The use of transgenics in the laboratory axolotl

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Abstract

The ability to generate transgenic animals sparked a wave of research committed to implementing such technology in a wide variety of model organisms. Building a solid base of ubiquitous and tissue-specific reporter lines has set the stage for later interrogations of individual cells or genetic elements. Compared to other widely used model organisms such as mice, zebrafish and fruit flies, there are only a few transgenic lines available in the laboratory axolotl (Ambystoma mexicanum), although their number is steadily expanding. In this review, we discuss a brief history of the transgenic methodologies in axolotl and their advantages and disadvantages. Next, we discuss available transgenic lines and insights we have been able to glean from them. Finally, we list challenges when developing transgenic axolotl, and where further work is needed in order to improve their standing as both a developmental and regenerative model.

Keywords

Ambystoma mexicanum; genome editing; laboratory axolotl; transgenics

1 | INTRODUCTION

The ability to edit the genome of vertebrates at will was a landmark discovery1 that has paved the way to a more detailed and accurate understanding of both cellular and molecular...
mechanisms during development, disease modeling and tissue regeneration. Transgenesis overcomes the limitation of ectopic mosaic expression of an externally delivered gene of interest and ensures its stable inheritance by virtue of integration in the germline of the animal. It also provides the opportunity to fluorescently label cells if the use of antibodies is not possible or not desired; this is particularly useful to visualize cell behavior in live animals. Due to its wide-reaching biomedical potential, transgenesis has been introduced in a broad range of model organisms including fruit flies, 2 zebrafish, 3 frogs, 4 mice, 1 and pigs. 5 While the methodology behind each subsequent protocol remains consistent, the exact manner by which the genome is altered must be tailored specifically to each species, accommodating their individual genomic needs.

The laboratory axolotl (Ambystoma mexicanum) is an excellent model for animal development and tissue regeneration studies due to their large clutch size (300–500 embryos/mating), ex vivo embryonic development, and tremendous tissue regeneration abilities. In the past, due to the absence of efficient transgenesis methodologies, research in the axolotl field was restricted to tissue transplantation and histochemical analysis. Similar to other model organisms, this changed when the first transgenic axolotl with a ubiquitously expressed fluorescent protein 6 was developed. Since then, axolotl transgenesis has come a long way and at present, a wide variety of transgenic lines are available for studies in this animal (Table 1). In this review, we will first provide an abbreviated history of transgenic techniques that have been adapted to produce various transgenic axolotl lines. Following that, we will highlight the application of these transgenic animals and the important lessons that we have learned from them. Finally, we will discuss the challenges associated with implementing transgenesis in the axolotl and highlight areas where further improvements are needed. Transgenic animals have also been developed in other salamander species, but they will not be part of this review. 7–13

2 | METHODS OF TRANSGENESIS

Commonly used techniques for generating transgenic animals can be broadly split into two categories: random transgenesis and targeted genomic modifications.

3 | RANDOM TRANSGENESIS

As the name suggests, in this method, foreign DNA is inserted at a random location within the genome. To do so, the cassette of interest is injected at the single cell stage of an embryo to allow for genomic integration before the first cell division. However, the process of genomic integration is inefficient and, in most cases, happens at a later stage of embryonic development, leading to mosaic F0 animals. To improve the integration efficiency, the cassette of interest is usually flanked by meganuclease restriction sites 14 or transposable elements, 15 while the plasmid DNA is co-injected with the corresponding restriction enzymes or transposase, respectively (Figure 1). The restriction enzymes used for this purpose are rare cutters. Among them, I-SceI meganuclease has gained popularity owing to its ability to recognize a 18-bp sequence which is extremely rare in the genome. 14 Using I-SceI-mediated transgenesis, the very first transgenic axolotl with ubiquitous eGFP expression was generated under the CAGGs promoter in 2006. 6 For transposase-based
transgenesis, plasmid DNA is coinjected with the transposase mRNA or protein. Some of the most common transposase methods utilize Tol2,\textsuperscript{16} PiggyBac (PB),\textsuperscript{17,18} or sleeping beauty (SB)\textsuperscript{19} transposons. We have tried I-SceI, Tol2, and PB-based transgenesis and they have all proven to label ~20% to 25% F0 axolotl.\textsuperscript{20} We usually select ~10 animals with the least mosaicism to grow as F0 breeders, which in most cases successfully integrate the cassette into the germline. In both meganuclease-based and transposase-based methods, multiple integrations of the cassette into the genome are possible, which may cause varying gene expression among siblings. Although we have not genotyped our lines for their insertions, we have noticed such varying expression in progenies of at least CAGGs: loxP-eGFP-loxP-Cherry animals that are widely used for lineage tracing purpose. Transposon elements are infamous for their ability to jump between various genomic locations (“local hopping”) even when the transposase protein is no longer present. Such unpredictable activity may lead to unstable expression of the cassette in the progeny\textsuperscript{21} and hence, caution must be exercised while using transposon-mediated transgenesis. Because of such “local hopping” concerns, our lab has preferred to use I-Sce-I over Tol2, but it is possible that our fears are unwarranted. Clearly, further work is needed in this direction to evaluate pros and cons of each method.

