Title
Xenopus as a Model for GI/Pancreas Disease

Authors
Matthew C. Salanga, Ph.D. (msalanga@mbl.edu)
Marko E. Horb, Ph.D. (mhorb@mbl.edu) **corresponding author

Mailing address:
National *Xenopus* Resource,
Eugene Bell Center for Regenerative Biology and Tissue Engineering,
Marine Biological Laboratory,
7 MBL St.
Woods Hole, MA 02543

Phone: 508-289-7627
Fax: 508-289-7900

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ABSTRACT

Diseases affecting endodermal organs like the pancreas, lung and gastrointestinal (GI) tract have a substantial impact on human welfare. Since many of these are congenital defects that arise as a result of defects during development broad efforts are focused on understanding the development of these organs so as to better identify risk factors, disease mechanisms and therapeutic targets. Studies implementing model systems, like the amphibian Xenopus, have contributed immensely to our understanding of signaling (e.g. Wnt, FGF, BMP, RA) pathways and gene regulation (e.g. *hhex*, *ptf1a*, *ngn3*) that underlie normal development as well as disease progression. Recent advances in genome engineering further enhance the capabilities of the Xenopus model system for pursuing biomedical research, and will undoubtedly result in a boom of new information underlying disease mechanisms ultimately leading to advancements in diagnosis and therapy.

Introduction

The pancreas and gastrointestinal (GI) tract are components of the digestive system, and are responsible for a number of physiological functions, including the breakdown of food for nutrient release, absorption of water, ions, minerals, and nutrients, glucose homeostasis, and hormone regulation. Dysfunction arising from cells, tissues, or systems within the digestive tract can result in a range of human diseases or syndromes affecting any of the physiological functions previously mentioned. Diseases like diabetes are of massive importance to human health and have a huge economic impact (estimated at $245 billion by the Centers for Disease Control and Prevention in 2012) resulting from the cost of therapies and lost productivity from patients suffering from direct and indirect consequences of impaired glucose homeostasis (heart disease, blindness) [1]. Many of these gastrointestinal disorders are congenital defects that arise as a result of aberrant development. The precise molecular mechanisms underlying the onset or progression of gastrointestinal disorders is of great importance for the development of therapies and early diagnosis, and the amphibian *Xenopus* offers numerous advantages for elucidating the molecular signals controlling how the GI tract and associated organs are specified during embryogenesis [2].

Single cell *Xenopus* eggs or embryos are large (~1.2mm *X. laevis*, 0.75mm *X. tropicalis*), making microinjection and other mechanical manipulations relatively simple. *Xenopus* are non-seasonal breeders, allowing for induction of ovulation every 3 months by injection of human chorionic gonadotrophin hormone into mature females. A typical brood size obtained from a single female may reach several thousands of eggs that can be fertilized in vitro using homogenized male testicle as a sperm source; in vitro fertilization is particularly advantageous for studies that benefit from synchronously developing embryos. *Xenopus* embryos develop rapidly providing a tractable time frame for studying early development and organogenesis. There are two commonly used *Xenopus* species in research, *X. tropicalis* (diploid) and *X. laevis* (tetraploid) species and sequencing of both genomes has been completed revealing large regions of synteny with mammals including humans [3-5]. *Xenopus* not only provides a remarkably broad experimental platform, but as an amphibian it also bridges the gap between costly mammalian models and the evolutionarily more distant zebrafish model [6, 7].

The GI tract and associated organs develop from the endodermal germ layer, which in *Xenopus* is derived from the vegetal hemisphere of the developing embryo. Fate map studies provide the necessary information to enable targeted manipulation of specific regions and organs in the developing endoderm [8, 9]. *Xenopus* embryos and tadpoles represent an excellent source for endoderm-derived organs, and precursor tissues for molecular analysis [10-13]. Aided by an expansive collection of expression data (e.g. in situ hybridization, microarray, RNA-seq) available to the community via the *Xenopus* website “Xenbase” (www.xenbase.org)

[14-16], and the release of genome wide stage specific transcriptomic datasets in addition to parallel proteomic expression data [17-20], provide substantial resources that can help shape hypotheses directed at understanding the mechanisms of development and disease.

