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Xenopus: leaping forward in kidney organogenesis

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Abstract

While kidney donations stagnate, the number of people in need of kidney transplants continues to grow. Although transplanting culture-grown organs is years away, pursuing the engineering of the kidney de novo is a valid means of closing the gap between the supply and demand of kidneys for transplantation. The structural organization of a mouse kidney is similar to that of humans. Therefore, mice have traditionally served as the primary model system for the study of kidney development. The mouse is an ideal model organism for understanding the complexity of the human kidney. Nonetheless, the elaborate structure of the mammalian kidney makes the discovery of new therapies based on de novo engineered kidneys more challenging. In contrast to mammals, amphibians have a kidney that is anatomically less complex and develops faster. Given that analogous genetic networks regulate the development of mammalian and amphibian nephric organs, using embryonic kidneys of *Xenopus laevis* (African clawed frog) to analyze inductive cell signaling events and morphogenesis has many advantages. Pioneering work that led to the ability to generate kidney organoids from embryonic cells was carried out in *Xenopus*. In this review, we discuss how *Xenopus* can be utilized to compliment the work performed in mammalian systems to understand kidney development.

Keywords

Xenopus; Kidney; Development; Pronephros; Nephron; Induction; Organoid

Xenopus: a unique model system for the study of nephrogenesis

Introduction

Kidneys are essential for the osmoregulation of bodily fluids. An organism achieves osmoregulation by controlling the input and output of water and ions in its body, thus

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regulating their concentration within body fluids. The nephron is the basic structural and functional unit within the kidney. Adult human kidneys are composed of approximately one million nephrons that regulate osmotic pressure by filtering and removing excess water and waste products from the blood. The ability of kidneys to regulate blood composition is dependent on the process of kidney development. Because mammalian model systems, such as that of the mouse, share many biological characteristics with human kidneys, they have been important resources for understanding human kidney organogenesis and are an invaluable tool for ultimately understanding human kidney development and disease. However, given that kidney development is highly conserved among vertebrates, exploiting some of the notable advantages of amphibian model systems, such as *Xenopus laevis* (African clawed frog), can facilitate our understanding of kidney organogenesis. The goal of this review is to provide insight into how experimental approaches in the simple, yet functional frog embryonic kidney can complement mammalian models to address open questions underlying the formation and function of the nephron.

Xenopus as a model system

Xenopus offers many advantages that can complement the well-established mammalian models used to study nephrogenesis. The *Xenopus* female lays a large number of eggs (100–500) that develop externally, allowing for easy manipulation and visualization of all stages of development. In addition to offering experimental simplicity, *Xenopus* embryos develop functional embryonic kidneys within 2–3 days of fertilization.

The previously characterized *Xenopus* cell-fate map allows for blastomere-specific microinjection and tissue-targeted disruption of genes [1–4]. Microinjection techniques can be used in combination with fluorescent tracers (chemical dyes and fluorescent constructs) as a method for generating a more detailed fate map and identifying stem and tissue progenitor cells. Furthermore, embryos can also be microinjected with morpholinos or CRISPR/Cas9 for knockdown/knockout experiments and mRNA for overexpression of genes of interest. These techniques make *Xenopus* a tractable model for pre-screening of DNA or mRNA constructs in order to identify important regulators of nephron formation that can then be further analyzed in mammalian systems. Given the recent availability of sequence data from patients with inherited kidney diseases, *Xenopus* offers the ability to understand the molecular consequences of these mutations fairly rapidly. Additionally, *Xenopus* can be used for large-scale chemical screens and identification of potential therapeutic agents. Rapid embryogenesis and the ability to generate large numbers of embryos allow for development of cost-effective high-throughput screens.

