Stem Cell Biology and Regenerative Medicine

Jeanne Wilson-Rawls
Kenro Kusumi *Editors*

Innovations in Molecular Mechanisms and Tissue Engineering

Humana Press

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ISSN 2196-8985 ISSN 2196-8993 (electronic)
Stem Cell Biology and Regenerative Medicine
ISBN 978-3-319-44994-4 ISBN 978-3-319-44996-8 (eBook)
DOI 10.1007/978-3-319-44996-8

Library of Congress Control Number: 2016957175

Printed on acid-free paper

This Humana Press imprint is published by Springer Nature
The registered company is Springer International Publishing AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Interest in regeneration has waxed and waned since Lazzaro Spallanzani first described salamander limb regeneration in the eighteenth century. Currently, regeneration is a highly researched area with potential applications derived from biomedical and engineering research that will impact future medical therapies. The availability of genomic and transcriptomic data from regenerative species, combined with new approaches to identify and culture stem cells, has led to an explosive growth in our understanding of the molecular mechanisms of regeneration. In this book, we bring together the latest insights into these mechanisms.

In the first chapter, Debuque and Godwin describe the history of molecular research in salamanders and discuss the latest findings on unique proteins that mediate regeneration. These authors also provide insight into the regeneration of multiple structures and tissues and the transgenic tracing of stem cells that contribute to newly made tissues. While amphibians demonstrate spectacular regenerative ability, the more limited tail regeneration in anole lizards is captivating because these reptiles are evolutionarily more closely related to humans than salamanders, yet retain a significant ability to regenerate nerves, skin, muscle, and cartilage. In the second chapter, Hutchins et al. update the most recent findings in this model species.

The immune response is important for wound healing and initiation of regeneration. In the third chapter, Lynch and coauthors focus on the integration of the inflammatory response and the regulation of stem cells in regeneration of skeletal muscle in mammals. The role of the immune response is also discussed in amphibians and reptiles in the abovementioned chapters. Further, the immune response and signaling pathways during wound healing versus regeneration of amputated mouse digit tips is discussed by Dawson et al. This fourth chapter reviews important differences that account for regeneration following loss of the digit tip, but not more proximal amputations, as seen in very young children.

The next chapters focus on regeneration of cartilage, the heart, and the central nervous system. In the fifth chapter, Lozito et al. discuss cartilage regeneration in amphibians and reptiles and the application of findings from these species to human cartilage repair. Tissue engineering efforts focus on the repair of cartilage defects and of damage due to degeneration, such as in osteoarthritis. In the sixth chapter, Judd and Huang provide a comprehensive discussion of cardiomyocyte regeneration in multiple model systems, including neonatal mice, with a focus on tissue engineering using stem cells. In the final chapter, Roussas et al. review traumatic brain injury and tissue engineering approaches to healing damage to the central nervous system.

In summary, this volume will appeal to readers interested in the broad overview of regenerative research, both in terms of species and tissues. Each chapter has a focus on molecular signals, the role of stem cells, and tissue engineering, making it a unique collection.

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Chapter 1
Research into the Cellular and Molecular Mechanisms of Regeneration in Salamanders: Then and Now

Ryan J. Debuque and James W. Godwin

1.1 Introduction

Regenerative medicine encompasses collaboration between scientists with diverse backgrounds in wound healing, immunology, developmental biology, stem cell science, tissue engineering and more recently, organic chemistry and nanotechnology. Research in this area aims to improve patient outcomes in the contexts of chronic diseases, ageing and acute injuries. Therapeutic efforts have focused on delivering single molecules, embryonic or adult stem cell derived tissues supported by artificial scaffolds to either directly replenish lost tissue or provide paracrine factors to enhance local wound healing [1]. Promising studies in mammalian in vitro and in vivo models following this strategy have spawned development of numerous clinical trials to varying levels of success but have yet to emulate major aspects of true regeneration exemplified in nature.

