Research proceedings on amphibian model organisms

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ABSTRACT

Model organisms have long been important in biology and medicine due to their specific characteristics. Amphibians, especially *Xenopus*, play key roles in answering fundamental questions on developmental biology, regeneration, genetics, and toxicology due to their large and abundant eggs, as well as their versatile embryos, which can be readily manipulated and developed in vivo. Furthermore, amphibians have also proven to be of considerable benefit in human disease research due to their conserved cellular developmental and genomic organization. This review gives a brief introduction on the progress and limitations of these animal models in biology and human disease research, and discusses the potential and challenge of *Microhyla fissipes* as a new model organism.

Keywords: Amphibian; Model organism; Life Science; Biomedicine; *Microhyla fissipes*

INTRODUCTION

Because many critical pathways and gene functions that govern organism development and apoptosis are highly conserved in different species, studies on model organisms can provide insight into basic biological processes (Fields & Johnston, 2005). Almost everything we know about the fundamental properties of organisms - how they grow and develop, how they express their genetic information, and how they use and store energy - has come from studies on model organisms. Not surprisingly, such studies have made important contributions to our understanding of human health and disease. These simple animals traditionally include the nematode worm (*Caenorhabditis elegans*), fruit fly (*Drosophila melanogaster*), zebrafish (*Danio rerio*), African clawed frog (*Xenopus laevis*), western clawed frog (*Xenopus tropicalis*) and mouse (*Mus musculus*), each a representative of the diversity of life (Chitramuthu, 2013; Fields & Johnston, 2005; LaBonne & Zorn, 2015). Model organisms usually exhibit certain key characteristics, which contribute to their viability in research, including small size and tractability in the laboratory, short generation time, high fertility rates, easy growth, and amenability to experimental manipulation. As model organisms, amphibians play key roles in developmental biology, regeneration, genetics, toxicology, and immunology research. *Xenopus*, including *X. laevis* and *X. tropicalis*, are important model organisms, especially for investigating fundamental questions on developmental and cell biology, due to their large, abundant eggs, readily manipulated embryos, and conserved cellular developmental and genomic organization. In addition, as anuran amphibians possess various skin secretions for defense against external stimuli, they are an optimal model for understanding special immune structures and functions as well as immune system conservation and differentiation among vertebrate taxa. In addition, salamander amphibians serve as an important vertebrate model for studying regeneration and tissue repair.

SALAMANDERS: THE REGENERATION MODEL

Salamanders have an incredible ability to regenerate a range of organs and tissues, even as adults. They can regenerate limbs, tails, spinal cords, jaws, gills, parts of the brain, retinas, irises, lenses, and sections of the heart, while anurans (frogs and toads) lose the ability to regenerate limbs as they approach metamorphosis (Brockes, 1997). These organisms thus offer the unique opportunity to discover the events and processes that occur and the genes that are expressed during successful regeneration.

Regeneration was first reported in salamander in 1768 by Spallanzani (Spallanzani, 1768). Our understanding of organ regeneration has advanced considerably since then through research on tissue morphology, cytology, and molecular pathways (Fei et al., 1987; Makanae et al., 2016; Morrison et al., 2015). Model organisms usually exhibit certain key characteristics, which contribute to their viability in research, including small size and tractability in the laboratory, short generation time, high fertility rates, easy growth, and amenability to experimental manipulation. As model organisms, amphibians play key roles in developmental biology, regeneration, genetics, toxicology, and immunology research. *Xenopus*, including *X. laevis* and *X. tropicalis*, are important model organisms, especially for investigating fundamental questions on developmental and cell biology, due to their large, abundant eggs, readily manipulated embryos, and conserved cellular developmental and genomic organization. In addition, as anuran amphibians possess various skin secretions for defense against external stimuli, they are an optimal model for understanding special immune structures and functions as well as immune system conservation and differentiation among vertebrate taxa. In addition, salamander amphibians serve as an important vertebrate model for studying regeneration and tissue repair.
When animals start regeneration, a typical regeneration blastema structure forms at the damaged site. This blastema consists of undifferentiated stem-like cells termed blastema cells, which are usually either unipotent or multipotent (Satoh et al., 2015). There are multiple means by which injured tissues can provide new cells for regeneration. Specifically, new cells can be produced by resident stem cells, by dedifferentiation (loss of differentiated characteristics) producing a dividing cell that acts as a progenitor cell, and by transdifferentiation, that is a change in state from one cell type to another (reviewed in Tanaka & Redden, 2011).