Kidney development and function

All vertebrates initially develop simple embryonic kidneys that eventually give rise to more complex adult kidneys. Kidney development begins during embryogenesis when the mesoderm is subdivided into paraxial, intermediate and lateral plate mesoderm. The kidney is derived from the intermediate mesoderm, and its development unfolds in consecutive stages, leading to a gradual increase in organizational complexity and number of nephrons. Both mammals and amphibians initially develop simple embryonic kidneys called pronephroi, with each pronephros consisting of a single nephron. The mammalian

pronephros is a nonfunctional structure of early development; however, its formation is required for the establishment of subsequent kidney forms. Amphibians also develop pronephroi, with one pronephros on each side of the tadpole. The amphibian pronephroi serve as fully functional kidneys essential for osmoregulation. Consequently, tadpoles with defects in the formation of the pronephroi develop edema [5–9]. In both mammals and amphibians, the pronephroi are eventually replaced by more complex kidney structures called mesonephroi, which contain numerous nephrons. While the mesonephroi represent the adult kidneys of amphibians, in mammals they serve as transient structures and are the precursors to the development of the adult kidneys, called the metanephroi.

Each *Xenopus* pronephros consists of a single large nephron that shares structural and functional similarities with the mammalian nephron (Fig. 1). For example, the *Xenopus* glomus and the glomerulus of the metanephric nephron in mammals are both responsible for blood filtration. Additionally, the *Xenopus* pronephric and the mammalian metanephric tubules and duct are both responsible for filtering and eliminating waste products, and the common developmental markers of nephric components are also conserved (Table 1) [10–36]. The similarities in segmentation and expression of molecular markers between the *Xenopus* pronephric and the mammalian metanephric nephron have been covered in detail in multiple reviews [37–42] and therefore will not be discussed here in great detail.

Kidney engineering: the in vitro-induced frog nephron is functional in vivo

Explant and transplantation models

Xenopus tissue transplants and explants have been successfully used to study pronephric development. Frog embryos are relatively large and have significant healing capacity, making them amenable to numerous microsurgical assays. The pronephric cells are specified by late gastrula stages, and isolated mesodermal explants of the presumptive pronephric region can form glomus tissue and nephric tubules in culture [43–45]. Additionally, the development of the pronephros can be initiated in vitro from a mass of undifferentiated cells called the animal cap [46]. The animal cap consists of pluripotent presumptive ectoderm cells derived from the late blastula, and it serves as a useful model for elucidating the mechanisms of kidney induction and development ex vivo. Activin A, a member of the transforming growth factor beta (TGF-β) superfamily, and retinoic acid (RA), a metabolite of vitamin A, are well-known regulators of differentiation and proliferation of mesodermal tissues. At specific doses, activin A and RA can induce the formation of nephric tubules in isolated animal cap explants. In vitro-generated pronephric organoids express known molecular markers involved in nephrogenesis (*lhx1*, *grem1*, *cret*, *osr1*, *osr2*, *hnf1b*, *pax8*) and closely recapitulate the process of in vivo renal tubulogenesis [47–49]. Transplantation experiments performed in frogs showed that in vitro-induced pronephroi are capable of replacing and restoring the function of the native pronephros in a host embryo in which both kidney rudiments had been removed (Fig. 2) [8]. Renal function in this study was assayed by suppression of edema. Although the success of these transplantations was not high (21 %), it is remarkable that pronephroi derived from reprogrammed immature ectoderm have the capacity to adapt and function in the bilaterally pronephrectomized host embryo.

The ultimate goal of regenerative medicine is to engineer renal tissue that will replace kidney function in patients following transplantation. Mouse and rat renal organoids derived in vitro from embryonic kidney rudiments are capable of in vivo implantation, and although short-lived, they do show some functional capability [50, 51]. Since in vitro-induced *Xenopus* pronephroi are capable of in vivo function, they are amenable for studies with the aim to improve survival and function of the cultured renal tissue. The frog kidney organoids can be used in combination with techniques to downregulate or overexpress genes, providing insights into the regulatory steps of in vitro kidney formation. Since the *Xenopus* renal organoids develop in serum-free media, the process of nephron assembly can be studied in response to controlled stimuli. The required use of animal serum containing growth factors for in vitro mammalian kidney cultures may make the results more difficult to interpret, and the variable composition of animal serum from different batches could trigger different experimental outcomes [51–53]. Moreover, *Xenopus* embryos and tissue explants can be used for high-throughput in vivo phenotypic drug screens and are suitable models for drug development [54–56].

