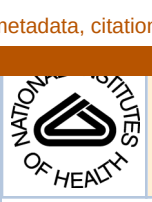

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Xenopus: An Emerging Model for Studying Congenital Heart Disease

Erin Kaltenbrun[1,2], **Panna Tandon**[1,3], **Nirav M. Amin**[1,3], **Lauren Waldron**[1,3], **Chris Showell**[1,3], and **Frank L. Conlon**[1,2,3,*]

[1]University of North Carolina McAllister Heart Institute

[2]Department of Biology, UNC-Chapel Hill, Chapel Hill, NC 27599

[3]Department of Genetics, UNC-Chapel Hill, Chapel Hill, NC 27599

Abstract

Congenital heart defects affect nearly 1% of all newborns and are a significant cause of infant death. Clinical studies have identified a number of congenital heart syndromes associated with mutations in genes that are involved in the complex process of cardiogenesis. The African clawed frog, *Xenopus*, has been instrumental in studies of vertebrate heart development and provides a valuable tool to investigate the molecular mechanisms underlying human congenital heart diseases. In this review, we discuss the methodologies that make *Xenopus* an ideal model system to investigate heart development and disease. We also outline congenital heart conditions linked to cardiac genes that have been well-studied in *Xenopus* and describe some emerging technologies that will further aid in the study of these complex syndromes.

Keywords

congenital heart disease; *Xenopus laevis*; *Xenopus tropicalis*; heart development; cardiac development; cardiomyocyte

Introduction

Xenopus as a Model System for Human Congenital Heart Disease

It is becoming increasingly clear that many forms of human disease are associated with defects in genes required for early steps in embryonic development. The African clawed frog, *Xenopus* shares surprising similarities with humans both genetically and anatomically. Thus, the molecular and cellular pathways through which these genes function can be elucidated using *Xenopus* to model vertebrate heart development and disease. *Xenopus* has numerous advantages as a model system in which to identify and characterize cellular and developmental processes. Unlike the mouse, the *Xenopus* embryo develops externally, and its early patterning and morphogenesis have been extensively studied. The *Xenopus* embryo is relatively large and is amenable to surgical manipulations, allowing defined regions to be excised and cultured in simple salt solutions in which the developmental and downstream transcriptional effects of exogenous growth factors can be determined. These classical approaches are complemented by molecular techniques that allow the overexpression or knockdown of specific gene products in the early embryo (Harvey and Melton, 1988; Heasman and others, 2000). In addition, transgenesis techniques are well established and

*Author for correspondence, 221 Fordham Hall, University of North Carolina, Chapel Hill, Chapel Hill, NC 27599, Ph: 919-843-5500, Fax: 919-843-3399, frank_conlon@med.unc.edu.

technologies are continually being optimized (Chesneau and others, 2008). Moreover, recent sequence annotation and assembly of the *Xenopus tropicalis* genome has demonstrated that it has long regions in which genes exhibit remarkably similar synteny relationships to those found in the human genome (Hellsten and others, 2010; Showell and Conlon, 2007). Specifically, regarding human congenital heart disease (CHD), *Xenopus* has unique advantages for studying cardiovascular development (Bartlett and Weeks, 2008; Evans and others, 2010; Warkman and Krieg, 2006). First, early *Xenopus* development can proceed in the absence of a functional circulation system, allowing defects to be extensively analyzed in living embryos. Second, *Xenopus* has a pulmonary system and a two-chambered atrium. Third, *Xenopus* has a well-established fate map that is not confounded by extensive cell mixing (Dale and Slack, 1987; Moody, 1987). Only three hours after fertilization, it is possible to identify the blastomeres that will give rise to the adult heart. Collectively, this unique set of attributes places *Xenopus* as an ideal model system for studying congenital heart defects and in this review we will describe the experimental tools available to researchers, together with the existing *Xenopus* models of human CHDs (Table 1).

Methods for studying heart development and disease in *Xenopus*

Protein Depletion and Overexpression

The advent of effective antisense techniques has enabled researchers to associate developmental processes with the genes that control them. The most extensively used of these techniques are the use of morpholino oligonucleotides (MOs), which inhibit the function of specific genes by preventing translation or splicing of messenger RNA (mRNA). This technique has resulted in the publication of many studies of heart development in *Xenopus* that have advanced our understanding of this process in vertebrates (Bartlett and Weeks, 2008; Brown and others, 2007; Brown and others, 2005; Christine and Conlon, 2008; Garriock and others, 2005a; Hilton and others, 2007; Inui and others, 2006; Kumano and others, 2006; Movassagh and Philpott, 2008; Nagao and others, 2008; Small and others, 2005; Zhang and others, 2005). MO antisense oligonucleotides are neutrally charged synthetic nucleic acid analogs that are stable, soluble, and bind to RNA with high affinity (Heasman and others, 2000; Moulton, 2007). In addition, they are resistant to nuclease degradation and have limited interaction with proteins (Bill and others, 2009; Eisen and Smith, 2008; Summerton, 2007). MOs are designed to reduce gene function in two ways. First, the MO can be designed to target sequences in the 5′ untranslated region close to the translation initiation codon of the gene to sterically block the attachment of the ribosomal machinery and inhibit protein translation (Heasman and others, 2000). Alternatively, MOs can be designed to target the splice junctions in the pre-mRNA strand, resulting in the incorporation of intron-encoded amino acids and, in many cases, early termination of translation via premature stop codons or by a shift in the reading frame of the subsequent sequence (Morcos, 2007).

Strategies other than MOs have been employed to inhibit gene function. These include antisense RNA injection (Harland and Weintraub, 1985; Melton, 1985; Dagle and Weeks, 2001), RNA interference in which the RNA is targeted for degradation by the binding of small inhibitory RNA molecules and recruitment of the RNA-induced silencing complex (Zhou and others, 2002; Summerton, 2007), DNA with chemically modified phosphate linkages that employs cellular RNase H to cleave the target RNA strand (Summerton, 2007), and peptide nucleic acid nucleotides which sterically block RNA translation in a similar way to MOs (Dagle and Weeks, 2001; Harland and Weintraub, 1985; Melton, 1985; Summerton, 2007; Zhou and others, 2002). However, due to few off-target effects, their binding success and their commercial availability, MOs have become a favored tool for studying gene function in vertebrate models (Knudsen and Nielsen, 1996; Summerton, 2007).

Protein overexpression can be as valuable a technique as protein depletion to determine the role of a particular gene in development. The microinjection of capped mRNA into the *Xenopus* embryo has commonly been used to study heart development in the context of globally increased function of the protein under investigation, the effects of lateral- and lineage-specific overexpression, and to study isoform-specific phenotypes (Campione and others, 1999; Goetz and others, 2006; Kitaguchi and others, 2000; Stennard and others, 2003). Overexpression of truncated or mutated proteins can also be utilized to reproduce and investigate phenotypes caused by mutations identified in human patients with CHD (Ataliotis and others, 2005; Bartlett and others, 2007). Furthermore, protein function can be manipulated in a spatio-temporal manner by injecting hormone-inducible constructs with timed dexamethasone application (Kitaguchi and others, 2000)(Afouda and others, 2008).