4 | TARGETED GENOMIC MODIFICATIONS

In contrast to random transgenesis, modifications at precisely defined positions in the genome are desired for many applications. This is facilitated by nucleases which generate double strand breaks (DSB) upon recognition of specific DNA sequences. The era of the targeted genome modifications in axolotl began with transcription activator-like effector nucleases (TALENs).\textsuperscript{22,23} TALENs were made by fusing a TAL effector DNA-binding domain to a nuclease domain which then cuts DNA. While this method was important in the early days of targeted genome modifications, it lacks the ease and precision offered by the CRISPR/Cas9 system,\textsuperscript{22,24,25} which quickly superseded it in almost all applications. Compared to the TALEN approach, the CRISPR/Cas9 system is highly versatile, less toxic, and more efficient, allowing for precise genomic modification while maintaining flexibility in the choice of cutting site. Furthermore, it is a lot easier to design and prepare gRNA as compared to the TAL-DNA binding domain. These important properties have made CRISPR/Cas9 a popular system among axolotl researchers. There are many excellent reviews written comparing TALENs and CRISPR/Cas9; we suggest readers refer to them to understand the subtlety of each method.\textsuperscript{23}

Following an artificially induced DSB along a chromosome, the cell initiates one of three DNA repair pathways: homology-directed repair (HDR), canonical non-homologous end joining (c-NHEJ), or microhomology-mediated end-joining (MMEJ, also called alternative NHEJ).\textsuperscript{26–29} In the absence of a homologous DNA sequence, the DNA repair machinery defaults to c-NHEJ, which often results in an insertion or a deletion (InDel) of nucleotides. This principle is used to develop targeted knock-out and knock-in animals in axolotl.\textsuperscript{30–32}

If a protein-coding gene needs to be knocked out, target sites are often chosen at the beginning of the open reading frame (ORF). Since the repair mechanisms are not perfect, DSB repair may cause a frameshift in the ORF due to InDels. Frameshifts result in either an altered protein sequence or a premature termination codon (PTC), both of which are
likely to produce a non-functional protein. When designing gene knockout strategies, it is advisable to avoid a target site in the middle or C-terminus of the protein, as it often leads to a PTC, which is known to activate nonsense mediated decay (NMD) of the mRNA. A recent study also suggests that NMD activation could lead to upregulation of homologous genes that share sequence similarity with the target ORF, resulting in mild to no phenotype in animals. Hence, if a target site must be chosen within the middle or C-terminus, then it is advisable to screen for in-frame mutants which do not activate NMD.

To generate a knock-in animal, a targeting construct whose cassette of interest has the same (or different) gRNA target site at its 5′ end is injected along with the gRNA and CAS9 protein (PNABio#CP03; or homemade CAS9-NLS protein) (Figure 2). CRISPR/Cas9 cuts both the genomic sequence and the targeting construct, thus activating c-NHEJ machinery to repair the damage. During the repair process, exogenous DNA is integrated in the genome due to its abundance, thus resulting in the insertion of the targeted gene into a defined genomic locus. Using this approach, we were able to knock-in cassettes up to 5 kb in length with up to 15% efficiency in F0 animals. Knock-in efficiency decreases with the increase in the size of the targeting construct. When inserting a small gene cassette, for example, the Cherry reporter gene, the knock-in efficiency of targeted gene can reach 15%. However, when inserting a large gene cassette, for example, a 5-kb Cre-ER^{T2} cassette, the knock-in efficiency is only about 5%. Since c-NHEJ repair is not perfect, in most cases gRNA targets are selected within introns or non-coding part of the genomic DNA to avoid frameshift of the ORF. To obtain a detailed overview of the possible knock-in strategies at the moment, we suggest reading the following publication.

Another drawback of this method is that the 3′ end of the cassette is left unchecked, possibly resulting in unwanted artifact integration along with the target construct. This may be particularly harmful when gene regulatory elements are located within the 3′ UTR. Therefore, in the future, a more targeted approach—such as homology-directed repair (HDR) pathway—is needed to generate axolotl knock-in lines, allowing cassette integration without any defects. We have not extensively tried HDR approaches in our labs. Furthermore, work on enhancer and gene traps have also not been attempted in axolotl, to the best of our knowledge. Therefore, HDR transgenesis, gene traps, and enhancer traps remain areas of exploration.