All animal system models have experimental advantages and limitations. For example, mammalian kidney culture systems allow direct observation of adult kidney development, but the simultaneous branching of multiple nephrons in metanephric kidneys can make it difficult to untangle the underlying molecular mechanisms of induction, patterning and tubulogenesis. The embryonic kidney of *Xenopus* is structurally simple, but does enable study of the induction signals and analysis of the cellular mechanisms at the single nephron level. Since the mammalian kidney is a complicated organ to grow de novo in culture, using the simple *Xenopus* pronephros to study the inductive and cellular events of kidney organogenesis can promote our understanding of the beginnings of its formation. For example, the animal cap induction experiments described above served as the basis for development of the protocol for induction of mouse and human stem cells into renal epithelia [57, 58].

Kidney nephrogenesis: in vivo imaging of the frog pronephros

Xenopus is a particularly useful model for in vivo studies of nephrogenesis. Given that *Xenopus* embryos develop outside of the mother and the developing kidney can be visualized through the surface ectoderm, the formation of the pronephric tubules can be visualized in whole embryos in vivo (Fig. 3). Whole-animal imaging allows individual renal progenitor cells to be tracked in their physiological environment as they differentiate, migrate and interact to assemble the pronephros.

In vivo pronephros morphogenesis

Kidney studies in mammalian models are carried out in either fixed tissue or organ culture, which has been the primary means of performing live imaging in mammalian systems. Previous studies using fixed tissues have suggested that mouse kidney tubules elongate through the process of convergent extension, a process that was first described in *Xenopus* [59]. Intercalation of neighboring cells with one another serves to extend the length of a tissue [59, 60] and, in the case of the kidney, forms a narrower and longer tubule. A study

from mice in fixed tissue suggests that Wnt9b produced in the ureteric bud and collecting ducts regulates the diameter of the developing kidney tubules through the regulation of convergent extension cell movements [61]. This study from the laboratory of T.J. Carroll was the first to suggest that vertebrate kidney tubule diameter is regulated by convergent extension. These experiments were carried out in fixed tissue, so this process had yet to be visualized through live imaging. Subsequently, studies in *Xenopus* implicated a role for convergence and extension in kidney tubulogenesis, and more recently, a study by Lienkamp et al. utilized live imaging in *Xenopus* kidneys to directly test the hypothesis that convergent extension movements are responsible for kidney tubulogenesis [62–64]. Kidney tissue was labeled using an injected mRNA encoding membrane-bound green fluorescent protein (GFP), and the movements of individual cells within the developing kidney were tracked in real time within their native environment in the embryo (Fig. 3). This experiment supported the conclusions drawn from the previous work by the Carroll research group [61] that was done using fixed mouse kidney tissue, showing that convergent extension movements are involved in kidney tubulogenesis.

Work in *Xenopus* also suggests how cell movements result in the convergence and extension of the kidney tubules. Live imaging of the forming pronephric kidney in *Xenopus* demonstrates that cells within the developing proximal and intermediate tubules undergo convergent extension by forming rosettes consisting of four to seven neighboring cells [64]. Rosettes, which were first described in the germ band epithelia of *Drosophila*, are pinwheel-shaped structures that form when groups of cells line up and then rapidly reorganize to elongate along a particular axis of a tissue [65]. Cells forming rosette-shaped structures have been described in mouse kidney tubules. Although it is postulated that the cell rosettes described in kidney tubules contribute to lumen development, the cellular movements that occur during rosette formation and resolution have not been determined in mice. The rosette structures visualized in real time in the developing *Xenopus* kidney are similar to the structures seen in the fixed tissue of the collecting duct of mice. In *Xenopus*, time-lapse imaging supports the findings that rosette structures in the kidney tubules are involved in convergent extension movements. Cells within a rosette constrict to form wedges with points that meet in the center of the rosette. Once the rosette is formed, the cells intercalate with one another, elongating on an axis roughly perpendicular to the axis in which the cells were originally oriented. These convergent extension movements serve to elongate the tubules of the developing *Xenopus* kidney. The unique ability to target developing kidney tissue in live *Xenopus* embryos has allowed researchers to clearly visualize how convergent extension movements and rosette formation and resolution form the tubules of the kidney.