Experimental biologists have been studying natural adult regeneration in many phyla for centuries and include vertebrates such as amphibians and teleosts reviewed in [2–6]. Urodele amphibians (commonly referred to as salamanders) are some of the oldest animals to be housed in laboratories and have contributed to many fundamental concepts and discoveries in experimental biology [7]. Regeneration is a property shared in all ten families but is best understood in a few species representing selected genera [8]. Evolutionary reasons for the robust

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J. Wilson-Rawls, K. Kusumi (eds.), *Innovations in Molecular Mechanisms and Tissue Engineering*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-3-319-44996-8_1

regenerative potential in salamanders, in comparison to mammals is poorly understood. Classically it has been hypothesized that mammals have lost the cellular machinery or processes required for *scarless* healing, a potential requirement for perfect tissue regeneration, possibly in favor of selecting for strategies to deal with a wider range disease pathogens [9–11]. An alternative reason centers around the idea that any species with the capacity to regenerate complex tissues may have acquired this ability through the selection of specific genes and is not an ancestrally shared property [12].

Regeneration in salamanders was first documented in 1776 by the Italian scientist Lazzaro Spallanzani who documented the regeneration of the forelimb, hindlimb, tail, gill and jaw [13]. This chapter encapsulates the major discoveries in the 240 years since, covering fundamental ideas originating from surgical manipulations, insights aided by molecular tools and potential outcomes anticipated using next generation sequencing and genome editing technologies.

1.2 Establishing the Tissue Requirements and Boundaries for Regeneration

1.2.1 The Regenerative Potential of Salamanders

Early inquiries into the regenerative potential in salamanders defined the limb, heart, brain, lens, tail, spinal cord, liver, jaw, bone segments, muscle, skin, and gills as tissues capable of growth after resection (See Table 1.1) [13, 78, 85, 102, 120, 135]. Studies concerning the regeneration of clinically relevant tissues (heart, spinal

Table 1.1 Regenerating tissues in salamanders

Tissue	Primary research references
Limb	[14–54][a] [18, 28, 39, 55–65][b] [66–68][c] [69–71][d] [72–75][e]
Heart	[76, 77][a] [78–84][b]
Brain	[85, 86][a] [87–90][b]
Lens/ Retina	[91][a] [92–96][b] [97][c] [98–101][e]
Spinal cord	[102–111][a] [112–115][b] [116][d] [117]
Liver	[118, 119][e]
Jaw	[120–122][b] [123][c] [124][d]
Bone	[125, 126][a] [127][c]
Muscle	[128–131][a] [131–134][b]

[a] Ambystoma
[b] Notopthalamus
[c] Cynops
[d] Pleurodeles
[e] Triturus

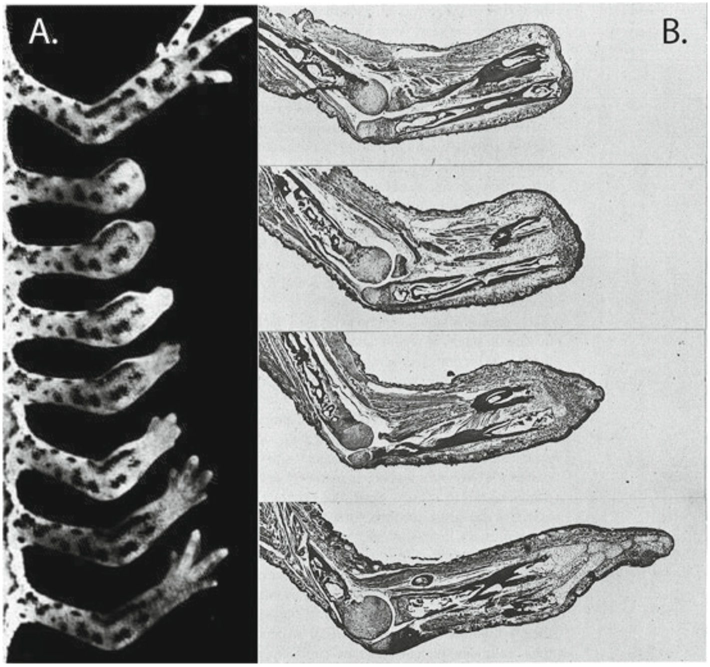

Fig. 1.1 Morphological and histological view of salamander limb regeneration. (**a**) Gross anatomical view of the successive stages of regeneration in the newt. (**b**) Histological sections of regenerating newt limbs. Following amputation, cells of the epidermis have migrated over to cover the wound and thicken to form a structure known as the wound epithelium (WE) or apical epidermal cap (AEC) (First top two images). Interactions between the WE and nerve provide mitogenic signals to cells beneath the WE to initiate a proliferative response resulting in the formation of a blastema (third image from the top). Proximal blastema cells differentiate and develop into new limb tissues such as bone, muscle and nerve to restore normal limb architecture. Adapted from [136]

cord, brain, and lens) were reported less frequently compared to the limb. Adult salamander limb regeneration is a unique property not observed in any other tetrapod. The limb is very amenable to complex procedures with low mortality risks and shares structural similarities to mammals. The process of limb regeneration after amputation has been well characterized and initially defined from early gross anatomical and histological observations (Fig. 1.1).