Xenopus Explants for Cardiogenic Assays

The *Xenopus* embryo is particularly amenable to tissue explant assays due to its unique ability to heal after microsurgery. In addition, *Xenopus* embryonic tissue can survive in the absence of added nutrients (due to the yolk contained in embryonic cells) allowing culture of tissue explants in a simple saline solution. The first use of *Xenopus* explants in a cardiogenic assay was performed by Horst Grunz in 1992 (Grunz, 1992) who demonstrated that the isolated blastopore lip, fated to give rise to notochord and somites but not the heart, gives rise to differentiated cardiac tissue when cultured in the presence of the growth factor-blocking compound suramin. This demonstrated the importance of growth factor signaling in negatively regulating the induction of cardiac cell fate in the dorsal marginal zone (DMZ), which restricts cardiac cell fate to two bilateral groups of cells in the dorsoanterior mesoderm of the gastrula. Similar experiments by Schneider and Mercola have shown that Wnt signaling can antagonize cardiac specification in DMZ explants. When an arc of dorsal marginal zone mesoderm containing the heart progenitors is excised from the equatorial region of the gastrula and cultured in isolation it gives rise to differentiated, beating cardiac tissue. However, overexpression of *wnt3A* and *wnt8* in these DMZ explants by injection of plasmid DNA into dorsal blastomeres results in a downregulation of cardiac marker expression, suggesting that inhibition of endogenous Wnt signaling might be required for proper heart induction (Schneider and Mercola, 2001). Indeed, when the Wnt inhibitors *dickkopf1* or *crescent* are expressed ectopically in non-cardiogenic ventral marginal zone (VMZ) explants, cardiac terminal differentiation can be induced. This results in the striking observation of beating cardiac tissue within the cultured explants (Schneider and Mercola, 2001). A role has also been identified for *wnt11*, encoding a non-canonical Wnt antagonist, in the induction of heart formation by the observation that it is sufficient to induce expression of cardiac markers and differentiation of contractile cardiac tissue in VMZ explants (Pandur and others, 2002). As WNT11 both inhibits β-catenin through the canonical pathway and activates JNKAs WNT11 also inhibits β-catenin through the canonical pathway, Pandur *et al*. injected mRNA encoding a dominant negative LEF-1 and observed that disruptioninhibition of β-catenin signaling alone fails to induce a contractile phenotype in VMZ explants. s. This result indicates that heart induction may require both 1) low levels of Wnt/β-catenin activity and, 2) activation of non-canonical Wnt/JNK signaling through WNT11 activation of the non-canonical Wnt signaling cascade through WNT11 is required for cardiac differentiation in this assay (Pandur and others, 2002). Alternately, it could reflect activity differences between TCF and LEF factors in β-catenin inhibition as another group demonstrated that injection of a dominant negative TCF3 was indeed sufficient to induce cardiogenesis in *Xenopus* animal caps.

Explants have also been used to investigate factors required for induction of cardiogenesis via a loss-of-function approach. For example, MO knockdown of *hex* (a transcription factor induced by antagonists of the canonical Wnt pathway) in DMZ explants resulted in loss of

cardiac markers, indicating that heart induction by Wnt antagonists relies upon activation of *hex* (Foley and Mercola, 2005).

The *Xenopus* animal cap also serves as a useful tissue for cardiogenic assays and can be used to examine the ability of various molecules to affect cardiac gene expression and differentiation. The animal cap consists of prospective ectoderm at the animal pole of a blastula embryo and is fated to become epidermal and neural tissues. Because the ectodermal cells of the animal pole are pluripotent, they can be induced to give rise to alternate cell lineages, including mesodermal derivatives such as the heart. Logan and Mohun demonstrated that cardiac muscle is induced in animal caps treated with high concentrations of the mesoderm-inducing factor activin (Logan and Mohun, 1993). A more recent protocol involving the dissociation and reaggregation of animal caps in the presence of activin results in beating animal cap explants that can form ectopic hearts in *Xenopus* adults following transplantation into the hosts at embryonic stages (Ariizumi and others, 2003). As with DMZ and VMZ explants, animal caps have been used to advance our understanding of the role of WNT signaling during cardiogenesis. Activin-induced expression of GATA factors in animal caps is abolished upon injection of an inducible form of β-catenin, suggesting that Wnt signaling may act to repress *gata* gene expression to restrict cardiogenesis (Afouda and others, 2008). In addition, inhibition of *gata4* and *gata6* by MO injection in animal cap explants results in decreased *wnt11* expression, whereas injection of inducible versions of *Gata4* and *Gata6* results in upregulation of *wnt11* expression, placing GATA factors in a regulatory pathway that links canonical and non-canonical Wnt signaling during cardiogenesis (Afouda and others, 2008; Pandur and others, 2002).

Finally, prospective cardiac tissue itself can be explanted from the embryo and used for cardiogenic assays in the absence of the rest of the embryo. This technique is particularly useful when it is necessary to bypass an early embryonic requirement for a gene to assess its later role in the developing heart. In short, tissue posterior to the cement gland including the heart field can be excised starting at stage 22 when the cardiac precursor populations form a ridge of tissue on top of underlying endoderm. In isolation, these explants will go on to form beating hearts in culture (Langdon and others, 2007; Raffin and others, 2000). Using this assay, a recent study identified a role for SHP-2, a protein tyrosine phosphatase that is disrupted in human CHD, in the maintenance of cardiac progenitors (Langdon and others, 2007).

Xenopus Transgenesis

The development of transgenesis in *Xenopus* has allowed investigators to introduce heritable genetic modifications into the frog genome, propelling *Xenopus* forward as both a genetic and developmental model. Transgenic procedures in *Xenopus* have primarily been used for promoter/enhancer analyses and for expressing transgenes in a tissue-specific manner with defined promoters. Early experiments involving microinjection of circular or linear DNA demonstrated that integration into the genome occurred in a significant number of injected embryos (Andres and others, 1984; Bendig and Williams, 1984; Etkin and others, 1984; Etkin and Roberts, 1983; Rusconi and Schaffner, 1981). The first characterization of transgenic frogs produced by this method showed that the resulting animals were mosaic and that the copy number was highly variable, even within cells from the same animal (Etkin and Pearman, 1987). It was not until the development of the Restriction Enzyme Mediated Integration (REMI) strategy that the problem of mosaicism was overcome (Kroll and Amaya, 1996b). Using this method, sperm nuclei are incubated with linearized DNA and a restriction enzyme along with egg extracts which promote DNA decondensation. The modified sperm nuclei are then injected into unfertilized eggs, where the foreign DNA is

believed to integrate randomly into the genomic DNA during the DNA repair process prior to the first cell division (Amaya and Kroll, 1999; Smith and others, 2006).

Other methods of transgenesis that utilize different core insertion techniques have been used with varying success. Transposable elements such as *Sleeping Beauty* or the *Tol2* transposon have been used as an alternative to REMI to facilitate transgene integration in *Xenopus* (Choo and others, 2006; Hamlet and others, 2006; Kawakami and others, 2004; Kawakami and others, 2000; Parinov and others, 2004; Sinzelle and others, 2006; Yergeau and others, 2009; Yergeau and Mead, 2007). The more commonly used transposon *Tol2* is an active and autonomous transposable element that can integrate into one or multiple sites in the genome and has been used successfully in zebrafish for insertional mutagenesis, although this method of transgenesis in *Xenopus* has not been as efficient as expected. Other groups have optimized the use of integrase-mediated transgenesis utilizing the bacteriophage ΦC31 and the more frequently used *I-SceI* meganuclease (Allen and Weeks, 2005; 2009). The *I-SceI* meganuclease, originally isolated from *Saccharomyces cerevisiae*, is used to digest transgene DNA containing *I-SceI* 18-bp recognition sites and this reaction mixture is injected into unfertilized eggs where it integrates randomly into the host genome. Copy number of integrated transgenes is relatively low, ranging from one to four, compared to the REMI method which typically results in the integration of transgene concatemers (two to six copies) at four to eight sites in the genome (Kroll and Amaya, 1996a; . Jacquier and Dujon, 1985; Ogino and others, 2006; Pan and others, 2006).

The developmental regulation of several cardiac genes has been characterized with *Xenopus* transgenesis, leading to a clearer picture of the complex gene regulatory networks that guide heart development. This commonly involves *in vivo* analysis of *cis*-regulatory regions that drive cardiac expression in the heart and is accomplished by inserting the promoter of interest upstream of a reporter transgene, such as green fluorescent protein (GFP), to follow transgene expression in the live embryo. This approach has been used to identify cardiac-specific regulatory elements for *atrial natriuretic factor* (*anf*), *cardiac α-Actin*, *myosin Light Chain 2* (*mlc2*), *myosin light chain 1v* (*mlc1v*), *nkx2–5*, *alpha myosin heavy chain* (α-*mhc*), and *tbx20* (Garriock and others, 2005b; Latinkić and others, 2004; Latinkić and others, 2002; Mandel and others, 2010; Small and Krieg, 2003; Smith and others, 2005; Sparrow and others, 2000). Additional studies examining the mechanisms of regulating these cardiac elements have revealed much about the signaling pathways that act on cardiac genes during development. Importantly, many of these regulatory elements are evolutionarily conserved as they are sufficient for cardiac-specific expression in other vertebrates including mice.