Modulating pronephros morphogenesis

In addition to using *Xenopus* embryonic kidneys to elucidate the cellular movements that underlie nephrogenesis, *Xenopus* embryos can be used to study the effect of a gene on kidney formation. For example, Lienkamp et al. used time lapse confocal imaging to assess the role of inversin in the developing *Xenopus* pronephros [66]. Mutations in inversin are known to cause type II nephronophthisis in humans, a juvenile genetic disorder known to cause defects in kidney development and renal failure. Inversin is also believed to facilitate a switch between the canonical Wnt signaling and planar cell polarity pathways [67].

Although inversin mutations in humans and mice cause kidney defects, the exact cause of these defects are unknown. The authors co-injected a translation-blocking inversin morpholino to knockdown inversin expression with mRNA encoding membrane-associated GFP and histone 2B-red fluorescent protein to label cell membranes and nuclei, respectively. Confocal time-lapse microscopy was used to track individual cell movements in the developing pronephros. Two types of cell movements were detected, with one type causing the proximal and early intermediate tubules to extend in a dorsal to ventral direction, and the other movement type consisting of cells moving from the distal portion of the intermediate tubules towards the head of the embryo. Combined, these two types of cell movements resulted in the proximal tubule elongating and extending in a ventral direction, with the distal-to-proximal cell movement leading to looping and extension of the intermediate tubule. Knockdown of inversin was found to decrease only the proximal and intermediate tubule extension in the dorsal to ventral direction. Although the authors tracked movements of individual cells in this study, they did not mention whether convergent extension movements are involved in the extension of the proximal or intermediate tubules. However, these cellular movements occur simultaneously with the convergent extension movements described in the paragraph above.

Lienkamp et al. also tested the role of inversin in elongation of the distal tubule [66]. The authors co-injected mRNA encoding the photo-convertible fluorophore Kaede with a translation-blocking inversin morpholino in *Xenopus* embryos to knockdown inversin expression, resulting in embryos displaying green fluorescence in the trunk and kidney region. Photo conversion of Kaede from green to red resulted in a red stripe on the side of the developing embryo. As the embryo developed, red cells in the distal tubule migrated beyond the original photo-converted stripe in a posterior to anterior direction at a similar rate as cells in an embryo injected with a control morpholino. These data suggest that inversin does not play a crucial role in posterior to anterior cell movement in the distal tubule, but instead it is necessary for proximal and intermediate tubule development. This information on cell movement during early nephrogenesis would have been difficult to obtain from fixed samples or from more complex model organisms.

To date, mouse kidney tubulogenesis has been primarily studied in fixed tissue specimens taken from embryos at various stages of development, and live imaging of *Xenopus* embryonic kidneys has supported work done in mice. Surgical removal and ex vivo culture of the complex mouse kidney has provided valuable information on early kidney development and supplemented work that was previously performed using fixed tissue [68–71]. Future work on kidney development and function could be complemented with *Xenopus*, which has the advantage of having a simple, fully functional model of a nephron: the pronephros. The use of a less complex model organism would allow the researcher to address concerns related to ex vivo organ culture, such as possible alterations of tissue development due to lack of normal environmental factors, as well as limited tissue life-span in culture. Therefore, in vivo imaging of *Xenopus* pronephric development can be used to verify and supplement the data collected in fixed tissue and ex vivo mouse studies.

