the primary cilium is present on principal cells of the tubule segment. We found interpolating multi-cilia cells and principal cells in the zebrafish distal pronephric duct. This mosaic cell pattern has been shown to be present in other anamniote vertebrates including marine teleosts [73], lampreys [74], and amphibians [75]. Notch signaling was required for the differentiation of speckled 4A6-positive cells in the posterior duct of Xenopus [22]. Our findings in zebrafish multi-cilia cells and the conserved pattern of cilia cells in amphibians [75] suggest that 4A6-positive cells are multi-cilia cells and that, in general, lateral inhibition may be involved in establishing the interpolating pattern of multi-cilia cells and principal cells in the ducts of anamniote vertebrates.

The renal collecting duct of mammalian kidneys comprises various kinds of intercalated cells (mediating acid and base transportation), principal cells (responsible for salt and water absorption), and inner medullary cells, which moderate all three types of transport. Inner medullary cells are “hybrid” cells—positive for both intercalated and principal cell markers [13,76,77]. Since jagged1 expression [23] and a similar mosaic pattern of intercalated cells and principal cells [13] were observed in the collecting ducts of mouse kidneys, it is tempting to speculate that Notch signaling is involved in the differentiation and patterning of these different cell types in the mammalian collecting duct.

The efferent duct transports material from the rete testis to the epididymis by motile cilia [78]. Similarly, multi-cilia cells and principal cells are found exhibiting a mosaic pattern in the efferent duct of reptile (turtle, [79]) and mammal (rat, [80]). It would be interesting to see how these two cell types differentiate and whether Notch signaling is involved in this differentiation process.

Materials and Methods
Zebrafish Lines. Fish were maintained and raised as described [81]. mi626, des538, hsp70:Gal4, and UASmy-notch1a-intra mutants or transgenic lines were described before [69,82,83].
Whole-mount and section in situ hybridization. Primer sets based on zebrafish cDNAs or ESTs were designed (rfx2, forward: CTCACCTTCCAGGCTCATTACG; reverse: CATAGGGTTTGGACCCCTGTAT; centrin2, forward: TCAAAATGGGCTCCTGCTTC; reverse: GACACACTAGGTCTTAAAGG; vhatp61, forward: TGCCATATGA-
The knockdown efficacy of the splicing MOs was checked by reverse transcriptase PCR (RT-PCR) with the following primers: notch3-sp MO, forward: GTAATCATGGAGACGGTGCG; reverse: GCATCTTGAATCAATGCACATTCCTCC; and notch3-utr MO, forward: CTTCTGCACTTTCTGGAGATTTAAAGAAG, reverse: CAGAAGCTCTAGATGCTGCCT, 0.29 pM; notch3-sp MO, forward: AATCGAGGATCTTGAATCAATGCACATTCCTCC; and reverse: AAATGAGTCCCGCC, 0.38 pM; notch3-utr MO, forward: AAAATGACCTGCACAATCATCCCTC; and reverse: her9-utr MO, forward: CTTCTGCACATTTCCGGTGGCAGGGATGATCG, 1.15 pM [62]; her9-utr (−62 to −38) MO: ATGAGAATATGAGGCCGTTGTGTT, 0.29 pM; and notch3-utr MO, forward: GTAGTGTAAAACGTGTCCTGTG, 2.30 pM.

To examine the knockdown specificity of jugged2a-atg MO and notch3-utr MO, we cloned the 5′ UTR of both jugged2a and notch3 into vectors equipped with SPOT INSIGHT (Diagnostic Instruments, Inc.) and examined with a Zeiss Axiosplan microscope (http://www.olympusamerica.com), and 3-D movies were generated using Fiji-ASW1.5 software.

**Fluorescent double in situ hybridization.** The method was as previously described [91] except that substrates fluorescein-tyramide and Cy3-tyramide were respectively replaced with Alexa 488-tyramide and Alexa 635-tyramide (Molecular Probes). The substrates were used at 100 pg/ml (100 ng/ml) in 0.1× SSC, 50% formamide, and 100 μg/ml nuclease P1.

**Immunohistochemistry.** Whole-mount antibody staining was performed on embryos fixed in 4% PFA or methanol; DMSO (80:20) was used as described [11,89]. The following antibodies and their dilution were used: acetylated tubulin and γ-tubulin, 1:500 (Sigma-Aldrich, http://www.sigmaaldrich.com); anti-Pcm1, 1:200 [30]; monoclonal Zo-1, 1:20; monoclonal αtubulin, 1:5 (Developmental Studies Hybridoma Bank, http://www.hybbrdx.org); and monoclonal phosphoacetyl (Ser10) (6G3), 1:40 (Cell Signaling Technology, http://www.cellsignal.com); rabbit anti-Pax2, 1:100 (Covance, http://www.covance.com); rabbit 488-anti-mouse and Alexa 635-anti-rabbit, 1:400 (Molecular Probes, http://probes.invitrogen.com); and Alexa 535-WGA, 1:1,000 (Molecular Probes). The TUNEL assay was performed as described in the product manual of the In Situ Cell Death Detection Kit AP (Roche, http://www.roche-diagnostics.com). Whole-mount embryos were embedded in Jung Tissue Freezing Medium (Leica, http://www.leica-microsystems.com), cryosectioned at 10 μm, and mounted in FluorSave reagent (Calbiochem, http://www.calbiochem.com) before in situ hybridizations were performed using a Zeiss Confocal LSM 510 or an Olympus Fluoview FV1000 microscope (http://www.olympusamerica.com), and 3-D movies were generated using Fiji-ASW1.5 software.

