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Xenopus Pronephros Development - Past, Present and Future

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Abstract

Kidney development is a multi-step process, where undifferentiated mesenchyme is converted into a highly complex organ through several inductive events. The general principles regulating these events have been under intense investigation and despite extensive progress many open questions remain. While the metanephric kidneys of mouse and rat have served as the primary model, other organisms also significantly contribute to the field. In particular, the more primitive pronephric kidney has emerged as an alternative model due to its simplicity and experimental accessibility. Many aspects of nephron development such as the patterning along its proximo-distal axis are evolutionarily conserved and are therefore directly applicable to higher vertebrates. This review will focus on the current understanding of pronephros development in *Xenopus*. It summarizes how signaling, transcriptional regulation, as well as post-transcriptional mechanisms contribute to the differentiation of renal epithelial cells. The data show that even in the simple pronephros the mechanisms regulating kidney organogenesis are highly complex. It also illustrates that a multifaceted analysis embracing modern genome-wide approaches combined with single gene analysis will be required to fully understand all the intricacies.

Keywords

amphibian; microRNA; patterning; pronephros; transcription; *Xenopus*

Organization of the Pronephros

The kidney is an essential organ to maintain water and salt homeostasis in the body and to excrete waste products [1–3]. Three kidney forms have developed in the course of evolution; the pro-, meso- and metanephros. The metanephros is only found in higher vertebrates and serves as the adult kidney. The mesonephros is formed in all vertebrates, but while it degenerates in higher vertebrates, it serves as the adult kidney in fish and amphibians. The pronephros is the most primitive kidney [4, 5]. It is only present in embryonic stages and is often non-functional. Exceptions are animals that spend the early phases of their life cycle in an aquatic environment. Due to the osmotic differences between the external and internal milieu, water has to be either retained (i.e. in salt water) or expelled (i.e. in fresh water). In the absence of this regulation embryos will either exsicate or develop edema and will ultimately die [3]. Despite the anatomical differences between the three kidney types, the basic functional unit, the nephron, is present in all of them. It is evolutionarily conserved at the structural and molecular level. While puzzling, this suggests that nephron development has only evolved once in the ancestral kidney, the archinephros [5].

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The amphibian pronephros can be subdivided into three distinct domains, the glomus, the tubules and the duct (Figure 1). The glomus carries out the blood filtration function and is composed of at least three cell types [4–7]: fenestrated endothelial cells that form capillary tufts [8]; mesangial-like cells that are probably involved in maintaining the three dimensional structure of the glomus, but have not yet been intensively studied; and finally, the podocytes, specialized epithelial cells that form extensive foot processes to establish the size exclusion barrier and prevent large molecules from exiting the blood stream [9]. Parietal epithelial cells are the only cell type not yet described in the pronephric glomus. These cells form the epithelial cell layer surrounding Bowman's space in the metanephric glomerulus. Since this structure is absent in the pronephros, parietal epithelial cells may not exist at all or have acquired a different identity.

From the glomus the ultrafiltrate enters the coelomic cavity and is transported via the so-called nephrostomes into the pronephric tubules. Nephrostomes connect the peritoneum to the proximal tubules and are lined by a specialized group of multi-ciliated cells. These are speculated to generate a swirling movement causing fluid influx into the tubules. Indeed, embryos with reduced or absent cilia develop edema as a consequence of impaired fluid transport [10]. The pronephric tubules themselves are subdivided into proximal, intermediate and distal segments [11, 12]. Each segment has a dedicated function, which is reflected by distinct cell morphologies [13] and gene expression profiles. The proximal tubules are divided into three domains, PT1, PT2 and PT3, and are responsible for reabsorbing ions, amino acids, glucose as well as water [11, 12, 14–16]. The intermediate tubules, IT1 and IT2, express marker genes found in the mammalian thin limb of the loop of Henle as well as the distal tubule. But since amphibians do not need to concentrate urine, an anatomical distinct loop of Henle is not formed. Instead, the function of the intermediate tubules has been adapted to salt and ion reabsorption only [11, 17]. The distal tubules, DT1 and DT2, are equivalent to the thick ascending limb of the loop of Henle (TAL) and the distal convoluted tubule (DCT), respectively. They are required for urine acidification, the reabsorption of magnesium ions and ammonium transport [11, 12, 18, 19]. The pronephric duct connects the distal tubules to the cloaca to excrete urine and is composed of one still poorly characterized cell type. In contrast to the collecting duct system of the metanephric kidney, there is no evidence for the existence of intercalated or principal cells in the pronephros [11].

