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Prospects for the *Xenopus* Embryo Model in Therapeutics Technologies

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Abstract: Access to suitable animal models is essential in the field of therapeutics technologies. Recently, lower vertebrates have emerged as attractive low-cost animal models which offer new exciting applications in functional genomics and therapeutics technologies. Amphibian embryos of the genus *Xenopus* have long served as important models for the study of vertebrate development. *Xenopus* is evolutionary significantly less distant to humans than fish models, which suggests that experimental findings gained with *Xenopus* will more accurately predict human biology. Numerous experimental advantages, including external development, large size, identifiable blastomeres, and their ability to withstand extensive surgical intervention and culture *in vitro*, have favored the use of the *Xenopus* model in the past. More recently, the introduction of a simple efficient method to disrupt gene functions and the rapid development of genomic resources has further increased the attractiveness of this low-cost, high-throughput model for the analysis of vertebrate gene functions. Using the *Xenopus* embryo as the primary animal model, our research in the field of therapeutics technologies has focused on the identification and validation of novel drug targets by employing genomic and transcriptomic information in the analysis of the molecular and cellular processes underlying kidney organogenesis and vascular development. Furthermore, our research on signaling pathways controlling cellular differentiation of embryonic tissues provides important insights that may ultimately lead to the development of novel cell-based therapies in regenerative medicine. Finally, we are exploring the possibility of employing the *Xenopus* embryos in chemical library screens to identify novel chemical modulators of organogenesis.

Keywords: Angiogenesis · Drug discovery · Genomics · Kidney organogenesis · *Xenopus*

Introduction

Research in the field of therapeutics technologies covers all aspects relating to the discovery, engineering, and delivery of therapeutics. This may include the following activities: identification of key determinants that regulate the biological processes underlying pathological conditions and their validation as drug targets; development of functional screens at the cellular, organ and/or whole organism level leading to the identification of novel drug targets; isolation and development of novel therapeutic agents; engineering of bioactive molecules with optimized pharmacokinetic profiles or improved targeting activities; development of novel diagnostic procedures and tools, or the establishment of global screening strategies to identify gene functions causing or contributing to human disease conditions. The field is currently undergoing profound change with the development of powerful profiling approaches based on information gained from the human genome sequence and with the introduction of low-cost, high-throughput animal models.

Here, our activities in the field of therapeutics technologies are reviewed. Our primary focus has been on chronic renal failure and cardiovascular diseases, which affect worldwide millions of patients. As a rational basis for the development of novel therapeutic approaches, we have been particularly interested in understanding the basis of kidney organogenesis and vascular development in the vertebrate embryo. Towards this goal, we have established the *Xenopus* embryo as an attractive, low-cost vertebrate model for large-scale identification of organ-specific gene activities and the rapid, high-throughput analysis of candidate gene functions. In addition, we are assessing possibilities of using *Xenopus* embryos for whole-organism-based drug discovery screens.

The *Xenopus* Embryo Model

The amphibian embryo has been a favorite animal model for embryologists since the early nineteenth century. Although many different species have been used for experimental work in the past, the African clawed frog *Xenopus laevis* [1] (Fig. 1A) has been the most popular amphibian species for embryological work since the early 1960s [2]. It ranks with the mouse, the chick and more recently the zebrafish among the most popular vertebrate species for research. By means of a simple injection of human chorionic gonadotropin hormone, a female *Xenopus* frog will lay thousands of eggs, which are large (1.4 mm diameter) and easily fertilized *in vitro* (Fig. 1B). Once fertilized, the eggs will develop to tadpoles completely outside of the mother in a simple salt solution. The rate of development is predictable and can be manipulated by temperature, which greatly facilitates experimentation. Furthermore, the

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embryos remain accessible to experimentation at all stages. They are easily manipulated, injected, grafted or labeled. In addition, fragments of early embryos and even isolated cells continue to develop if incubated in simple salt solutions. After experimental manipulation, the embryos can be readily analyzed by localizing mRNA transcripts using whole-mount *in situ* hybridization (*e.g.* Fig. 2B, C) or by localizing proteins using immunohistochemical techniques. Embryonic development is rapid and cleavage divisions, gastrulation, morphogenesis, and organogenesis occur within 48–72 h. By this point the organism possesses discrete organs and tissues such as the brain, sensory organs, a heart, blood vessels, a liver, a pancreas, and two kidneys (Fig. 1C). *Xenopus* organs are remarkably similar to their human counterparts at the anatomical, physiological and molecular levels, facilitating the study of vertebrate biological processes that are inaccessible to invertebrate model organisms.

Given these favorable features, the *Xenopus* embryo model has been widely used for studying basic cell biological processes and for understanding vertebrate development. Cell biological studies have greatly contributed to our understanding of cell cycle control, gene regulation, chromosome structures and regulation, cytoskeletal dynamics, and signal transduction. Embryological studies have provided important insight into the mechanisms of axis determination, mesoderm induction, early fate decisions, morphogenesis, and early organogenesis.

Xenopus laevis would seem to be the ideal all-purpose vertebrate research organism, except for the fact that it is not ideal for genetics given its pseudotetraploid genome and its relatively long generation time. The *Xenopus laevis* genome (haploid chromosome number, n = 18) contains four copies of many genes. The duplicated genes may retain partially or wholly overlapping functions complicating loss-of-function studies or mutagenesis screens. Recently, however the closely related diploid species *Xenopus* (formerly *Silurana*) *tropicalis* has emerged as a potential ideal amphibian model organism [3]. *Xenopus tropicalis* retains many of the experimental advantages of its larger relative *Xenopus laevis*. *Xenopus tropicalis* has with 3–5 months a shorter generation time compared to 6–8 months required for *Xenopus laevis*, which favors the development of stable transgenic lines and the breeding of homozygous mutant lines. With 1.7×10^9 base pairs per haploid nucleus (n = 10), the genome size of *Xenopus tropicalis* is about half the size of the mouse genome. Phylogenetic studies of vertebrate genomes place *Xenopus tropicalis* close to its relative *Xenopus laevis* with an estimated divergence time of about 30 million years (Myr) ago, which is less than the evolutionary distance between mouse and rat (Fig. 1D). Finally, all evidence to date indicates that *Xenopus tropicalis* contains a typical diploid tetrapod genome [4].