Foregut specification

Many congenital malformations of the gastrointestinal tract are a direct result of abnormal development during embryogenesis. Deciphering the molecular mechanisms that control how endodermal organs are specified during early development is helpful in understanding how these anomalies arise, and *Xenopus* provides unparalleled access for embryological manipulations at these early stages. The foregut arises from the anterior endoderm and gives rise to many different organs, including the liver, pancreas, duodenum and lungs [21]. Several recent studies have used *Xenopus* to decipher the molecular mechanisms controlling how these organs become subdivided in the early endoderm and identified interactions between the Wnt and BMP pathways. Microinjections into different *Xenopus* blastomeres at early cleavage stages allows for accurate targeting of specific molecules to distinct regions of the developing gastrointestinal tract, and can be combined with transplantations and explants of these regions to ascertain the effects in different conditions [22].

Using these approaches several reports showed that Wnt signaling activity must be excluded from the anterior endoderm to allow for proper formation of liver and pancreas [13, 23-25]. The Zorn lab used elegant transplantations of anterior and posterior endoderm combined with overexpression of key Wnt components to show that Wnt signaling must be repressed during the initial stages of foregut development (Fig.1a). In addition, they were able to control the timing of activation using hormone-inducible proteins, and this allowed them to identify a narrow time window when Wnt must be inhibited, from the end of gastrulation at stage 11 to mid-neurula stage 15 [23]. This led them to identify the *hhex* gene as the mediator of foregut specification, and through promoter analysis they identified the transcription factor Vent2 as the key repressor of *hhex* expression in the posterior endoderm [23]. In agreement with this, the RNA binding protein Staufen 2 was recently shown to be necessary for *hhex* expression in the anterior endoderm, and its loss led to ectopic expression of *vent2* in the anterior endoderm [26]. Subsequent studies revealed that Wnt signaling was suppressed by the secreted Wnt antagonist Sfrp5 [25]. These studies led to a model where Wnt signaling regulated hindgut development, while suppression of Wnt promoted foregut development.

However, a recent study revealed that low levels of Wnt signaling are actually required for foregut development [27]. In that study they were able to create multiple thresholds of Wnt signaling using a combination of *fzd7* knockdown (to eliminate Wnt signaling) with different doses of the small molecule BIO, which inhibits GSK3 thereby promoting Wnt signaling. They found that complete elimination of Wnt signaling in the anterior endoderm by *fzd7* morpholino inhibited foregut development, while treatment with low levels of BIO in the *fzd7* morphants led to restoration of *hhex* expression [27]. More recently, BMP signaling was also found to play an important role in the process. By comparing the gene expression profiles of anterior endoderm explants, either alone or with mesoderm, BMP signaling from the mesoderm was found to be required at these early stages to maintain foregut progenitors [28••]. They found that Sizzled (Szl) was induced in the endoderm by mesodermal BMP signals and that loss of *szl* resulted in agenesis of liver, thyroid, pancreas and lung. These studies point to the importance of Wnt and BMP signaling for proper development of foregut organs.

In parallel retinoic acid (RA) signaling has been shown to promote development of foregut organs. A recent study by the Chen lab identified a direct RA target gene, N-myc downstream regulated gene 1a (*ndrg1a*), as being required for proper specification of the pancreas

esophagus, stomach and duodenum [24•]. They showed that *ndrg1* was expressed in the dorsal anterior endoderm and that it acted to repress Wnt signaling. Using TALEN mediated genome editing they demonstrated that mutations in ndrg1 resulted in pancreas, stomach and duodenum hypoplasia, with no effect on the liver; a similar phenotype was found in the morpholino knockdown embryos as well. These results provide a link between RA signaling and Wnt repression in the anterior endoderm and provide mechanistic insight into how the foregut is patterned.