These structural and molecular analyses support the notion that the pronephros is functionally very similar to the nephrons present in the metanephric kidney. The remainder of this review will focus on the mechanisms regulating pronephros development (Figure 1A, 2). While there are two model organisms extensively used in the study of the pronephric kidney, *Xenopus* and zebrafish, and both have distinct advantages and disadvantages, this review will solely focus on the work performed in *Xenopus*.

Signaling in the Pronephros

In *Xenopus*, pronephros development starts around stage 12.5. The pronephric anlage is induced from the intermediate mesoderm by the surrounding tissues. Mesenchymal-epithelial transition results in the formation of a tubular organ that is reorganized by a series of highly coordinated morphogenetic movements. Upon terminal differentiation of the renal epithelial cells the pronephros become functional at stage 38 (approximately two days post fertilization, Figure 1A). Studies in chick and mouse have implicated multiple signaling pathways including BMP, Wnt, FGF and GDNF in the development of the meso- and metanephric kidney [20, 21]. Interestingly, the signaling molecules that govern pronephros formation have turned out to be evolutionarily conserved and have similar functions in the three kidney types.

GDNF/Ret signaling components are expressed in the pronephric region ([22, 23], our own unpublished observations), but their roles have yet to be defined. Studies in *Ambystoma*, another pronephric model organism, suggest a role for GDNF in duct elongation [24]. Such a function is clearly different from the better-described role in the regulation of the ureteric bud outgrowth/branching in the metanephros and it will be interesting to see whether this is relevant for higher vertebrates. In contrast to GDNF, other ligands such as Wnt4 or Fgf8 have analogous functions in both the pronephros and metanephros [25–28].

Among the multiple Wnt molecules expressed during early *Xenopus* development, four are so far connected to pronephros formation: *wnt11* and *wnt11r* are expressed in the developing somites and *wnt11r* can later even be found in the pronephric duct by stage 37 ([29] and our unpublished observations). During pronephros induction the somite-derived *wnt11/11r* ligands signal to the underlying intermediate mesoderm and promote tubule and duct formation [29]. Wnt4 can be detected in the pronephric anlage as early as stage 19 and is required for proximal tubule development [26]. Finally, Wnt6 is present in the pronephric anlage by stage 23, but its precise role has not yet been defined [30]. From all the Wnt receptors only Frizzled-8 has been studied in pronephric kidney development and has been shown to regulate epithelial differentiation [31]. Moreover, at least some of these Wnt molecules function via the canonical Wnt signaling pathway, since β -Catenin-dependent transcription is required in the pronephros [32].

Other signaling pathways also play crucial roles. Fgf8 is expressed in the pronephric primordium between stages 22 to 30 and is required for pronephric tubule and duct development [25, 33]. The same process also requires Bmp signaling, but the responsible BMP ligand is still unknown [34]. Notch signaling is involved in the specification of a subpopulation of cells in the pronephros. Delta1, Serrate1, Notch1, the glycosyl transferases lunatic fringe and radical fringe, as well as the Notch-dependent transcription factor Hey1 regulate proximo-distal patterning. They determine whether cells are fated towards podocytes and proximal tubule or distal tubule [6, 35–37]. In addition, another Notch ligand, Jagged2, seems to determine the multi-ciliated cells of the nephrostomes (our unpublished observations) similar to studies reported in zebrafish [38, 39].