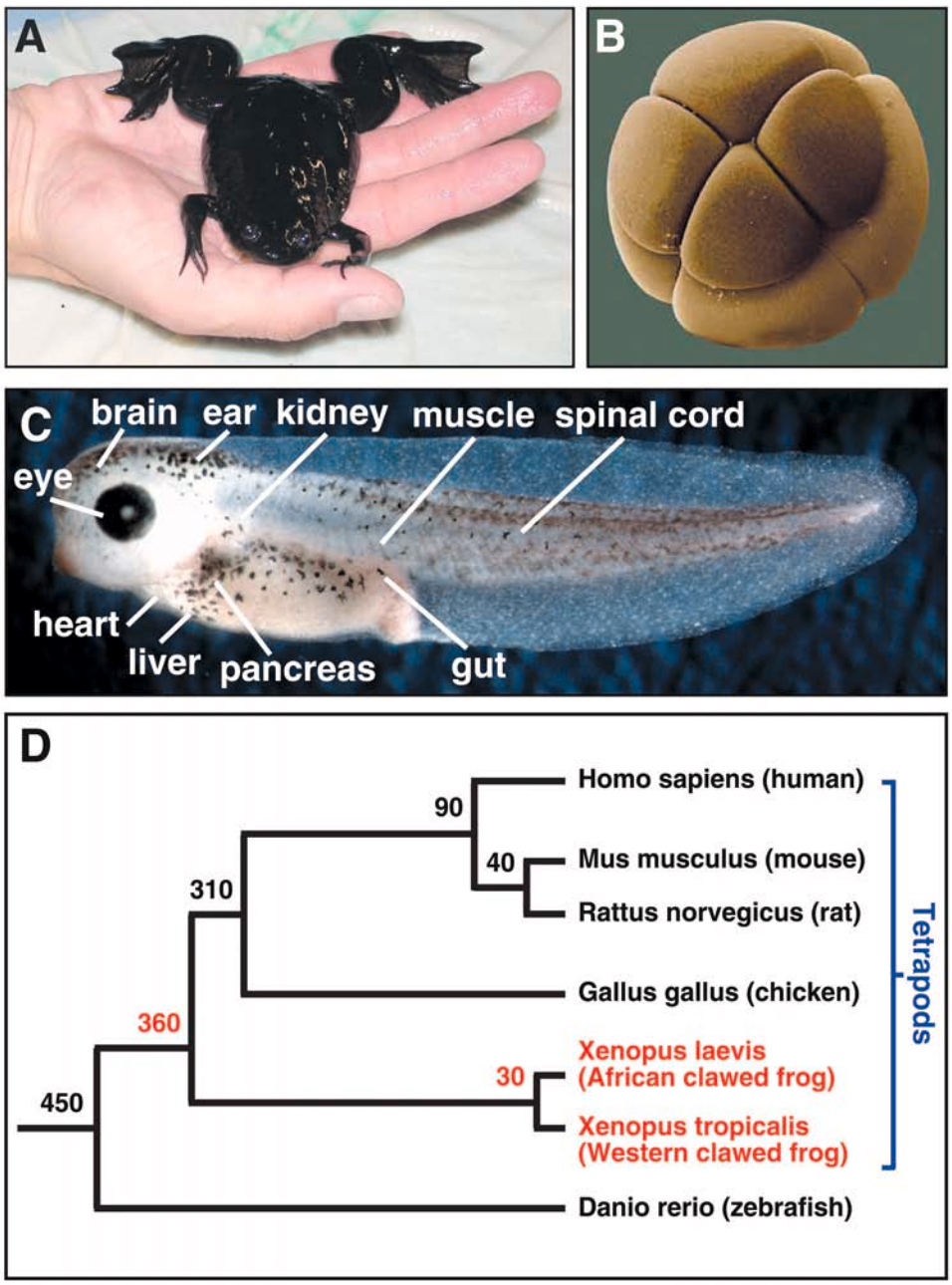

Fig. 1. **The *Xenopus* embryo model**. (**A**) *Xenopus laevis*, the African clawed frog, adult female. A female will lay more than thousand eggs, which can be fertilized *in vitro* to generate a large population of synchronized embryos. (**B**) *Xenopus laevis* embryo at the 8-cell stage (stage 4; 135 min post fertilization). Due to their large size, single blastomeres can be easily injected with synthetic mRNA, antisense oligonucleotides, recombinant proteins or antibodies. (**C**) *Xenopus laevis* late tadpole stage embryo (stage 41; 3 days post fertilization). Most organs of the vertebrate body are present and are performing their physiological functions. (**D**) Evolutionary relationship between various vertebrate species used as model organisms to study organogenesis, disease mechanisms and tumorigenesis. The numbers at the nodes are estimated divergence times in million of years. The relationship and divergence times are largely based on a molecular clock study of 658 proteins [2].

With the recent establishment of the zebrafish model, one might ask whether there is a need for a further lower vertebrate model for genetic studies. As *Xenopus*, zebrafish embryos offer the advantages of externally developing embryos, which are available in large numbers. These features overcome many of the limitations of the mouse system, such as internal development and small litter size, in studying embryonic development and organogenesis. Although zebrafish embryos are excellent for some manipulations, particularly blastomere injections and transplantation experiments, the embryos are relatively fragile and one cannot perform explant experiments that have made *Xenopus* such a powerful embryological model system. Despite the fact that many valuable zebrafish

