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***Xenopus* as a Model Organism for Birth Defects – Congenital Heart Disease and Heterotaxy**

Anna R. Duncan¹ and Mustafa K. Khokha²

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Abstract

Congenital heart disease is the leading cause of birth defects, affecting 9 out of 1000 newborns each year. A particularly severe form of congenital heart disease is heterotaxy, a disorder of left-right development. Despite aggressive surgical management, patients with heterotaxy have poor survival rates and severe morbidity due to their complex congenital heart disease. Recent genetic analysis of affected patients has found novel candidate genes for heterotaxy although their underlying mechanisms remain unknown. In this review, we discuss the importance and challenges of birth defects research including high locus heterogeneity and few second alleles that make defining disease causality difficult. A powerful strategy moving forward is to analyze these candidate genes in a high-throughput human disease model. *Xenopus* is ideal for these studies. We present multiple examples demonstrating the power of *Xenopus* in discovery new biology from the analysis of candidate heterotaxy genes such as GALNT11, NEK2 and BCOR. These genes have diverse roles in embryos and have led to a greater understanding of complex signaling pathways and basic developmental biology. It is our hope that the mechanistic analysis of these candidate genes in *Xenopus* enabled by next generation sequencing of patients will provide clinicians with a greater understanding of patient pathophysiology allowing more precise and personalized medicine, to help them more effectively in the future.

Keywords

Xenopus; human genetics; disease model; heterotaxy; congenital heart disease; galnt11; nek2; BCOR

1. Birth Defects

Birth Defects, or structural malformations of the body that occur during embryological development, have a massive impact on the health and welfare of children. Approximately 8 million children are born with serious birth defects each year – roughly 6% of births worldwide (1). Importantly, an additional, untold number of birth defects lead to stillbirths

Corresponding author: Dr. Mustafa Khokha, mustafa.khokha@yale.edu.

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and miscarriages, which can have a devastating effect on parents hoping to start a family (2, 3). In the United States, during the first year of life, birth defects are the leading cause of pediatric hospitalizations (4), medical expenditures (5), and death (6). Further, birth defects rank as a leading cause of death among children aged 1–4 years (#2 cause of death), 5–14 years (#3) and 15–24 years (#6) (7). Therefore, given the impact birth defects have on children, there is a pressing need to improve diagnosis and treatment. Unfortunately, for the vast majority of birth defects, we lack a molecular understanding of the pathophysiology. Advances in human genomics are offering exciting avenues to address the causes of birth defects, but challenges remain prompting clinicians, developmental biologists, and geneticists to urge the NIH to address the problem of birth defects and their impact on child health (8). Here, we outline some of these challenges and strategies to integrate patient driven gene discovery with a powerful disease model, *Xenopus*.

2. Congenital Heart Disease

Of the birth defects, congenital heart disease (CHD) is the most common and the most life threatening. CHD affects 9 in every 1000 live births and 1.3 million newborns annually worldwide (9). CHD is associated with high rates of morbidity and mortality in those affected and typically requires surgical intervention early in life (10). Finally, the care costs for patients with CHD in the United States exceeds 1.75 billion dollars annually (11).

Recent medical and surgical advances have permitted a greater number of infants with complex CHD to survive to adulthood. There were at least 117,000 CHD adult survivors living in the U.S. in 2000; this number has increased greatly over the past fifteen years and is predicted to rise by approximately 5% per year (12, 13). Coincident with increasing survival for children with CHD is the recognition of the associated medical and surgical disorders they may harbor. These range from hemodynamic instability and arrhythmias to infertility, pulmonary disease and significant neurodevelopmental disorders (14, 15).

Emerging data suggest that both the rate and type of CHD vary significantly across the world, ranging from 6.1/1000 live births in the U.S. to 9.3/1000 in Asia. Although access to health care may contribute to global differences in CHD, both environmental and genetic factors most certainly play a role (9). In the age of molecular medicine, identifying those genetic mechanisms responsible for CHD is a priority for both physicians and developmental biologists. To do so, *Xenopus* is an outstanding model and has made major contributions to our understanding of cardiac development (16, 17).

