Xenopus: Experimental Access to Cardiovascular Development, Regeneration Discovery, and Cardiovascular Heart-Defect Modeling

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Xenopus has been used to study a wide array of developmental processes, benefiting from vast quantities of relatively large, externally developing eggs. *Xenopus* is particularly amenable to examining the cardiac system because many of the developmental processes and genes involved in cardiac specification, differentiation, and growth are conserved between *Xenopus* and human and have been characterized in detail. Furthermore, compared with other higher vertebrate models, *Xenopus* embryos can survive longer without a properly functioning heart or circulatory system, enabling investigation of later consequences of early embryological manipulations. This biology is complemented by experimental technology, such as embryonic explants to study the heart, microinjection of overexpression constructs, and, most recently, the generation of genetic mutations through gene-editing technologies. Recent investigations highlight *Xenopus* as a powerful experimental system for studying injury/repair and regeneration and for congenital heart disease (CHD) modeling, which reinforces why this model system remains ideal for studying heart development.

Studies in amphibians have formed the basis of cardiac biology in vertebrates for >80 years, yielding many of our most important insights (Taylor 1931; Nieuwkoop 1947; Chuang and Tseng 1957; Jacobson 1960, 1961; Monnickendam and Balls 1973). The frog has several advantages over other species for studying heart development. For example, mice are genetically tractable but are difficult for live imaging or studying biochemistry of the heart. Fish are an outstanding system for live imaging, but their small size and clutch numbers make systems-level proteomic approaches difficult. *Xenopus* has an advantage over these species in that a suite of novel tools exists that will allow—in a single organism—integration of systems-level genomic and proteomic analyses with quantitative live imaging of cardiac cell behaviors.

Classical fate-mapping studies have shown that at a mere 3 hours after fertilization, at the 32-cell stage, four blastomeres in the dorsal equatorial region of *Xenopus* are fated to be-

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come the adult heart (Fig. 1; Dale and Slack 1987; Moody 1987a,b). As in all embryos with yolk-filled blastomeres, cells do not undergo extensive mixing before gastrulation. Instead, the descendants of the four blastomeres remain as a coherent group of cells that lie juxtaposed between the organizer and the underling endoderm; these two tissue types induce the cardiac lineage (Fig. 1). During gastrulation, the cardiac cells are the first cells to become specified and determined (Symes et al. 1994; Nascone and Mercola 1995; Mercola 1999; Mohun and Leong 1999; Zhu et al. 1999; Kolker et al. 2000; Mohun et al. 2000).