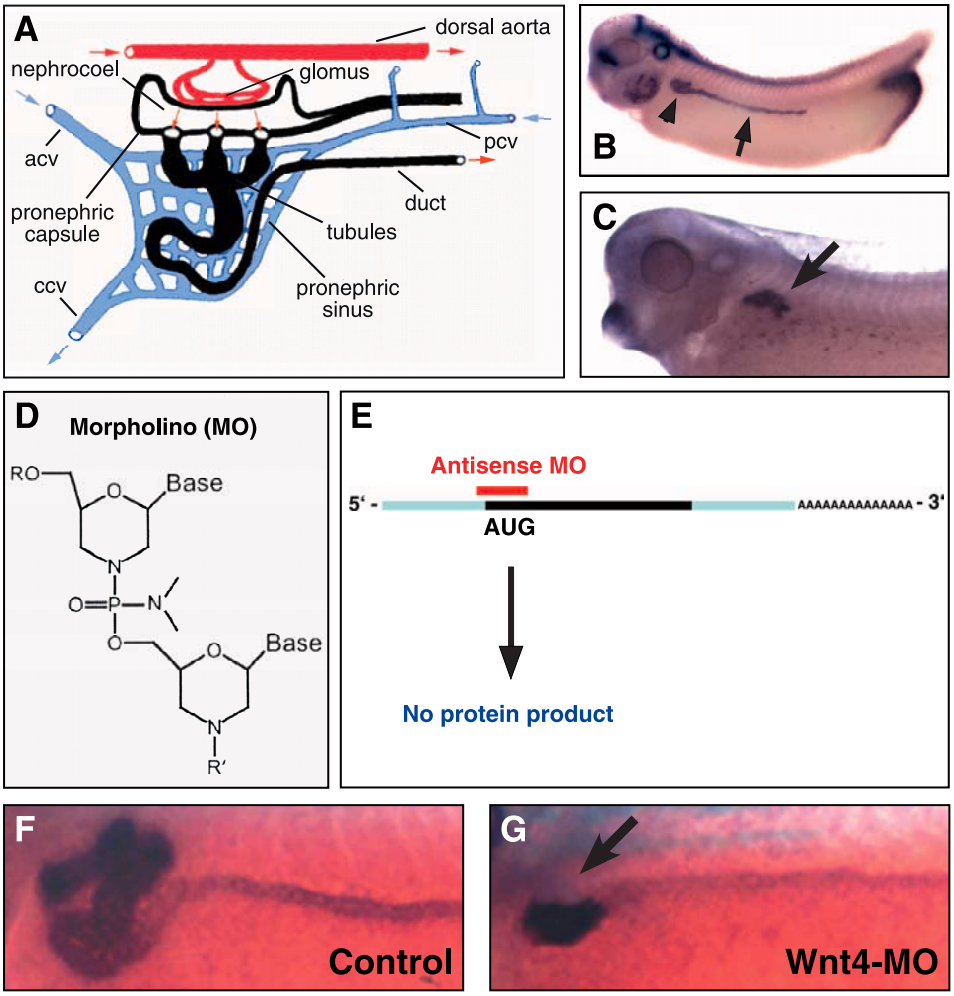

Fig. 2. **Kidney organogenesis in *Xenopus*.** (**A**) Schematic representation of the *Xenopus* pronephric kidney. Abbreviations: acv, anterior cardinal vein; ccv, common cardinal vein; pcv, posterior cardinal vein. (**B–C**) Molecular markers of the developing pronephric kidney in *Xenopus*. Whole mount *in situ* hybridizations of embryos (**B**, stage 31; **C**, stage 36) analyzed with antisense RNA probes are shown. (**B**) Expression of the transcription factor Pax2 is found in the eye, ear, brain, and spinal cord. In the pronephros, Pax2 expression is detected in both developing tubule (arrowhead) and duct epithelia (arrow). (**C**) Close-up view of a *Xenopus* embryo stained with SLC15A2, which encodes the proton oligopeptide transporter 2 (PEPT2). In the pronephric kidney, SLC15A2 expression (arrow) is confined to the proximal segments of the nephron. (**D–E**) Antisense morpholino-based knock-down technology. (**D**) Structure of a morpholino oligonucleotide (morpholino-phosphorodiamidate). R and R' denote continuation of the oligomer in the 5' and 3' direction, respectively. (**E**) Mechanism of antisense morpholino inhibition. Sequence-specific binding of the antisense morpholino (MO) to the AUG initiation codon of the mRNA blocks translation. (**F–G**) Wnt4 is required for pronephric tubule formation in *Xenopus*. (**F**) Pronephric kidney of a control embryo visualized by *in situ* hybridization for Pax2. (**G**) Pronephric kidney of an embryo injected with an antisense MO against Wnt4 (Wnt4-MO). Note the absence of pronephric tubules (arrow). The figure shown in panel A is taken from [29] and those shown in panels F and G are from [19].

mutations have been isolated to date, an ancestral genome duplication, which occurred in an ancient teleost fish about 235 Myr ago [5], makes zebrafish a less than ideal system for genetics. While zebrafish and mammals share many similarities at the anatomic as well as the molecular level, they are nevertheless distant relatives evolving separately since about 450 Myr (Fig. 1D). In contrast, the evolutionary distance between *Xenopus* and humans is significantly smaller as their genomes diverged about 360 Myr ago. Surprisingly, this is only 50 Myr prior to the estimated date for the divergence of bird and mammalian genomes. Given these facts, it is therefore likely that the findings gained from studies of organogenesis and other developmental processes in the *Xenopus* embryo model will more accurately relate to the situation in humans than those gained in the zebrafish model. This also emphasizes the unique and important role that the *Xenopus* model is expected to play in functional genomics and biomedical research.

Xenopus Genomics

The recent efforts to establish genomic resources for *Xenopus*, particularly large expressed sequence tag (EST) databases, and the decision to sequence the *Xenopus tropicalis* genome are already becoming an extraordinary resource for the *Xenopus* community in general. The status of *Xenopus* genomics is rapidly advancing with the help of the Trans-NIH *Xenopus* Genome Initiative, the Joint Genome Institute, the Washington University Genome Sequencing Center, and the Welcome Trust Sanger Institute [6].

In 2002, the Joint Genome Institute (JGI) of the Department of Energy at Walnut Creek (USA) initiated a genome sequencing project for *Xenopus tropicalis* (*http://genome.jgi-psf.org/xenopus*). Two sets of whole genome shotgun libraries have been produced and are being sequenced at 5–6× depth. Several genome assemblies have been released to date. The most recently assembly was released in February 2004 and includes approximately 4.7× sequence coverage. The sequence drafts are available for homology searches at the JGI website. A final draft assembly based on ~8× sequence coverage and incorporating other data including end sequences of bacterial artificial chromosomes and physical map information will be completed in the first half of 2005. Efforts are also on the way to initiate sequencing of the *Xenopus laevis* genome (Don Brown, personal communication).

Expressed sequence tags (EST) are single sequence traces derived from a random set of cDNAs, which are generated from reverse-transcribed mRNAs. Large collections of ESTs are an important resource for gene discovery, oligonucleotide-based gene knock-down studies, the construction of DNA microarray chips, and gene mapping studies in the context of the *Xenopus* genome project. Until recently, the sequencing of ESTs from *Xenopus* has however lagged behind the efforts in many other vertebrate model systems. This has however dramatically changed over the last three years [6–8]. Dozens of cDNA libraries were generated from tissues of the two *Xenopus* species. The cDNA libraries represent oocytes and developmental stages from fertilized eggs to tadpole stages and eight adult organs including kidney and heart. All EST sequences are submitted to GenBank and are also accessible through dbEST, the EST database at the National Center of Biotechnology Information (NCBI; *http://www.ncbi.nim.nih.gov/dbEST*). Currently, dbEST lists over 430'000 *Xenopus laevis* and 420'000 *Xenopus tropicalis* ESTs (Table). Taken together, more than 850'000 *Xenopus* ESTs are presently known and *Xenopus* collectively now ranks third of all vertebrate model organisms. In terms of available EST data, it is currently the best-understood non-mammalian vertebrate organism.