3. Heterotaxy

Heterotaxy (Htx) is a cause of 3% of CHD occurring in 1 in 10,000 newborns, and leads to a particularly severe form (18, 19). Heterotaxy is an abnormal development of the left-right (LR) axis, which leads to incorrect position and organization of the internal organs. The heart, whose function depends on its LR asymmetry, can be severely affected by abnormal development of the LR axis (18), and patients with Htx are at high risk for increased post-operative and respiratory complications, arrhythmias, and complications due to other congenital malformations (20, 21). Positioning of the internal organs can be divided into three categories: *Situs solitus* or a normal positioning of the internal organs; *situs inversus*,

in which the organs are a mirror image; and finally, *situs ambiguus*, in which there is no clear specificity of the organs along the LR axis. Htx falls within the category of *situs ambiguus*.

Studies in many developmental model organisms have identified a conserved left-right (LR) patterning program that determines proper cardiac situs. At the end of gastrulation, asymmetric development begins at the Left-Right Organizer (LRO; mouse node, zebrafish Kupffer's vesicle, and gastrocoel roof plate [GRP] in frog; shown in figure 1), which forms in the dorsal posterior region of the embryo (22). In cells of the LRO, inner motile monocilia (red cilia) beat to create a leftward flow of extracellular fluid (blue arrows) (23, 24).

According to the two-cilia model, immotile cilia (green and purple cilia) on surrounding cells act as sensors, detecting the flow (purple cilia) driven by motile cilia (red cilia) and eventually translating it into asymmetric gene expression (25, 26). *Coco* (CERL2), a nodal antagonist, is one of the earliest genes that is asymmetrically expressed (27, 28). *Coco* expression leads to nodal inhibition and decreased phosphorylated Smad2 on the right (29). Phosphorylated Smad2 eventually activates *pitx2* on the left side of the embryo, which is an essential step in organ *situs* determination (29, 30). *Pitx2* is also involved in the organogenesis of the heart, gut and lungs (31, 32). Following activation of these developmental pathways, cardiac precursor cells in the lateral plate mesoderm fuse at the midline to form a straight heart tube (33, 34). This central region and first heart field will eventually form the left ventricle. One end of the tube will become the outflow tracts and atria, and the other end will become the right ventricle and inflow tracts. This linear tube eventually loops to the right, establishing cardiac asymmetry. A complex signaling network is essential for this asymmetry to occur (35).

4. Patient Driven gene discovery

Classically, the work of Afzelius showed that cilia especially motile cilia are a significant cause of heterotaxy, which has been replicated in a host of subsequent work (36). However, more recently a genomic analysis of patients with heterotaxy demonstrated that copy number variants (CNVs; insertions or deletions in the genome) could also be causative of heterotaxy (27). By evaluating 262 heterotaxy subjects and 991 controls, we previously identified relatively small CNVs affecting 61 different genes. Further evaluation of these genes showed that 7 were strongly expressed in the LRO of the frog and 5 of 7 tested demonstrated severe cardiac looping defects in *Xenopus*. This paper highlighted that mutations in pathways as diverse as Tgf-beta-receptor-2 to the glycosyl transferase, Galnt11, and the nuclear pore protein, Nup188, may lead to heterotaxy. This study helped to change the perception of heterotaxy causation, showing researchers and clinicians, that genes outside of the typical realm can affect LR development. In fact, in many cases, connecting a molecular mechanism between these genes and what is known about LR patterning was challenging, and offered an exciting avenue of research (37).

The Pediatric Cardiac Genomics Consortium has taken patient driven gene research a step further, and has focused not only on heterotaxy, but on multiple forms of congenital heart disease. Their aim is to define the genetic causes of congenital heart disease in patients affected all over the world through whole exome sequencing of the proband and each of

their parents (trio sequencing) (38, 39). They have enrolled more than 10,000 patients so far. Recently, they examined 362 different probands, with 70 having heterotaxy and the others having conotruncal defects (defects of the large outflow vessels emerging from the heart – aorta and pulmonary), left ventricular obstruction, or other severe complex congenital heart disease. They found that a large percentage of these patients had *de novo* mutations with an enrichment in histone methylation or ubiquitination genes. Using mouse embryos to identify expression levels in the heart, they showed that genes with *de novo* mutations in patients had higher levels of expressivity in the heart when compared to controls. The *de novo* mutations tended to be protein altering and highlighted how changes in chromatin can alter cardiac development (38).