One of the benefits of transgenesis is the ability to tightly control the spatial and temporal expression of a transgene during development with tissue-specific promoters. Several cardiac promoters that drive expression throughout the developing heart have been used with great success to misexpress a gene of interest specifically in the heart-forming region. Using transgenic embryos expressing *bmp4* under the control of the *mlc2* promoter, Breckenridge *et al*. demonstrated that ectopic expression throughout the developing heart results in randomization of the direction of cardiac looping, indicating that asymmetric BMP4 signaling is required for proper cardiac looping (Breckenridge and others, 2001).

Congenital Heart Disease

Atrial Septal Defects: *Nkx2.5* and *Gata4*

Atrial Septal Defects (ASD) are relatively common and account for 10% of all human congenital heart defects (Hoffman and Kaplan, 2002). ASD refers to a failure of the atrial septum to fully separate the right and left atrial chambers after birth and is often accompanied by other forms of CHD including cardiac conduction system abnormalities.

The cardiac transcription factor *Nkx2.5* is mutated in a number of patients with non-syndromic ASD, suggesting a critical function for this gene in the septogenesis process (Benson and others, 1999; Schott and others, 1998).

Nkx2.5 encodes a homeodomain protein that is highly conserved from *Drosophila* to human. *Nkx2.5* is expressed early in development, and in combination with other cardiac transcription factors, helps define the cardiogenic field in which it plays an essential role in the specification of cardiac progenitors from the cardiogenic mesoderm. Cardiac expression of *Nkx2.5* persists into adulthood, however, a complete understanding of the role NKX2.5 plays during the later stages of heart formation has been complicated by the fact that targeted disruption of murine *Nkx2.5* results in embryonic lethality at E9-E10, prior to heart looping (Lyons, 1995). Interestingly, a recent study in *Xenopus* demonstrated that injection of two truncated forms of *Xenopus laevis* NKX2.5, corresponding to two human NKX2.5 point mutations identified in patients with cardiac defects including ASD and atrioventricular conduction delays, results in atrial septal and conduction system defects (Bartlett and others, 2007; Schott and others, 1998). Significantly, this study focused on the effects of injecting mutant forms of *nkx2.5*on internal changes to the hearts of stage 46 embryos when the *Xenopus* heart is fully looped with chambers, septae, valves, and a functional conduction system (Bartlett and others, 2007). The results suggest that early expression of mutant *nkx2.5* in the frog can lead to a late phenotype that includes cardiac defects consistent with those seen in human disease.

NKX2.5 has also been shown to physically interact with the cardiac zinc finger transcription factor GATA4, an additional gene mutated in in patients with ASD (Durocher and others, 1997; Garg and others, 2003; Lee and others, 1998; Pehlivan and others, 1999; Sepulveda and others, 1998). The NKX2.5-GATA4 interaction synergistically activates cardiac promoters during the cardiogenic program, suggesting that deficiencies in either member of this transcriptional complex can result in ASDs (Durocher and others, 1997; Lee and others, 1998; Sepulveda and others, 1998). Studies utilizing the embryonic carcinoma P19 cell line have shown *Gata4* to be essential for cardiac differentiation. However, *Gata4* null mice can generate differentiated cardiac myocytes that express contractile proteins but are deficient in ventral morphogenesis, resulting in a failure of cardiomyocytes to form a linear heart tube at the ventral midline (cardia bifida) (Grepin and others, 1997; Grepin and others, 1995; Kuo and others, 1997; Molkentin and others, 1997). Recent studies in *Xenopus* complement the mouse work and demonstrate with MOs that *gata4* is dispensable for cardiac specification but essential for proper heart morphogenesis downstream of the induction of the myocardium (Haworth and others, 2008). Interestingly, when GATA4, GATA5, and GATA6 are all depleted from *Xenopus* embryos, *myosin heavy chain* expression is completely lost from most morphant embryos, suggesting that there is GATA factor redundancy in the regulation of myocardial differentiation, providing a possible explanation for the persistent presence of differentiated cardiomyocytes in $Gata4^{-/-}$ mice (Peterkin and others, 2007).

DiGeorge Syndrome: *Tbx1*

DiGeorge syndrome (DGS) is a congenital disorder that has many overlapping characteristics with velo-cardio-facial syndrome and conotruncal anomaly face syndrome due to shared deletions within chromosome band *22q11.2* (Baldini, 2004; Yamagishi and Srivastava, 2003). For this reason, these syndromes are collectively known as *22q11* deletion syndrome (*del22q11DS*). Approximately 80% of neonates displaying *del22q11DS* have congenital heart defects that include Tetralogy of Fallot, persistent truncus arterious, and cardio-facial abnormalities (Baldini, 2004; Di Felice and Zummo, 2009; Epstein, 2001; Momma, 2010; Starr, 2010; Yamagishi and Srivastava, 2003).

DGS is one of the most prevalent chromosomal microdeletion genetic disorders. The region of chromosome *22q11.2* that is deleted in DGS encompasses 1.5 to 3 Mb and includes 24–30 genes (Baldini, 2004; Epstein, 2001; Yamagishi and Srivastava, 2003). A heterozygous mouse genetic model in which the orthologous chromosomal region affected in DGS is deleted displays similar phenotypes as those in human patients (Lindsay and Baldini, 2001; Lindsay and others, 1999). The deleted region frequently includes the locus encoding the T-box transcription factor *Tbx1*. In a genetic analysis screen, five patients were identified who exhibited DGS phenotypes and had *Tbx1* mutations but not chromosomal microdeletions, suggesting that *Tbx1* may be contributing to the DGS phenotype in these individuals (Yagi and others, 2003). DGS abnormalities have been correlated with disrupted pharyngeal and neural crest patterning during development. Subsequently, *Tbx1* was shown to be expressed in the pharyngeal arches, and mouse genetic models have demonstrated that *Tbx1* haplo-insufficiency disrupts the development of the fourth pharyngeal arch arteries, possibly in conjunction with FGF8 signaling (Ataliotis and others, 2005; Baldini, 2004; Chapman and others, 1996; Kochilas and others, 2002; Kochilas and others, 2003; Lindsay and others, 2001b; Merscher and others, 2001; Sauka-Spengler and others, 2002; Showell and others, 2006; Vitelli and others, 2002). In addition, *Tbx1* plays a role in growth and septation of the outflow tract (OFT). Conditionally ablating *Tbx1* in the *Nkx2.5* domain of the secondary heart field results in mild pharyngeal defects and a severe defect in aorto-pulmonary septation of the OFT that is associated with neural crest migration defects and reduced proliferation of cells in the secondary heart field (Waldo and others, 2001; Xu and others, 2004). FGF signaling may be involved in the latter event as there is a reduction in *Fgf10* expression in the secondary heart field in *Tbx1*-null mice. Further, *Fgf10* is a direct transcriptional target of *Tbx1 in vitro* (Waldo and others, 2001; Xu and others, 2004). Interestingly, the defect in OFT septation suggests a dose-dependent role for *Tbx1* because this phenotype can be partially rescued upon reestablishing *Tbx1* expression (Jerome and Papaioannou, 2001; Lindsay and others, 2001a; Xu and others, 2004).