During neurulation, the cardiac cells move as two distinct populations on either side of the embryo toward the forward end of the mesoderm sheet as it engulfs the yolk and encompasses the embryo (Fig. 1). The cells converge to form a single sheet at the ventral anterior midline, where they form a tube positioned along the anteroposterior axis (Figs. 1 and 2). The linear heart tube is bilaminar, composed of an outer myocardium and an inner endocardium
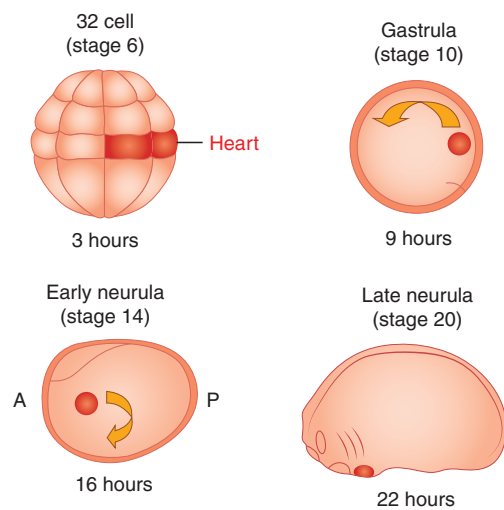
Figure 1. Schematic of early *Xenopus laevis* heart development. Stages are shown above embryos, and hours of development at room temperature are shown beneath. Blastomeres at the 32-cell stage fated to become heart tissue are labeled in red and their decedents labeled in red at gastrula, early neurula, and late neurula. (A) Anterior, (P) posterior.
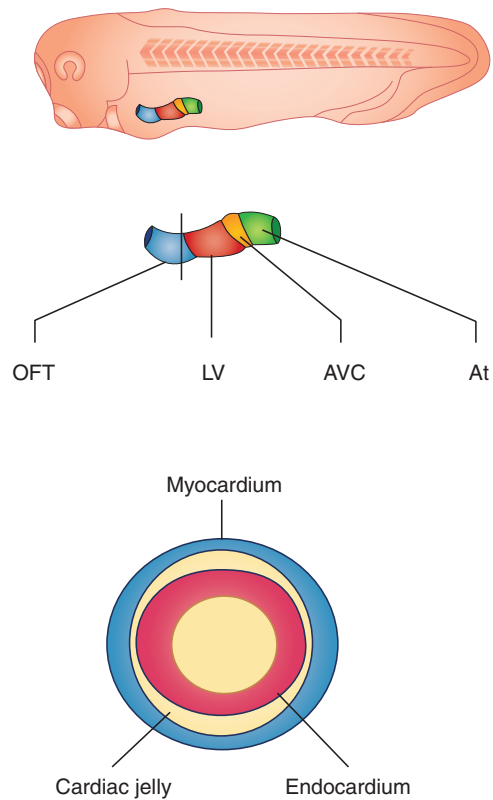
Figure 2. (*Top*) A schematic of *Xenopus laevis* heart development at the 32-cell stage showing the position of the linear heart tube. (*Middle*) Specific relative positions of cell type along the heart tube outflow tract (OFT), left ventricle (LV), atrioventricular canal (AVC), and atrium (At). (*Bottom*) Section of the heart tube with black solid lines in the middle of the schematic showing that the heart is comprised of a bilaminar heart tube with an inner endocardium and an outer myocardium, which are separated by extracellular cardiac jelly.

(Fig. 2; Mohun and Leong 1999; Kolker et al. 2000; Mohun et al. 2000).

As with all vertebrates, the blood in *Xenopus* flows from the tail to the head. As development proceeds, the cardiac tube begins to undergo looping. Collectively, this activity folds the heart, bringing the inflow and outflow tracts near each other to make the proper connections with the developing vascular system and to place the two developing atria on top of the single ventricle. The atria will then undergo a slow septation

process, leading to the formation of a three-chambered heart (Mohun and Leong 1999; Kolker et al. 2000; Mohun et al. 2000).

UNDERPINNING CLASSICAL EMBRYOLOGY AND EXPERIMENTAL BIOLOGY

The large amphibian embryos introduced above provide a distinct experimental advantage. The large embryos allow for observation of the conserved vertebrate body as it develops, and the associated large embryonic cells (called blastomeres) allow the experimental researcher to use lineage tracing and targeted tissue- and organ-specific delivery of experimental reagents by microinjection (e.g., target the red blastomeres in the 32-blastomere embryo in Fig. 1). The large embryos also lend themselves to powerful, so-called "cut-and-paste" experiments to study the role of tissue interactions, particularly in heart development, and "just cut" embryonic explant experiments to study, for instance, the development of heart tissue in relative isolation. Such approaches can be used to separate cardiogenic from noncardiogenic mesoderm (i.e., dorsal marginal zone [DMZ], ventral marginal zone [VMZ]) (reviewed by Afouda 2012) to study the function of inducers and repressors of heart development (e.g., Fig. 3; Foley and Mercola 2005). Many of the fundamental discoveries made in *Xenopus* are a result of these intrinsic advantages of the experimental model. For instance, the important role of Wnt signaling in cardiogenesis, both canonical β-catenin-mediated (Schneider and Mercola 2001) and noncanonical (Pandur et al. 2002), was discovered in *Xenopus*, as well as bone morphogenetic protein (BMP) (Breckenridge et al. 2001) and later fibroblast growth factor (FGF) signaling (Deimling and Drysdale 2011). These discoveries, together with findings in other models such as chick (Marvin et al. 2001; Wittig and Münsterberg 2019), gave rise to an often reproduced figure in textbooks (Fig. 4) regarding signaling pathways, and also inhibition of signaling pathways, specifying anterior and posterior lateral plate mesoderm and subsequently cardiogenic versus hemangiogenic tissue differentiation. Combined with state-of-the art molecular approaches, *Xenopus* research continues to provide unique opportunities for current and future pioneering discoveries relevant to cardiovascular biology and medicine.

