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Abstract
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Keywords
germline transgenic axolotls, gene expression, stem cells, cartilage, spinal cord regeneration, regeneration phenotype, Cre/loxP system

Disciplines
Biology | Cell Biology | Developmental Biology | Structural Biology

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The salamander is the only tetrapod that regenerates complex body structures throughout life. Deciphering the underlying molecular processes of regeneration is fundamental for regenerative medicine and developmental biology, but the model organism had limited tools for molecular analysis. We describe a comprehensive set of germline transgenic strains in the laboratory-bred salamander Ambystoma mexicanum (axolotl) that open up the cellular and molecular genetic dissection of regeneration. We demonstrate tissue-dependent control of gene expression in nerve, Schwann cells, oligodendrocytes, muscle, epidermis, and cartilage. Furthermore, we demonstrate the use of tamoxifen-induced Cre/loxP-mediated recombination to indelibly mark different cell types. Finally, we inducibly overexpress the cell-cycle inhibitor p16INK4a, which negatively regulates spinal cord regeneration. These tissue-specific germline axolotl lines and tightly inducible Cre drivers and LoxP reporter lines render this classical regeneration model molecularly accessible.

INTRODUCTION

Generations of biologists have been fascinated by salamander limb and tail regeneration. Important grafting experiments delineated the fundamental tissue interactions involved that provide a rich basis for a molecular understanding of regeneration (Bryant and Iten, 1977; Dunis and Namenwirth, 1977; Steen, 1968; Stocum, 1975; Wallace and Wallace, 1973) for reviews, see Nacu and Tanaka, 2011; Stocum and Cameron, 2011). For example, after limb amputation, cells from the muscle, connective tissue, skeletal, peripheral nerve, and epidermis all contribute to the progenitor cell zone, called the blastema, which will reconstitute all limb tissues. The progenitors from these diverse tissues remain largely separate and show divergent behaviors, for example, during limb patterning (Kragl et al., 2009; Nacu et al., 2013). Therefore, a mechanistic understanding of regeneration requires the control of gene expression in specific cell types. Furthermore, because genes involved in regeneration may also be implemented during development, inducible gene expression is important.

Although several vertebrate models of regeneration are available, there are compelling reasons to study regeneration in the salamander. First, the graftability of salamander tissue provides a uniquely powerful approach to lineage trace cells and to test cell-autonomous versus -nonautonomous events (Muneoka et al., 1986; Pescitelli and Stocum, 1980). Second, the distinct anatomy of the zebrafish fin from the tetrapod limb limits what we can infer from fins to limbs (Sordino et al., 1995). Xenopus, a valuable molecular model of regeneration, regenerates the developing hindlimb only prior to metamorphosis (for review, see Yakushiji et al., 2009). This regeneration occurs before full differentiation and could involve progenitor cell sources distinct from those utilized to regenerate a fully differentiated limb (Dent, 1962). Therefore, it is important to study regeneration in multiple vertebrates.

Detailed molecular analysis of regeneration has been difficult because it is an adult phenotype with a complex starting point involving the response of many tissues to injury. It is likely that only a subset of cells in any given adult tissue contributes to the blastema. Electroporation of plasmid DNAs and morpholinos has been used to analyze important phenotypes during salamander limb regeneration, but this technique provides transient, variable transfection (Echeverri and Tanaka, 2005; Kumar et al., 2007; Mercader et al., 2005). Adenovirus, vaccinia virus, and pseudotyped retrovirus have also been applied, but the limited cargo size and toxicity have limited their use and have not yet been widely adopted (Kawakami et al., 2006; Morrison et al., 2010; Roy et al., 2000; Whited et al., 2013). Sustained expression from genomic integration is extremely important for the study of salamander limb regeneration. Successful germline transgenesis has been reported for axolotl (Sobkow et al., 2006) and has been applied to the Japanese newt and Iberian ribbed newt (Casco-Robles et al., 2010; Hayashi et al., 2013). Moreover, germline EGFP and nuclear Cherry-expressing transgenic axolotls were used in combination with tissue...
grafting to provide important proof that the regenerating limb blastema is a mixed population of tissue-restricted progenitors (Kragl et al., 2009). For further molecular dissection of regeneration, however, conditional induction of gene expression in a time and/or cell-type-dependent fashion is essential. Recently, Whited and colleagues described a system to induce gene expression in axolotls using an IPTG system, whereas this system is promising for acute control of gene expression, the continuous presence of inducer is required to sustain expression that could limit its use for cell tracking during regeneration (Whited et al., 2012). It is not yet known how well the system can be combined with cell-type-specific control of gene expression. Furthermore, the effectiveness of the system was not shown in germline-transmitted F1 animals where full-genomic integration of the transgene may alter its expression properties. In larval axolotls, full-limb regeneration occurs over 2–3 weeks, whereas in fully adult animals, the process can take over 10 weeks. Therefore, a sustainable gene induction system such as the Cre/loxP system would be valuable for regeneration studies, but tamoxifen-inducible systems were characterized as too leaky for axolotl transgenics (Whited et al., 2012).