Tbx1 has been identified in many vertebrates including *Xenopus laevis* and *Xenopus tropicalis*. In these model systems, the expression domains of *tbx1* replicate those seen in other vertebrates, namely the pharyngeal arches, otic vesicle, and mesenchyme surrounding the OFT (Ataliotis and others, 2005; Chapman and others, 1996; Kochilas and others, 2003; Sauka-Spengler and others, 2002; Showell and others, 2006). A dominant interfering mutant of *tbx1* injected into *Xenopus* embryos results in very similar phenotypes to those of mice deficient in *Tbx1*, including pharyngeal defects, unlooped heart, pericardial edema, and a reduction in anterior structures. These defects can be rescued by co-injecting wild-type *tbx1* mRNA. To lineage trace the fate of TBX1-deficient cells, Ataliotis *et al*. co-injected β-*galactosidase* mRNA and the dominant interfering mutant *tbx1* mRNA into *Xenopus* embryos and identified a requirement for TBX1 in cells that contribute to pharyngeal mesoderm (Ataliotis and others, 2005). Additionally, recent advances in *Xenopus* transgenesis have enabled researchers to analyze cardiac and craniofacial phenotypes in embryos with reduced functions of specific genes, effectively generating models of CHD such as DGS. Using the active promoter of *mlc1v* to drive GFP, craniofacial and cardiac muscle formation was followed in *Xenopus* embryos injected with the dominant interfering mutant of *tbx1*, enabling real-time visualization of cardiac structural defects in developing embryos (Smith and others, 2005).

Holt-Oram Syndrome: *Tbx5*

Holt-Oram Syndrome (HOS), also known as heart-hand syndrome, is a congenital autosomal dominant disorder that primarily affects the heart and upper limbs (Holt and Oram, 1960). HOS is the most common heart-hand syndrome, affecting nearly 1 in 100,000 total births (Basson and others, 1994). Approximately 75% of patients with HOS experience cardiac

defects, most commonly ASD, ventricular septal defects (VSD), and/or defects in the cardiac conduction system (Basson and others, 1994; Benson and others, 1996; Cross and others, 2000; McDermott and others, 2005; Newbury-Ecob and others, 1996). Atypical phenotypes have also been discovered and characterized, and phenotypic expression is variable even within families (Brassington and others, 2003; Garavelli and others, 2008; Lehner and others, 2003; McDermott and others, 2005; Newbury-Ecob and others, 1996; Sletten and Pierpont, 1996). HOS is often caused by mutations in the coding region of the T-box transcription factor *Tbx5* on chromosome *12q.24.1* (Basson and others, 1997; Basson and others, 1999; Li and others, 1997). More than 70% of patients with HOS have a mutation in the *Tbx5* coding exons, and 85% of these mutations are *de novo* (McDermott and others, 2005). Most HOS mutations are predicted to result in haploinsufficiency of *Tbx5* (Basson and others, 1999; Li and others, 1997).

Mice lacking *Tbx5* do not survive past E10.5 due to arrested cardiac development caused by impaired cardiac differentiation (Bruneau and others, 2001). Heterozygous $Tbx5^{del/+}$ mice display subtle defects in the paw and wrist, enlarged hearts with ASD, cardiac conduction defects, and a variety of additional complex cardiac defects reminiscent of patients with HOS. The expression of several cardiac genes is reduced in mice lacking *Tbx5*. Two of these genes, *ANF* and *Cx40*, are also reduced in mice expressing 50% of the normal TBX5 levels (Moskowitz and others, 2004). Similar heart and limb defects are observed in the orthologous *Tbx5* zebrafish mutant *heartstrings*, suggesting that both the expression domain and protein function of *Tbx5* are conserved among vertebrates (Garrity and others, 2002). In *Xenopus*, *tbx5* is first expressed in the migrating heart primordia and eye anlage of the late neurula embryo. Its expression is maintained in the primitive heart tube, although its expression becomes more graded after looping of the heart, with higher expression in the ventricle than the atria (Horb and Thomsen, 1999; Showell and others, 2006). Consistent with work in other organisms, *Tbx5* was demonstrated to be critical for proper heart morphogenesis in *Xenopus* (Brown and others, 2005; Horb and Thomsen, 1999). Overexpression of a dominant negative hormone-inducible form of TBX5 blocks heart tube formation, whereas knockdown of *tbx5* expression by MO results in reduced cardiac cell number and an unlooped heart tube (Brown and others, 2005; Horb and Thomsen, 1999). The decrease in cardiac cell number in the *tbx5* morphant embryos was demonstrated to result from a proliferation defect caused by a delay or arrest in the G1/S phase of the cell cycle, implicating a role for TBX5 in cardiac cell cycle control (Goetz and others, 2006). These results from *Xenopus* as well as those from human studies (Hatcher and others, 2001), suggest that HOS defects may in part arise from a decrease in cell cycle progression and cardiac cell proliferation in *Tbx5*-expressing regions. In addition, work in a number of model systems has demonstrated a conserved role for *Tbx5* in the regulation of cardiac-specific gene expression (Brown and others, 2005; Bruneau and others, 2001; Garrity and others, 2002; Hatcher and others, 2001; Hiroi and others, 2001; Liberatore and others, 2000; Plageman and Yutzey, 2004).

Spectrum of Congenital Heart Defects: *Tbx20*

Tbx20 is a member of the T-box family of transcription factors and is one of the first genes to be expressed in the cardiac lineage along with *Nkx2.5*, *Gata4*, and *Tbx5*. In all species examined, expression of *Tbx20* is maintained throughout the primary heart field, in both myocardium and endocardium, as development proceeds and persists in the adult heart. Kirk *et al*. were the first group to identify mutations in human *Tbx20* in patients with familial CHD (Kirk and others, 2007). The two mutations identified are both in the T-box DNA binding domain and segregate with a spectrum of cardiac pathologies including ASD, VSD, valve disease, pulmonary hypertension, and cardiomyopathy. Loss-of-function mutations were the first to be identified. However, several other groups have since identified new

Tbx20 mutations with both loss- and gain-of-function that are associated with CHD (Liu and others, 2008; Posch and others; Qian and others, 2008). In addition, upregulation of *Tbx20* expression has been noted in patients with Tetralogy of Fallot (Hammer and others, 2008). The wide range of defects associated with mutant or misregulated *Tbx20* may be the result of the expression of *Tbx20* in both myocardium and endocardium, where endocardial cushions give rise to valves and the interventricular septum.

Although the early expression of *Tbx20* in cardiogenic mesoderm suggests a role in the specification, migration, and/or differentiation of cardiac progenitors, a requirement for TBX20 is not evident until the early stages of heart morphogenesis, as shown by studies in fish and frogs (Brown and others, 2005; Szeto and others, 2002). Upon MO knockdown of *Tbx20* in zebrafish and *Xenopus* embryos, morphant embryos display unlooped heart tubes and pericardial edema, but express markers of cardiac specification and differentiation, indicating an essential role for TBX20 in cardiac morphogenesis. In addition, TBX20-depleted *Xenopus* hearts have reduced cardiomyocyte cell numbers and fail to properly form chambers (Brown and others, 2005). Likewise, mice lacking *Tbx20* undergo normal cardiac specification and differentiation, but development is arrested in the primary linear heart tube stage, and chamber differentiation is not initiated (Cai and others, 2005; Singh and others, 2005; Stennard and others, 2005; Takeuchi and others, 2005). There also appears to be a proliferation defect in *Tbx20* null hearts that is thought to be mediated by a loss of repression of *Tbx2*, thereby allowing aberrant repression of the cell cycle gene *N-Myc* in chamber myocardium (Cai and others, 2005; Singh and others, 2005). The misregulation of *Tbx2* in *Tbx20* mutant hearts may partially explain the loss of cardiomyocytes seen in *Xenopus* and mouse *Tbx20* mutants.