5 | AVAILABLE TRANSGENIC AXOLOTLS

Similar to any methodologies, while performing animal transgenesis, a decision of what kind of genomic modification is needed depends upon the ultimate purpose for which the animals are being developed. A common goal that is usually achieved with transgenic animals is to visualize the tissues of interest using an endogenously expressed fluorescent protein, understand cell lineage relationships using cell tracing, and decipher the role of a gene or regulatory element during development, regeneration, normal homeostasis, or disease. Thus, one of the most important factors when designing a cassette for a transgenic animal is the selection of promoters and regulatory elements, which can be broadly categorized as either ubiquitous or tissue-specific. These categories are further diversified based on the cassette these regulatory elements are driving, which we will cover in the subsequent sections.
6 | UBIQUITOUS FLUORESCENT REPORTERS

Ubiquitous fluorescent reporter animals are generated by regulatory elements that drive fluorescent gene expression in all different tissues across the body. In other model organisms, a wide variety of ubiquitous regulatory elements such as CMV (cytomegalovirus),\(^4\) CAGGs (a combination of CMV (C), chicken b-actin (A) and the rabbit beta-globin splice acceptor (G)),\(^36\) UBIC,\(^37\) PGK,\(^38\) EF1\(^39\) and SV40\(^40\) have been used to ensure ubiquitous expression of a cassette. We have tried all of the above promoters in the axolotl and found that CAGGs consistently gives a reliable and most ubiquitous expression. However, we have also noticed that the CAGGs promoter leads to a very strong gene expression in most cell types, including muscles, but very low expression in the neural and muscle progenitors.\(^34,41\) Hence, identifying a truly ubiquitous promoter or an equivalent of the murine Rosa26 locus\(^42\) that drives uniform gene expression in all tissues remains a challenge. Nevertheless, many important transgenic animals have been developed using the CAGGs promoter (Table 1), and they have provided useful insights into the process of tissue regeneration.

Such ubiquitous fluorescent reporter animals serve as an excellent tool for embryonic and tissue grafting purposes to achieve a more detailed understanding of biological processes. For instance, by using a CAGGs:eGFP transgenic embryo as a donor, Kragl et al achieved clean labeling of tissue anlage in the axolotl limb.\(^43\) Tracing of individual tissues during axolotl limb regeneration showed that the axolotl blastema is not made up of pluripotent cells but is instead a mix of tissue-restricted progenitors, which are only capable of contributing to their own lineage—for example, muscles, nervous system, epidermis and connective tissue cells each contributed to their own lineage during limb regeneration. However, such grafting approaches could not delineate nuances within certain tissues; within connective tissue, dermal fibroblasts seem to have contributed to the skeletal cells, but it remained unclear how skeletal and periskeletal cells contributed to the regeneration. To understand such subtlety, advance genetic tools such as tissue-specific inducible Cre-loxP is needed. Nevertheless, this study provided important insights on lineage restriction during limb regeneration. Since then, many different studies have used the grafting approach to provide a further detailed understanding of evolution, development, and tissue regeneration in axolotl\(^30,44–48\)

7 | TISSUE-SPECIFIC FLUORESCENT REPORTERS

Expression of a protein can be restricted to certain tissues by using tissue-specific promoters to achieve tissue-specific gene expression and circumvent the need for tissue grafting approaches. In many cases, regulatory elements are conserved, and it is possible to use those of other species to achieve tissue-specific expression in axolotl. For example, it was possible to label the entire neuronal population in the axolotl using the mouse β3-tubulin regulatory elements.\(^49\) Similarly, using mouse 2',3'-cyclic nucleotide 3' phosphodiesterase (CNP), Xenopus keratin 12 (KRT12), Xenopus cardiac actin (Car-Act) and Xenopus collagen2-alpha1 (Col2A1), made it possible to label Schwann cells and neural progenitors, outer epidermis, muscle, and cartilage cells, respectively, in axolotl.\(^49\) Although such exogenous regulatory elements are not 100% conserved in axolotl, in many cases they display

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stereotypic tissue-specific expression as reported in other model organisms. However, often such regulatory elements fail to drive faithful expression in axolotl, particularly when there is significant sequence divergence. Under such circumstances, researchers have to rely on regulatory elements from the axolotl’s complex genome, which can be identified by homology mapping between species. For example, the upstream sequence of Sox2 from the axolotl genome was used in the initial attempt to generate a reporter line for neural progenitors. However, it only showed restricted expression within the telencephalon. The most likely reason for this is that the Sox2 regulatory elements are distributed across multiple enhancers in the genome and the upstream regulatory sequence is not sufficient to mimic endogenous Sox2 gene expression. With the development of knock-in technology, it is now possible to generate specific fluorescent reporter lines for tissues in which elements from other species do not work reliably or are dispersed across hundreds of kilobases. Such Sox2 knock-in animals showed broader expression in neuromast cells besides brain and spinal cord neural progenitors, suggesting that targeted knock-in could be more reliable and specific as compared to random transgenesis.

Regulatory elements can also be used to study pathways that activate them. In axolotl, retinoic acid response element (RARE) was used to drive expression of GFP to report activity of the retinoic acid (RA) pathway during limb regeneration. The current notion of the field suggests that the RA pathway controls proximal-distal identity of the limb, in that a gradient of RA imparts proximal identity of the limb. Contrary to this notion, during limb regeneration, RARE:GFP animals showed the highest activity of the RA pathway in the apical ectodermal cap (AEC), suggesting a unique role of RA during limb regeneration.