Molecules and mechanisms that can transform wound healing responses into blastema induction responses have been previously investigated. Cooperative inputs of fibroblast growth factor (FGF)- and bone morphogenetic protein (BMP)-signaling can substitute for the nerve functions in accessory limb model blastema induction (Makanae et al., 2016). Extracellular signal-regulated kinase (ERK) activation is required for re-entry into the cell cycle of post-mitotic salamander muscle cells. Remarkably, while long-term ERK activation is found in salamander myotubes, only transient activation is seen in their mammalian counterparts, suggesting that the extent of ERK activation could underline differences in regenerative competence between species (Yun et al., 2014).

It is essential, therefore, to explore the molecules and mechanisms of tissue regeneration, and thus provide a theoretical basis for the regeneration of human organs and the suppression of cancer.

**AMPHIBIANS: THE IMMUNITY MODEL**

Vertebrate immune systems are classically categorized into two interconnected types: that is, innate and adaptive immune systems. Amphibians occupy a key phylogenetic position in vertebrates and evolution of the immune system and share many features of cellular immunity with mammals (Xiang et al., 2014; Zhao et al., 2014). As mammals, the effector cells of amphibian innate immunity eliminate infected cells by phagocytosis, via macrophages, neutrophils, and dendritic cells or by natural killer-mediated direct cytotoxicity. Moreover, the humoral side of innate immunity in amphibians includes epithelia-secreted antimicrobial peptides and some serum peptides, such as those of the complement system. In addition, frogs have a thymus where T-cells differentiate and a spleen where B- and T-cells accumulate, while leukocytes such as neutrophils, basophils, eosinophils, monocytes, and macrophage-like cells are also found in the blood (Robert & Ohta, 2009).

Amphibian skin plays a key role in everyday survival and the exploitation of a wide range of habitats and ecological conditions, and is thus a model system for diseases affecting vertebrate mucosa. Bioactive components of amphibian skin secretions, especially biologically active peptides, have been extensively studied. Granular glands in the skin of anuran amphibians synthesize and secrete a remarkably diverse array of antimicrobial peptides (AMPs), 10-50 residues in length, that are released onto the outer layer of the skin as an effective and fast-acting defense against harmful microorganisms (Li et al., 2007). Xu & Lai (2015) summarized the sequence and structure of AMPs in 26 genera. Sitaram et al. (2002) characterized tigerinin 1 from the skin secretions of the Indian frog, *Rana tigrina*, which exhibited impressive activity against a variety of clinical bacteria. Furthermore, esculentin-1a produced by the skin of the green edible frog, *Pelophylax lessonae/ridibundus*, was found to rapidly kill both planktonic and biofilm forms of *Pseudomonas aeruginosa* via pronounced membrane-perturbing activity (Luca et al., 2013). Therefore, exploration of the structures, biological functions, and mechanisms of active peptides from amphibian skin secretions is important for developing new therapeutic agents.

Adaptive immunity represents the most recent branch of the immune response system from an evolutionary point of view due to its appearance in the gnathostomes. The high complexity of the adaptive immune system is based on the intact B-cell receptor (BCR)-T-cell receptor (TCR)-major histocompatibility complex (MHC). Transcriptome analysis of *Bombina maxima* skin and blood identified the transcripts of BCR, TCR and MHC, suggesting an immune system nearly parallel to that of mammals (Zhao et al., 2014). However, some studies have suggested that the adaptive immune response to some pathogens is weak (Fites et al., 2013, 2014; Xu & Lai, 2015). The MHC is an adaptive feature of the immune system that likely evolved in basal tetrapods. Genetic relatives share MHC alleles, which encode T-cell repertoire, so their immune systems should recognize similar arrays of pathogens. The MHC is genetically diverse in most populations, with this variation likely responsible for the patterns of morbidity and mortality observed when a population is challenged with a given pathogen (Barnabe et al., 2008). Immune system genes show higher rates of adaptive evolution than non-immune genes across a range of taxa, including crustaceans, insects, anurans, birds, and primates (Savage et al., 2014). Gene synteny is helpful for identifying divergent genes, such as those involved in immunity. For example, in *Xenopus*, as in mammals, cluster of differentiation 8β (CD8β) retains proximity to CD8α, whereas CD4 is closely linked to Lag3 and B genes (Chida et al., 2011). Ongoing whole genome mutagenesis allows one to search for genes critically involved in immune functions. Due to their special phylogenetic position and living environment, frogs provide a valuable platform for investigations on detailed immune responses and adaptive evolution.