The frequent occurrence of cardiac defects resulting from perturbations in the complex regulatory network guiding the cardiomyogenic program highlights the importance of understanding the interactions that occur between members of this network. *Xenopus* embryos co-injected with MOs against *tbx20* and *tbx5* display a more severe cardiac phenotype than single mutants, indicating that TBX20 and TBX5 cooperate to regulate cardiac morphogenesis (Brown and others, 2005). TBX20 physically interacts with the cardiac transcription factors NKX2.5, GATA4, GATA5, and TBX5 and, in transcription assays, TBX20 synergistically activates cardiac promoters in the presence of NKX2.5, GATA4, and ISLET1 (Brown and others, 2005; Stennard and others, 2003; Takeuchi and others, 2005). Surprisingly, in transient transcription assays, the shorter TBX20b isoform, which is terminated shortly after the T-box domain, is more effective at activating reporter gene expression than the longer TBX20a that is the predominant isoform expressed in the heart during development (Stennard and others, 2003). To determine which TBX20 isoform promotes changes in morphogenesis and gene expression *in vivo*, *Tbx20a* and *Tbx20b* mRNAs were injected into *Xenopus* embryos. Overexpression of *Tbx20a*, but not *Tbx20b*, results in multiple developmental defects, including shortening of the anterior/posterior axis and secondary axis formation (Stennard and others, 2003). *Tbx20* mRNAs were also injected into explanted *Xenopus* animal pole caps resulting in an upregulation of the early mesoderm marker *Xbra* and the cardiac marker *Nkx2.5* in the *Tbx20a*–injected, but not *Tbx20b*–injected, caps (Stennard and others, 2003). These studies suggest that the C-terminal domain of TBX20a is essential for TBX20 activity in the embryo and highlight the utility of *Xenopus* embryo assays for investigating the biological relevance of *in vitro* findings.

Noonan Syndrome: *Shp-2*

Noonan syndrome is one of the most common forms of CHD. The disorder leads to several cardiac developmental abnormalities including ASD, VSD, pulmonary stenosis, and hypertrophic cardiomyopathy (Noonan, 1968; 1994). Noonan syndrome was shown to be

associated with mis-sense mutations in *SHP-2* in approximately half of affected individuals (Kosaki and others, 2002; Maheshwari and others, 2002; Tartaglia and others, 2002; Tartaglia and others, 2001). *Shp-2* mis-sense mutations are associated with a gain-of-function and are thought to result in prolonged downstream activation of several growth factors including epidermal growth factors (EGFs), fibroblast growth factors (FGFs), and platelet-derived growth factor (Feng, 1999; Feng and others, 1994; Qu, 2000; Tartaglia and others, 2001; Van Vactor and others, 1998; Zhang and others, 2000). Interestingly, patients with acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), and juvenile myelomonocytic leukemia (JMML) carry a second, mostly mutually exclusive, somatically introduced subset of mis-sense mutations in *Shp-2*, strongly suggesting a genotype-phenotype relationship between *Shp-2* mis-sense mutations and disease (Bentires-Alj and others, 2004; Kratz and others, 2005; Loh and others, 2004; Musante and others, 2003; Tartaglia and others, 2003). However, the cellular and biochemical basis for the role of SHP-2 in Noonan syndrome, AML, ALL, and JMML is unknown.

Shp-2 is a widely expressed non-receptor tyrosine phosphatase comprised of two tandemly arranged SH2 domains and a protein tyrosine phosphatase (PTP) domain. *Shp-2*, also known as *Sh-Ptp2*, *Ptpn11*, *Ptp1d*, and *Ptp2c*, is the vertebrate homologue of the Drosophila gene *corkscrew* (*csw*). The sequence, expression pattern, and function of *Shp-2* are highly conserved throughout evolution. For example, *Xenopus* and human orthologues display 94% sequence identity, and as in fly and mouse, *Xenopus shp-2* is believed to be ubiquitously expressed (Langdon and others, 2007; Tang and others, 1995). Moreover, several animal models have suggested a critical role for *Shp-2* in vertebrate development. For example, mice expressing an internal deletion of the amino-terminal (N-SH2) domain of *Shp-2* die at late gastrulation and display several mesodermal abnormalities including heart and vascular defects (Saxton and others, 1997; Saxton and Pawson, 1999; Yang and others, 2006). In addition *Shp-2* mutant cells derived from homozygous mutant embryos show that *Shp-2* is required for full and sustained activation of the MAPK pathway in response to FGF, thus demonstrating that SHP-2 functions downstream of the FGF/MAPK pathway *in vivo* (Saxton and others, 1997; Saxton and Pawson, 1999). Consistent with these findings, studies in *Xenopus* have shown that a dominant negative form of *Xenopus Shp-2* can completely block mesoderm formation in response to both MAPK and FGF (Tang and others, 1995). Furthermore, *in vitro* and tissue culture studies have shown that *csw/Shp-2* interacts directly with the FGF inhibitor SPROUTY, leading to SPROUTY phosphorylation and inactivation (Hanafusa and others, 2004; Jarvis and others, 2006).

In the mouse and chick, SHP-2 is required in the EGF pathway for formation of cardiac valves. However, because approximately one-third of patients with Noonan-associated heart defects appear to undergo normal valvulogenesis (Chen and others, 2000; Krenz and others, 2005), it remains unclear if SHP-2 is required downstream of other receptor tyrosine kinase receptors for other aspects of heart development. To address whether SHP-2 functions in cardiac pathways in addition to EGF and valvulogenesis and to bypass the early embryonic requirements for SHP-2, Langdon et al. (2007) used a *Xenopus* cardiac explant assay and chemical SHP-2 inhibitors to demonstrate that SHP-2 is required for the survival of actively proliferating cardiac progenitor populations but not those that have exited the cell cycle. It was further demonstrated that SHP-2 is directly phosphorylated on specific residues *in vivo* in response to FGF signaling, that SHP-2 co-immunoprecipitates with the FGF receptor adaptor, and that a constitutively active Noonan-associated *Shp-2* mutation can rescue cardiac defects induced by FGF inhibition. Collectively, these studies imply that SHP-2 functions in the FGF/MAPK pathway to maintain survival of proliferating populations of cardiac progenitor cells. However, it remains to be determined why mis-sense mutations in *Shp-2* lead to a tissue-specific effect in animals and humans.

Heterotaxy and Cardiac Looping Defects: *Zic3*

Heterotaxy (*situs ambiguus*) is a spectrum disorder in which the position of thoracic and abdominal organs is abnormal. Heterotaxy malformations are thought to arise from defective left-right patterning during embryonic development.

Establishing laterality in the embryo is a complex process involving a multitude of spatio-temporal signaling events (Boorman and Shimeld, 2002; Mercola, 1999). Initially, cells in the left-right coordinator (posterior notochord in mammals, gastrocoel roof plate in *Xenopus*, and Kupffer's vesicle in zebrafish) adjacent to the organizing node develop specialized motile cilia that generate a leftward fluid flow and an asymmetrical morphogen gradient (Blum and others, 2009; Sutherland and Ware, 2009; Tabin and Vogan, 2003). The subsequent lateralized expression of nodal, a member of the transforming growth factor β (TGFβ) family, is then thought to be involved in specifying left-right asymmetry via the notch signaling pathway (Krebs and others, 2003; Raya and others, 2003). *Pitx2*, a paired homeobox transcription factor, also plays a crucial role in organ symmetry, particularly in heart looping, downstream of nodal signaling (Ryan and others, 1998).

One of the first major morphological symmetry-breaking events in vertebrates occurs when the relatively symmetrical heart tube undergoes a rightward (dextral) bend, after which a complex process of looping and septation results in the mature multi-chambered heart (Boorman and Shimeld, 2002; Manner, 2000; 2009; Mercola, 1999). Cardiac looping defects are commonly observed in cases of heterotaxy, and these defects account for approximately 3% of all CHDs. Other common heart phenotypes seen in heterotaxic patients include ASD, VSD, transposition of the great arteries, double outlet right ventricle, single ventricle, and aortic arch defects (Belmont and others, 2004; Bowers and others, 1996; Lin and others, 2000; Sutherland and Ware, 2009). Numerous cases of familial clustering of heterotaxy have been identified, suggesting autosomal inheritance of the disorder. However, X-linked inheritance has also been shown, involving mutations in the conserved zinc-finger transcription factor gene, *Zic3* (Gebbia and others, 1997; Ware and others, 2004). Heart defects and altered nodal expression are observed in *Zic3* mutant mice (Purandare and others, 2002). In *Xenopus* embryos, *zic3* is expressed in the mesoderm of the gastrulating embryo in a left-right (L–R) symmetrical fashion, however, unilateral right-sided overexpression of *zic3* is sufficient to disturb the L-R axis, resulting in abnormal heart and gut looping and affecting the lateral expression of *pitx2* and *nodal related 1 (XnrI)* (Kitaguchi and others, 2000). *Xenopus zic3* is therefore considered to have a conserved early role in transducing signals from the left-right organizer and establishing asymmetry (Kitaguchi and others, 2002; Kitaguchi and others, 2000).