Epithelial morphogenesis

Not only is *Xenopus* useful for studying patterning and cell fate specification, but it provides a useful model to study morphogenesis of the developing gut. In the last few years several groups have identified key components of signaling pathways required to establish and maintain proper gastrointestinal epithelium [25, 28-30]. Two of these studies linked Wnt and BMP patterning events to tissue morphogenesis [25, 28•]. In the first study they showed that *sfrp5* was required to establish proper foregut epithelium and they were able to identify that both canonical and noncanonical Wnt signaling was critical for specification and morphogenesis, respectively. As Sfrp5 inhibits Wnt ligands it is able to interfere with both canonical and noncanonical Wnt pathways. Since knockdown of *sfrp5* resulted in defects in both patterning and morphogenesis of the foregut, the Zorn lab was able to identify whether each or both defect was due to perturbations of the canonical or noncanonical Wnt pathway. They showed that epithelial integrity (and not patterning defects) was restored in *sfrp5* morphants by blocking noncanonical Wnt signaling with mutant Dsh constructs or using a JNK inhibitor. Conversely, patterning defects, but not epithelial integrity, were restored in *sfrp5* morphants by blocking the canonical Wnt pathway using Dkk1 or mutant Dsh constructs. They went on to show that Wnt11 was the target of Sfrp5 for both pathways [25], while a more recent study showed that Wnt5 was a target for the noncanonical Wnt pathway [31].

A recent publication from the Nascone-Yoder lab further extended the results and showed that JNK activity controlled the rearrangement of endodermal cells underlying elongation of the gut tube in *Xenopus* [30•]. They found that JNK activity maintained cell-cell adhesion by stabilizing microtubules necessary for gut tube elongation and they confirmed that JNK was not required for patterning the digestive tract. Similarly, *shroom3*, a known regulator of epithelial morphogenesis in the neural tube, was identified as a key regulator of epithelial morphogenesis in the developing *Xenopus* gut [29]. In that paper they demonstrated another strength of *Xenopus* to find a Pitx1 enhancer element in the *shroom3* promoter and show that both *pitx1* and *shroom3* were required for epithelial cell shape changes. These results demonstrate the strength of using *Xenopus* to interrogate signaling pathways responsible for patterning and morphogenesis of the developing gastrointestinal tract.

Pancreas development

The pancreas is an endodermaly-derived organ that initially develops from a single dorsal bud and two ventral buds, which by three days post-fertilization in Xenopus embryos, fuse together to form a single pancreas [32]. This differs from mammals, where one of the ventral bud regresses before fusion at E12.5 in mice [33] and six weeks of fetal development in humans [34]. Developmental defects occurring at these early stages of pancreas formation can lead to clinically recognizable anomalies, including annular pancreas or pancreas divisum that result from inappropriate ventral pancreas formation and dorsal pancreas agenesis [35].

Xenopus provides an excellent model in which to study development of dorsal and ventral pancreatic buds and identify the molecular genetic differences between them. For example, by producing chimeric embryos where either the dorsal or ventral pancreatic bud was labeled we

demonstrated that the ventral pancreatic bud cells migrate extensively into the dorsal bud after fusion while dorsal pancreatic bud cells do not (Fig.1b) [11••]. By isolating individual dorsal and ventral pancreatic buds we compared their gene expression profiles and identified several new genes involved in regulating cell fate specification and proliferation in the developing pancreas [11, 36, 37]. We showed that the tetraspanin, *tm4sf3*, was localized to the ventral pancreas and regulates fusion of the dorsal and ventral buds [11], whereas the RNA binding protein Celf3 controls proliferation in the developing endoderm through translational activation of key cell cycle genes. By producing different fusion proteins between Celf3 and Celf1 we identified the linker region of Celf3 as responsible for the translational enhancement activity on cyclin A2 mRNA [36]. More recently, it was shown that the transcription factor Hhex plays a critical role in regulating formation of the ventral pancreatic bud, but does not influence dorsal pancreas formation [38]. These results demonstrate the utility of *Xenopus* for rapid identification and functional analysis of new genes involved in regulating development of the dorsal and ventral pancreatic buds.