**XENOPOUS IN SCIENTIFIC RESEARCH**

Here, the genus *Xenopus* refers to two species – that is, *X. laevis*, a classic allotetraploid frog used by researchers for several decades, and *X. tropicalis*, a diploid frog more recently adopted due to its easy genetic manipulation. *X. laevis* and *X. tropicalis* are species of African aquatic frog of the family Pipidae. *Xenopus* is an invaluable tool in vertebrate embryology and development, basic cellular and molecular biology, genomics, neurobiology, and toxicology, and as a model for human diseases (Horb, 2014). *Xenopus* eggs and embryos are outstanding tools in basic biology and biomedical research.
First, *Xenopus* lay abundant eggs year-round in response to mammalian hormones. Second, their embryos tolerate extensive manipulation ranging from very delicate procedures, such as transplantations of single cells, to extensive ‘cut and paste’ operations that challenge large sections of the embryo with new environments (Harland & Grainger, 2011). Third, a range of materials, such as nucleic acids, proteins, and intact nuclei, can be easily injected into whole embryos or specific cells. Fourth, cell-free extracts from *Xenopus* oocytes allow for in vitro studies on fundamental aspects of cellular and molecular biology, such as cell cycle, cellular components, ion transport, and channel physiology. Fifth, developing larvae and tadpoles are transparent, which facilitates the detection of tissue and organ development by visual inspection under a dissection microscope.

*Xenbase* (http://www.xenbase.org), the *Xenopus* model organism bioinformatic database, is a crucial resource that integrates diverse genomic, expression and functional data available from *Xenopus* research. The National *Xenopus* Resource (NXR) provides a facility for the breeding of *X. laevis* and *X. tropicalis*, maintenance of genetic stocks, which are available to researchers, as well as development of new experimental tools and husbandry techniques. Recently, ORFeome, which provides a comprehensive set of full-length, end-sequence validated, high-quality open reading frame clones in the Gateway cloning system, was established in *Xenopus* for functional genomics and disease modeling (Grant et al., 2015). These resources all provide support for studies on this model.

Developmental biology

*Xenopus* is an established and powerful model organism for the study of embryogenesis in vertebrates. Their relatively large embryos (in size and number) enable transplantation and microinjection, which has led to key discoveries not only on the functional role of inducers and inhibitors in vertebrate embryos, but also on their molecular mechanisms in vertebrate cells. Gurdon et al. (1958) conducted different nuclear transplantation experiments to demonstrate that mature *Xenopus* cells could be reprogrammed. Undifferentiated cells, collectively known as the animal cap, are present in the blastula of *X. laevis*. This region comprises approximately 1,000 cells and is capable of inducing myocardial cell differentiation following the activation or overexpression of factors such as GATA4 and Wnt11 (Kinoshita et al., 2010). Using *Xenopus* as a model, Clau-Ulitz et al. (2013) established the genetic cascade specifying the emergence of adult hemangioblasts and built a gene regulatory network for the programming of these cells. Nieuwkoop (1985) investigated inductive interactions during early *X. laevis* development and their animal cap assay has enabled investigators to hone in on the most intractable problem in developmental biology: embryonic induction. Over the last two decades, with the development of molecular biology, a growing number of inducers and inhibitors for specific differentiation have been identified. Neural inducers such as noggin, chordin, and follistatin have been found to induce neural differentiation in isolated *Xenopus* animal caps (Hemmati-Brivanlou & Melton, 1994; Lamb et al., 1993; Sasai et al., 1995). *Xenopus* has also been one of the foremost vertebrate models for unraveling the functions of β-catenin, such as dorsal accumulation and activation of a cascade of regulatory genes by β-catenin complexes and high mobility group (HMG) box transcription factors, which are critical for specification of the dorsal axis (Moon, 2001).