**Transmission electron microscopy.** Embryos were fixed with 2% paraformaldehyde and 4% glutaraldehyde in 100 mM cacodylate buffer for 3 h and post-fixed with 2% osmium tetroxide in 100 mM sodium cacodylate buffer for 1 h at 4 °C. Embryos were then dehydrated through a series of 30%, 50%, 70%, 90%, and 100% ethanol, and finally in propylene oxide prior to infiltration with epoxy resin and polymerized at 65 °C overnight. Ultra-thin sections were cut on a Reichert ultramicrotome (http://www.leica-microsystems.com) and examined with a transmission electron microscope (JEM1010, JOEL, http://www.jeol.com) at 100 kV.
Figure S2. Pcm1 and γ-Tubulin Colocalize
Antibody staining of (A) Pcm1 and (B) γ-tubulin on transverse section of 36-hpf zebrafish pronephric duct revealed that they are (C) colocalized in the apical site of the duct epithelial cell. Arrowheads point to staining of the individual basal body, and arrows point to the staining of multiple basal body. Bar scale: 10 μm.

Figure S3. notch1a, jagged2a, and her9 Are Expressed in the Distal Duct at the Time of Cell-Fate Determination
(A and B) Fluorescent double in situ hybridization of notch1a and myoD revealed that notch1a is expressed in the pronephric duct spanning from somite 10 to 14 (arrows) at 18 ss. (C and D) Fluorescent double in situ hybridization of jagged2a and myoD revealed that jagged2a expressing single cells are found in the pronephric duct spanning from somite 8 to 14 (arrows) at 24 hpf. (G and H) Fluorescent double in situ hybridization of her9 and myoD revealed that her9 is expressed in the pronephric duct spanning from somite 10 to 12 (arrows) at 18 ss. (I and J) Fluorescent double in situ hybridization of jagged2a (green), slc4a2/ae2 (red, anterior), and retl (red, posterior) revealed that jagged2a-expressing single cells are found in the distal duct between the proximal duct (marked by slc4a2/ae2; [27]) and the cloaca (marked by retl) ([11]).

Figure S4. Specificity of jagged2a-utr and notch3-utr Morpholinos on Targeting the 5' UTR of jagged2a and notch3 and the Effectiveness of the notch3-sp Splicing Morpholino
(A–C) Specificity of the jagged2a morpholino. (A) Injection of jagged2a-utr-GFP mRNA at 250 pg produced green fluorescence, (B) coinjection of 0.29 pM jagged2a-utr-MO with 250 pg of jagged2a-utr-GFP mRNA inhibited GFP production, and (C) coinjection of 0.29 pM 5mis-match-jagged2a-utr-MO with 250 pg of jagged2a-utr-GFP mRNA did not inhibit its production.

(D–F) Multi-cilia cell probed with rfx2 at 24 hpf in (D) wt embryos, (E) jagged2a-utr mutants, and (F) 5mis-match-jagged2a-utr morphants. Note that the number of multi-cilia cells was increased in jagged2a-utr morphants (Table 1, 93%; n = 231) but not in 5mis-match-jagged2a-utr morphants (97%; n = 35).

(G–I) Specificity of the notch3 morpholino. (G) Injection of notch3-utr-GFP mRNA at 250 pg produced green fluorescence, (H) coinjection of 0.38 pM notch3-utr-MO with 250 pg of notch3-utr-GFP inhibited GFP production, and (I) coinjection of 0.38 pM 5mis-match-notch3-utr-MO with 250 pg of notch3-utr-GFP did not inhibit its production.

(J–L) Multi-cilia cells probed with rfx2 at 24 hpf in (J) wt embryos, (K) notch3-utr morphants, and (L) 5mis-match-notch3-utr morphants. Note that the number of multi-cilia cells was increased in notch3-utr morphants (Table 1, 97%; n = 33) but not in 5mis-match-notch3-utr morphants (100%, n = 30).

(M) Molecular analysis of the effectiveness of the notch3-sp splicing morpholino. RT-PCR of ten embryos generates a 320-bp notch3 fragment in control embryos, bridging part of exon 1 to part of exon 2 at 24 hpf (lane 3) and 48 hpf (lane 4). notch3-sp morpholino-injected embryos analyzed with the same primer sets at 24 hpf (lane 1) and 48 hpf (lane 2) show a larger amplicon of 1,800 bp caused by a nonsplicing of intron 1 and other aberrant splicing variants. Lane L: 100-bp ladder. Bar scale: 1,000 μm (A–C and G–I) and 100 μm (D–F and J–L).

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