Here, we describe tissue- and time-dependent control of gene expression in germline transgenic axolotls via Cre/loxP methodology. We provide a set of germline transgenic animals driving the GFP reporter behind different cell-type-specific promoters relevant for regeneration research. We further employ Cre/loxP to conditionally initiate gene expression in a time and cell-type-specific manner. As a proof of principle for phenotype analysis, we demonstrate the inducible overexpression of the tumor suppressor p16INK4a. It has been proposed that the p16INK4a/ARF locus first arose in mammals and is not present in the salamander, a potential basis for the restricted regeneration capacities of mammals (Pajcini et al., 2010).

To generate germline transgenic lines expressing EGFP in neurons, we implemented the mouse βIII-tubulin regulatory sequences by injecting a previously characterized mouse BAC construct in which the gene for a GAP43-EGFP fusion protein sequence had been integrated with its own poly(A) signal downstream of the coding sequence (Attardo et al., 2008). Injection was performed without SceI meganuclease, and 12 animals out of 343 injected eggs showed neural-specific expression over large portions of the body (approximately >80% of cells) (Table 1). Six animals were grown up for germline transmission and mated to a nontransgenic mate. One of the animals displayed a germline transmission rate of 24%. Figure 1A shows whole-mount images of a live F1 progeny showing GFP signal in nerve fibers. To confirm the specificity of expression to neurons, we immunostained tail and limb sections for βIII-tubulin (Figures S1A and S1B available online). Excellent (100%) colocalization of EGFP signal with immunofluorescence signal was observed, confirming the specific expression of the transgene. Abundant expression was observed in the spinal cord (arrow), as well as the dorsal root ganglion (arrowhead) and peripheral nerve tracts (asterisks) (Figure S1A), indicating that central and peripheral neurons expressed the transgene.

For marking of glial cells including oligodendrocytes and Schwann cells, we implemented the mouse CNP promoter (Glaser et al., 2005). After coinjection of the construct with SceI meganuclease, we obtained 7 strongly expressing F0 animals out of 758 originally injected. Three were mated, and all showed germline transmission. Figure 1B shows the nerve tracts expressing EGFP in the limb of a typical live F1 animal. Out of the three mated founders, the F1 progeny of one had uniform EGFP expression in putative Schwann cells and oligodendrocytes, whereas the progeny of the other two founders showed mosaic EGFP expression.