The amphibian model system has historically been used to study left-right patterning since the early 1900s by Spemann and colleagues. Subsequently, the *Xenopus* model was established, and has proven ideally suited to study the role of left-right laterality, using well-accepted techniques such as lineage tracing and fate mapping, in the process of heart development (Blum and others, 2009; Gormley and Nascone-Yoder, 2003). One of the first events in *Xenopus* embryonic development is cleavage at the one-cell stage to form two blastomeres, the descendents of which will contribute almost exclusively to either the left or the right side of the embryo. This feature enables researchers to independently alter signaling events or gene expression unilaterally to determine their effect on asymmetry and to conduct left-right lineage tracing experiments, particularly of the heart region (Branford and others, 2000; Chen and others, 2004; Dagle and others, 2003; Jahr and others, 2008; Kitaguchi and others, 2002; Kitaguchi and others, 2000; Ramsdell and others, 2006; Toyoizumi and others, 2006). Recent work in *Xenopus* has demonstrated that cell lineages in the heart display a high degree of asymmetry, and that defects in left-right patterning alter

cardiomyocyte allocation and differentiation in the heart, leading to cardiac malformations (Chen and others, 2004; Ramsdell and others, 2006). The *Xenopus* model is therefore an optimal organism to study the fate of cardiac cell populations and to determine how specific genes such as *Zic3* may be involved in establishing laterality in the heart and their roles in heterotaxic phenotypes.

Axenfeld-Reiger Syndrome: *Pitx2* and *FoxC1*

Axenfeld-Rieger syndrome (ARS) is a complex autosomal dominant disorder primarily characterized by anomalies of the anterior segment of the eye, face, teeth, and umbilical stump. Congenital heart defects, including ASD, pseudotruncus arteriosus, and mitral valve and intraventricular septal defects have also been reported in a number of patients with ARS (Akkus and Argin, 2010; Antevil and others, 2009; Aysenur Pac and others, 2008; Baruch and Erickson, 2001; Bekir and Gungor, 2000; Calcagni and others, 2006; Cunningham and others, 1998; Davies and others, 1999; Grosso and others, 2002; Maclean and others, 2005; Mammi and others, 1998; Weisschuh and others, 2008). Linkage analyses have identified four different loci in humans, *4q25*, *6p25*, *13q14*, and *16q24*, each of which has been independently associated with ARS. Further analyses of *4q25* and *6p25* in patients with ARS have uncovered mutations in two genes, *Pitx2* and *Foxc1*, respectively (Amendt and others, 2000; Hjalt and Semina, 2005; Maclean and others, 2005).

Pitx2 is a highly conserved homeodomain transcription factor that is expressed asymmetrically in the left lateral plate mesoderm in chick, zebrafish, *Xenopus*, and mouse embryos (Campione and others, 1999; Ryan and others, 1998). At heart-forming stages, *pitx2* expression continues to be restricted to the left half of the heart tube in *Xenopus* embryos. In mouse, *Pitx2* is expressed in the left side of the heart tube and in the left ventricle, OFT, and atrium during heart looping (Ryan and others, 1998). The defects observed in *Pitx2* null and hypomorphic mice, such as altered looping of the heart, absence of atrial septation, and dysmorphic ventricular septation, recapitulate the defects observed in human ARS patients with *Pitx2* dysfunction (Gage and others, 1999; Lin and others, 1999; Lu and others, 1999).

The use of *Xenopus* has been instrumental in understanding the dual role of *Pitx2* in heart development, firstly in directing the looping of the heart tube and secondly in controlling the morphogenesis of the cardiac chambers. Misexpression of *pitx2* by injection of its mRNA on the right side of the *Xenopus* embryo results in a reversal of heart looping, showing the conserved role of *pitx2* in directing this event. The restricted expression of *pitx2* is likely to be downstream of the TGFβ signaling family, as bilateral injections of mRNA encoding nodal or activin results in bilateral expression of the gene (Campione and others, 1999). Further, injection of a dominant negative form of the activin type II receptor into *Xenopus* embryos alters *pitx2* expression levels and subsequent heart looping. These findings are supported by similar experiments in chick (Ryan and others, 1998). Of the three isoforms of *Pitx2* present during development, experiments in *Xenopus*, zebrafish, and mouse demonstrate that *Pitx2*c is the isoform that is specifically expressed in heart (Essner and others, 2000; Schweickert and others, 2000). The injection of modified antisense oligonucleotides that mediate degradation of *pitx2*c mRNA in *Xenopus* embryos results in cardiac defects that are very similar to phenotypes observed in *Pitx2* mutant mice, including abnormal atrial septation, extracellular matrix restriction, abnormal positioning of the atrial and ventricular chambers, and restriction of ventricular development. These tadpoles also exhibit dramatic straightening of the OFT, followed by a rightward migration (Dagle and others, 2003). This study demonstrates the conservation of *pitx2* function in *Xenopus* cardiac development and its relationship to ARS. It has recently been shown that *Pitx2* patterns the second heart field and is required to specify the left versus right atrium (Ai and others, 2006;

Galli and others, 2008; Liu and others, 2002). It will be interesting to determine if *Xenopus* can be exploited as a useful model for testing the effects of various ARS-derived mutations on *Pitx2* function during second heart field development and for further identifying the mechanisms by which *Pitx2* functions.

Foxc1 is a member of the forkhead family of transcription factors and is expressed in endothelial and mesenchymal cells of the developing heart as well as in endocardial cushions derived from cardiac neural crest cells (Iida and others, 1997; Kume and others, 2001; Seo and others, 2006; Winnier and others, 1999). *Foxc1* transcripts have also been detected in the second heart field and in the proepicardium (Seo and Kume, 2006). In the newly formed heart, *Foxc1* is expressed in the atrial septum, the venous, aortic and pulmonary valves, and the mitral and tricuspid valves (Swiderski and others, 1999). Consistent with its widespread expression in the heart, FOXC1 plays a critical role in heart valve formation and atrial septation as suggested by the cardiac defects noted in mice mutant for *Foxc1*. Specifically, *Foxc1* homozygous mutants display interruption or coarctation of the aortic arch, VSD, and pulmonary and aortic valve dysplasia (Winnier and others, 1999). Mice lacking both *Foxc1* and the closely related Fox transcription factor *Foxc2* have even more severe cardiac abnormalities consisting of hypoplasia or lack of the OFT and right ventricle as well as the inflow tract, and dysplasia of the OFT and atrioventricular cushions. These mice also have an abnormally formed epicardium, reduced cell proliferation, and increased apoptosis of neural crest cells (Kume and others, 2001; Seo and Kume, 2006; Winnier and others, 1999).

Foxc1 has been identified in *Xenopus* and is present in cardiac lineages (Gessert and Kuhl, 2009; Koster and others, 1998). Depletion of *foxc1* during early *Xenopus* development results in downregulation of adhesion molecules involved in mesoderm development and increased apoptosis, correlating with the phenotypes observed in the mouse mutants (Cha and others, 2007). It remains to be determined if reduction of FOXC1 levels in *Xenopus* has similar effects on cardiac morphology to those observed in mouse knockouts and patients with ARS. However, the early phenotypes of FOXC1 depletion during *Xenopus* development provide a model in which to investigate the phenotypic changes that result from *Foxc1* disruption.

CHARGE Syndrome: *Chd7*

CHARGE syndrome (Coloboma, Heart defects, choanal Atresia, Retarded growth and development, Genital abnormalities, and Ear anomalies) is a complex disease associated with a number of cardiac abnormalities including Tetralogy of Fallot, atrioventricular canal defects, and aortic arch anomalies (Davenport and others, 1986). The majority of individuals with CHARGE syndrome have mutations in the coding region of *chromodomain helicase DNA binding protein 7* (*Chd7)* (Aramaki and others, 2006; Delahaye and others, 2007; Jongmans and others, 2006; Kaliakatsos and others, 2010; Lalani and others, 2006; Vissers and others, 2004; Wessels and others, 2010; Wincent and others, 2008).