NCBI's UniGene database (*http://www.ncbi.nim.nih.gov/entrez/query.fgci?db=uni*

Table. Public EST entries by model system

Species	No. of EST entries
Homo sapiens (human)	5'657'997
Mus musculus + *domesticus* (mouse)	4'235'142
Rattus sp. (rat)	661'663
Danio rerio (zebrafish)	532'545
Gallus gallus (chicken)	495'089
Xenopus laevis (African clawed frog)	434'889
Xenopus tropicalis (Western clawed frog)	423'107
Drosophila melanogaster (fruit fly)	382'439
Caenorhabiditis elegans (nematode)	298'805

Note: Numbers are taken from the database of "Expressed Sequence Tags" (dbEST; release of July 30, 2004) at the National Center of Biotechnology Information (*http://www.ncbi.nlm.nih.gov/dbEST*).

gene) uses the sequence information from well-characterized genes and ESTs to generate non-redundant sets of clusters, each representing a unique gene. To date, about 24'000 UniGene entries are reported for *Xenopus laevis* and 15'000 for *Xenopus tropicalis*. UniGene clusters provide a basis for the selection of candidate complete open reading frame (ORF) cDNA clones for complete nucleotide sequencing. The *Xenopus* Gene Collections database (XGC; *http:xgc.nci.nih.gov*) currently lists the sequences of 5'221 and 1'513 full-length cDNA clones for *Xenopus laevis* and *Xenopus tropicalis*, respectively. In a separate EST sequencing project, the Wellcome Trust Sanger Institute (*http://www.sanger.ac.uk*) has recently reported a set of approximately 36'000 unique expressed sequences for *Xenopus tropicalis*, which apparently represent about 7'000 full-length clones [8]. At present, the degree of redundancy between the two *Xenopus tropicalis* databases is not known. Nevertheless, the UniGene clusters and non-redundant full-length cDNAs serve as an important resource for gene mapping projects, prediction of exon-intron structures, large-scale gene expression screens and gene discovery searches. *Xenopus* EST databases have also been very valuable for developing microarrays. Microarray technology has emerged as a major research tool enabling global gene expression profiling. Prototype microarrays for *Xenopus laevis* have been reported previously [9], and in late 2003, the first commercial oligonucleotide microarray (Affymetrix's GeneChip *Xenopus laevis* Genome Array) has appeared on the market offering the possibility of simultaneously monitoring the expression of 14'400 mRNA transcripts.

A Toolbox of Methods to Analyze Gene Functions in *Xenopus*

Whole genome sequences are now available for several vertebrate model organisms including human, mouse, rat and chicken. Furthermore, the zebrafish and *Xenopus tropicalis* genomes are nearing completion. Scientists are now faced with the challenge to identify all genes present in the vertebrate genome sequences and assign functions to each gene. *Xenopus* embryos are ideally suited for functional genomics leading to the elucidation of the role of genes in embryogenesis and organ development. Gene functions can be easily assessed in the *Xenopus* embryo through simple gain- and loss-of-function approaches. Most importantly, the toolbox of methods available in *Xenopus* to manipulate gene functions has recently been expanded by the introduction of morpholino oligonucleotides for transient gene knock-down studies [10].

Ectopic gene expression relies on the ability of *Xenopus* embryos to translate injected, synthetic mRNA. This ability has been exploited for gain-of-function screens, where the misexpression of mRNAs is used to identify gene products inducing specific phenotypic changes. The mRNAs can be misexpressed in broad or more restricted domains, depending on the developmental stage at which they are injected into single blastomeres of early *Xenopus* embryos. For example, genes can be ectopically expressed in broad domains by injecting mRNAs into the early cleavage-stage embryo. Such screens have led to many important contributions, such as the identification of the major classes of inducing factors (TGFß/BMP, FGF and Wnt) used by all animals [11].

Ablation of gene expression is central to the dissection of genetic mechanisms in model organisms. Targeted gene inactivation experiments in the mouse have revolutionized our understanding of gene functions during development. This approach is however dependent on embryonic stem cell technology that is currently only available for the mouse. Furthermore, the significant costs (>$50'000 per gene) and the time (>1 year) required for the breeding of homozygous mutant progeny make this approach unsuitable for genome-wide screens. The experimental approaches to disrupt gene functions in *Xenopus* have until recently been limited to overexpression of mRNAs encoding dominant-negative mutants [12]. While this approach has been used very successfully in the last decade to disrupt functions of various genes in *Xenopus* embryos (*e.g.* [13][14]), it is not universally applicable. First, the development of true dominant-negative mutants has failed for many proteins. Second, the overexpression of dominant-negative mutants may also impair the functions of structurally related proteins. Alternatively, the injection of inhibitory antibodies into *Xenopus* embryos has been used in some cases to block the activity of gene products [15]. This strategy is however limited to cases where suitable antibodies with high binding affinities are available. Finally, RNA interference (RNAi) using short, double-stranded RNA (small interfering RNA, or siRNA) was recently introduced for suppressing gene expression in mammalian cells. Generally, siRNA induces degradation of target mRNA in a sequence-specific manner, leading to posttranscriptional silencing of gene expression. *In vivo* RNA-based interference has been reported to work in zebrafish and *Xenopus* with highly variable and controversial results [16]. Current RNAi methods do therefore not appear to reliably inhibit gene functions in lower vertebrate embryos.

The introduction of morpholino phosphorodiamidate oligonucleotides (also called morpholinos) offer a new alternative to study loss of gene activity in zebrafish and *Xenopus* [10][17]. Morpholinos are synthetic DNA analogs that can bind to and block the translation of mRNA *in vitro*, in cultured cells, and in vertebrate embryos [18]. Morpholinos have morpholine moieties in place of the standard riboside moieties and contain a neutral charge backbone due to the replacement of the phosphorodiester linkages with phosphorodiamidate intersubunit linkages (Fig. 2D). They are usually designed as 25-mer to anneal against the leader sequence of the targeted mRNA transcript. With high affinity for RNA, morpholinos display little toxicity and are resistant to endonucleases. After injection into single blastomeres of embryos, they function through a RNase-H-independent mechanism to inhibit protein translation (Fig. 2E). In zebrafish, many known mutations have been phenocopied [17] and experiments in *Xenopus* embryos have demonstrated that mouse knock-out phenotypes can be accurately induced by morpholino injections [19]. Taken together, morpholinos provide a simple, effective and rapid method to study gene functions.

It is therefore likely that the application of morpholino technology will increase the pace of functional analysis of many *Xenopus* genes emerging from the large-scale EST projects and the sequencing of the *Xenopus* genomes.

Transgenesis in *Xenopus*

The study of late gene functions is in general difficult to achieve in vertebrate model systems. In the mouse, difficult and laborious procedures are required to generate conditional knock-out strains. In *Xenopus*, methods to affect gene functions such as mRNA, morpholino oligonucleotide or antibody injections are all essentially transient as the genome remains unaltered and the injected material decays with time. One of the most significant technical advances in the field of *Xenopus* embryology in recent years has been the development of methodology for transgenesis [20]. Known as restriction mediated insertion (REMI) transgenesis, the method involves mixing transgene DNA with purified and permeabilized sperm nuclei, along with a small quantity of restriction enzyme, and then injecting the mixture into unfertilized eggs with the aim of transferring one sperm nuclei per egg. This method is capable of producing hundreds of non-mosaic transgenics in the parent (F_0) generation and transgenes are reliably transferred through subsequent generations. Since all embryonic cells, including the germ cells, carry the transgene, experiments can be performed without the need to generate stable transgenic lines. This is a major advantage over transgenesis in zebrafish and mouse, where the breeding is required to establish transgenic lines. *Xenopus* transgenesis offers the ability to study transgene expression in living embryos using tissue-specific promoters and green fluorescent protein as a reporter. Furthermore, Cre and FLP conditional mutagenesis systems and tetracycline-inducible constructs are being developed for *Xenopus* [21]. Finally, the transgenesis technique in *Xenopus tropicalis* has also been used for gene trapping and insertional mutagenesis [22]. Collectively, these methods are powerful new tools to elucidate late gene functions in organogenesis and to validate novel drug targets emerging from the various vertebrate genome projects.