1.2.2 Tissue Requirements for Limb Regeneration

Many of the experiments performed during this era aimed at identifying methods to prevent or perturb limb regeneration. Loss of function studies carried out by scientists at the time utilized two main methods, surgical removal of specific tissues in the limb or ablation of blastema cells via irradiation [14]. Key findings using these methods were the identification of the nerve and wound epithelium as essential tissues for regeneration. The clearest example for the requirement of wound epithelium came from experiments demonstrating a blockade of limb outgrowth by grafting a flap of intact skin over the site of amputation [15, 16]. Failure of this outgrowth was attributed to a reduction in cellular proliferation after the first week of regeneration, within the blastema (mound of progenitor cells forming at the amputation site) [55]. First reported in 1823, de-nervation of the limb either prior to or at the time of amputation results in the formation of a scar-less stump [137]. Subsequent studies both in the salamander and anuran amphibians identified that limb outgrowth is dependent on density of nerve tissue, not type of innervation and that signals from the nerve control blastema outgrowth [17, 56, 72, 138–141]. Additional experiments supporting this idea originated from experiments where nerves were resected and deviated towards foreign areas to produce supernumerary limbs [74, 142, 143].

1.2.3 Grafting Tissues to Understand Positional Identity During Limb Regeneration

Historically salamanders have been known to tolerate both allografts and xenografts without acute rejection, which has allowed the design of long term regeneration studies featuring tissue grafts [144, 145]. In particular this technique has been useful for understanding ideas regarding positional identity and memory during regeneration of a tissue. In the case of the limb, regeneration occurs across three dimensional axis (proximal-distal, anterior-posterior and dorsal-ventral). Most experiments examining positional memory have looked at the proximal-distal axis (shoulder-wrist). One example is the experiment performed by Goss, who implanted a distal amputated limb into the flank after which resection of the elbow joint (originally proximal) displayed outgrowth of distal skeletal elements (wrist) [146].

Another example was the finding that intercalary regeneration (replacement of missing structures between two juxtaposed tissues) is unidirectional and proceeds in a proximal-distal fashion (referred to as the law of distal transformation) [19, 57]. Other approaches to studying positional identity involved the use of grafting blastemas from different levels along the PD axis onto the dorsal side of proximal stumps to observe the displacement of the grafted tissue back to its original position and then proceeding with limb outgrowth [20].

Further work using tissue-grafting experiments established the concept of positional discontinuity during the early stages of regeneration as a requirement for outgrowth. Originating from studies in invertebrate models, positional discontinuity is achieved when tissues from opposite sides of an axis confront each other (e.g. dermis from the anterior side of an amputated limb meets with the posterior side) [147]. Experiments focusing on the relationship of cells along transverse axes of the limb (anterior-posterior and dorsal-ventral) demonstrated this requirement by inducing supernumary tissues to form by rotating tissues of a stump following amputation or rotating a blastema and grafting them to a stump [21, 22, 57, 58, 148]

1.3 Molecular Mechanisms and Cellular Dynamics of Regeneration

1.3.1 Identifying the Molecular Mechanisms Underlying Limb Regeneration

Experimental approaches for dissecting molecules that regulate limb regeneration were inspired by research conducted in the late 1970s by Niazi and Saxena who first reported the abnormal effects of vitamin A on limb regeneration in tadpoles [149]. Repeated in the axolotl shortly after, Maden was able to show that retinoic acid and its derivatives were able to reject the law of distal transformation and cause proximal limb elements to regenerate from a distal amputation [23]. Subsequent studies later found that regeneration along the transverse axis of the limb was also perturbed and have implicated additional roles for retinoic acid signaling in other regenerating tissues [18, 150, 151].