The National Birth Defects Prevention Study is also investigating the causes of heterotaxy. They have taken both a genetic and epidemiologic approach to their patient cohort, in hopes of understanding all factors contributing to laterality. Their study has examined 517 patients, 73.1% with heterotaxy and 26.9% with *situs inversus totalis*. Their research shows that heterotaxy is more than twice as prevalent as *situs inversus totalis*, and that it is more likely to occur in premature infants that are small for gestational age (SGA) and born to non-white mothers that are less than 20 years old. This study also shows that the prevalence of heterotaxy and laterality defects has not changed over time. This study highlights the primary population that has been affected by heterotaxy, is a population that is already at risk (39).

Population based studies have examined DNA from dried blood spots from newborns in order to unravel the genetic basis of their cardiac defects. In 2015, Reigler *et al* published a study looking at causes of heterotaxy in New York State, and found that copy number variants could be utilized to genotype a broad spectrum of the population (40). This study identified 20 new candidate genes for heterotaxy, including, BMP2 and MNDA, the first associated with bone development and TGF-beta signaling, and the second, strongly associated with myeloid differentiation with a possible role in apoptosis (40). This study, like those previously discussed, provides many new candidate genes with unknown mechanism that need to be further understood in order to better serve this patient population.

This comprehensive range of research methodologies and findings highlight the vast number of ways that the genetics of heterotaxy is being better understood. The next step is to understand how these genes function, and why their mutations lead to abnormal LR development of the heart. Importantly, a large number of genes have already been identified; therefore, to do this efficiently, we need to be able to analyze these genes in a rapid, high-throughput human disease model such as *Xenopus*.

5. Challenges to Birth Defects Research – CHD and Heterotaxy

While these studies have been remarkable in identifying candidate genes for heterotaxy and congenital heart disease, they also illustrate major challenges, since there appear to be many genes that cause birth defects. In genetics, the gold standard for defining a gene as causative of disease is that different alleles of the same gene are identified repeatedly in unrelated patients. Nearly all of the genes identified to date in congenital heart disease or heterotaxy

do not have second alleles to define causality. In retrospect, this may not be surprising. Patterning the heart and/or LR axis is a complicated process that almost certainly requires many genes in order for it to occur correctly. Therefore, one would expect high locus heterogeneity or many genetic loci that could contribute to congenital heart disease and heterotaxy. Identifying second alleles will likely only become possible when massive patient populations are analyzed. While sequencing costs are falling, another massive drop in sequencing cost would be necessary in order to analyze the patient numbers necessary to find second alleles in the majority of CHD/heterotaxy or by extension birth defect patients.

Until then, patient driven gene discovery must be one part of a two pronged attack on birth defects. Discovering the genes is essential. If there were a list of genes that caused congenital heart disease, then it could be used for diagnosis, providing parents with critical information on why their child suffers from birth defects. The technology, exome or genome sequencing, is in hand but an understanding of the variant information and their relation to birth defects is lacking. Molecular diagnosis would then enable genetic counseling, prognosis, and potentially improve patient care (Figure 2) (8). Clinically, patients with a similar birth defect often have dramatically different clinical outcomes. Perhaps, this is because therapies are tailored on phenotype rather than genotype as the latter is not available or understood. However, based on the genomic analyses above, it is clear that many diverse genes can cause birth defects, suggesting that we as clinicians are overly “lumping” different diseases together and that therapeutic gains could be achieved if we understood the differences in our patients’ genotypes.

While this is a lofty goal, it is complicated by two factors: 1) challenges in assigning disease causality and 2) an actual understanding of the disease process. As described above, due to high locus heterogeneity, assigning disease causality for a candidate gene is difficult. Then, how can we tailor our therapies without certainty that a disease is caused by a gene variant? Further, the genes identified are either novel to cardiac development, novel to embryonic development, or have no known function. In these cases, a lack of mechanism also complicates assigning disease causality since we cannot identify a parsimonious mechanism for pathophysiology. Therefore, until large patient populations can be sequenced cost effectively, we need to discover the mechanisms of these birth defect *candidate* genes. By discovering a birth defect mechanism, we bolster the evidence of disease causality. To do this we need a human disease model with sufficient throughput to accommodate the massive number of candidate genes. *Xenopus* is exactly such an ideal model.