Dissecting *Xenopus* Kidney Organogenesis: Establishing a Rational Basis for Renal Cell Replacement Therapies

Loss of kidneys or the progression of chronic kidney disease from renal failure to end-stage renal disease (ESRD) is incompatible with life. Chronic kidney disease is defined according to the presence of kidney damage and the level of remaining kidney function, regardless of the type of kidney disease. In addition, many drugs can injure the kidney and cause progressive renal insufficiency [23]. Chronic kidney disease is a significant medical problem affecting roughly 25 million people alone in the United States. It is also a risk factor for cardiovascular disease, including myocardial infarction, atherosclerosis, stroke, and hypertension. The number of patients currently undergoing treatment of end-stage renal disease in the United States is about 400'000, but this is projected to soar in the next years primarily due to a decline in mortality and the growth of the number of diabetic patients [24]. Life can be prolonged in human patients either by dialysis, a procedure with considerable morbidity, or kidney transplantation. The latter is usually the preferred therapeutic treatment as it provides a better quality of life for many patients. However, transplantation is limited by the increasing lack of suitable organs. One possible solution to the problem is the use of undifferentiated or partially developed kidney precursor cells for replacement therapy. Renal precursor cells could serve as a potential source for regenerating kidney cells [25]. Successful implementation of such strategies requires a detailed understanding of both the molecular and cellular processes underlying kidney organogenesis and the nature of renal stem cells.

The adult mammalian kidney is a complex organ composed of at least 26 specialized cell types, which fulfill specific physiological functions in glomerular filtration, reabsorption and secretion of fluid and solutes [26]. These cell types are derived from embryonic precursor tissues and have to be precisely assembled into the key structures of the kidney including glomeruli, tubular epithelia and the interstitial tissues to sustain life. Careful analysis of kidney development is now providing cues how this complex assembly process is achieved.

Development of the vertebrate kidney is an intricate well-orchestrated process that requires the formation of three distinct excretory organs, the pronephros, mesonephros, and metanephros, which are all derived from the intermediate mesoderm [27]. The pronephros represents the simplest form of a vertebrate kidney, but largely degenerates in mammalian embryos. Formation of the pronephros is however necessary in all vertebrates as it initiates subsequent steps of kidney development. Interestingly, embryos of lower vertebrates, such as zebrafish and *Xenopus*, develop fully functional pronephric kidneys to sustain the larvae's life outside of the mother [28][29]. The *Xenopus* pronephros is a simple excretory system that very much resembles the mammalian kidney at the anatomical level as well as in regard to the corresponding patterns of gene expression (Fig. 2A–C). Induction of pronephric tissues can also be achieved in an organ culture system prepared from presumptive ectoderm of *Xenopus* embryos [30]. Finally, *Xenopus* A6 cells, isolated from adult kidney [31], have been used successfully to explore epithelial cell polarity and the regulation of renal solute transport. Therefore, the *Xenopus* embryo model combined with the pronephric organ culture system and renal cell lines represents an attractive set-up to define the conserved mechanisms governing the development of all vertebrate kidney forms. Our recent contributions within the area of kidney organogenesis will be reviewed briefly.

Pax Genes in Early Renal Development

Patients suffering from renal-coloboma syndrome display optic nerve dysplasia and congenital renal hypoplasia, which are caused by mutations in the PAX2 gene [32]. This important finding stimulated our initial interest in studying Pax genes in the *Xenopus* embryo model and we hoped to gain a deeper understanding of the role of Pax genes during vertebrate kidney organogenesis. Towards this end, we have cloned and characterized *Xenopus* cDNAs encoding Pax2, Pax5 and Pax8 [33][34]. Embryonic expression of *Xenopus* Pax2 was detected in all developing renal epithelia, the optic stalk and other structures of the developing nervous system (Fig. 2B). This is largely comparable with the situation in mammals suggesting that embryonic Pax2 expression has remained evolutionary conserved. Surprisingly, our research on the other Pax2-related genes led to the identification of Pax8 as the earliest gene product expressed in the developing pronephric primordium [34]. Pax8 expressing pronephric precursor cells emerge in the embryonic trunk soon after the completion of gastrulation. Our findings were recently confirmed by studies in the mouse, establishing a crucial function for Pax8 in nephrogenesis [35]. Analysis of the genetic program controlled by Pax8 in the renal primordia will be of particular interest in future studies. Furthermore, the isolation and characterization of Pax-8 expressing renal progenitor cells will be pursued.

Wnt Signaling in Nephrogenesis

Wnt proteins form one of the major families of secreted ligands that play key roles in developmental signaling by controlling stem cell maintenance, cell fate determination and cell differentiation [36]. Wnt ligands bind and signal *via* seven trans-

membrane domain receptors of the Frizzled (Fz) family. Several findings implicate Wnt signaling in kidney development [37]. Wnt4 is expressed in the condensing mesenchyme and pretubular aggregates, which will give rise to the tubular epithelia of the mammalian metanephric kidney. Interestingly, mice homozygous mutant for Wnt4 form metanephric kidneys devoid of renal tubules [38]. This indicates that Wnt4 acts as a mesenchymal signal that is involved in the transition of renal mesenchyme to epithelium. While metanephric tubulogenesis is critically dependent on the signaling molecule Wnt4, it was unknown whether Wnt4 signaling is equally required for the formation of renal epithelia in the other embryonic kidney forms. We therefore investigated the expression of Wnt genes during pronephric kidney development in *Xenopus* [19]. Wnt4 was found to be associated with developing pronephric tubules, but absent from the pronephric ducts. Onset of pronephric Wnt4 expression coincided with mesenchyme-to-epithelium transformation. We used a morpholino antisense oligonucleotide-based gene knock to disrupt Wnt4 gene function (Fig. 2D, E). *Xenopus* embryos injected with antisense Wnt4 morpholinos developed normally, but marker gene and morphological analysis revealed a complete absence of pronephric tubules (Fig. 2F, G). Pronephric duct development was largely unaffected indicating that ductogenesis may occur normally in absence of pronephric tubules. Our results indicate that, as in the metanephric kidney, Wnt4 is critically required for tubulogenesis in the pronephric kidney indicating that a common, evolutionary conserved gene regulatory network may control tubulogenesis in different vertebrate excretory organs.