8 | CONSTITUTIVE GENE OVEREXPRESSION

Transgenic approaches are also useful to study gene function following its overexpression in a ubiquitous or tissue-restricted manner. For example, the laboratory axolotl has a white (d/d) phenotype as compared to the dark (D/D) phenotype observed in wild type animals. The reason for the d/d phenotype is the lack of pigment cell migration from the neural crest during embryonic development. Recently, Woodcock et al identified a mutation in the Edn3 gene which causes the white (d/d) phenotype. A constitutive gene expression approach using both ubiquitous (Actb2) and tissue-specific (Krt5) promoters could rescue the white mutant, suggesting that loss-of-function mutations in Edn3 underlies the traditional phenotype.

9 | SPATIO-TEMPORAL CONTROL OF GENE EXPRESSION

The genetic systems which provide spatiotemporal control over gene expression are crucial for indelible labeling of cell types as well as studying cellular lineages and gene functions. Some of the most commonly used genetic systems are FLP/FRT, UAS/GAL4, LacI/IPTG-inducible, tetracycline-inducible (tet), and inducible Cre/loxP. Among all, the inducible Cre/loxP system has been widely adapted in axolotl due to its ease of use and reliability.
The inducible Cre/loxP system provides spatiotemporal control over gene expression and has revolutionized lineage tracing and gene function studies in a variety of model organisms. It uses a P1 bacteriophage-derived Cre enzyme, which is capable of site-specific recombination at a pair of loxP sites. To achieve spatial control of the gene expression, Cre is expressed through a tissue-specific promoter, which then allows for site-specific recombination in the cell type of interest. To achieve the temporal control over recombinase activity, Cre is fused to the ER\textsuperscript{T2} sequence, which sequesters Cre in the cytoplasm by binding to HSP90. The interaction between Cre-ER\textsuperscript{T2} and HSP90 can be disrupted using 4-hydroxytamoxifen (4-OHT), allowing nuclear translocation of the Cre-ER\textsuperscript{T2}, wherein it carries out the recombinase activity. To achieve a tighter control over Cre activity, the enzyme is often fused to the ER\textsuperscript{T2} sequence at both its N- and C-terminus (ER\textsuperscript{T2}-Cre-ER\textsuperscript{T2}). In axolotl, we generated transgenic lines under at least three different promoters using single and double ER\textsuperscript{T2} sequences and we found that in all cases, a single ER\textsuperscript{T2} sequence is always leaky and allowing background level recombination, even in the absence of tamoxifen (Figure 3D). In contrast, we see no leakiness in the transgenic lines which use the same promoter alongside a double ER\textsuperscript{T2} sequence (Figure 3A). That being said, the single ER\textsuperscript{T2} sequence allows up to 90% conversion rate in the cell type of interest, whereas with the double ER\textsuperscript{T2} sequence it is often less than 40%. The conversion rate is also significantly influenced by the activity of the promoter. For example, Prx1 regulatory elements which acts as a marker for limb connective tissue cells, are poorly active in a mature limb (Figure 3B,E) whereas they display high activity during limb bud formation and limb blastema formation (Figure 3C,F). Hence, the single ER\textsuperscript{T2} sequence should be used when maximum expression of a transgene is required, while the double ER\textsuperscript{T2} is more suitable if high tissue-specificity with no leakiness is desired.

Although only a few studies using the tissue-specific inducible Cre/loxP system have been carried out so far in the axolotl, all have provided great insights into the process of tissue regeneration. The inducible Cre-loxP allows for clean and indelible labeling of tissue types, which can then be used to study cellular lineages or to isolate cells from a tissue sample using FAC-sorting and studying their molecular profile. For instance, inducible Cre/loxP was used in a comparative study of axolotl and newt limb regeneration which demonstrated muscle formation in axolotl relies solely on satellite cell populations, whereas in newts, muscle fiber dedifferentiation acts as the cellular source. Recently, in our efforts to understand the cellular source of the blastema, we developed inducible Cre lines that label various sub-compartments of connective tissue (Table 1). Our work showed that fibroblasts are the major source of blastema tissue and are capable of forming all different subtypes of connective tissue. Furthermore, FAC-sorting Prx1-labeled fibroblasts from Prx1:TFPnls-T2a-ER\textsuperscript{T2}-Cre;CAGGs:loxP-eGFP-loxP-Cherry axolotl and analyzing them using single-cell transcriptomics showed that axolotl limb regeneration proceeds via fibroblast dedifferentiation. A more complex loxP system—a Limbow line—was also developed and proved useful for clonal analysis of axolotl digit tip and limb regeneration. Considering that axolotl can regenerate many different body parts and that there are many cell types involved in each, it would be important to develop tissue-specific
inducible Cre lines that can label other tissues, including the epidermis, muscle fibers, cardiomyocytes, neurons, endothelium, and a range of immune cells.