RESULTS

Tissue-Specific Germline Transgenic Reporter Strains

Because grafting experiments indicated that each tissue contributes uniquely to regeneration (Kragl et al., 2009), a molecular analysis of regeneration requires controlling gene expression specifically in different tissues. We implemented tissue-specific promoters from different animal species to successfully establish germline transgenic axolotl strains showing faithful, cell-type-specific expression of the EGFP gene (Table 1; Figure 1). Transgenic strains were produced by injection of plasmid or BAC DNA into one- to two-cell embryos as previously described (Khattak et al., 2009; Sobkow et al., 2006). Injected animals were grown to larval stages and examined via fluorescence protein expression for the extent of expression. Only animals with apparently greater than 80% expression along the body were raised to sexual maturity and then mated to nontransgenic mates (see Table 1 for numerical details for each transgenic strain). The F1 progenies were examined for EGFP expression in the expected cell/tissue types as live whole mounts. To confirm specificity and penetrance of expression, limbs and tails were cross-sectioned and immunostained for respective proteins. We only propagated strains that showed strict colocalization of the EGFP with the respective marker proteins and no extraneous expression. We found this to be an important aspect of the screening process because random integration of the transgenic constructs leads to position effects that can yield mosaicism in the progeny.

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The limbs and tails of F1 progeny from the uniform founder were cross-sectioned and immunostained with an anti-MBP antibody confirming high (100%) colocalization of EGFP signal with MBP+ structures (Figures 1B, S1C, and S1D). EGFP expression was observed in both the central spinal cord (arrow) (Figure S1C), as well as in DRG (arrowhead) and peripheral nerve tracts (asterisks) of the tail and limb (Figures S1C and S1D), indicating that oligodendrocytes and Schwann cells expressed the transgene. Higher-magnification confocal imaging of a nerve tract in the limb confirmed expression of EGFP in the nucleus and cytoplasm of MBP+ cells wrapping around the axon (Figure S1E).

To further propagate these confirmed lines, F1 progenies were raised to sexual maturity and crossed with white nontransgenic hosts to check for germline transmission and specific uniform EGFP expression in F2 progeny. The F1 male and females were mated to establish “homozygous” lines that result in 100% transgenic progeny when mated to a nontransgenic animal (Table 1).

**Application: Tracking Neurons and Muscle during Limb Regeneration**

The ingrowth of nerves is an essential aspect of limb regeneration (Kumar and Brockes, 2012), but the process has so far been followed by quantitative electron microscopy...
and immunofluorescence. The optical clarity of the “white” axolotl strain allows visualization of fluorescent cells in live animals (Echeverri et al., 2001). Here, to demonstrate the feasibility of following axonal outgrowth during limb regeneration, we amputated limbs of larval \( \beta_{III}\text{-tub}:\text{GAP43-EYFP} \) animals and followed them over time in the same live animal (Figures 2A–2C). During regeneration, we observed ingrowth of thin axonal fibers already in the early 6 day blastema (Figure 2A). Further infiltration of axons was observed by the Notch stage of regeneration (Figure 2B) and consolidation of larger axonal bundles infiltrating the forming digits at the Palette stage (Figure 2C). These data indicate that robust innervation occurs early during blastema formation, and fiber termini extend well toward the distal epithelium. In the future, more detailed imaging studies can elucidate the dynamics of nerve-epithelial interactions that are crucial for proper limb outgrowth (Kumar et al., 2007; Mullen et al., 1996).

Another important aspect of limb regeneration is determination of proximal versus distal identity. It was recently shown that skeletal/connective tissue-derived blastema cells regenerate limb elements only more distal to the amputation plane, whereas muscle-derived blastema cells have the potential to regenerate muscle tissue of all limb segments (Nacu et al., 2013). In the previous studies, muscle labeling was performed by grafting of presomitic mesoderm from constitutively expressing \( \text{CAGGs:EGFP} \) donor embryos into white hosts. Here, we demonstrate the utility of the \( \text{CarAct:EGFP} \) transgenic animal for revealing the potency of muscle to regenerate cells in all limb segments. To track muscle versus other limb tissues, a \( \text{CarAct:EGFP} \) animal was first crossed to the \( \text{CAGGs:Cherry nuc} \) animal (expresses nuclear Cherry in all cells) to generate double-transgenic \( \text{CarAct:EGFP}; \text{CAGGs:Cherry nuc} \) that express both transgenes. Midbud hand blastemas were grafted onto the upper-arm stump of a white host animal, in a classic intercalation assay (Figure 2D) (Iten and Bryant, 1975; Stocum, 1975). Limbs of normal morphology regenerated (Figure 2E). Examination of fluorescence signal showed that the muscle-specific EGFP signal (that comes only from the transplanted wrist blastema) was observed in muscle of upper limb, lower limb, and hand (Figures 2D and 2E). In contrast, the nuclear Cherry signal, which in whole mount is most noticeable in the skin-derived cells, was largely confined to the hand. These results show that muscle progenitors from a hand blastema can form upper- and lower-arm muscle.
Use of Cre/loxP System for Tissue and Time-Dependent Control of Gene Expression