6. *Xenopus* as a model organism for human disease

The frog model, *Xenopus*, is a rapid, efficient, and *in vivo* system to study LR patterning and cardiac development (17, 41, 42). There are two species of *Xenopus* typically used in developmental biology—*X. laevis* and *X. tropicalis*. *X. laevis* is allotetraploid while *X. tropicalis* is a true diploid, making *X. tropicalis* a more convenient organism for loss of function studies via gene depletion or modification. *X. laevis*, on the other hand, is the “gold standard” for gain of function studies and biochemistry due to the massive amount of egg and embryonic material that can be isolated. *Xenopus* embryos can be easily generated in large numbers. Gene expression can then be manipulated early in development through

depletion or overexpression techniques. The hearts of embryos can then be studied, just three days after fertilization. Cardiac looping can be examined by simply looking at the heart through transparent skin. The conservation between human and *Xenopus* allows us to model human biology more closely than other model systems. For example, the zebrafish heart is two chambered, atrium and ventricle, while the *Xenopus* heart is three-chambered, with two atria and an atrial septum. Also *Xenopus* has a highly trabeculated single ventricle, atrioventricular valve, and an outflow tract more closely resembling mammals. *Xenopus* also has a very low “background rate” of cardiac abnormalities allowing for robust assays.

In *Xenopus*, microinjection of embryos is straightforward, and mRNA or morpholinos (MOs) can be used for gain or loss of function studies, respectively. In addition, CRISPR based gene modification is so efficient that biallelic deletions occur in a few hours after microinjection and can reveal even early zygotic phenotypes (43). By targeting one cell at the two-cell stage, embryos can be selected where the right or left side is manipulated, while the other side serves as an internal control. This is not possible in other heterotaxy disease models (41). Lastly, *Xenopus* is the closest vertebrate model to humans that retains the advantages of speed and cost when compared to zebrafish and mouse. For all of these reasons, *Xenopus* is an ideal system to model human congenital heart disease.

The molecular mechanisms of multiple congenital heart disease genes, such as CHD7 and Tbx20, in addition to the heterotaxy candidate genes Galnt11, Nek2, and BCOR have all been unraveled in *Xenopus* and will be discussed in further detail below.

7. *Xenopus* and Congenital Heart Disease

Xenopus have been used broadly in congenital heart disease research, ranging from the study of arrhythmias to cardiomyopathies and genomic syndromes. This research has been invaluable, and has increased our understanding of cardiac development, embryonic signaling and human disease.

One of the genes that *Xenopus* has been essential in describing is CHD7. Mutations in CHD7 lead to CHARGE Syndrome, a syndrome characterized by congenital heart disease, retinal anomalies, ear malformations, choanal atresia, and intellectual disabilities. CHD7 was identified through a genomic screen of patients affected with CHARGE. 10 out of 17 patients analyzed had mutations present in the gene, CHD7, an ATP-dependent chromatin remodeler, on chromosome 8 (44). A study by Bajpai *et al* in *Xenopus* showed that CHD7 plays an essential role in neural crest migration (45). Neural crest migration is conserved between *Xenopus* and humans. If CHD7 or its ATP-domain are depleted, neural crest migration is altered, preventing expression of the genes Sox9, Twist and Slug, essential for neural crest specification. Depletions in CHD7 lead to a CHARGE syndrome in *Xenopus*-embryos with cardiac looping defects, colobomas, and multiple craniofacial and cartilage anomalies similar to those observed in the patient population affected (45). *Xenopus* is the perfect model system for CHARGE syndrome, since it allows rapid gene depletion and analysis in addition to visual confirmation of the organ systems affected.