In addition, studies on *Xenopus* have contributed to the molecular dissection of major signaling pathways (i.e., Wnt, BMP, activin, FGF) for embryogenesis. *Xenopus* research established the link between β-catenin and Tcf/LEF and revealed some of the first direct Wnt/β-catenin target genes in vertebrates (siamois, Xnr3, twin, fibronectin, engrailed-2, Xnr5, Xnr6, and slug) (Chien et al., 2009; Yang et al., 2002). FGF-signaling is essential during the late blastula stage for the gastrula ectoderm to undergo neural differentiation provoked by neural inducers (Delaune et al., 2005). The distinctions of transforming growth factor β (TGFβ) family signaling through Smad2 and Smad1 were also first clearly documented in *Xenopus*, with the roles of these signaling pathways in embryo organization predating loss-of-function experiments in other vertebrates (Whitman, 1998).

Amphibian metamorphosis shares many similarities with mammalian development during the perinatal period. Therefore, *Xenopus* provides an ideal system for studying precocious induction in vivo and characteristic features of post-embryonic development, such as morphogenesis, tissue remodeling, gene reprogramming, and programmed cell death (Tata, 1996). During metamorphosis, thyroid hormones (T3) regulate the expression of Wnt5a/Ror2 to induce some larval epithelial cells to become adult stem cell analogs (Ishizuya-Oka et al., 2014). Heimeier et al. (2010) identified 17 larval-specific genes that might represent molecular markers for human colonic cancer by gene expression study during metamorphosis, which helps to understand intestinal organogenesis and human disease.

Investigating cell cycle mechanisms

Because large volumes of extracts can be prepared from eggs and oocytes of a single frog, and the cell-free nature of these extracts recapitulates the complex events of the cell cycle in vitro, they can be fractionated to identify structural and regulatory components. For over 20 years, oocytes, eggs, and early embryos of *Xenopus* have contributed to answering questions concerning the mechanisms that underlie cell cycle transitions - the cellular components that synthesize, modify, repair, and degrade nucleic acids and proteins, the signaling pathways that allow cells to communicate, and the regulatory pathways that lead to selective expression of subsets of genes (Goloh et al., 2011). Essential cell cycle regulators such as INCENP, securin, geminin, and sororin were identified and characterized using functional screens in *Xenopus* extracts (reviewed in Grant et al., 2015). Maturation promoting factor, consisting of two subunits, cdc2 kinase and either cyclin B1 or B2, was originally characterized as an activator present in unfertilized *Xenopus* eggs, which could induce germinal vesicle breakdown when microinjected into resting oocytes. Moreover, the cdc2 protein has been shown to contain three regulatory
phosphorylation sites to regulate entry into and exit from mitosis and meiosis (Li et al., 1995). Khoudel et al. (2008) blocked DNA replication by inhibiting either replication licensing or S phase cyclin-dependent kinase (CDK) activity, and found that Mcm2-7 plays a central role in coordinating the nuclear structure with DNA replication.

Xenopus is not limited to in vitro investigations on cell cycle functions. The developing Xenopus embryo also presents an interesting in vivo system to study the regulation of proliferation, particularly in view of the changes in cell cycle regulation during early development (Hardwick & Philpott., 2015; Saka & Smith, 2001; Woodland, 1974). The developing Xenopus embryo has a single cdk inhibitor, p27Xic1, to regulate the cell cycle, which functions during the neuronal commitment stage and is necessary for primary neurogenesis, independent of cdk2 inhibition (Vernon et al., 2003).

Regeneration research
Xenopus laevis can regenerate larval tails and limbs by formation of a proliferating blastema and can regenerate eye lenses by transdifferentiation of nearby tissues, while also formation of a proliferating blastema and can regenerate eye early development (Hardwick & Philpott., 2015; Saka & Smith, 2001; Woodland, 1974). The developing Xenopus embryo has a single cdk inhibitor, p27Xic1, to regulate the cell cycle, which functions during the neuronal commitment stage and is necessary for primary neurogenesis, independent of cdk2 inhibition (Vernon et al., 2003).