To study regeneration, an inducible expression system is important for two reasons. First, when using genetic fate mapping, irreversible activation of marker expression in a given cell type is important because the cell expressing a given marker gene in the mature tissue may, during regeneration, turn off the marker. Second, when studying gene function, inducible expression is important because genes functioning in regeneration may also have earlier roles in development. Considering the long timescales of regeneration, a system that induces sustained expression would be desirable. We therefore focused on testing the Cre/loxP system. We first generated a germline transgenic loxP reporter strain where the CAGGs promoter drives a floxed EGFP-STOP cassette followed by the Cherry gene - CAGGs:LP-EGFP-STOP-LP-Cherry (Figure S2). A total of 11 putative founders were raised to sexual maturity. Seven were mated to white nontransgenic hosts, and all yielded germline transmission with a frequency in the range of 5.4%–54%. Only one founder had mosaic EGFP expression as determined by cross-sectioning the limbs and tails of F1 progeny and staining the sections with DAPI to ascertain that EGFP is expressed in every cell.

To evaluate whether CRE-mediated recombination can work on the genomically inserted transgene, we electroporated a Cre expression plasmid into the mature limb and tail of the F1 progeny of loxP reporter. After 3 days, we observed robust Cherry expression only in animals that had received Cre plasmid (Figures S2D and S2H), and not in control un-electroporated animals (Figures S2B and S2F). No Cherry expression was observed when PBS and/or empty plasmid (pUC19) was electroporated into the tail and limb of the loxP reporter animal. Similarly, no spontaneous Cherry expression was observed after limb/tail amputation and...
during regeneration of the PBS/pUC19 electroporated animals (Figure S3).

Interestingly, coelectroporation of the split Cre constructs (Casanova et al., 2003) together also induced robust recombination and Cherry expression, whereas electroporation of the single constructs did not, bringing up the possibility of making CRE activity dependent on expression of two promoters (Figure S4).

**Cell-type-Dependent Control of Cre Activity in Sox2+ Neural Stem Cells**

We next sought to control Cre expression in a cell-type-specific manner. We isolated a 15 kb genomic clone for the axolotl Sox2 gene that is expressed in the neural stem cells of the CNS (Li et al., 1998; McHedlishvili et al., 2012; Zappone et al., 2000). The clone included 11.68 kb upstream of the coding sequence, the Sox2 coding sequence and 2.66 kb downstream sequence of the coding sequence. Recombining was used to incorporate a Cre \( \text{ERT}^{12} \)-T2A-nucGFP cassette by replacing the Sox2 coding sequence. The resulting construct was injected into eggs together with SceI meganuclease. \( \text{Cre}-\text{ERT}^{12} \) is a highly used method for induction of CRE activity via the tamoxifen metabolite, 4-hydroxytamoxifen (4-OHT) (Metzger and Chambon, 2001). If progeny from a germline-transmitting animal showed robust expression of nuclear GFP in the ventricular, SOX2+ cells of the brain (Figures 3C–3G). When crossed to the CAGGs:LP-EGFP-STOP-LP-Cherry reporter, the F1 progeny displayed strong Cherry expression in the absence of tamoxifen, indicating leaky but cell-type-specific activity of the \( \text{CRE}-\text{ERT}^{12} \) (Figures 3I and 3J). Because recombination was occurring continuously even in the absence of tamoxifen, the distribution of Cherry+ cells was broader than the nucGFP because the Sox2+ Neural Stem Cells