WIDENING ACCESS TO STEM-CELL(-LIKE) EXPERIMENTS

One further type of *Xenopus* embryonic explant deserves further mention: the so-called "animal cap" and—specifically for the field of heart development—its use as an "activin cap." The animal cap is the tissue from the animal pole of the *Xenopus* embryo, which is removed from the rest of the embryo before gastrulation by straightforward microdissection (Fig. 3). Importantly, this extracted tissue retains pluripotency
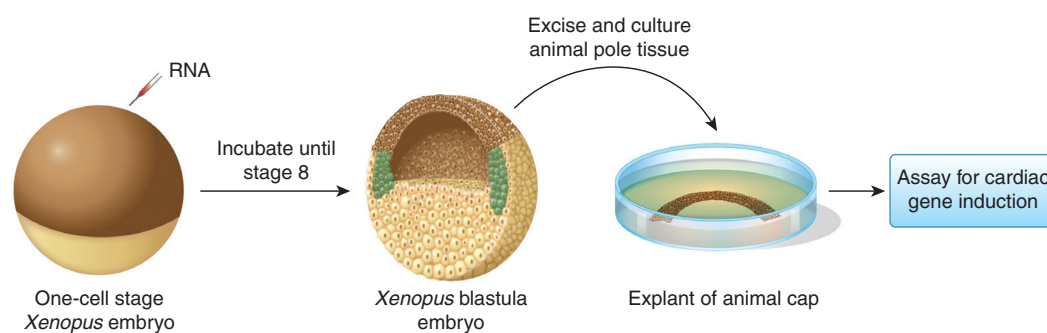
Figure 3. Schematic of *Xenopus* animal cap assay. (*Left-hand* panel) a one-cell-stage embryo injected with mRNA or DNA is then allowed to develop to stage 8 (pre-gastrula) stage embryo, at which time point the ectodermal or animal cap is removed and placed in isolation, cultured, and assayed for the presence, absence, or type of cardiac tissue.
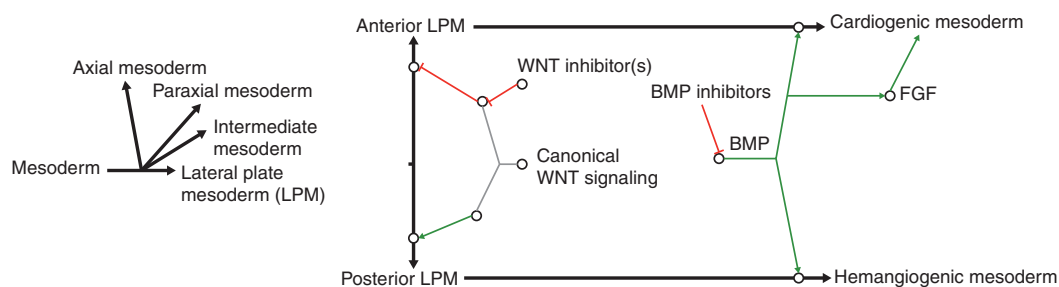
Figure 4. Schematic outlining role of signaling pathways and inhibition of signaling pathways in regulating cell fate specification in the mesoderm toward cardiac differentiation. (BMP) Bone morphogenetic protein, (FGF) fibroblast growth factor. (Figure based on data in Gilbert and Barresi 2016.)

and can be used in stem-cell approaches inexpensively (Furue and Asashima 2004). Remarkably, treatment of this pluripotent animal cap with activin (resulting in "activin caps") leads to differentiation of autonomously beating heart tissue (Fig. 3; Kinoshita et al. 2010; Afouda 2012). This heterologous heart tissue differentiation stem-cell(-like) system can be combined with microinjection experiments and transcriptomics analysis to study the regulatory mechanisms driving vertebrate heart development (Afouda et al. 2018).