The inducible Cre/loxP also provides a great tool for gene function studies. A gene can be overexpressed in its native form or in a mutant form under the loxP control through a tissue-specific inducible Cre driver. Within mutant forms, many enzymes and receptors can also be expressed in a constitutive-active or dominant-negative form to mimic the disease pathology and allow for study of their role in development and regeneration. Using this approach, when a cell cycle inhibitor p16\(^{INK4A}\) was induced in CAGGs:loxP-eGFP-loxP-p16\(^{INK4A}\)-T2a-Cherry; CAGGs:ERT\(^{2}\)-Cre-ERT\(^{2}\)T2A-eGFPnuc double transgenic animals and it resulted in a decline of tail regeneration ability in axolotl.\(^{49}\) Although this straightforward system is easy to adapt, its use has been limited primarily owing to the cost associated with maintaining an axolotl colony. However, with the rejuvenated interest in axolotl tissue regeneration research, we are expecting many more studies using spatiotemporal gene manipulation in the near future. It is also important to keep in mind that Cre-loxP approaches lead to irreversible recombination, meaning turning off gene expression is not an option. Hence, if a temporary gene expression is desired, ON/OFF systems such as TET3G or UAS/GAL4 may be more useful.

### 11 | OTHER GENETIC SYSTEMS

Apart from inducible Cre/loxP, other genetic systems such as the tetracycline-inducible TET3G\(^{59}\) or the binary UAS/GAL4\(^{57}\) system are useful when leakiness is a concern or temporary gene expression is required. These are routinely used to perform gene function studies in various model organisms. To this end, Whited et al. tried to establish a TET-2G system in axolotl but found that in vivo induction of the gene was only possible at a lethal concentration.\(^{58}\) It remains to be seen if the TET3G system works better than the TET-2G system in axolotl.\(^{59}\) In another attempt to achieve transient expression, Whited et al. established an inducible system for gene function interrogation based on the Escherichia coli-derived Lac-repressor/IPTG system.\(^{58}\) In this system, the gene of interest is placed under the control of the lac operator and its expression can be induced by adding the lactose analog IPTG to the water (Figure 4). In the absence of IPTG, the Lac repressor binds the lac operator and the gene is repressed. Following its administration, IPTG binds to Lac repressor and leads to a conformation change preventing its binding to the operator and allowing gene expression. This system was successfully used to show that thrombospondin-4 plays an important role in patterning skeletal structures during limb regeneration in axolotl.\(^{58}\) Although more aspirational at this stage, a combination of CRISPR-mediated knockin and lox sites can be used to generate conditional mutant phenotypes (Figure 5). Such technologies have been used in other model organisms\(^{68,69}\) and may be useful to dissect mechanisms of regeneration in axolotl. Recently, a light activable myosin motor (OptoATV) was used to control activity of myosin motors in axolotl blastema, which proved its crucial role in axolotl limb regeneration.\(^{70}\) Although this study used electroporation of a construct, it shows the potential of optogenetics in tissue regeneration research. For example, a transgenic line with photoactivable Cre\(^{71}\) could be developed and tested to achieve further spatial restriction over recombinase activity of Cre.
cell ablation systems

Spatiotemporal control over gene expression can also be used to drive the expression of a toxin or toxin-producing enzyme to selectively kill cells in which it is expressed. Precisely targeted ablation of certain cell populations is useful for determining the role of a given cell type within a tissue or organ. Such transgenic animals are commonly used in other model organisms such as zebrafish and mouse to study the role of progenitor cells during development and regeneration. Some of the most commonly used systems in these models are the conditional expression of nitroreductase (NTR), Herpes simplex virus thymidine kinase (TK), Diphtheria toxin A (DTA), and a combination of DTA and conditional expression of DTA receptors (Figure 6). Currently, there is no such proven system in axolotl, and it would be interesting to see which method works with reliability and precision to perform cell ablation.

13 | Constitutive gene knock-out

In the past, forward genetic approaches have played a key role in elucidating gene function. However, with the emergence of genomic data, reverse genetics, and sequence-specific gene engineering, scientists are able to target specific nucleic acid sequences and test gene function hypotheses that they were previously not able to. The gene knock-out is a crucial tool for elucidating gene function. Studying how certain molecular pathway functions in the absence of a protein can contribute to unraveling the role of a gene. In one such study, the role of Sox2 was shown in neural stem cell amplification during tail regeneration in axolotl. In other studies, a crucial role of thrombospondin and Eya2 was identified during axolotl limb regeneration. Successful generation of genomic knock-outs in axolotl using the CRISPR/Cas9 system was shown to reach mutation frequencies of up to 100%, although, we have noticed very poor cutting efficiencies with some gRNAs. Thus, it is important to design more than one gRNA and validate each of them before undertaking further studies. Due to the ease of use and flexibility in choosing target sites, CRISPR/Cas9 is very easy to implement and we are expecting a huge surge in gene knock-out animals in the coming years.