**Tamoxifen-Inducible Expression in CollagenII-Expressing Vertebral Cells**

To combine cell-type-specific and temporal induction of gene expression, we placed the \( \text{Tet}^{12} \)-Cre-\( \text{ERT}^{12} \)-T2A-nucEGFP cassette behind the Xenopus Col2a1 promoter that drives expression in skeletal cells (see Figure 1E), and germline-transmitting animals were produced. Five putative founders were raised to sexual maturity, and one transmitted through the germline showing nuclear EGFP expression plasmid, we observed strong Cherry expression in the absence of tamoxifen (Figures S5G and S5H). Therefore, we tried a多名 ways to attenuate the CRE-ERT activity including fusion with a nuclear export signal and fusion with a ubiquitinylation signal, but neither decreased the background activity sufficiently (Figures S5I–S5L). However, we found that the \( \text{ERT}^{12} \)-Cre-\( \text{ERT}^{12} \) fusion (Zhang et al., 1996) did elicit tight, tamoxifen-inducible CRE activity, as seen by no Cherry expression after the electroporation of the construct in the absence of tamoxifen (Figures S5M and S5N) but robust expression of Cherry after electroporation and subsequent tamoxifen induction (Figures S5O and S5P). We therefore produced a germline transgenic CAGGs: \( \text{ERT}^{12} \)-Cre-\( \text{ERT}^{12} \)-T2A-nucEGFP (Cre-\( 2 \times \text{ERT}^{12} \)) animal (Figures 4A and 4B). When double-CAGGs:ERT\( ^{12} \)-Cre-\( \text{ERT}^{12} \)-T2A-nucEGFP/+;CAGGs:loxP-EGFP-STOP-\text{loxP-Cherry}/+ animals were bred (Figure 4C), we observed no recombination prior to 4-OHT induction (Figures 4D and 4F). After a single intraperitoneal injection of 4-OHT, we observed robust Cherry expression as assayed in limb and tail tissue (Figures 4E and 4G). Some EGFP expression persisted after induction even in Cherry-positive cells. This is presumably due to the loxP reporter harboring multiple integrated copies of the transgene or due to perdurance of the GFP. We conclude that the \( \text{ERT}^{12} \)-Cre-\( \text{ERT}^{12} \) fusion provides tight, temporal control of gene expression in the axolotl.
expression in cartilage of F1 progeny when crossed with white nontransgenic host. In this animal, the head and tail cartilage showed particularly strong expression of the transgene. The germline-transmitting animal was crossed to the \( \text{loxP} \) reporter animals (Figure 5A). In the F1, double-transgenic progeny, no Cherry expression was observed in the head or tail tissue prior to tamoxifen administration (Figures 5B and 5D). After tamoxifen induction, bright areas of Cherry-positive cells were observed in the head cartilage and in the notochord/cartilage ventral to the spinal cord (Figures 5C and 5E).

In conclusion, we have demonstrated that cell-type and temporal control of gene expression is efficient and possible in germline transgenic axolotl lines. This will be useful to track cells during axolotl regeneration and development.

**Use of Tamoxifen-Inducible Cre/loxP System to Overexpress p16\(^{\text{INK4a}}\)**

The Cre/loxP system is a potentially powerful tool to test the role of molecular pathways during regeneration by overexpression of genes. It has been proposed that one rationale...
for the robust regeneration in the salamander is its lack of the growth inhibitor gene products p16\(^{INK4a}/ARF\). We therefore aimed to apply Cre/loxP-based induction to determining the effect of p16\(^{INK4a}\) overexpression during regeneration. Temporal control of gene expression was important because the p16\(^{INK4a}\) may be expected to strongly repress cell division during normal development. To overexpress p16\(^{INK4a}\) during regeneration, we fused the human p16\(^{INK4a}\) sequences with T2A-Cherry and cloned the fusion construct behind the floxed GFP cassette (floxed p16-Cherry). In order to elicit induction of p16\(^{INK4A}\) expression just prior to regeneration, this animal was crossed to a 4-OHT-inducible CAGGs:ERT2-Cre-ERT2-T2A-nuc-EGFP transgenic animal (Figure 6A). p16\(^{INK4A}\) gene expression was initiated with a single intraperitoneal injection of 4-OHT. Cherry expression was observed 5 days after 4-OHT induction in double-transgenic animals (Figure 6C), and p16\(^{INK4A}\) ectopic expression was independently confirmed using an antibody against human p16 (data not shown). Tails from Cherry-expressing and -nonexpressing control animals were amputated, followed, and the length of regenerated spinal cord was measured as a discrete indicator of regeneration (Figures 6B and 6C). At 4 days after tail amputation, a significant inhibition of spinal cord regeneration was observed in animals overexpressing human p16\(^{INK4A}\) (Figure 6D).