Transcription in the Pronephros

The *Xenopus* pronephric anlage arises from the intermediate mesoderm and can be identified by the expression of the transcription factors *Lhx1/Lim1* and *Pax8* as early as stage 12.5 [40, 41]. Shortly thereafter, three other transcription factors, *Pax2*, *Wt1* and *Hnf1 β* are detected [40–44]. Interestingly, *Pax2* is confined to the future tubular domain, while *Wt1* marks the glomus area, a fact that may be caused by a crosstalk between *Wt1* and *Pax2* as shown in mouse and zebrafish [45–47]. Gain-of-function studies have suggested that these transcription factors are able to induce ectopic kidney structures and/or modulate the preexisting pronephric patterning [40, 48–51]. Unfortunately, - with the exception of *Wt1* [6, 36] - knockdown studies for these transcription factors in *Xenopus* have not yet been reported and their precise contribution to tubular development is still unclear. Two other transcription factors, *Osr1* and *Osr2* have recently been shown to function upstream of this transcriptional cassette and are required to establish the pronephric anlage [52].

In addition to the early inductive events, the subdivision of the developing nephron into functionally distinct segments has recently gained attention. This analysis was originally confined to structural proteins but has been expanded to transcriptional regulation of nephron patterning. The zinc finger transcription factor *Evi1* is expressed in the distal tubule and pronephric duct [53]. The *Iroquois* genes, *Irx1*, *Irx2* and *Irx3* are expressed in the proximal tubular segment, PT3, and the intermediate tubule, IT1 and IT2 [17]. Importantly,

the discovery of the pronephric expression of the *Iroquois* genes in *Xenopus* prompted the discovery of their metanephric expression in mouse in the S3 segment of the proximal tubule and the thick ascending limb of the Loop of Henle (TAL). Moreover, loss- and gain-of-function studies in *Xenopus* point to an instructive role for these transcription factors in the specification of the corresponding nephron segments [17, 53, 54]. Other transcription factors, such as Gata3 and Hnf1 α are expressed in the pronephric duct and proximal tubules, respectively, but their role in pronephros development has not yet been addressed [10, 55, 56].

The best-studied example on the cooperativity of transcription factors in pronephros specification is the development of podocytes, highly specialized cells involved in the formation of the slit diaphragm of the glomerulus. Studies in several model organisms have implicated multiple transcription factors in podocyte development, yet their precise role and crosstalk was unclear until recently [6, 57]. In *Xenopus*, at least six transcription factors (Wt1, Foxc2, Lmx1b, Mafk, Tcf21, Hey1) are expressed in the developing podocytes of the pronephros [6, 36, 58, 59]. Systematic knockdown of the transcription factors either individually or in combination revealed an orchestrated gene regulatory network similar to the ones described in sea urchin and *Drosophila* development [60, 61]. As previously suggested [36], Wt1 was instrumental in podocyte development, but could not explain all aspects thereof. Only the simultaneous knockdown of Wt1 and Foxc2 was sufficient to abolish all podocyte development. Furthermore, the other transcription factors contributed in distinct ways that often could only be observed in double or triple knockdowns ([6] and our unpublished observations). This gene regulatory network of podocyte development provides a basic framework to expand on. In particular, it will be useful to integrate the vast amount of data generated by high throughput sequencing approaches such as RNA-seq and ChIP-seq.

In the future, it will be important to extend these kinds of studies to other cell types present in the kidney. It is highly likely that a similar complex regulation takes place and the podocyte gene regulatory network may serve as an initial step in grasping the complexities of transcriptional regulation.

Post-transcriptional Regulation in the Pronephros

Besides transcription and signaling, post-transcriptional control mechanisms have emerged as a third tier in the regulation of cell fate determination. This area encompasses multiple and diverse processes, among which microRNAs (miRNAs) are the most prominent. miRNAs are small non-coding RNAs that bind to the 3'UTR of a given mRNA to inhibit translation and promote mRNA degradation. While their role in kidney development has not yet been extensively studied, the *Xenopus* pronephros has proven to be a very valuable model to dissect out this mechanism. Global inhibition of miRNA biogenesis resulted in delayed terminal differentiation of the renal epithelial cells, increased apoptosis, and defects in podocyte patterning ([62, 63] and our unpublished observations). Moreover, one particular miRNA family, *miR-30*, seems to play a unique role in the pronephros. It targets Lhx1, one transcription factor involved in the initial specification of the pronephric kidney, and restricts its activity once epithelial differentiation has taken place [62].