COMPLEMENTARY APPROACHES USING *XENOPUS LAEVIS* AND *XENOPUS TROPICALIS*

The traditional species associated with *Xenopus* research is *X. laevis*. It has particularly large em-

bryos, even among *Xenopus*, and is robust in its husbandry and recovery of normal embryonic development after quite rough experimental manipulation. Its cousin, *X. tropicalis*, has a simpler genome organization, which benefitted many high-throughput genomic studies and transcriptome analyses (Table 1; Fig. 5; Hellsten et al. 2010; Kashiwagi et al. 2010; Owens et al. 2016). Genome sequencing and analysis has shown that *X. laevis* is not a tetraploid but rather is an allotetraploid frog possessing two diploid genomes, the L and S genomes, derived from the mating of two distinct ancestral species 17–18 million years ago (Session et al. 2016). Approximately 56% of genes in the present-day *X. laevis* genome are duplicated between the L and S genomes as a result of the allotetraploidy event (Session et al. 2016). Thus, the two species are very closely related and often can be used interchangeably be-

Table 1. High-throughput genomic studies and transcriptome analyses of *Xenopus laevis* and *Xenopus tropicalis*

	X. laevis	*X. tropicalis*
Size of adults (♂; ♀)	~7 cm; ~10 cm	~3 cm; ~5 cm
Size of embryo (diameter; volume)	~1–1.4 mm; ~1–2 mL	~0.6–0.8 mm; ~0.2–0.5 mL
No. of chromosomes	18 chromosomes	10 chromosomes
Size of genome	3.1×10^9 bp	1.7×10^9 bp
Generation time	~13 months	~4 months
Brood size	≥1000 embryos	≥300 embryos

X. laevis is allotetraploid, believed to have emerged after hybridization of two *X. tropicalis*–like diploid ancestors (but not directly *X. tropicalis* itself). The 18 chromosomes of *X. laevis* can still be clearly ordered into two subgenomes (chromosomes 1L to 9L and 1S to 9S, respectively, presumably derived from either prehybridization diploid ancestor). Chromosomes 1L to 8L and 1S to 8S in *X. laevis* correspond to *X. tropicalis* chromosomes 1 to 8, whereas sequences on *X. tropicalis* chromosomes 9 and 10 correlate with the ninth chromosomes from either subgenome in *X. laevis* (therefore now often called chromosomes 9_10L and 9_10S, respectively) (Session et al. 2016). Under ideal laboratory conditions, the generation time of *X. laevis* has been reduced to 8 months and that of *X. tropicalis* to 3 months (Hirsch et al. 2002).

Figure 5. *Xenopus laevis* flanked by smaller cousin *Xenopus tropicalis*. (Photo by Atsushi Suzuki.)

cause most probes work in both species. Possible subtle but consequential differences in regenerative potential in the heart, which are currently debated (see below) may further benefit from this two-species approach (Fig. 4).

KNOCKDOWN, KNOCKOUT, KNOCKIN, AND TRANSGENICS

The use of *Xenopus* for gain- and loss-of-function experiments has been legendary because of its ease of experimental manipulation. This traditional strength has been complemented with efficient transgenesis approaches (see recent review in Horb et al. 2019) and gene editing (see recent reviews in Tandon et al. 2017; Deniz et al. 2018), including knockin (Aslan et al. 2017). These approaches have already proven particularly powerful in *Xenopus*, not least because analysis can begin in transheterozygotes of the F_0 generation only hours after CRISPR-Cas9 technology application (Blitz et al. 2013) and in targeted mosaics (Naert et al. 2016).

REGENERATION

In their early stages, both *X. tropicalis* and *X. laevis* appear able to regenerate heart tissue. However, in adults, *X. tropicalis* but not *X. laevis* reportedly undergo cardiac regeneration on injury. Because side-by-side studies have not been conducted, the differences in adults may simply represent differences in protocols or the relative age of the adult frogs. However, they might instead be the result of genetic differences between the two species in abundance of proteins associated with antigen-specific adaptive immunity (Liao et al. 2017, 2018; Marshall et al. 2017, 2018, 2019).