Chd7 expression has been characterized in the chick and mouse, where it has been found to be expressed mainly in the neural ectoderm and branchial arches (Aramaki and others, 2007; Bosman and others, 2005; Hurd and others, 2007). Mutant *Chd7* mice display defects in neural stem cell proliferation, olfaction, and some cardiac defects, including formation of the interventricular septum (Adams and others, 2007; Bosman and others, 2005; Hurd and others, 2007; Layman and others, 2009). It has been proposed that the multiple phenotypes in CHARGE syndrome are caused by defects in neural crest cell (NCC) migration (Siebert and others, 1985). Recently, *Chd7* was found to be expressed in *Xenopus* NCCs as well as in human NC-like cells. MO knockdown of *chd7* in *Xenopus* perturbs the migration of NCCs

to the pharyngeal arches. This phenotype is partially rescued by injecting human *Chd7* mRNA, suggesting that the molecular function of CHD7 is well-conserved. In addition, overexpression of human *Chd7* with a substitution of a conserved lysine residue in its ATPase domain results in a dominant-negative effect (Bajpai and others, 2010). This dominant negative effect recapitulates the major features of CHARGE syndrome described above, including abnormal positioning of the truncus arteriosus and OFT. Analysis of markers associated with NCCs reveal that *chd7* is not required for the induction or survival of NCCs, but for their specification. The expression of *sox9*, *twist*, and *slug*, genes that mark multipotent, migrating NCCs, is severely perturbed in *chd7* downregulated embryos (Bajpai and others, 2010; Sauka-Spengler and Bronner-Fraser, 2008). These studies in *Xenopus* have provided a powerful *in vivo* model in which to study the role of NCCs in heart development and the CHDs that result from improper NCC migration and specification. More recently, the chromatin remodeling factors CHD8 and BRG1 have been shown to physically interact with CHD7 (Bajpai and others, 2010; Batsukh and others, 2010) and *Brg1* was demonstrated to play a role in regulating cardiac growth and differentiation (Hang and others, 2010). The roles of *Chd8* and *Brg1* in a *Xenopus* model of CHARGE syndrome could aid in the understanding of this complex syndrome.

Future Directions and Emerging Technologies in *Xenopus*

Investigating a Role for the Epicardium in Congenital Heart Disease

The epicardium is a mesothelial sheet of cells surrounding the myocardium of the developing looped heart in many vertebrate organisms (Hirakow, 1992; Ho and Shimada, 1978; Jahr and others, 2008; Manner and others, 2001; Pombal and others, 2008; Serluca, 2008; Viragh and Challice, 1981). The epicardial structure arises from the pro-epicardial organ (PEO), which is situated on the sinus venosus. These mesothelial cells cluster and bridge over towards the ventricular surface of the heart and migrate onto the myocardial surface as an epithelial-like sheet. Subsequently, subsets of epicardial cells undergo epithelial-mesenchymal transition (EMT) and migrate into the sub-epicardial space and myocardium where they differentiate into various cell populations including fibroblasts and smooth muscle cells of the coronary vasculature (Lie-Venema and others, 2007; Manner and others, 2001; Winter and Gittenberger-de Groot, 2007). The epicardium is thought to play a mitogenic role in cardiomyocyte growth and has been shown to be important for cardiac repair in adult zebrafish (Lepilina and others, 2006). Recently, adult human epicardium-derived cells (EPDCs) were demonstrated to have a paracrine role in improving mammalian cardiac function when co-transfected with cardiomyocytes into an infarcted murine heart (Winter and others, 2007; Winter and others, 2009). Thus, the epicardium may have the potential to stimulate cardiac repair and regeneration, given the right conditions.

Various congenital heart diseases display abnormalities that may arise from improper epicardium formation or differentiation of EPDCs. In avian embryos in which either the pro-epicardial organ is ablated or the epithelial-mesenchymal transition of EPDCs is disrupted, defects are seen in the compact layer of the myocardium, while the inner curvature of the heart is wider and often displays a double outlet right ventricle, indicative of a heart looping defect (Gittenberger-de Groot and others, 2000; Lie-Venema and others, 2005; Manner and others, 2005). Ventricular non-compaction is also seen when genes involved in epicardium formation, e.g., *Wt1* and *RXRα*, are knocked out in the mouse (Merki and others, 2005; Moore and others, 1998). In addition to their roles in regulating the development of the compact ventricular and atrial myocardia, EPDCs are involved in the development of cardiac structures associated with the conduction system. The annulus fibrosus, which plays an important insulating role in the cardiac conduction system, is derived from the epicardium, and EPDCs also influence the formation of the peripheral Purkinje fiber network from ventricular cardiomyocytes (Eralp and others, 2006; Zhou and others, 2010).

Electrophysiological cardiac defects, such as Wolff-Parkinson-White syndrome and Mahaim tachycardia, may therefore have an origin in improper epicardium or EPDC formation.

EPDCs also contribute cells to the atrioventricular cushions and valves, and disrupting epicardium formation can lead to aberrant valve formation (Gittenberger-de Groot and others, 2000; Gittenberger-de Groot and others, 1998; Manner and others, 2005; Perez-Pomares and others, 2002). It is conceivable that disorders in which the valve leaflet has not fully delaminated - for example, Ebstein's anomaly (tricuspid valve leaflet) - might result from defects in epicardial patterning or signaling (Attenhofer Jost and others, 2005; Lie-Venema and others, 2007). Ventricular non-compaction may also be indicative of an epicardial defect as shown by mouse knockout models of genes involved in epicardium formation, e.g., *Wt1* and *RXRα*, or in PEO ablation studies in chick (Manner and others, 2005; Merki and others, 2005; Moore and others, 1998; Sucov and others, 2009). Interestingly, the *Fog-2* knockout mouse, which has an epicardial defect phenotype, displays many of the anomalies described above - including tricuspid atresia, thin myocardium, double outlet right ventricles, and VSD (Clark and others, 2006; Svensson and others, 2000; Tevosian and others, 2000).

To date, little is known about how the epicardium develops and functions in *Xenopus* embryos. Scanning electron microscopy has demonstrated the presence of the PEO on the right side of the septum transversum in *Xenopus* embryos, similar to other vertebrates (Jahr and others, 2008). Furthermore, genes characteristic of the vertebrate PEO and epicardium are conserved in *Xenopus* (Jahr and others, 2008). The *Xenopus* model lends itself to studying the epicardium and potential defects in valve formation and conduction systems due to the ease of manipulating gene function and the established techniques of tissue explanting, antisense MO microinjection, lineage tracing, and transgenics. Recent advances in live imaging have enabled researchers to utilize the *Xenopus* model to visualize cardiac development in real time and to use non-invasive electrical recording, Doppler optical cardiograms, and optical coherence tomography to study heart structure, conduction, and blood flow to determine the role of the epicardium in these processes (Bartlett and others, 2004; Kieserman and others, 2010; Mariampillai and others, 2007; Yelin and others, 2007).

In Vivo Imaging of the Developing *Xenopus* Heart

Xenopus embryos are very well-suited for live imaging of dynamic developmental processes due to their large size and external development. Because of the large size of the embryos, individual cells in the embryo are larger, which allows visualization of the subcellular localization and dynamics of a given fluorescent fusion protein. For example, live confocal imaging has been successfully used to demonstrate the dispersal of individual fluorescent myeloid cells throughout the *Xenopus* embryo (Kieserman and others, 2010). A combination of transgenesis and advanced imaging tools makes this type of approach feasible in the living animal. Yolk opacity in the early *Xenopus* embryo presents a challenge for imaging of deeper tissues. However, cells and/or deep tissues can be visualized easily after microsurgery and subsequent culture of tissue explants. High resolution imaging of the structure and function of the developing myocardium will be critical to complete our understanding of how the heart develops in three and four dimensions, and thus how developmental defects can arise in this system.