Another example for post-transcriptional regulation in the kidney is the RNA-binding molecule Bicaudal-C (Bicc1). Bicc1 is required for the maintenance of the pronephric tubules and duct and in its absence embryos develop a polycystic kidney disease (PKD)-like phenotype [10, 64]. Bicc1 regulates mRNA stability and translation of Polycystin-2 (Pkd2), one of the genes implicated in human forms of PKD [64]. Interestingly, Bicc1 antagonizes the effects of another miRNA family, *miR-17* and the double knockdown of Bicc1 and

miR-17 in *Xenopus* rescued - at least partially - the *Bicc1* morphant phenotype [64]. While the precise molecular mechanism of *Bicc1* activity is still unknown, these studies clearly suggest that post-transcriptional regulation has an underappreciated role in pronephric kidney development.

Outlook

The pronephros has long been thought to be a rudimentary organ whose development does not provide insights into the development of the metanephric kidney. However, recent findings suggest that the opposite holds true. Most of the processes that regulate the formation of a functional pronephros are evolutionarily conserved. They are not only active in the pronephros, but are also reiterated during the development of the individual nephrons found in the metanephric kidney (and probably also the mesonephros). The best examples are the two transcription factors, Pax2 and Pax8. In the frog, they regulate early aspects of pronephros specification [40, 41]. Similarly, in mouse Pax2 and Pax8 are required for nephric duct formation [65], but are also crucial in the renal vesicle [66]. As such, the pronephros is a valuable asset. *Xenopus* offers several advantages to the field of kidney development: a functional pronephros develops within two days after fertilization; individual fertilizations yield an abundance of eggs that facilitate experiments with high statistical significance; gain- and loss-of-function analyses (including double and triple knockdowns) can be performed in a time- and cost-efficient manner; the sequence of the *Xenopus tropicalis* genome [67] provides the framework for genome-wide assays such as RNA-Seq or ChIP-Seq [68].

Moreover, one main problem in exploring cell type specification in the metanephric kidney is that the nephrons (up to 1.5 million in humans) develop asynchronously. While the deeper medullary nephrons are already terminally differentiated, the more cortical ones are only beginning to be specified. As a consequence, it is often difficult to pinpoint the temporal regulation of cellular differentiation to individual genes. The pronephros provides an elegant solution to this heterogeneity problem. It consists of two nephrons located on the left and right side of the body that are specified and patterned synchronously and even become functional at the same time.

In the future, it will be imperative that the pronephric kidney research field moves beyond demonstrating evolutionary conservation and instead tackles novel questions that have yet to be addressed in any organism. Obviously, the pronephric kidney cannot be used to address all questions pertaining to kidney development. E.g. the outgrowth and branching of the ureteric bud tree is restricted to the metanephric kidney and analogous processes do not occur in the pro- or mesonephros. But there are ample opportunities. How is the border between different nephron segments established and subsequently maintained even under adverse conditions (e.g. injury)? Is post-transcriptional regulation a key player in the homeostasis of kidney physiology as suggested by the presence of long 3'UTRs and multiple miRNA binding sites in many renal function genes [63]? What are the mechanisms underlying epithelial-mesenchymal transition in renal fibrosis? How can one regenerate a functional kidney/nephron from mesenchymal stem cells? Based on such questions we believe that the golden age of pronephros development may just have begun.

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Figure 1. The Amphibian Pronephros

(A) Schematic of pronephros development from an undifferentiated pronephric anlage to terminal differentiated renal epithelial cells. The color scheme depicts the progressive differentiation of the cells and the different nephron segments (modified from [69]). (B) Illustration of the glomus (red), the nephrocoelom, the nephrostomes (blue-gray) and the tubules (blue, adapted after [4]).

INDUCTION → PATTERNING → HOMEOSTASIS

Figure 2. Regulation of Pronephros Development

List of ligands, receptors, transcription factors and post-transcriptional regulators shown to function in the induction, patterning or homeostasis of the pronephric kidney in *Xenopus*. See text for details.