In a next step, we have begun to investigate which Fz proteins may cooperate with Wnt4 to promote tubulogenesis during kidney organogenesis. Towards this end, we have identified cDNAs encoding *Xenopus* orthologues for nine out of the ten Fz genes present in mammalian genomes. We have subsequently performed a comprehensive *in situ* hybridization study on *Xenopus* embryos to determine the subset of Fz genes with renal expression. Gain- and loss-of-function studies of candidate Fz genes are in progress. Using pharmacological approaches, we are also addressing the question of the nature of the intracellular signaling pathways activated by Wnt proteins in renal development. Furthermore, we are interested in determining the target genes activated by Wnt4 signaling during renal tubulogenesis. Taken together, we believe that a profound understanding of the signaling pathways and genes controlling renal tubulogenesis in the vertebrate embryo will have a direct impact on development of novel therapeutic strategies, such as the engineering of renal stem cells or progenitors in tissue culture.

Large-scale Gene Discovery Screens Identify Molecular Markers of the Segmented Nephron

The kidney fulfills essential physiological functions by regulating uptake of solutes, fluid balance, osmolarity and disposal of metabolic waste products. These tasks occur in a highly compartmentalized fashion along the nephron and are mediated by intrinsic transporter systems (channels, pumps, and transporters). For example, proteins of the solute carrier (SLC) gene family play a critical role in ion and nutrient transport and serve to maintain solute homeostasis inside and outside of cells [39]. Renal expression of SLC genes is usually highly specific and restricted to discrete segments of the nephron consistent with their physiological functions. The molecular mechanisms underlying the strict segmental expression of different transport system along the nephron remain however elusive. The *Xenopus* pronephric kidney represents a simplified model to address this problem experimentally. In a first step, we addressed whether key transport systems of the adult kidney are expressed in the developing pronephric kidney. To date, we have completed studies on the expression of Na,K-ATPase subunits [40], on SGLT-1L [41], a member of the SLC5 gene family, and on the ClC family of chloride channels (S. Eid and A. Brändli, manuscript in preparation). Collectively, our present results strongly support the notion that physiologically relevant transport processes are confined to specific segments of the nephron in the *Xenopus* pronephric kidney in a manner largely comparable to the adult mammalian kidney.

The isolation and characterization of *Xenopus* ESTs has become a powerful resource for gene discovery. We have therefore initiated a large-scale screen of *Xenopus* EST databases to systematically identify ESTs encoding *Xenopus* SLC proteins. To date, the human genome appears to contain more than 300 SLC genes, which constitute 43 distinct SLC gene families [39]. EST database screens performed in the laboratory have led to the identification of more than 1300 *Xenopus* SLC ESTs, representing about 160 distinct SLC genes (D. Raciti and A. Brändli, unpublished observations). We are currently determining by whole mount *in situ* hybridization the temporal and spatial expression profiles of all SLC genes identified to date. One of the aims of the study is to develop a comprehensive map of SLC gene expression during pronephric kidney organogenesis. Remarkably, many of the *Xenopus* SLC genes, such as the proton oligopeptide transporter SLC15A2 (PEPT2) (Fig. 2C), are expressed in a highly regionalized manner in the nephron reminiscent of their mammalian counterparts.

Many of the human SLC genes encode orphan transporters, *i.e.* transporters for which substrates have not been identified yet [42]. Detailed information on the expression of the cognate *Xenopus* SLC genes may therefore provide important insights about the possible physiological roles of their human counterparts. Finally, access to a collection of fully characterized, segment-specific marker genes is an essential prerequisite for future studies on the molecular basis of nephron segmentation. Ultimately, we hope to understand the molecular mechanisms and signaling pathways that pattern the nephron and give rise to the diversity of cell types of the mature vertebrate kidney. Again, this knowledge will be important for the development of renal cell-based therapies in regenerative medicine.

The *Xenopus* Embryo: An Emerging Model for Studies on Angiogenesis

Embryonic blood vessels develop through a combination of two related processes. The *de novo* formation of blood vessels by endothelial progenitors is termed vasculogenesis, while angiogenesis describes the sprouting and maturation of new vessels from pre-existing vasculature [43]. Concurrently, arteries and veins have to differentiate during these processes. The regulation of vascular development is complex and involves several known genes and signaling pathways. Best understood is the role of vascular endothelial growth factor-A (VEGF-A) and its receptors (Flt1/VEGFR1 and Flk1/VEGFR2), which play essential and evolutionary conserved roles in vasculogenesis [44]. In addition, it has become apparent that abnormalities of vascular growth in the adult body play critical roles in a variety of disease [45]. Angiogenesis is an important element of tumor growth, and gene products that are involved in blood vessel formation during embryogenesis have been targeted by a number of anticancer drugs. In coronary artery disease, a lack of functional blood supply causes heart attack. It is therefore essential to identify factors that promote, inhibit and modify blood vessel growth in order to treat disease with pathologic blood vessel formation.

Research by us and others have shown that the *Xenopus* embryo is an extremely useful model for analyzing vascular gene functions and mechanisms of angiogenesis because of the ease of its manipulation and visualization [14][46][47]. Interestingly, the *Xenopus* embryo shares a greater vascular similarity with mammals than do other model systems, such as zebrafish [47]. The development of the embryonic vasculature can be easily visualized using live embryo microangiography, where a fluorescent dextran is injected into the embryonic cir-

culation (Fig. 3A, B; R. Kälin and A. Brändli, unpublished data). Alternatively, DiI-labeled acetylated low-density lipoproteins can be used to label endothelial cells and characterize the vasculature in live *Xenopus* embryos [47]. In the future, transgenic embryos that express fluorescent reporter proteins under the control of vascular-specific promoters are expected to become available. This will offer an attractive alternative to fluorescent dye injections for the *in vivo* analysis of the developing vasculature.

Novel Molecular Markers of *Xenopus* Vascular Development

In situ hybridization provides a powerful method for investigating the developing vasculature. However, only a handful of molecular markers specific for endothelial cells of the *Xenopus* embryo have been fully characterized to date [48]. Given the intense biomedical interest in human angiogenesis, any vertebrate model system will be judged by how well it predicts human biology. We therefore decided to identify and characterize genes encoding molecular markers of angiogenesis commonly used in human pathology. Exploiting the *Xenopus* EST databases, we have identified and sequenced cDNAs encoding *Xenopus* orthologues of the platelet-endothelial cell adhesion molecule PECAM/CD31, and the receptor tyrosine kinases Flt1/VEGFR1, Flk1/VEGFR2, and Flt4/VEGFR3 (R. Kälin, I. Senn, and A. Brändli; manuscript in preparation). Characterization of the embryonic expression profiles by whole mount *in situ* hybridization has revealed specific expression of these genes in the developing *Xenopus* vasculature (Fig. 3C, D). Vascular expression of the *Xenopus* marker genes was identical with their mammalian counterparts validating also at the molecular level the *Xenopus* embryo model as a suitable model for research on vertebrate blood vessel development.