Mutations in transcription box genes, such as Tbx20, have also been shown to cause congenital heart disease. Tbx20 was identified through a screen of 352 probands. Mutations

in Tbx20 were identified in two probands, both with family histories significant for congenital heart disease (46). These mutations lead to dilated cardiomyopathy, atria septal defects and mitral valve malformations. Tbx20 is thought to act through its regulation of Tbx2, by promoting its presence at the atrioventricular canals, and inhibiting its expression within the chambers. This regulation is necessary for cardiac chamber differentiation (47). Research by Mandel *et al* has looked upstream of Tbx20, in order to understand its regulatory mechanism (48). Their research shows that Tbx20 is a target of the BMP signaling pathway. Smad1 binds specifically to Tbx20 enabling its function. Blocking nuclear localization of Smad1 expression led to decreased Tbx20 expression in cardiac cells. However, it does not prevent Tbx20 expression in other tissues, such as the brain, highlighting the cardiac specificity of this pathway (48). Together, this data explains how BMP activation leads to Smad1 binding to Tbx20, which regulates Tbx2, enabling chamber differentiation. This research helps to explain disease causation, linking familial congenital heart disease to a basic embryonic pathway.

8. Galnt11

In Fakhro *et al's* 2011 study, a patient with right atrial isomerism, or bilateral cardiac symmetry, was identified with a copy number deletion of GALNT11 (37). Galnt11 controls the initiation of GalNAc-type O-glycosylation, however, its target for glycosylation and any role in LR patterning were previously unknown (49). Using *Xenopus* as a model organism, the mechanism of *galnt11* was first examined through cardiac looping, since the patient had heterotaxy. Depletion of *galnt11* led to cardiac looping defects as well as abnormal *pitx2c*, a global marker of LR patterning normally expressed in the left lateral mesoderm. Therefore, LR patterning defects previously identified in the heterotaxy patient, were readily recapitulated in frogs. As discussed above, the LR axis is initiated early in development at the level of the LRO. At the LRO, a leftward flow is generated by motile cilia, which is then sensed by immotile cilia. This sensation of flow leads to gene regulation and the initiation of asymmetry. One gene that changes during this time period is *coco*. Initially, *coco* is expressed bilaterally at the LRO. Once leftward ciliary flow begins, *coco* is down regulated on the left side. This typical *coco* progression did not occur when *galnt11* was knocked down; instead, *coco* remained bilaterally expressed. This suggested that *galnt11's* role was essential for the initiation of LR asymmetry and likely related to ciliary movement or signaling. Unexpectedly, when fixed LRO's from *galnt11* depleted embryos were compared to control embryos, the cilia appeared normal, providing no additional insight into the LR patterning process (50).

Fortunately, cilia are also plentiful on the *Xenopus* embryonic epidermis. There, multiciliated cells beat to create extracellular fluid to sweep bacteria and other pathogens off the surface of the embryo much like multiciliated cells of the respiratory tract. As these cilia are plentiful, briskly beating to create fluid flow, and readily visualized without dissection, Boskovski *et al* decided to examine these cilia in the context of *galnt11* depletion. Surprisingly, *galnt11* depletion led to no obvious defects in cilia structure or beating; however, the density of multiciliated cells seemed dramatically higher with *galnt11* depletion compared to control embryos (50). Chris Kintner's group had shown elegantly that Notch signaling is essential for the proper spacing of multiciliated cells across a field of

embryonic epidermis; too little Notch signaling and multiciliated cells become more dense, too much Notch signaling and the multiciliated cells become too sparse (51).

Boskovski and colleagues then demonstrated that *galnt11* also affects the density of multiciliated cells in a similar fashion. They then attempted to rescue the *galnt11* depletion LR phenotype with Notch pathway members. While the delta ligand had a minimal effect, both the intracellular domain of Notch and a constitutively active suppressor of hairless efficiently rescued the *galnt11 pitx2c* phenotype suggesting that *galnt11* affects the Notch pathway directly. In collaboration with Henrik Clausen and colleagues, they then demonstrated that a peptide spanning the juxtamembrane region of Notch is efficiently glycosylated by Galnt11 *in vitro* in addition to two EGF repeats on the Notch extracellular domain. Juxtamembrane glycosylation actually enhances Notch peptide cleavage suggesting a mechanism for activating the Notch pathway. While this placed *galnt11* in the Notch pathway, a mechanism to explain abnormal laterality with *galnt11* knockdown was still unclear (50).