Historically, the morphology of the *Xenopus* embryonic heart has been studied in fixed embryos with a combination of confocal microscopy and 3D reconstruction of serial sections through the heart (Kolker and others, 2000; Mohun, 2000). As even slight morphological or dynamic changes in the heart can result in myocardial dysfunction, it is of vital importance to examine these changes *in vivo* in the developing embryo. One area in which this is particularly relevant is in the characterization of defects in the cardiac

conduction system. Human CHD is often complicated by atrioventricular conduction abnormalities. However, a thorough understanding of the defects in embryonic heart contraction as they result from a genetic or morphological abnormality is lacking due to the difficulty of examining these defects *in vivo*. It has recently been demonstrated that the *Xenopus* embryo is amenable to noninvasive live video analysis of the conduction system, allowing one to examine the properties of chamber contraction *in vivo* (Bartlett and others, 2004). Moreover, *Xenopus* cardiac electrophysiology shares many characteristics with the human conduction system, making it an ideal model in which to analyze the physiology of cardiac conduction defects. As a proof of principle, this methodology has been used to examine the microscopic timing of heart contraction in embryos injected with two human *Nkx2.5* mutants and resulted in the identification of a number of conduction defects including a delay in the AV interval and a distinct tachycardia (Bartlett and others, 2007). This type of study, when applied to other human mutations such as those in *Tbx5* that cause Holt-Oram Syndrome, can improve our understanding of these complex human pathologies.

Protein Interactions and Biochemical Function

As discussed previously, transgenesis has primarily been used to study the regulation of gene expression and has revealed much about the transcriptional regulation of cardiac development. However, it has been used relatively little as a tool for isolating protein complexes. Because protein complexes mediate the majority of cellular processes, knowledge of the composition and function of cardiac-specific protein complexes will provide key insights into their tissue-specific activity in the heart. With the advent of proteomics-based approaches to identify endogenous protein complexes, there are a variety of convenient *in vivo* tags such as GFP that can be used to genomically label any protein of interest. Thus, the generation of transgenic *Xenopus* lines that express tagged versions of proteins will provide virtually unlimited material for applications such as mass spectrometry and protein localization studies. Additionally, tagged proteins carrying known CHD-causing mutations could be utilized to characterize changes in their ability to form complexes with other cardiac proteins. As discussed previously, deficiencies in members of cardiac transcriptional complexes often lead to congenital heart malformations, illuminating the need to investigate the functional role of these complexes during cardiac development.

Genetic Approaches in *Xenopus tropicalis*

To date, most studies that have used *Xenopus* as a model system to examine cardiac development have used the pseudotetraploid species *Xenopus laevis* and have relied upon well-established nucleic acid microinjection techniques for depletion or over-expression of proteins in the developing embryo. As our understanding of the genetic basis of inherited cardiac disease increases, there will be a greater need to use genetic approaches in *Xenopus*. Genetic techniques enable precise experimental manipulation that can be used to gain a deeper understanding not just of individual gene functions, but also of the interconnectivity between genes in genetic networks (through enhancer/suppressor gene interaction studies, for example). These interconnections, coupled with variation in the genetic background, may ultimately explain the range of disease type and severity often observed in patients with particular CHDs (Basson and others, 1994; Newbury-Ecob and others, 1996; Sznajer and others, 2007). The use of loss-of-function or gain-of-function alleles of endogenous genes that are generated by mutagenesis also circumvents the undesirable features of MO-based inhibition and mRNA-based expression that result from the inherent limitations in controlling where and when MOs or mRNAs are active in the embryo. Specifically, the role of mutations that affect the biochemical function of a protein can be examined without inappropriately expressing the mutant protein in cells in which the native protein is not expressed, or at times at which it would not normally be present.

The diploid *Xenopus tropicalis* is much more amenable to genetic analysis than the pseudotetraploid *X. laevis*. Fortunately, the vast majority of reagents and techniques developed by researchers for *X. laevis* can be adapted for use with *X. tropicalis*, primarily due to the extremely close similarities between the two species at the genetic and embryonic levels. Even complex reagents such as microarrays developed in one *Xenopus* species have been shown to be usable in both, although species-specific reagents are becoming increasingly available (Chalmers and others, 2005). A key factor encouraging researchers to adopt *X. tropicalis* is the availability of a high-quality genome sequence and the resources that have stemmed from it, including a simple-sequence repeat map of more than 1,500 polymorphic markers that allows mutations to be mapped to relatively small regions of the genome following their isolation in forward genetic screens (Hellsten and others, 2010; Xu and others, 2008) (http://tropmap.biology.uh.edu/index.html). The first two mutations mapped in *X. tropicalis*, *Muzak* and *Dicky Ticker*, both affect cardiac function and are located in the myosin heavy chain gene *myh6* and the muscle-specific chaperone gene *unc45b*, respectively (Abu-Daya and others, 2009; Geach and Zimmerman, 2010). These early successes validate *X. tropicalis* as a model in which novel cardiac genes can be identified through phenotype-based forward genetic screens. The primary importance of this work will be to gain a better understanding of the developmental genetics of cardiac cell type differentiation and morphogenesis. However, studying the effects of mutations in disease-associated genes is also likely to advance our understanding of the etiology of congenital heart abnormalities.

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Table 1

Xenopus models of human congenital heart disease

Gene Name	Disease	Cardiovascular Manifestations	*Xenopus* Model	*Xenopus* Cardiac Phenotype	Refs
Tbx1	DiGeorge Syndrome	TOF, persistent truncus arteriosus	overexpression of dominant negative	unlooped heart, pericardial edema	(Ataliotis and others, 2005)
Tbx5	Holt-Oram Syndrome	ASD, VSD, cardiac conduction system defects	overexpression of dominant negative	unlooped heart	(Horb and Thomsen, 1999)
			protein depletion	unlooped heart, loss of cardiac cells	(Brown and others, 2005)
Tbx20	NA	ASD, VSD, valve disease, pulmonary hypertension, cardiomyopathy, TOF	protein depletion	unlooped heart, pericardial edema, loss of cardiac cells	(Brown and others, 2005)
Nkx2.5	NA	ASD, cardiac conduction system defects	overexpression	enlarged heart	(Cleaver, 1996)
			overexpression of dominant negative	reduced heart	(Fu, 1998; Grow and Krieg, 1998)
			overexpression of mutant mRNA	ASD, cardiac conduction system defects	(Bartlett and others, 2007)
Gata4	NA	ASD	protein depletion	partial fusion of heart fields, cardiac bifida, abnormal cardiac looping	(Haworth and others, 2008)
Shp2	Noonan Syndrome	ASD, VSD, pulmonary stenosis, hypertrophic cardiomyopathy	chemical inhibition	unfused heart fields, loss of cardiac cells	(Langdon and others, 2007)
Zic3	Heterotaxy	cardiac looping defects, ASD, VSD, transposition of the great arteries, double outlet right ventricle, ventricle and aortic arch defects	overexpression	abnormal cardiac looping	(Kitaguchi and others, 2002; Kitaguchi and others, 2000)
Pitx2	Axonfeld-Reiger Syndrome	ASD, pseudotruncus arteriosus, mitral valve and intraventricular septal defects	overexpression	abnormal cardiac looping	(Campione and others, 1999)
			overexpression of dominant negative	abnormal cardiac looping	(Campione and others, 1999)
			protein depletion	abnormal atrial septation, defects in atrial and	(Dagle and others, 2003)

Gene Name	Disease	Cardiovascular Manifestations	*Xenopus* Model	*Xenopus* Cardiac Phenotype	Refs
				ventricular chamber position, restriction of ventricular development	
Chd7	CHARGE Syndrome	TOF, atrioventricular canal defects, aortic arch defects	protein depletion	defects in neural crest cell migration	(Bajpai and others, 2010)
			overexpression of mutant mRNA	abnormal positioning of truncus arteriosus and OFT	(Bajpai and others, 2010)

TOF, Tetralogy of Fallot; ASD, atrial septal defects; VSD, ventricular septal defects; NA, Not Applicable; OFT, outflow tract

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