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Using constitutive or dominant negative gene products, both the BMP and Notch signaling pathways have been shown to be essential for tail regeneration, furthermore, BMP is upstream of Notch and has an independent effect on regeneration of muscle (Slack et al., 2004). Researchers have found that pathways involved in development also play important roles in regeneration. For example, TGFβ signaling plays a critical role in wound healing of the tail (Ho & Whitman, 2008). FGF ligands are upregulated in the regeneration bud of the tail as early as 24 h after amputation, and their receptors, Fgr1 and Fgr2, are present in the regenerating tail (Lin & Slack, 2008). Regeneration of limb buds is most successful during the early stages of limb differentiation and then declines as metamorphosis proceeds (Dent, 1962). Yokoyama et al. (2000) found that regeneration capacity depends on the mesenchyme to supply both signals and progenitor cells and that the non-regenerative epidermis retains the capacity to respond to these stimuli. Extensive transcriptome changes in regenerative tadpoles 1 d after spinal cord injury, while this was only observed 6 d after injury in non-regenerative froglets, and genes related to neurogenesis and axonal regeneration were differentially regulated after injury in regenerative and non-regenerative stages (Lee-Liu et al., 2014). With the ever-increasing interest in regenerative medicine, the next 10 years will be an exciting time for regeneration research, and the advantages of the Xenopus system and advanced genetic manipulation ensure that Xenopus will continue to lead the way.

As a human disease model
When using animal models in biomedical research to gain insights into human developmental biology, disease pathology, and novel therapeutics, it is important to be aware of their evolutionary distances to humans. The smaller the evolutionary distance, the more reliable the results from model organism studies will be translated to medicine. Importantly, Xenopus bridges the gap between costlier and less tractable mammalian models and the evolutionarily more distant zebrafish model, and as such is uniquely positioned to inform conserved biological processes relevant to human health.

Out of 20 000 protein genes of X. laevis, there are at least 1 700 orthologs of human disease genes (Hellsten et al., 2010). Currently, the Xenopus model organism system has been used to study cancer; GI/pancreatic diseases, cardiovascular diseases, neurological diseases, immunological diseases, muscle atrophy, and human ciliopathies (Salanga & Horb, 2015; Schweickert & Feistel, 2015; Ymlahi-Ouazzani et al., 2010). Mutations found in Xenopus genetic screens often appear to be linked to human syndromes (Abu-Daya et al., 2009). When a wild-type human MeCP2 gene was overexpressed in Xenopus tectal neurons in vivo, these neurons were found to develop fewer, albeit longer, dendrites compared with normal tectal cells (Marshak et al., 2012). Furthermore, in Xenopus, as in humans and rodents, variations in MeCP2 activity cause redistribution between close- and long-range network connections, which is one of the landmark circuit abnormalities in autism spectrum disorders (Geschwind, 2009). Key transcription regulators are efficiently conserved between Xenopus and humans (Amir et al., 1999), allowing the human MeCP2 gene to interact with native Xenopus pathways to reveal disease mechanisms, thus ultimately leading to advancements in diagnosis and therapy (Marshak et al., 2012; Pratt & Khakhali, 2013). In X. laevis embryos, for example, Irx5 modulates the migration of progenitor cell populations in branchial arches and gonads by repressing Sdf1 (Bonnard et al., 2012).

Genetic and genomic research
Genetic research on X. laevis is challenged by its allotetraploid genome (reflected in four copies of many genes) and its generation time of over a year. Thus, researchers have turned to a genetically tractable amphibian species, X. tropicalis, a West African relative of X. laevis, with a small, diploid genome and much shorter generation time (just under three months for males) (Hirsch et al., 2002a). Xenopus tropicalis also promotes multigenerational studies, taking advantage of efficient transgenic methods in Xenopus (Harland & Grainger, 2011). This has enhanced the use of Xenopus in different assays, for example in scoring genes by transgenic embryos and generating transgenic lines (Hirsch et al., 2002b), perturbing gene function by tissue or stage-specific expression of designer constructs (Hartley et al., 2001), and defining key regions of enhancers (Ogino et al., 2008). In addition, the long fertility period of Xenopus greatly simplifies maintenance of stocks for backcrosses and test crosses relative to other animal models. The X. tropicalis genome sequence was first published in 2010, filling the gap between mammals and fish and revealing the extraordinary synteny between frog and human genomes. The Xenopus genome contains genes similar to at least 1 700 genes that, in humans, are associated with disease (Hellsten et al., 2010). Thus, understanding these genes in frogs could help
biologists understand how they are involved in human disease.