14 | Conditional knock-outs

Although gene knock-outs are necessary for functional genomics, homozygous germline mutations often lead to embryonic lethality or developmental defects. For this reason, methods that enable in vivo gene knock-out in a spatial and temporal manner are crucial, and conditional knock-out animals can serve as an alternative in such scenario. The previously described Cre/loxP system is the most commonly used tool for this purpose. To create Cre-mediated conditional gene knock-outs, one or more exons of the gene of interest are placed between two loxP sites. These transgenic animals are then bred with animals expressing the Cre enzyme under the control of a tissue-specific promoter, leading to targeted, permanent gene disruption. Such conditional loxP alleles can also be crossed with inducible tissue-specific lines to achieve further spatiotemporal control over gene knockout. The current knock-in technology is not adequate to do such complex insertion in axolotl.
and requires further improvement so as to achieve insertions at two different loci without disrupting gene function.

15 | NATURALLY OCCURRING MUTANTS

While we discussed diverse variety of transgenic animals that can be generated in the lab, there is also a wide variety of naturally occurring mutant animals that are available for research. Their phenotypes show great diversity and include but are not limited to albino (a/a), twisted gills (t/t), short toes (s/s), cardiac lethality (c/c), and vasodilation (v/v). In fact, the white axolotl strain (d/d) most commonly used for research has a naturally occurring mutation in the *Edn3* gene, as we discussed earlier.55 With the advancements in genomics and gene editing, now it is possible to characterize each of these mutant animals. In one such study, a mutation within *Tnnt2* was identified as a reason for cardiac lethality (c/c).78 The full list of mutant animals is available at the Ambystoma Genomic Stock Center (AGSC) (https://ambystoma.uky.edu/12-educationresources/9-mutant-genes).

16 | CHALLENGES

Axolotls are easy to breed and lay large clutches of eggs, which is an advantage for transgenesis.79 However, the slow development, frequently mosaic F0 animals, and late age of sexual maturity (8–12 months) represent significant challenges for researchers working with axolotl. Typically, embryos are screened for a fluorescence gene and raised as a potential founder. At the moment, there is no possibility to screen for non-fluorescent cassettes due to the mosaicism in F0 animals and hence, further improvement in transgenesis methodology which reduces mosaicism and allows work with F0 animal is needed. It takes approximately a year to grow up embryos as breeders and obtain an F1 transgenic line which shows uniform expression due to germline-transmission. However, in some instances, F0 breeders may show a poor germ-line transmission rate in F1, and researchers may be forced to wait for F2 to do an actual experiment. A female breeder also requires a three-month rest period between matings, significantly stretching wait time for experiments. However, with the availability of a sex determination protocol,80 it is now possible to pre-screen potential breeder and founder animals for their sex. Unlike zebrafish, axolotls are large animals (~25 cm in adulthood) and require significant space for housing. Currently, we lack robust methods for freezing sperm in axolotl that allows sperms to be functional after thawing and needs to be developed. Thus, every transgenic line must be continuously maintained and bred in an aquatic facility. Considering that at least 10 animals are needed to maintain a transgenic line, this number quickly grows, and the expense to run such an aquatic facility becomes unaffordable for small labs. The lack of cryopreservation also affects the distribution of transgenic axolotls. Altogether, generating a transgenic animal requires a long-term commitment and significant use of human and housing resources.

One of the major resources for axolotl researchers is the colony maintained at the Ambystoma Genetic Stock Center (AGSC) at the University of Kentucky. The AGSC provides research laboratories worldwide with animals, along with housing, breeding and disease treatment information. The AGSC is also responsible for collecting and distributing transgenic animals. The lack of similar facilities in other parts of the world has created
challenges in efficient distribution of transgenic axolotls to laboratories outside the USA. The community also lacks a consolidated database to search for available transgenic animals. Currently, they are made available by individual laboratories that developed them. At the moment, very few transgenic animals are currently available for supply by the AGSC. In fact, many of the early transgenic animals were generated outside of the USA. Although these transgenics were shared with laboratories across Europe, one of the major problems has been establishing mechanisms in place for their import to the AGSC. We have recently managed to place these mechanisms in place and have successfully imported transgenic animals from Europe. We have initiated contacts with the AGSC to supply them with these animals, as both we and AGSC are committed to supplying the wider research community with animal resources. It is also important that the entire community commits to deposit published transgenic animals with the AGSC, otherwise we may remain at the mercy of catastrophe and could lose these precious lines which are currently available in only a few selected labs. With new transgenic animals being generated by different laboratories, there is also a need for capacity expansion, not just at the AGSC, but in every laboratory working with axolotls. In recent years, the salamander community has organized regular meetings and established platforms for communications. These community driven efforts will pave the way for generating transgenic lines that are needed but unavailable at the moment.