Research spawning from the influence of retinoic acid aimed to utilise the molecular tools of the early 1990s to elucidate roles for candidate genes regulating limb regeneration. Inspiration for choosing candidates to examine came from a plethora of studies on vertebrate limb development, which had well defined morphogenetic signalling zones. Blastema outgrowth and patterning shares many structural similarities to a developing limb thus it is logical to assume that the same molecules have similar roles. Indeed such a hypothesis is supported with several studies elucidating roles or identifying expression patterns of genes belonging to several developmental signalling pathways such as Hox, Fgf, Hh, Bmp and Wnt [25–27, 68].

A molecular explanation for retinoic acid's control across the PD axis came with the identification of Prod1 [59]. Identified in a subtractive cDNA screen of cultured newt blastema cells, Prod1 is known to be expressed at the cell surface and regulated by retinoic acid and Meis homeoprotein during limb regeneration [29, 30, 59]. It is one of the few salamander proteins to have its structure solved and is present in nine salamander species spanning four families [152, 153]. Interestingly this gene is required for pre-axial digit formation and has no known mammalian orthologues making it one of the few known salamander specific genes involved in limb regeneration [12, 28]. Prod1 is also indirectly involved in nerve dependent regeneration where it has been shown to bind to the newt orthologue/paralogue of anterior gradient protein 2 (nAG) [60]. nAG is expressed first at severed nerve sheaths, secreted by Schwann cells and subsequently in gland cells of the wound epithelium. Over-expression of this protein is sufficient to stimulate blastema cell outgrowth of de-nervated limbs, rescue limb regeneration, and provide novel a molecular pathway to study nerve dependent regeneration [60].

1.3.2 New Insights to Cellular Contributions During Limb Regeneration

Advancements in imaging in cloning have allowed the production of genetic tools to fluorescently label whole animals or tissues and visualise cellular dynamics during regeneration [154]. Grafting fluorescent donor tissues into wild-type is one of the most common strategies applied when studying cellular contribution during regeneration, a feature accessible to few models [31, 32, 130, 131]. One study to take advantage of this strategy is the landmark paper by Kragl and colleagues who determined the heterogeneity of cells in the blastema and their restricted nature to contribute to tissues differing from their embryonic origin [33]. Another example was the deployment of a suite of HOXA antibodies staining donor GFP connective tissue blastema cells during limb regeneration, overturning fundamental concepts regarding segment formation along the proximal distal axis [34–36].

Research into the potential contributions of the immune system to regeneration has historically been limited with most studies examining its relationship in the wound healing response. Clear documentation of infiltrating leukocyte kinetics and the influences of peripheral lymphoid organs was reported in the 1980s [61, 155]. Many immune-modulating drugs and procedures have been screened for effects on outgrowth with other studies implicating roles for known mammalian genes involved with wound healing [37, 62–64]. Renewed efforts in this area have seen the development and adaptation of modern techniques to study the immune system in the salamander [156, 157]. Recent evidence using the axolotl suggests that cells of the evolutionarily conserved innate immune system, particularly macrophages have roles through all phases of regeneration [38]. Macrophage depletion after blastema formation allows regeneration to complete, but is delayed. Macrophage ablation prior to amputation has been shown to block limb regeneration but re-amputation following replenishment resets normal limb regeneration indicating a temporal requirement for these cells prior to blastema formation [38]. Failed limb regenerations are characterised by collagen rich scars, decreased cellular proliferation, and alterations to key regeneration associated genes such as MMPs, and TGF-β [37, 38, 65]. Further work also implicated a role for dependent immune-surveillance and clearance of senescent cells in the regenerating limb [39].

1.3.3 Mechanisms of Outgrowth During Regeneration Found to be Tissue Specific

A key concept of regenerative medicine is that mechanisms governing cellular outgrowth are not universal and should be considered tissue specific (Fig. 1.2). Generally speaking, replacement of a new structure requires cells to arise and undergo several rounds of rapid proliferation. The mode through which this is conducted can be through the recruitment and activation of stem-progenitor cells or stimulating resident and neighbouring post-mitotic cells to re-enter the cell-cycle. One example of the former is regeneration of the spinal cord. Regeneration of the spinal cord following resection activates resident neural stem cells to mobilise locally and then migrate along the anterior-posterior axis. These cells serve as a multi-potent source for all neural cells in the regenerated tissue [103–105]. Molecules implicated for regulating in this process include planar cell polarity genes and microRNAs both conserved and unique to the salamander