The *Xenopus* model system has been further strengthened by recent advances in several powerful genome editing techniques, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR/Cas 9 system (Guo et al., 2014; Lei et al., 2012; Young et al. 2011).

Biochemistry, cell biology and toxicology

*Xenopus* oocyte extracts have served for years as an important cellular expression system in studies on biochemistry and electrophysiological properties of ion channels, solute carriers, ATP-driven transporters, and signaling receptors (Schmitt et al., 2014). By treating adult females with a relatively simple course of hormone injections, oocyte maturation is initiated and egg laying is encouraged. These eggs can then be collected to harvest egg extracts for further work.

Having been used for decades by researchers in academia as well as the US Environmental Protection Agency (EPA) to assay toxic and teratogenic effects of environmental chemicals, the *Xenopus* tadpole is not strange to the field of embryo toxicology (Berg et al., 2009; Dumpert & Zietz, 1984; Saria et al., 2014). The tadpole has served as a workhorse for these studies mostly because their metamorphosis from tadpole to frog depends entirely on the thyroid hormone (TH) (Damjanovski et al., 2000), and TH inhibitors are one of the most prevalent environmental contaminants and endocrine disruptors. In addition, environmental estrogens, including nonylphenol, octylphenol, methoxychlor, antiandrogen, p,p'-DDE, and synthetic androgen 17α-methyltestosterone, alter early development in *X. laevis* by disrupting hormone-sensitive processes (Bevan et al., 2003). There is no doubt that the many unique advantages of *Xenopus* and other amphibians will ensure their position as a fundamental vertebrate model. It is likely that future contributions from *Xenopus* research will lead to rapid progress in biology and biomedicine.

The genomic architecture and expression profiles of *Xenopus* are, however, not likely representative of amphibians as a whole. *Xenopus* are members of the family Pipidae, an early divergent group of anurans, with 95% of extant frog species actually belonging to the clade Neobatrachia, a much more recent radiation that diverged from Pipidae approximately 300 million years ago (Pyron & Wiens, 2011). Furthermore, *Xenopus* live in the water all its life, and the development mechanism and physiological function of this sepecie is aquatic adaptive without information for the function, mechanism and evolution from aquatic to terrestrial. It is clear that no single model organism can fill all requirements for future research. Therefore, additional model organisms are needed for specific issues.

**POTENTIAL OF MICROHYLA FISSIPES**

*Microhyla fissipes* is a typical tailless anuran from the family Microhylidae suborder Neobatrachia. It is widely distributed in eastern Asia and Southeastern Asia and is of small size with a strong survivability (Figure 1). It can produce about 240-450 eggs at one time, which are large enough (0.8-1.0 mm) for microinjection. The embryos develop rapidly and hatch after ~24 h. Moreover, the tadpole is transparent (Figure 1A) and takes only 20-30 d to complete metamorphosis (Fei et al., 2009). Furthermore, *M. fissipes* is diploid (2n=24) (Chen et al., 1983). These characteristics suggest that *M. fissipes* would be a good species with which to study developmental biology, adaptive mechanisms from aquatic to terrestrial lifeform, environmental toxicology, and human disease.

![Figure 1](https://example.com/figure1.png)

**Figure 1** Photograph of tadpole (A) and adult (B) *Microhyla fissipes*

At present, research progress has been made in the feeding, breeding, embryonic development, and metamorphosis of *M. fissipes*.

*Microhyla fissipes* tadpoles are mainly fed on cooked egg yolk and *Artemia salina*, froglets are primarily fed on *Oncyhiums fimeitayius linnaeus* and *Folsomia candida*, and adult frogs are mainly fed on *Pseudaletia separata*, *Plutella xylostella* and *Drosophila*. In future, suitable fodder will be gradually added for domestication of this species, which will help to promote its storage in the lab.