Perspectives

The cellular movements responsible for kidney tubulogenesis are conserved between mammals and frogs. The ease of manipulating gene expression in *Xenopus* through knockdown or overexpression coupled with the ability to perform time-lapse imaging makes *Xenopus* a promising model system to study the effects of perturbations in gene expression on cell movements during nephron assembly. CRISPR/Cas9 genome editing has also been successfully used to create knockout *Xenopus* frogs, allowing researchers to selectively knockout genes suspected to play a role in kidney tubulogenesis [71–74]. In addition, *Xenopus* proximal tubules have been reported to regenerate after surgical removal, making *Xenopus* a promising model of vertebrate kidney regeneration after acute kidney injury [75]. Similarly, animal cap explant assays take advantage of the ability to explant tissue from a *Xenopus* embryo and induce kidney development ex vivo. These assays, coupled with the new transgenic lines expressing fluorescent proteins in the kidney and live imaging technologies that are currently available, make kidney engineering and regeneration experiments in *Xenopus* feasible [42]. Additionally, *Xenopus* is a good candidate for multi-model studies involving human genome-wide association studies, mouse organoid cultures and cell culture work. In this context, the ease of tissue manipulation and live imaging techniques available in *Xenopus* can complement the mutant lines available in mice and cell cultures. Taken together, *Xenopus* is a unique vertebrate model system for the study of kidney development.

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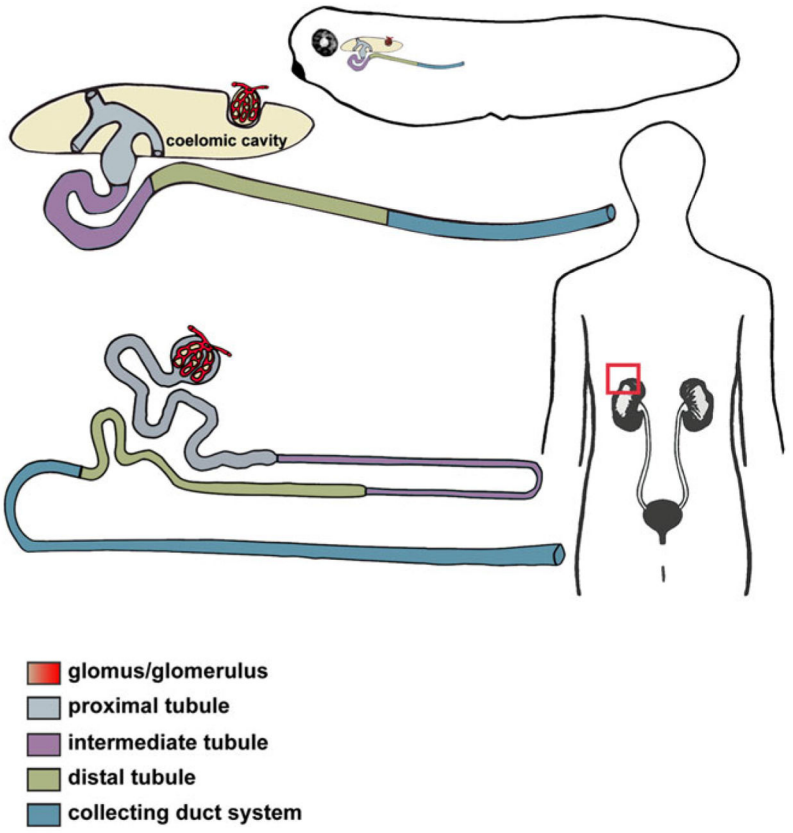

Fig. 1.