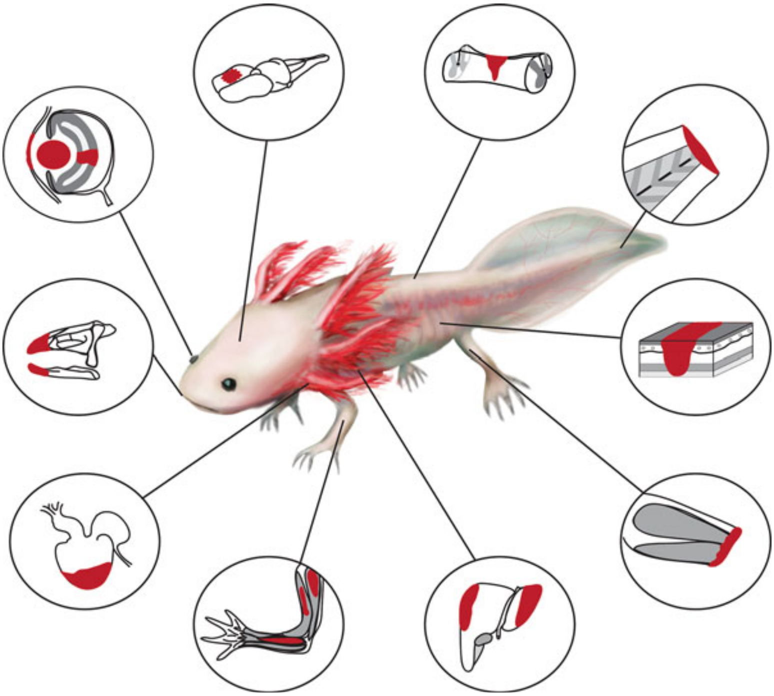

Fig. 1.2 A range of clinically relevant tissues can be regenerated in adult salamanders. This figure illustrates a generalized summary of regenerating tissue in salamanders using various species as outlined in Table 1.1. The tissues identified so far include: brain, spinal cord, tail, skin, limbs, liver, skeletal muscle, heart, jaws, and ocular tissues such as retina, cornea, and lens. Variation in modes of regeneration and adult capacity are outlined in the text. Injury site is highlighted in *red*. Axolotl image provided and adapted with permission from Memuco© artist services and IUCN Arkive

[106, 107, 109, 158]. Similar mechanisms have been observed in the case of the regenerating telencephalon and dopaminergic neurons of the mid-brain. Following mechanical removal or chemical ablation, cells within these tissues have been shown to cause rapid proliferation and neurogenesis from spatio-temporal restricted zones [86–90].

In contrast many studies have reported that the salamander lens and heart utilise the second mode of replacing cells, which interestingly has limited examples in mammalian regeneration [76, 79, 92]. One of the most studied areas of salamander biology is lens regeneration. After lentectomy, pigment epithelial cells originating from the dorsal iris, re-enter the cell cycle, lose their pigmentation and other differentiated characteristics, before undergoing trans-differentiation into new lens tissue. This trans-differentiation is accompanied by the activation of sequential lens development gene expression, reviewed in detail elsewhere [159]. It should be noted that this process can be repeated almost indefinitely as mounting evidence from both histological and molecular studies suggests that lens regeneration is not affected by age or the number of times it is removed [93, 94]. Similarly cardiomyocytes can lose many of their differentiated characteristics and proliferate following ventricle resection, replacing up to 20 % of the original ventricle tissue [76, 80]. Signals initiating cell-cycle re-entry have yet to be identified, however one known important regulator is components of the extracellular matrix, which has shown to undergo rapid changes during the early stages of heart regeneration [80–82].

An intriguing aspect to keep in mind is the potential for identical tissues to make use of different mechanisms between species. Already two examples for this have emerged. The first being skeletal muscle where axolotls deploy activated resident satellite cells to contribute to the regenerate whereas the myofibers of the red spotted newt re-enter the cell cycle [131]. The second example is in the case of the lens, where newts replace cells from only the dorsal iris compared with contributions from either the dorsal or ventral iris as seen in the axolotl, though this potential is lost shortly after hatching [91].

Distinguishing the regeneration specific signals from the background noise arising from amputation associated wound healing and trauma, is extremely difficult. Ideally studies elucidating molecular signals from essential regenerative tissues (e.g. nerve or wound epithelium) should reduce irrelevant signaling that could mask the identification of key pathways and obscure accurate interpretation. One available assay that addresses these criteria is the accessory limb model, which produces ectopic limbs by deviating nerves to positionally discontinuous skin grafts [40]. This unique gain of function ectopic outgrowth assay in the salamander is an extremely useful tool in a model where majority of functional experiments involve loss of function studies. Indeed several molecules have been tested in this system and should gain future utility testing novel candidate genes required for limb regeneration [41–44].