Multiple studies have previously implicated Notch signaling in LR patterning (52–56). However, some of these studies were conflicting, while others implicated different steps in the LR patterning cascade. Boskovski *et al* chose to look at this problem independently. They noticed that their GALNT11 patient had right atrial isomerism and that depletion of *galnt11* in *Xenopus* led to midline A-loops, as if the hearts simply failed to receive any laterality signal. Conversely, overexpression of *galnt11* led mostly to L-loops, a phenotype similar to *situs inversus* often seen in patients with primary ciliary dyskinesia (PCD). Therefore, gain of *galnt11* could be explained by cilia that failed to move (all immotile or sensory cilia); alternatively, loss of *galnt11* could be explained by cilia that failed to sense (possibly because they all began moving). The Two Cilia Model proposes that there are two types of cilia in the LRO (25, 26); however, the signal to specify these two cilia types, immotile (presumably sensory) and motile cilia was unknown. Boskovski *et al* hypothesized that *galnt11* via Notch signaling might act as this switch specifying the two types (50).

To test this hypothesis, Boskovski, Yuan and colleagues performed live imaging of the LRO cilia by dissecting explants from *Xenopus* embryos where cilia were labeled with arl13b-mCherry. They were then able to distinguish motile and immotile cilia clearly. Gain of Notch led to an increased number of immotile cilia, while depletion of either *galnt11* or *notch1* led to a much higher percentage of motile cilia in the LRO. This result suggested that Galnt11 via Notch1 signaling is necessary for the balance between motile and immotile (sensory) cilia, and thus providing the missing signal for the Two Cilia model (50).

While the goal of these studies was to understand the disease mechanism in the heterotaxy patient, Boskovski *et al* also discovered critical regulators of fundamental signaling pathways in embryonic development. Patient driven gene discovery in a CHD patient, led to an exciting breakthrough in our understanding of the regulation of Notch signaling and LR patterning. Also importantly clinically, they established a plausible disease mechanism lending further evidence that GALNT11 is a strong candidate gene for heterotaxy and congenital heart disease.

9. Nek2

In 2011, Fakhro *et al* also identified Nek2, in a copy number duplication in a patient with heterotaxy (37). Nek2, or Never in Mitosis kinase 2, is a serine threonine kinase that is highly conserved in vertebrates and plays multiple roles in the cell cycle. It has been shown to phosphorylate centrosomal linker proteins in order to cause disjunction of the centrosome and eventual mitotic spindle formation (57, 58). It has also been shown to phosphorylate Nup98, leading to its dissociation from its nuclear pore complex and eventual break down of the nuclear envelope (59). Endicott *et al* have shown that Nek2 can also regulate ciliary formation and reabsorption supporting a role for Nek2 as a candidate gene for heterotaxy (60).

Endicott *et al*'s research first confirmed that depletion or overexpression of Nek2 lead to LR patterning defects in *Xenopus. coco*, the first marker of asymmetric gene expression, is abnormal in these cases suggesting a role in the LRO and possibly due to cilia. Nek2 plays critical roles in the cell cycle. Further, Endicott and colleagues show that Nek2 is necessary for centriole formation and regulation. Second, they show that Nek2 is crucial for ciliary reabsorption, highlighting that Nek2 plays an invaluable role in the full life cycle of cilia. Therefore, Nek2 appears to affect LR patterning via cilia, a result consistent with prior known roles of Nek2 in centriole disjunction, since the centriole acts as the base of the cilia (60).

In addition to the LRO cilia, *nek2* also eliminates the cilia of multiciliated cells of the embryonic epidermis. This result provides an additional clinical correlation for Nek2. As described above, single and multiciliated cells play different roles. Monocilia help to form the LR axis during development, and if altered, heterotaxy can occur. In contrast, multiciliated cells are present throughout the respiratory tract and play an important role in the clearance of debris and prevention of infections. One often thinks of primary ciliary dyskinesia (PCD) as the genetic cause of *situs inversus* with frequent respiratory infections. However, this model shows that patients with mutations in Nek2 may have a similar phenotype to those with PCD- such as frequent respiratory and sinus infections. PCD patient are also at risk for infertility, which will be something to consider in patients found to have Nek2 mutations. On the other hand, loss of function of *galnt11* leads to an *increased* number of multiciliated cells on the embryonic epidermis of *Xenopus* and while this result needs to be tested in mammalian respiratory track, it suggests that loss of *galnt11* might have a very different respiratory complication rate than loss of *nek2* (if any) (50, 60). Therefore, we strongly speculate that genotype may prove critical for predicting complications that could arise in patients and may explain why some patients with similar phenotypes (ie Heterotaxy) have such different clinical courses (ie different rates of respiratory complications).