Xenopus is also a useful platform for investigating the temporal activity of pancreatic transcription factors in controlling specification of individual cell fates. A recent study revealed that transient expression of the pancreatic endocrine transcription factor Ngn3 (using a hormone-inducible version) for 1-4 hours during early neurula stages promoted development of beta and delta cells and not alpha cells, while similar transient expression at mid-neurula stages promoted development of alpha, beta and delta cells [39•]. The authors then went on to isolate endodermal tissue four hours after Ngn3 activation and identified many new downstream targets of Ngn3; using morpholinos to block translation they showed that several of these new factors were essential for endocrine pancreatic cell development downstream of Ngn3. Other studies in *Xenopus* have also demonstrated the sufficiency of specific pancreatic transcription factors (Pdx1 and Ptf1a) to promote ectopic pancreatic fates either in the early endoderm or through transdifferentiation of liver to pancreas [12, 40-42]. Moving forward, *Xenopus* provides an excellent platform to define the pancreatic gene regulatory network and can be used to interrogate these relationships in high temporal resolution. The ability to combine gain-of-function and loss-of-function techniques in developing endoderm highlight the benefits that *Xenopus* can offer for elucidating the molecular mechanisms controlling pancreatic cell fate specification.

Diabetes

Xenopus has also proven useful in elucidating the function of candidate genes related to diabetes and establishing the significance of specific gene mutations identified in humans. Neonatal diabetes is a form of diabetes that affects infants in their first year of life. Mitchell-Riley syndrome (MRS) is a neonatal diabetes syndrome that presents with hypoplastic pancreas and gall bladder, and intestinal atresia [43, 44]. MRS has been linked to several distinct mutations in the gene encoding the transcription factor, Regulatory Factor X 6 (Rfx6)[45]; these studies however did not address whether these specific point mutations altered Rfx6 function. Studies in *Xenopus* were performed to establish whether these specific mutations altered Rfx6 function, interfering with proper pancreas development and thus could contribute to the development of neonatal diabetes [46••]. First, they established that knockdown of *rfx6* using antisense morpholinos to block translation of *rfx6* mRNA resulted in pancreatic and endodermal defects, showing that *rfx6* functioned similarly in *Xenopus*. Next, they showed that wild type *rfx6* rescued these specific defects thus identifying an assay to test the function of mutant *rfx6* versions. Last, they generated three different mutant versions of *rfx6* that reproduced the human mutations found in patients and tested their ability to rescue the *rfx6* knockdown phenotype; two of these were single point mutations that changed one amino acid. They found that none of these mutants versions were able to rescue the *rfx6* knockdown phenotype like the wild type *rfx6*. This

important result provided direct evidence that these specific point mutations functionally impaired Rfx6 pointing to a molecular mechanism responsible for the disease and demonstrates how *Xenopus* can be used to directly establish whether specific mutations identified in patients is causative to the disease.

Defects in the pancreatic beta cell ATP-sensitive K+ (KATP) channel (Kir6.2/SUR1) have been associated with both neonatal diabetes and type 2 diabetes [47, 48]. The KATP channels are multimeric complexes comprised of four potassium channel Kir6.2 subunits (encoded by the KCNJ11 gene) that form the inner channel and four sulfonylurea receptor 1 (SUR1) subunits (encoded by the ABCC8 gene) [47]. This hetero-octameric protein complex plays an important role in regulating insulin secretion. The KATP channels on the beta cell membrane are open in a state of low glucose; as blood glucose levels increase, glucose enters the beta cell and is subsequently metabolized leading to an increase in intracellular ATP. The increase in ATP triggers closure of the KATP channels and activation of voltage sensitive calcium channels leading to calcium mediated insulin secretion. Loss-of-function mutations in either subunit, resulting in channel closure, leads to an increase in insulin secretion and subsequent hyperinsulinism, while gain-of-function mutations result in a decrease in insulin secretion and subsequent diabetes [49]. Sulfonylurea drugs are used to treat diabetes and these drugs increase insulin secretion by binding to SUR1 leading to closure of the KATP channel [50].