Transgenesis in axolotl also comes with challenges due to its enormous genome size (32 Gbp). The large genome represents a major challenge for the application of targeted DNA-modifying enzymes for transgenesis. Moreover, genes are often spread across hundreds of kilobases with an average gene length of 427 kb (personal communication, Sergej Nowoshilow). Understanding the gene architecture in such long sequences can be daunting. The recent advances in axolotl genome assemblies have mitigated some of these issues and provided a clean reference for designing the target site for gene modifications. Interestingly, the axolotl genome is largely made up of repetitive elements and displays an absence of large scale genome duplication, which is an advantage while designing targets for genome modification. However, there are instances of gene duplications in axolotl, which may generate redundant function and affect knockout studies. Hence, researchers should remain cautious of them while designing gene knockout targets. The current genome assemblies can be accessed at the Sal-site (https://ambystoma.uky.edu-genome-resources) which is hosted by the AGSC, and at the Axolotl-omics website (https://genome.axolotl-omics.org/) which is hosted by the Tanaka lab. More community driven efforts are needed to unify these assemblies, but further efforts are underway in this direction as well.

17 | OUTLOOK

The remarkable regenerative abilities of the axolotl make it an invaluable model system for elucidating the molecular mechanisms of tissue regeneration. Although significant progress has been made in the field of axolotl transgenesis, clearly more work needs to be done, including the establishment of cryopreservation techniques for transgenic lines, their efficient distribution, and improvement of existing transgenic methodologies. Both more funding and awareness are needed to support and augment the existing aquatic
infrastructure. Even though axolotl transgenesis comes with its fair share of challenges, their continued use will provide more nuanced understanding of tissue regeneration.

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FIGURE 1.
A typical example of a cassette for random transgenesis. The gene of interest (green) is driven by a gene regulatory element (promoter/enhancer) that allows either a ubiquitous or tissue-specific expression depending on the regulatory element (blue). The flanking I-Sce-I site or transposon element (eg, Tol2) facilitates integration of the cassette into the genome when co-injected with the I-Sce-I meganuclease or transposase (eg, Tol2 transposase), respectively.
FIGURE 2.
Methods for generating a transgenic animal using CRISPR/Cas9. A, Strategy to generate c-NHEJ dependent knock-out animal. Once a target genomic sequence (cyan) has been selected, guide RNA (gRNA) can be synthesized and co-injected with the CAS9 enzyme to generate a transgenic knock-out animal. B, Strategy to generate c-NHEJ-dependent knock-in animal. Injecting a plasmid that contains the same target genomic sequence followed by the gene of interest (green) alongside the gRNA and Cas9 enzyme will generate a transgenic knock-in animal. PAM (protospacer adjacent motif) (orange) sequences are required for a Cas9 nuclease to create double strand break. Indels (purple)
FIGURE 3.
Effect of double ERT2 and single ERT2 sequence, and promoter activity on conversion efficiency in axolotl. Stereoscopic DIC images overlaid with fluorescence of, A-C, Prrx1:TFPnls-T2a-ERT2-Cre-ERT2; CAGGs:loxP-eGFP-loxP-Cherry, (double ERT2), D-F, Prrx1:TFPnls-T2A-ERT2-Cre; CAGGs:loxP-eGFP-loxPCherry (single ERT2) transgenic axolotl. A,B,D,E, Unamputated limb, A,D, before 4-OH-tamoxifen (4-OHT) treatment, B,E, 30 days after first 4-OHT treatment. C,F, 30 days post amputation (Amp.) regenerated limb. 4-OHT treatment was given during early stages (first treatment at 3 days post amputation) of blastema formation. 2 μM 4-OHT treatment was given three times by bathing on every alternate day. Converted cells (cherry), Scale bar: 500 μm
FIGURE 4.
Schematic model of the IPTG/LacI system. A, Lac repressor binds to the lac operator and blocks movement of RNA polymerase II. B, In the presence of IPTG, the lac repressor fails to bind to the lac operator and allows RNA polymerase II mediated transcription of the gene of interest.
FIGURE 5.
A possible strategy to generate conditional point mutation knock-in using the Flex system. Using the CRISPR/Cas9 system the wild type allele is replaced by a targeting vector containing wild type exon2, point mutated exon2 and two sets of different lox sites (e.g., loxP (blue) and lox2272 (grey)). Such a line could be combined with tissue-specific inducible Cre line to achieve spatiotemporal control of the mutant genotype. Upon 4-hydroxy tamoxifen treatment Cre activity causes two recombination events, leading to replacement of wild type exon2 by the point mutated exon2'. In step 1, the blue set of lox sites facing each other causes flipping of the flanked sequence. This leaves the grey lox sites with the same orientation causing the excision of the intervening sequence, containing the wt exon2.
FIGURE 6.
Possible strategies to perform cell ablation in a targeted cell type. A, A tissue specific promoter drives expression of a protein (e.g., Caspase3) or a toxin (diphtheria toxin) that is responsible for cell death. In such strategy cell ablation occurs as soon as certain cell types are specified and expresses toxin. As an alternative strategy, B, receptor (diphtheria toxin receptor) that binds to toxin (diphtheria) or, C, enzyme (nitroreductase) that produces toxin upon drug (metronidazole) administration is expressed under tissue specific promoter. In such strategies, administration of a toxin or a drug is done at a timepoint when cell ablation is needed, thus providing temporal control over cell ablation. Tissue-specific promoter provides spatial control over cell ablation in all three strategies.
TABLE 1