The mating season for *M. fissipes* is from March to September, though it peaks from early May to the end of June. During the mating season, males attract females mainly through croaking. Once drawn, the male embraces the female for several hours, climbing onto her back and performing amplexus breeding behavior. Spawning occurs in the water, during which time the female will lay her eggs and the male will release his sperm concurrently, with the fertilized eggs eventually hatching into tadpoles. This spawning occurs several times and lasts about 10 min. *Microhyla fissipes* can be induced to lays eggs in response to luteinizing hormone-releasing proteins.
hormone A3 (LHRH-A3) (Ningbo Sansheng Pharmaceutical Co., Ltd. Zhejiang, China) at a concentration of 0.3 μg/g from March to October, while half these concentrations are sufficient for males (unpublished data).

The fertilized eggs are yellow and float on the water. Embryonic development of *M. fissipes*, like other amphibians, includes early and post-embryonic development. Considering the extent of morphological changes observed for other amphibians, especially anurans (Gosner, 1960; Shimizu & Ota, 2003), 45 developmental stages have been determined for *M. fissipes*. The early embryonic period (stages 1-28), from fertilization to completion of spiracle, lasts for 82.6 h at 23.5±0.6 °C (water temperature), and the larval period (stages 29-45), from limb formation to metamorphosis, takes 38 d at 24.6±0.8 °C (water temperature). Raising the water temperature will accelerate embryonic development in *M. Fissipes* (data unpublished). During embryonic development, the larvae of *M. fissipes* are transparent and total length reaches a maximum at stage 40 (unpublished data).

To investigate gene regulated metamorphosis, the transcriptomes of three key developmental stages of *M. fissipes* (pre-metamorphosis (PM), metamorphic climax (MC), and completion of metamorphosis (CM)) were deep-sequenced on the Illumina platform by Novogene (Beijing). A total of 34 938 unique transcripts were annotated, 2 293 differentially expressed genes were identified from comparisons of transcriptomes, and these genes showed stage-specific expression patterns. The stage-specific transcripts were detected by comparison in pairs. We found proto-oncogene could be attributed to the cellular proliferative activity of organisms in the PM stage. At the MC stage, transcripts associated with extracellular matrix (ECM), and ECM remodeling were attributed to the morphological changes that accompany larval transitions. Detected unigenes important in metamorphosis can be considered as candidates to further elucidate the molecular mechanisms underlying metamorphosis in *M. fissipes*. Unexpectedly, we found that thyroid hormone receptor α (TRα) was highly expressed in *X. laevis* and *Bufo gargarizans* at PM but showed low expression in *M. fissipes*. Correspondingly, *M. fissipes* spent a shorter amount of time attaining metamorphosis onset and had a smaller body size than either *Xenopus* or *B. Gargarizans* (Zhao et al., 2016). In contrast, TRβ was highly expressed during metamorphosis in *M. fissipes*, *X. laevis* and *B. gargarizans*. This implies that TRβ is essential for initiating metamorphosis, at least in *M. fissipes*. Thus, our work clarifies the roles of unliganded TRα in regulating tadpole growth and timing of metamorphosis, which may be conserved in anurans, and the role of liganded TRβ in launching metamorphosis (Zhao et al., 2016).

CONCLUSIONS

Compared with other model animals, research on *M. fissipes* is in its infancy and further studies on breeding, feeding, morphology, physiology, gene functions and molecular mechanisms of metamorphosis, and function, mechanism and evolution from aquatic to terrestrial are needed for this species. At present, the key aim is to establish an inbred line, and to set the feeding and breeding standards for lab rearing. The species *M. fissipes* is cheap and convenient for laboratory breeding due to its small body size, as well as rapid embryonic development and metamorphosis, which has facilitated its use in developmental biology. Furthermore, *M. fissipes* belongs to the clade Neobatrachia, and thus may represent 95% of extant frog species. As a typical anuran, *M. fissipes* metamorphoses quickly from transparent tadpole to terrestrial froglet, which can also be used to explore the functional evolution of Anura from aquatic to terrestrial lifeform. The unusual characteristics of *M. fissipes* provide a novel inroad to address the mechanisms of spatiotemporal scaling during evolution. Additionally, its skeletal muscles, which undergo tremendous remodeling at metamorphosis, are an ideal model for studying muscle fiber apoptosis, differentiation, and adaptation of muscle function from aquatic to terrestrial lifeform. Therefore, our future work will focus on the basic biology of *M. fissipes* so as to detect its potential as a novel model in terrestrial adaptation mechanisms, lung development, muscle remodeling during metamorphosis, and other foundational biological and biomedical research.

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