Understanding the physiological impact specific mutations have on channel activity is a key area of research in diabetes, and *Xenopus* oocytes provide a good platform to investigate the function of these channel proteins in the presence or absence of single gene mutations and/or pharmacological treatment. The *X. laevis* oocyte is largely transcriptionally silent, however the oocyte maintains a pool of maternally transcripts which provide a source for protein translation [51, 52]. A single female contains thousands of oocytes that are easily isolated and can be cultured for several days. The large size of the oocyte lends itself well to physiological perturbations allowing for direct measurement of channel activity via molecular or electrophysiological assays under a range of conditions including mutant protein, chemical inhibitors, or activators [53, 54]; exogenous channel coding mRNAs can be delivered by microinjection and the protein products are processed and trafficked to the membrane [55].

Several recent studies used *Xenopus* oocytes to investigate key interactions between sulfonylurea drugs and different variants of KATP channel subunits. In one such study they identified a novel heterozygous mutation of the Kir6.2 gene (i.e. W68R) in a patient with remitting and relapsing neonatal diabetes. To test if the W68R could be the cause of diabetes observed in the patient, the authors overexpressed wildtype SUR1 and wildtype or mutant Kir6.2 by mRNA injection in *Xenopus* oocytes. Using whole cell electrophysiology methods (i.e. patch-clamp single channel recording) the authors determined that this W68R mutation, replacement of tryptophan with arginine, reduced the ATP sensitivity of the channel [56•]. They went on to show that the sulfonylurea compound, tolbutamide maintained canonical channel blocking activity, despite lowered ATP mediated inhibition on Kir6.2, suggesting that patients harboring the W68R mutation could benefit from sulfonylurea therapy [56]. Another report from the same lab examined four different Kir6.2 mutations (i.e. R210C, G334D, I296L and V59M) associated with neonatal diabetes [57]. They expressed different combinations of wild type and mutant versions in *Xenopus* oocytes and measured channel activity using patch-clamp single channel recording in excised patches. During recording, patches were treated with gliclazide, another sulfonylurea drug, to block channel activity. Their results indicated that the G334D mutation rendered Kir6.2 free of any ATP mediated inhibition, thus in the presence of sulfonylurea the channel remained active [57]. These results help to explain why patients with a G334D mutation do not respond to sulfonylurea therapy. Subsequent studies reported on the

interplay between sulfonylureas and Mg-nucleotides on Kir6.2/SUR1 in *Xenopus* oocytes. Briefly they found that when sulfonylureas were bound to their target on SUR1 they reduced Mg-binding to SUR1 decreasing normal channel-opening activity [58]. These studies represent rigorous physiological investigations, aided by the utility of the *Xenopus* oocyte, to dissect the mechanistic basis for efficacy of the sulfonylurea family of anti-diabetic drugs, and sheds light on why certain monogenic mutations lend themselves well to sulfonylurea treatment while others do not.

Lung and thyroid development

In addition to its well known role as a model for early patterning events and posterior foregut development, *Xenopus* has emerged as a good model system for studying development of the respiratory system [59, 60]. Unique among aquatic model systems, *Xenopus* embryos rapidly develop lungs in the first few days of embryogenesis, which at the molecular and histological level are similar to early mouse lungs [60••]. Recent studies have revealed that the key signaling pathways Wnt, FGF and BMP implicated in mammalian lung development are conserved in *Xenopus* lung development [60-64]. Using small molecule inhibitors and dominant negative FGFR1 constructs two studies showed that FGF signaling was required for specification of lung tissue, but not thyroid [62, 64]. Shifley et al also showed that both MEK and PI3K pathways downstream of FGF are essential for lung development [64]. Using an RA antagonist, RA was also shown to be necessary for lung specification, but only during the early stages [61, 62]. Interestingly, embryos treated with RA showed ectopic lung marker expression in the presumptive thyroid, suggesting that RA is a key regulatory switch controlling lung versus thyroid development.