Recently, Federspiel et al. (2019) conducted a direct comparison of the adult cardiac proteomes of four model vertebrates with dual circulatory systems: the pig (*Sus scrofa*), the mouse (*Mus musculus*), *X. laevis*, and *X. tropicalis*, which were all compared with human. Surprisingly, a significant increase in protein abundance in a vast array of cell-cycle proteins was observed in *X. laevis* versus *X. tropicalis* in age-matched female hearts. Thus, one alternative explanation for the ability of adult *X. tropicalis* to regenerate is their ability to induce cardiac cell-cycle genes on injury. Also noteworthy, the investigators further observed a significant increase in proteins that control metabolic growth including the TOR pathway in *X. tropicalis* versus *X. laevis* (Fig. 5). It will be exciting to learn whether these differences underlie the observed differences in the ability of the two species to undergo adult regeneration.

HEART DISEASE MODELING

Xenopus has proven to be a great asset for defining the molecular and cellular underpinnings of several human congenital heart disease (CHD) states, including Holt–Oram disease (Horb and Thomsen 1999; Garg et al. 2003; Brown et al. 2005; Goetz et al. 2006; Puskaric et al. 2010; Herrmann et al. 2011; Steimle et al. 2018), Tbx20-related CHD (Brown et al. 2003, 2005; Stennard et al. 2003; Showell et al. 2006; Mandel et al. 2010; Kaltenbrun et al. 2013), and Nkx2.5-related heart disease (Biben and Harvey 1997; Jiang et al. 1999; Raffin et al. 2000; Small et al. 2000; Jamali et al. 2001a,b; Shiratori et al. 2001; Kasahara et al. 2003; Small and Krieg 2003; Stennard et al. 2003; Bartlett et al. 2007). In addition, it has recently proven to be a powerful tool in defining the molecular source of CHD. One of the central issues facing clinicians in treating CHD is to try to decipher from massive patient data sets which human differences in DNA or RNA sequence represent naturally occurring single-nucleotide polymorphisms (SNPs) and which are somatic mutations in coding regions of potential disease-causing genes (Musunuru et al. 2018). One approach in addressing this issue has come from pioneering

work by the Khokha laboratory, which has used a high-throughput CRISPR-Cas9 system to screen potential human mutations in *Xenopus* for those that affect essential cardiac genes (Fakhro et al. 2011; Boskovski et al. 2013; Duncan and Khokha 2016; Garfinkel and Khokha 2017; Griffin et al. 2018; Kulkarni and Khokha 2018; Kulkarni et al. 2018; Sempou et al. 2018; Robson et al. 2019; Sempou and Khokha 2019). This unbiased, patient-driven gene discovery approach has led to the identification of new genes and protein pathways that may have been missed through other approaches, as well as providing a genetic resource for studying the normal and disease states (Fakhro et al. 2011; Boskovski et al. 2013; Duncan and Khokha 2016; Garfinkel and Khokha 2017; Griffin et al. 2018; Kulkarni and Khokha 2018; Kulkarni et al. 2018; Sempou et al. 2018; Robson et al. 2019; Sempou and Khokha 2019).

CONCLUSION

There is no single best model for studying heart development and disease. The accessibility of embryonic tissues and the bounty of experimental sample material available for state-of-the-art analysis methods undoubtedly make *Xenopus* an important part of an approach that complements other model systems. Moreover, *Xenopus* has the unique advantage of combining a matchless suite of novel tools that will allow—in a single organism—integrating systems-level genomic and proteomic analyses with quantitative live imaging of cardiac cell behaviors. These studies can be conducted at the level of single cells, in stem-cell-like and organoid-like explants, or even in whole embryos and animals. By applying this innovative experimental toolbox, *Xenopus* will continue its impressive track record as an important experimental system for groundbreaking novel discoveries in vertebrate heart development, regeneration, and disease.

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