List of available transgenic lines, methods used to develop them

| Serial No. | Construct for transgenesis                      | Method of transgenesis | Publication          |
|------------|-------------------------------------------------|------------------------|----------------------|
| **Ubiquitous fluorescent reporter**               |                    |                        |                      |
| 1          | Ubiquitous fluorescent reporter                  |                        |                      |
| 2          | CAGGs:eGFP                                       | I-SceI                 | Sobkow et al., 2006  |
| 3          | CAGGs:CherryNuc                                  | I-SceI                 | Kragl et al., 2009   |
| **Tissue-specific fluorescent reporter**          |                    |                        |                      |
| 4          | CAGGs:β3Tubulin:eGFP                             | I-SceI                 | Khattak et al., 2013 |
| 5          | CAGGs:CNP:eGFP                                   | I-SceI                 | Khattak et al., 2013 |
| 6          | CAGGs:Col2A1:eGFP                                | I-SceI                 | Khattak et al., 2013 |
| 7          | CAGGs:KRT12:eGFP                                 | I-SceI                 | Khattak et al., 2013 |
| 8          | CAGGs:CarAct:eGFP                                | I-SceI                 | Khattak et al., 2013 |
| 9          | CAGGs:RARE:GFP                                   | I-SceI                 | Khattak et al., 2013 |
| 10         | CAGGs:Sox2:Sox2-ORFΔ-T2A-Cherry                  | CRISPR-Knock-in        | Fei et al., 2017     |
| 11         | CAGGs:HoxA13:Cherry                              | CRISPR-Knock-in        | Fei et al., 2017     |
| **Cre drivers**                                   |                    |                        |                      |
| 12         | CAGGs:ER-Cre-ER-T2A-eGFPnuc                      | I-SceI                 | Khattak et al., 2013 |
| 13         | AxSox2:Cre-ER-T2A-αGP                            | I-SceI                 | Khattak et al., 2013 |
| 14         | Col2A1:ER-Cre-ER-T2A-eGFPnuc                     | Tol2                   | Khattak et al., 2013 |
| 15         | Pax7: Pax7-ORFp-Δ-T2A-Cherry                     | CRISPR-knock-in        | Fei et al., 2017     |
| 16         | Sox2: Sox2-ORFp-Δ-T2A-Cherry                     | CRISPR-knock-in        | Fei et al., 2017     |
| 17         | Prx1:TPPnls-T2A-Cre-ER-T2                       | I-SceI                 | Current manuscript   |
| 18         | Prx1:TPPnls-T2A-Cre-ER-T2                       | I-SceI                 | Current manuscript   |
| 19         | Col1A2:TPPnls-T2A-Cre-ER-T2                      | I-SceI                 | Current manuscript   |
| 20         | Col2a1:TPPnls-T2A-Cre-ER-T2                      | I-SceI                 | Current manuscript   |
| 21         | Eya2:Eya2-P2A-Cre                                | CRISPR                 | Sousounis et al., 2020|
| **Ubiquitous loxP reporters**                     |                    |                        |                      |
| 22         | CAGGs:LP-eGFP-LP-Cherry                          | I-SceI                 | Khattak et al., 2013 |
| 23         | CAGGs:LP-eGFP-LP-Tomato                          | I-SceI                 | Khattak et al., 2013 |
| 24         | CAGGS:Limbow 2.1                                 | I-SceI                 | Currie et al., 2016  |
| **LoxP for gene function analysis**               |                    |                        |                      |
| 25         | CAGGs:LP-eGFP-LP-p16 ΔNK4a-T2A-Cherry             | Tol2                   | Khattak et al., 2013 |
| **LacI for gene function analysis**               |                    |                        |                      |
| 26         | CAGGs:LacI-pO-thrombosporin-4                    | —                      | Whited et al, 2012   |
| **Gene knock-out**                                |                    |                        |                      |
| 27         | Tyrosinase Gene                                  | CRISPR                 | Fei et al., 2014     |
| 28         | Sox2 Gene                                        | CRISPR                 | Fei et al., 2014     |
| 29         | EGFP Gene                                        | CRISPR                 | Flowers et al., 2014 |
| 30         | Brachyury Gene                                   | CRISPR                 | Flowers et al., 2014 |
| Serial No. | Construct for transgenesis | Method of transgenesis | Publication                  |
|-----------|----------------------------|------------------------|------------------------------|
| 31        | Thrombospondin Gene        | TALEN                  | Kuo et al., 2015<sup>55</sup> |
| 32        | Eya2 Gene                  | CRISPR                 | Sousounis et al., 2020<sup>77</sup> |
|            | **Gene overexpression**    |                        |                              |
| 33        | Krt5:edn3                  | Tol2                   | Woodcock et al., 2017<sup>55</sup> |
| 34        | Actb2:edn3                 | Tol2                   | Woodcock et al., 2017<sup>55</sup> |