One of the strengths of *Xenopus* is the ease with which epistatic relationships between signaling pathways can be tested by simple treatment of embryos with small molecules or inducible proteins at different times. Taking advantage of this, Rankin et al showed that Wnt acts downstream of FGF signaling to induce Nkx2.1 in presumptive lung tissue [60]. They activated FGF signaling using a drug inducible FGFR1 construct and analyzed whether constitutive FGF signaling was sufficient to induce lung development in the presence of Wnt knockdown. Activation of FGF signaling on its own was sufficient to induce ectopic Nkx2.1 expression in the foregut endoderm, while it had no effect in the presence of Wnt morpholinos [61]. Similarly, they showed that the transcription factors Osr1 and Osr2 function downstream of FGF, but upstream of Wnt signaling in lung development [61]. Additionally, they found that Osr1/Osr2 function also played a role in repressing bmp4 expression in the lateral plate mesoderm, linking these three signaling pathways. They showed that Osr1/Osr2 promoted lung specification by repressing *bmp4* expression in the lateral plate mesoderm thus promoting Wnt and RA signaling. Although only recently emerging as a model for lung development, *Xenopus* provides a unique platform for understanding the signaling network controlling specification of early lung buds.

Genome editing and the future of disease research in *Xenopus*

In the last three years there has been an explosion of progress in the field of disease modeling primarily due to the emergence of genome editing tools that allow researchers to generate mutations in precise regions of the genome virtually at will. Two genome editing tools that have received much attention as of lately, are Transcriptional Activator Like Effector Nucleases (TALENs) and the Conserved Repetitive Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system [65]. In both systems, administration of the genome editing tools, usually be microinjection, result in sequence specific targeting of host genomic DNA. The TALEN and CRISPR system are, in most cases, engineered to have nuclease activity, such that appropriate targeting of the locus of interest will be cleaved by the

TALEN or Cas9 proteins resulting in double stand breaks (DSB) in the genomic sequence, which are subsequently repaired in an error prone fashion by non-homologous end-joining often leading to insertion and/or deletion errors (INDELS) that result coding sequences that are defective (i.e. frameshift, premature stop) [66-68].

TALENs and the CRISPR/Cas9 system have been used in *Xenopus* to generate mutations with much success [69-73]. For example Lei and colleagues used TALENs targeting several loci including the pancreatic master regulator Ptf1a resulting in pancreatic agenesis [72]. More recently Guo et al. provided a comprehensive report using the CRIPSR/Cas9 system for targeting a number of endoderm and pancreas specific factor, including Ptf1a, Hhex, Elastase, and Pdx1 [69•]. These studies highlight the usefulness in combining the *Xenopus* experimental platform with genome editing tools. The next level of innovation surrounding genome editing with the goal of modeling human disease is in the development of an efficient method for homology directed repair of TALEN/CRISPR induced DSB [68]. Investigators in nearly all model systems are contributing to these efforts and several reports of success have circulated including one using *Xenopus* [74]. Despite these considerable successes it is not completely clear what the effective rate for incorporation of donor genetic material is, and the efficacy for insertion into the precise locus absent from off targets (i.e. random insertions). Nonetheless these reports show incredible promise and once methods are widely established the technique will undoubtedly revolutionize monogenic disease characterization and modeling.