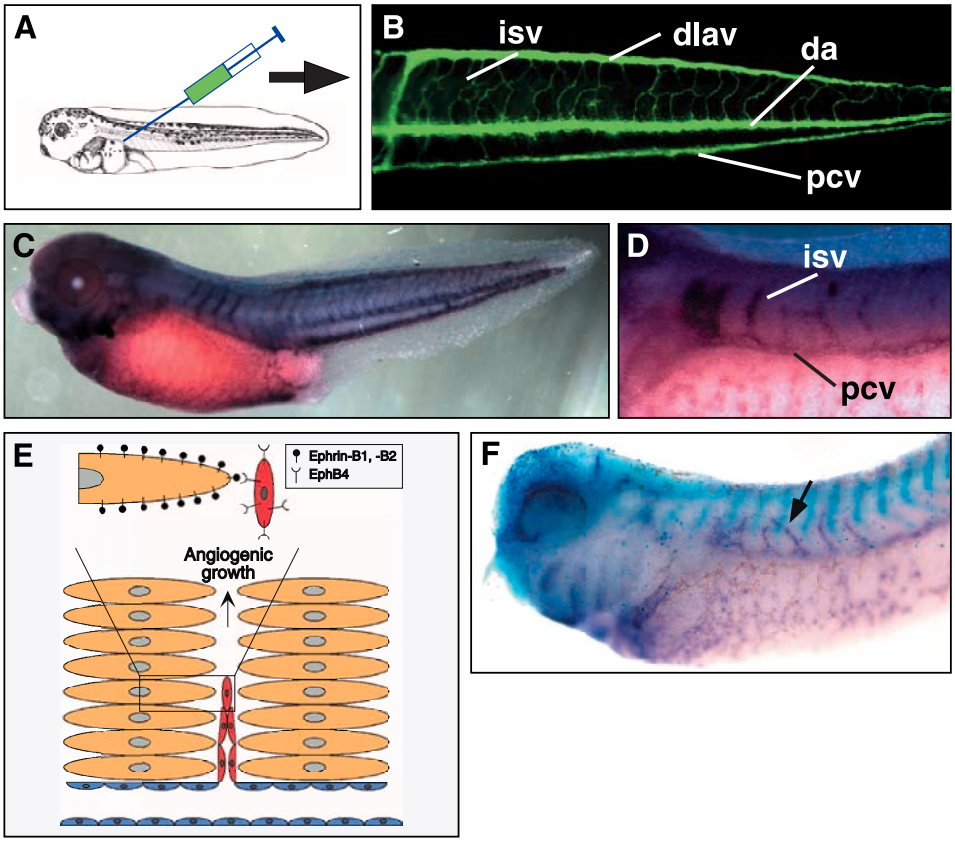

Fig. 3. **The vasculature of *Xenopus* embryos**. (**A–B**) Visualizing the *Xenopus* vasculature using microangiography. (**A**) A solution of dextrans that are conjugated with a fluorescent dye is injected into the circulation of *Xenopus* embryos. A fluorescence microscope is used to visualize the circulatory system. (**B**) Microangiogram of the tail of a stage 45 embryo injected with a fluorescein-labeled dextran solution. The dorsal aorta (da), posterior cardinal vein (pcv), dorsal longitudinal anastomosing vessels (dlav) and intersomitic vessels (isv) can be visualized in living *Xenopus* embryos. (**C–D**) Molecular markers of the developing vasculature in *Xenopus*. Whole mount *in situ* hybridizations of embryos (**C**, stage 40; **D**, stage 35) analyzed with antisense RNA probes are shown. (**C**) Expression of the *Xenopus* orthologue of PECAM1 (also known as CD31), which encodes a member of the immunoglobulin superfamily that is specifically expressed in endothelial cells. (**D**) Close-up view of the trunk of a *Xenopus* embryo stained for VEGFR2 (Flk1), a tyrosine kinase that is a receptor for vascular endothelial growth factor A (VEGF-A). Intersomitic vessels (isv) are sprouting from the posterior cardinal vein (pcv) into the intersomitic spaces. (**E–F**) EphB4 and its ephrin-B ligands regulate angiogenic growth of intersomitic veins. (**E**) Model summarizing the expression of EphB4 and its ligands ephrin-B1 and ephrin-B2 in the embryonic trunk. The receptor tyrosine kinase EphB4 is expressed in the posterior cardinal vein and intersomitic veins. Ephrin-B1 and ephrin-B2 are expressed in the adjacent somitic tissue. Interaction of EphB4 expressing endothelial cells with ephrin-B expressing somitic tissue restricts growth of intersomitic vessels to the intersomitic spaces. (**F**) Disruption of EphB4 signaling by overexpression of a dominant-negative EphB4 mutant results in aberrant projection of intersomitic veins (arrow). Blood vessels are visualized by hybridization with an antisense probe directed against the endothelial marker gene Msr. Figures shown in panels E and F were taken from [14].

Eph Signaling in Embryonic Angiogenesis

The cues and signaling systems that guide the formation of embryonic blood vessels in tissues and organs are poorly understood. Members of the Eph family of receptor tyrosine kinases and their cell membrane-anchored ligands, the ephrins, have been assigned important roles in the control of cell migration during embryogenesis, particularly in axon guidance and neural crest migration [49]. We investigated the role of EphB receptors and their ligands during embryonic blood vessel development in *Xenopus laevis* [14]. In a survey of tadpole-stage *Xenopus* embryos for EphB receptor expression, we detected transcription of EphB4 receptors in the posterior cardinal veins and their derivatives, the intersomitic veins. Vascular expression of other EphB receptors could however not be observed suggesting that EphB4 is the principal EphB receptor of the early embryonic vasculature of *Xenopus*. Furthermore, we found that ephrin-B ligands are expressed complementary to EphB4 in the somites adjacent to the migratory pathways taken by intersomitic veins during angiogenic growth (Fig. 3E). Disruption of EphB4 signaling by dominant negative EphB4 receptors in *Xenopus* embryos resulted in intersomitic veins growing abnormally into the adjacent somitic tissue (Fig. 3F). Our findings demonstrated that EphB4 and B-class ephrins act as novel regulators of angiogenesis possibly by mediating repulsive guidance cues to migrating endothelial cells. Furthermore, we showed that the intersomitic vein model is particularly useful for studying subtle perturbations in angiogenesis. Ongoing studies are aimed at determining the role of ephrin-B ligands in guiding intersomitic vessel growth using *in vivo* and *in vitro* approaches. Furthermore, we are assessing the potential application of ephrin-B molecules in blood vessel engineering. Taken together, our studies firmly establish the *Xenopus* embryo as a simple cost-effective, and high-throughput model that is ideally suited for further research on gene products with pro- and antiangiogenic properties.

The *Xenopus* Embryo Model in Drug Discovery and Validation

The sequencing of the human genome has revealed a large number of potential targets for drug development. Novel candidate drugs require extensive testing in animal models. While mammalian models are expensive to house and time-consuming to use, inexpensive invertebrate models (*C. elegans*, *Drosophila*) lack many anatomically comparable structures, such as cardiovascular system or excretory organs, which are characteristic of the vertebrate body. Lower vertebrate models such as zebrafish or *Xenopus* may therefore represent cost-effective alternatives for screening and drug candidate evaluation. Besides these applications, there may be new emerging opportunities for whole organism-based small-molecules screens in drug discovery. Traditional drug screens are performed using cell lines or *in vitro* protein binding assays, however neither represent the normal physiology of a multicellular organism. The use of vertebrate embryos in these screens would allow for the selection of compounds that are bioactive in a whole organism and in cells functioning in the context of their natural environment, *i.e.* tissues and organs. For example, embryological assays could be developed to screen for drugs that promote renal cell fates or prevent angiogenesis.