10. BCOR

Heterotaxy can also be part of a larger syndrome, such as Oculo-facial-cardiac-dental syndrome (OFCD). Patients with OFCD have been found to have mutations in the X-linked gene, BCOR, the binding partner of BCL-6 (61). Recent studies using *Xenopus* have unraveled the mechanism of why BCOR depletion leads to heterotaxy and OFCD syndrome.

In 2007, Hilton *et al* identified two patients with OFCD that had heterotaxy involving multiple organ systems (62). To evaluate a possible role of BCOR in laterality defects, they depleted BCOR in *Xenopus*. Their results showed that a large percentage of embryos had cardiac and gut looping defects, suggesting that their patient's multisystem heterotaxy was also due to the mutation in BCOR. They looked upstream at the gene *Pitx2c*, which is typically expressed in the left lateral plate mesoderm and is important for left-sided organ development. Their research demonstrated that when BCOR is depleted, *pitx2c* is no longer expressed at the left lateral plate mesoderm. This result showed that BCOR is necessary for the expression of *pitx2c* and establishment of the LR axis (62).

In 2010, Sakano *et al* took Hilton *et al*'s research another step further. They showed that during development in *Xenopus*, BCOR forms a transcriptional repressor complex with BCL6. Together they inhibit the Notch target gene *ESR1*. This inhibition is essential, because if *ESR1* is expressed, it will block the expression of *pitx2c* in the left lateral plate mesoderm. Since *pitx2c* is essential for LR axis differentiation, depletion of BCOR will have laterality defects in *Xenopus* (63).

Most recently, Tanaka *et al* have shown that *ESR1* binds to a region on the *pitx2* locus called the left side-specific enhancer (ACE). The binding of *ESR1* to the *pitx2* locus leads to the recruitment of histone deacetylase 1 (HDAC1). When *ESR1* and HDAC1 are present, *Xnr1* can no longer activate *Pitx2* gene expression preventing its expression in the left lateral plate mesoderm. Taking a step back, this means that it is necessary for *ESR1* to be inhibited by the BCL6-BCOR repressor complex, in order for *Pitx2* expression to occur on the left side (64). Overall, this research demonstrates how the genetics of a clinical syndrome in combination with an efficient and powerful human disease model system like *Xenopus* can unravel a complex regulatory system in development biology.

11. Conclusion

GALNT11, NEK2 and BCOR, all highlight how complex signaling pathways and the basic physiology of LR development can be further understood through patient driven gene discovery when coupled with mechanism discovery in an efficient human disease model like *Xenopus*. By understanding the mechanisms of each of these genes, the heterotaxy patient population will also be better served. Clinicians will be able to use this information not only to define diseases more precisely to themselves and parents, but also anticipate additional risks based on the underlying pathophysiology. While our original intent was reverse translational (that is bedside to bench), we now hope to exploit this information to reverse the flow of information from the bench back to the bedside (translational research) to provide precision-based medicine for the patients affected. In order to do that, a fast and efficacious model system, such as *Xenopus*, is essential as inexpensive sequencing of patients is now in hand.

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Figure 1. Ciliary flow and signaling in the gastrocel roof plate (GRP) of the frog

a.) The black dashed line outlines the GRP. The red lines represent the inner motile cilia, and the green and purple represent the outer immotile cilia. The blue arrows show the leftward fluid flow across the GRP that is produced by the motile cilia and sensed by the immotile cilia.

b.) *coco* is initially expressed bilaterally. Ciliary flow reduces the expression of *coco* on the left side. *Coco* inactivates *Nodal* on the left, which leads to *pitx2c* expression on the left side of the embryo and ultimately plays a role in organ situs and asymmetric development.

Figure 2. Model for patient centered gene discovery, analysis and ultimately personalized medicine

This model highlights how DNA can be taken from a patient with congenital heart disease. His or her genome can then be sequenced and geneticists can analyze it for likely causative mutations. Embryologists can then use *Xenopus* as a model system to understand gene function. This knowledge can then be taken back to the patient in order to personalize medicine.