Xenopus pronephric and human metanephric nephrons share a conserved segmentation pattern. A schematic representation of an enlarged *Xenopus* pronephric nephron (*top*) and mammalian metanephric nephron (*bottom*), with the distinct tubular compartments labeled. The glomus/glomerulus filters the fluid from the blood plasma through capillary walls into the proximal tubule. The drawing depicts a slight difference in organization of the glomar/glomerular anatomy between the amphibian pronephric and mammalian metanephric nephrons. In mammalian metanephric nephrons, the glomerulus is integrated into the Bowman's capsule near the proximal tubule, while the *Xenopus* glomus projects into a body cavity (coelomic cavity). Three branches of pronephric tubule (nephrostomes) use ciliary action to force the glomar filtrate down the pronephric tubule. Together, the tubule segments (proximal, intermediate and distal) and the collecting duct system (connecting tubule and duct) facilitate the process of filtrate reabsorption and transport of wastes products for secretion

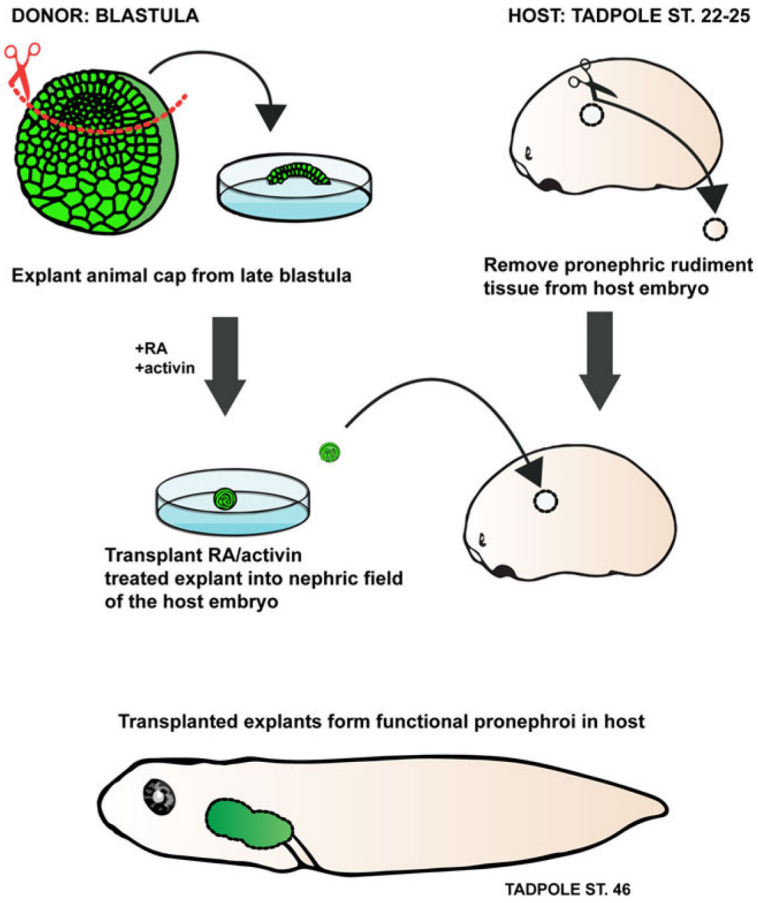

Fig. 2.

Xenopus in vitro-induced kidney organoids. Isolated animal cap (presumptive ectodermal tissue) explants (*green*) treated with activin and retinoic acid (*RA*) differentiate to form pronephric organoids in culture. The animal cap tissue treated with activin and RA can be transplanted into a host embryo which lacks kidney primordia. The in vitro-induced pronephric tissue is capable of functioning in vivo and compensates for the loss of the host kidneys by suppressing edema formation