1.4 Entering the era of Next Generation Sequencing and Genome Editing

1.4.1 Unraveling the Salamander Genome and High-Throughput Sequencing Studies in Regeneration

Salamanders are known to have some of the largest genomes amongst all vertebrates with some species approximately containing between 14 and 120 Gb, compared with a genome size of around 3.2 Gb in humans [160, 161]. Characterized by high percentages of transposable elements, the genomic gigantism observed across the salamander family are hypothesized to have originated from a shared period of genome expansion during the Jurassic era [162, 163]. As such, complete genome assemblies are lacking in all families, which is perhaps the biggest drawback for any prospective academics interested in working with the model. Several resources have been put in place to obtain sequence information, largely derived from transcriptome and proteomic analysis from tissues across multiple species [45, 46, 83, 161, 164]. In addition online repositories are available and regularly updated with omics data from the latest studies [165–167].

Many experiments have already utilized next generation sequencing technologies for high through-put transcriptome analysis during limb, spinal cord and lens regeneration [47–49, 95, 110]. One example was the time course analysis performed by Knapp and colleagues examining the transcriptional changes over the course of limb regeneration [45]. This approach revealed that gene expression follows a similar pattern as seen in morphological studies with signature wound healing genes first among those upregulated, followed by amputation associated regenerative genes and then finally, genes implicated in limb development [45]. Studies utilizing proteomics have also been conducted across multiple tissues and species and have contributed major findings such as the identification of novel newt specific CCN, a protein located in the endocardium that is specifically upregulated during the early stages of heart regeneration [83, 168, 169].

Ultimately sequenced based inquiry into the genetic networks of regeneration will require a complete genome assembly however progress towards obtaining genomic information in any species has been understandably limited. Encouragingly the first characterization of the axolotl genome has been documented [170]. Estimated to be 32 Gb in size, the axolotl genome provides an example of the difficulties associated with assembling sequences from large genomes as well as the potential approaches used to overcome current computational limitations [170].

1.4.2 Genome Editing Technologies

Innovations over the last decade in the field of molecular biology have provided a multitude of options to genetically modify the salamander Table 1.2. The application of Sce1-meganuclease or Tol2-transposase technology has produced germline transgenics expressing ubiquitous fluorescent proteins in various salamander species [154, 175, 176, 179]. Several transgenic reporters have been developed to track the fates of tissues such as nerve, Schwann cells, muscle, epidermis and cartilage or signaling molecules like retinoic acid [51, 52]. In addition it is now possible to temporally control gene expression within specific cells thanks to the development of

Table 1.2 Genetic tools available in salamanders

Genome editing tool		References
Non-germline vectors		
Viruses	Vaccinia virus	[171][a]
	Adenovirus	[133, 172][b]
	Pseudotyped virus	[173][a]
	Foamy virus	[174][a]
Germline transgenics		
Ubiquitous reporters	CAGGS;EGFP	[154, 175, 176][a,c,d]
	CAGGS;CherryNuc	[33][a]
	CAGGS;LP-EGFP-LP-Tomato	[51][a]
	CAGGS;LP-EGFP-LP-p16-T2A-Cherry	[51][a]
	CAGGS;ER-Cre-ER-T2A-EGFP-nuc	[51][a]
Tissue specific reporters	B3Tubulin:EGFP	[51][a]
	CNP;EGFP	[51][a]
	Col2a1:EGFP	[51][a]
	Krt12:EGFP	[51][a]
	CarAct;EGFP	[51][a]
	AxSox2;cre-ert2-T2A-GFP	[51][a]
	Col2A1:ER-Cre-ER-T2A-EGFP-nuc	[51][a]
Signaling molecule reporters	RARE;EGFP	[52][a]
Loss of function genetics		
TALENs	Tryosinase	[177][d]
	Sox2	[111][a]
	Thrombospondin-1	[54][a]
	Prod1	[28][b]
CRISPR	Brachyury	[178][a]
	Sox2	[111][a]

[a] Ambystoma
[b] Notophthalamus
[c] Cynops
[d] Pleurodeles