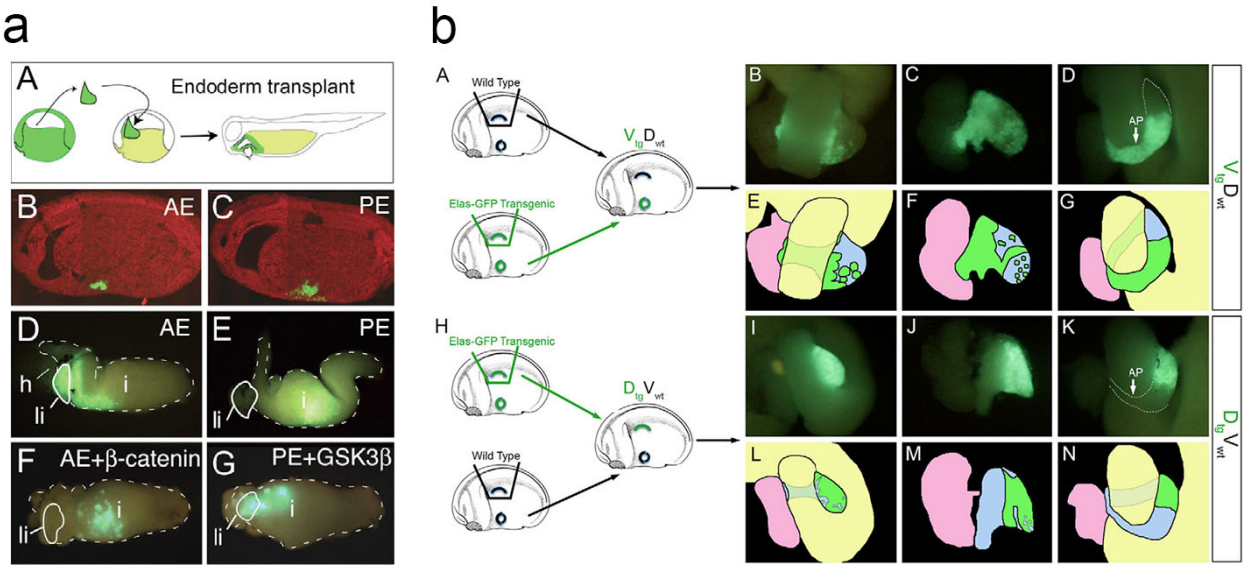
One practical challenge for disease modeling in *Xenopus* is the breeding and maintenance of mutant animals, since most *Xenopus* laboratories are not equipped to raise many different lines. The National *Xenopus* Resource (NXR) benefits researchers from *Xenopus* and non-*Xenopus* laboratories by providing access to an expanding repertoire of *Xenopus* lines available for delivery or in house use [75]. The NXR facilitates disease modeling research through a multitude of services, including custom mutant lines and a research hotel service. In the custom mutant service the NXR designs, clones and injects the CRISPR and TALEN constructs and then grows the animals to adulthood. A research hotel service enables investigators to come to the Marine Biological Laboratory for short-term visits and utilize NXR infrastructure, animals and expertise for a wide range of *Xenopus* laboratory technique, including genome engineering. Investigators can utilize these tools to generate custom animal lines necessary for their own research program, while leaving the challenge of raising these animals to the NXR [75]. Resources like the NXR provide a center for rapid disease modeling in *Xenopus*, and a mechanism for delivering these valuable new tools to the greater research community.

Concluding remarks

The purpose for this review is to provide the reader with an overview of recent studies and advancements for understanding human pancreas and gastrointestinal disease using the *Xenopus* as a platform for discovery. The major take home message from this review is the diversity of research questions that can be addressed using *Xenopus* to address the mechanistic basis of disease in the developing gastrointestinal tract. Implementation of gene editing techniques will only enhance the utility of this model for human disease modeling.

Figure Legend

Figure 1. Two examples illustrating how transplantations are used in Xenopus. a) Transplantation of labeled anterior or posterior endodermal fragments expressing specific Wnt signaling factors (beta catenin or Gsk3b) into an unlabeled host embryos at gastrula stages established that Wnt signaling acted directly on the endoderm. (Reproduced with permission from Development [23]) b) To establish the behavior of dorsal versus ventral pancreatic bud cells, chimeric embryos were made between wild type and transgenic Elas-GFP Xenopus embryos by transplanting dorsal pieces at the tail bud stage. These experiments revealed that the ventral pancreatic bud cells migrate extensively after fusion, while the dorsal bud cells did not. (Reproduced with permission from Development [11])



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