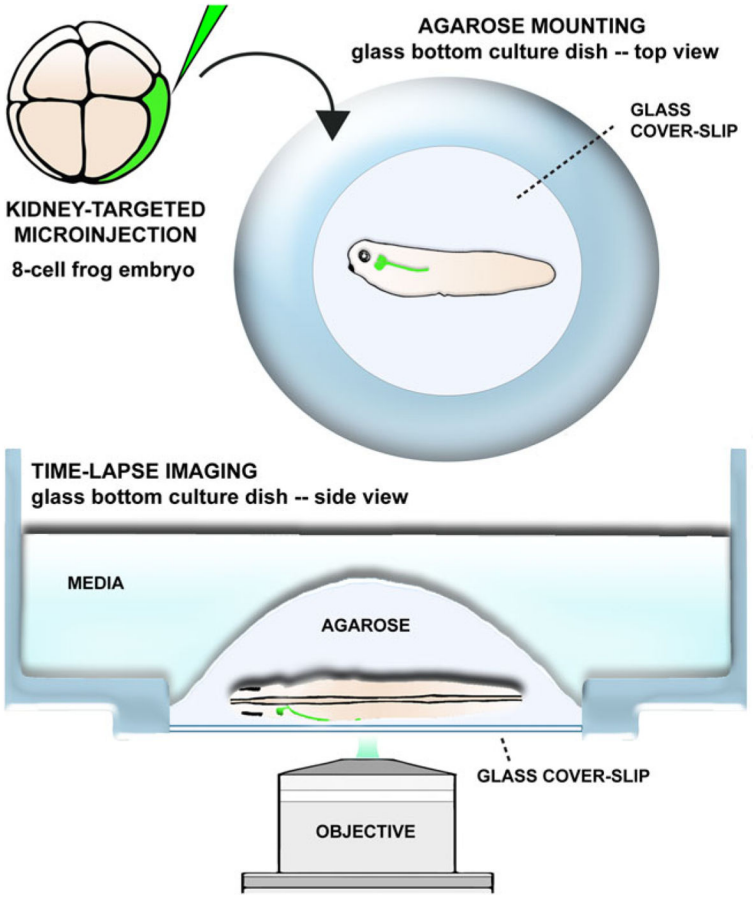

Fig. 3.

In vivo imaging of the frog pronephros. *Xenopus* fate-map of early development permits microinjections of blastomeres fate-mapped to kidney cell progenitors and it can be utilized for targeted delivery of fluorophores to developing pronephros. Microinjected embryos can be immobilized in low melting-point agarose and subjected to time-lapse imaging. This sample preparation allows for in vivo analysis of cell behaviors and molecular dynamics underlying pronephros formation

Table 1

A list of the key molecular markers[a] expressed during development of *Xenopus* pronephros [10, 11]

Gene symbol	Gene name	Expression	Role in pronephric development	References
lhx1; lim1	*lim homeobox 1*	PR, G, PTD	Required for establishment of the pronephric rudiment Depletion impairs development of glomus and tubules	[12–14]
hnf1b	*hnf1 homeobox b*	PR, G, PTD	Regulates pronephros development	[15–20]
pax8	*paired box 8*	PR, G, PTD	Overexpression promotes ectopic tubulogenesis Morpholino knockdown causes a complete absence of tubule	[11, 21, 22]
pax2	*paired box 2*	PR, G, PTD	Morpholino knockdown leads to impaired tubulogenesis, mainly of the proximal tubules Required following establishment of the pronephric rudiment	[21–23]
wt1	*wilms tumor 1*	PR, G	Overexpression impairs pronephric rudiment specification and formation of proximal tubules Morpholino knockdown inhibits *wnt4* expression	[24–27]
gdnf	*glial-cell derived neurotropic factor*	PTD	Not established	[28, 29]
bmp4	*bone morphogenetic protein 4*	PR, PTD	Required for pronephric development	[30]
bmp7	*bone morphogenetic protein 7*	PR, PTD	Required for pronephric development	[31–33]
wnt4	*wingless-type MMTV integration site family, member 4*	PR, G, PTD	Morpholino knockdown causes absence of pronephric tubules with minimal effect on duct formation Overexpression induces formation of ectopic nephrostomes and glomus at the expense of tubules	[25, 29, 33]
osr1	*odd-skipped related transcription factor 1*	PR, G, PTD	Morpholino knockdown impairs renal development Overexpression promotes ectopic kidney development	[34]
osr2	*odd-skipped related transcription factor 2*	PR, G, PTD	Morpholino knockdown impairs renal development Overexpression promotes ectopic kidney development	[34]

PR, Pronephric rudiment; G, glomus; PTD, pronephric tubules and duct

[a]The listed genes are shared between amphibians and mammals and are essential for proper kidney formation in both mouse and frogs