A number of recent studies have focused on using zebrafish embryos for chemical library screening [50] and drug validation [51]. The potential of *Xenopus* embryos as an alternative to zebrafish has to date not been explored thoroughly. With regard to whole organism-based screening, the *Xenopus* embryo model shares many of the advantages of zebrafish embryo, such as small size, rapid extra-uterine development, and inexpensive, simple culture conditions, but it is significantly closer to mammals than zebrafish (Fig. 1D). Proof of concept experiments performed in our laboratory indicate that *Xenopus* embryos can be readily used to test identified compounds that affect cell differentiation (M. Kretz, C. Héligon, and A. Brändli, unpublished observations). Small-molecule compounds are frequently poorly soluble in water and are therefore dissolved in organic solvents, such as dimethyl sulfoxide (DMSO). We found that *Xenopus* embryos develop without any externally visible defects in the presence of 1% DMSO. Thus, test compounds – usually dissolved in DMSO at 5 mg/ml – can be screened at final concentrations of 50 µg/ml, which corresponds to compound concentrations in the micromolar range. Furthermore, phenylthiourea (also known commonly as PTU), a tyrosine inhibitor that is routinely used to inhibit pigment production in zebrafish, was shown to work efficiently in *Xenopus* embryo (Fig. 4A–C). This indicates that *Xenopus* embryos share permeabilities to small molecules that are comparable to zebrafish. Taken together, our findings provide solid evidence that the *Xenopus* embryo model is well suited for whole-organism based drug screening. Fig. 4D shows a simplified scheme of a phenotypic chemical screen to identify small-molecule compounds from a chemical library that are able to modify organogenesis and cell differentiation. In addition, the screening procedure could be made more specific and sensitive using transgenic *Xenopus* expressing a fluorescent reporter gene in a tissue-specific manner. The screening approach is not limited to externally added small molecules, because simple injection techniques can be used to introduce chemicals into the cytoplasm of fertilized eggs. Recently, this approach has been used successfully to validate candidate small-molecule antagonists of glycogen synthase kinase-3 (GSK-3) and the oncogenic Tcf/ß-catenin protein complex in the *Xenopus* embryo [52]. Limitations of whole-organism based chemical screening strategies lie in the amount of compounds that can be screened by one person per day and in the challenge to identify the molecular targets of the small molecules discovered in the screens. Finally, unlike genetic knock-outs or antisense-based gene disruption strategies, small molecule inhibitors can be administered at any stage of development to assess a gene function. The use of such chemical inhibitors in place of classical loss-of-function approaches will therefore be particularly interesting to study gene functions at late stages of development and in the adult organism.

Perspectives

Suitable animal models are essential tools in most areas of therapeutics technologies, which includes the discovery, engineering, and delivery of therapeutics. While researchers in the field have traditionally favored mammalian animal models, lower vertebrates, such as the *Xenopus* embryo, have recently emerged as attractive, complementary systems amendable to large-scale screening strategies, which were previously not possible with mammals. Novel genomic technologies, the development of efficient methods for generating transgenic embryos and high-throughput gene knock-down techniques are now allowing the study of organogenesis and late development and will lead to the identification and validation of novel drug targets. Furthermore, these novel approaches in combination with the traditional advantages of *Xenopus* will allow researchers to continue to make an extensive contribution to functional studies in the future. These may lead to the disclosure of new principles about how organs do form. Other complex phenomena, such as origin and maintenance of stem cells and regulation of tissue differentiation are also becoming more ac-

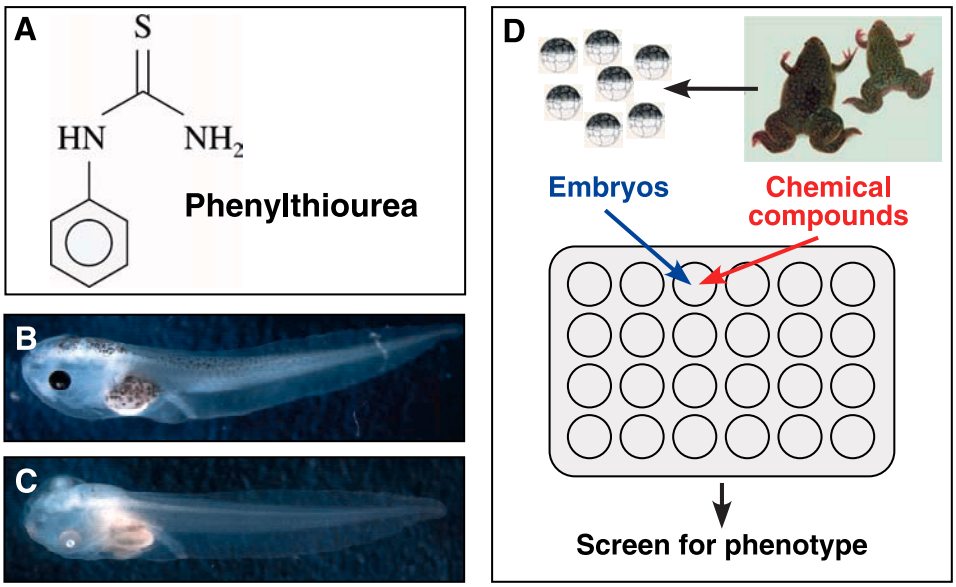

Fig. 4. **Chemical modifiers of *Xenopus* development**. (**A–C**) Effects of a small molecular compound on the development of *Xenopus* embryos. (**A**) Chemical structure of phenylthiourea (PTU). (**B**) Untreated control embryo at stage 45. (**C**) Embryo (stage 45) exposed to 0.5 mM PTU from stage 20 on. The embryo completely lacks pigment-containing melanosphores as well as pigmentation in the eye. (**D**) Screening for chemical modifiers of *Xenopus* development. More than thousand embryos can be obtained from a single *Xenopus* female. These are arrayed in a 24-well plate (or 48-well plate), which will hold up to 10 (5) embryos per well. Small-molecule compounds from a chemical library are added to the growth medium of each well. Embryos are incubated at room temperature and screened with a stereomicroscope for developmental defects.

cessible to experimental manipulation. The central challenge for the future will be to translate the basic principles gained by studies in the *Xenopus* embryo model into effective novel therapeutic approaches for the clinic. With regard to drug discovery, the *Xenopus* embryo model offers new opportunities for large-scale small-molecule screens in the context of a whole organism. Finally, the rapid acquisition of efficacy, toxicity and specificity data in the setting of a whole vertebrate organism may significantly facilitate the drug development process.

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