DISCUSSION

Here, we have contributed a number of tools and insights into the use of germline transgenic animals for salamander regeneration research by generating germline transgenic animals for cell-type-specific control of gene expression. By employing BACs, heterologous promoters, and axolotl genomic sequences, we have generated a set of animals that drives EGFP in different cell types of the nervous system—neurons (\(\beta\)-III-tubulin), glia (Cnp), and neural stem cells (Sox2). These will provide an invaluable resource for studying brain and spinal cord, as well as peripheral nerve regeneration in these animals. We have also generated animals driving EGFP in muscle, cartilage, and epidermis.

Importantly, we have combined cell-type-specific expression with tight temporal control of gene expression using...
the Cre/loxP system. These are critical tools for the molecular analysis of regeneration because cells from several different tissues contribute to the blastema and remain as distinct progenitor cell pools during regeneration (Kragl et al., 2009). Furthermore, the marker used to initiate gene expression in the mature tissue may not be maintained during regeneration. Therefore, the Cre/loxP system is particularly valuable for regeneration studies. A critical aspect of the Cre/loxP or any induction system is leakiness of the inducer. We therefore scanned a number of means to tightly induce the CRE activity. In contrast to Whited et al. (2012), we observe strict tamoxifen-inducible gene expression when employing the doubly regulated ERT2-Cre-ERT2 sequences in F0 and after germline transmission to F1 in combination with ubiquitous promoters (CAGGS) or a tissue-specific promoter (COL2A1). We can speculate on several sources for the difference in leaky versus non-leaky expression in the two settings. First, we have observed that injection liquids present in the glass microinjection needle (such as plasmid DNAs for transgenesis or tamoxifen for injection into the animals) are often left behind in the micropipette holder after use, and these reagents can be carried over into subsequent injections with new glass microcapillaries via aerosol. Therefore, if several plasmids are screened in sequence, the presence of contaminating Cre-ERT2 DNA from previous injections remaining behind in the micropipette holder and then being transferred to new microcapillaries could have confounded the previous results. Similarly, use of the microinjection device for tamoxifen injection yielded “background” recombination when the DMSO control sample was injected after the tamoxifen samples without cleaning the micropipette holder in between, but not vice versa. We therefore clean

Figure 5. Temporal Induction of Gene Expression in Co2A1+ Cells Using Col2A1:ERT2-Cre-ERT2-T2A-EGFP-nuc Transgenic Axolotls
(A) Scheme of mating between loxP reporter animal (CAGGS:loxP-EGFP-STOP-loxP-Cherry) and the driver animal (Col2A1:ERT2-Cre-ERT2-T2A-EGFP-nuc).
(B and D) Double-transgenic animal shows no Cherry fluorescence in head (B) and tail (D) before tamoxifen induction. High-exposure times were used for imaging so that overall tissue architecture could be seen.
(C and E) Double-transgenic head (C) and tail (E) images after tamoxifen induction. Clear Cherry expression is observed only in the skeletal elements of the head and vertebral column in the tail. Half-exposure time from control (C and E) was used.
(F–I) Cross-section of head showing colocalization of Cherry (F) with Collagen type II antibody staining (G). DAPI delineates nuclei in blue. (I) represents merged image.
(J) High-resolution image of inset marked in (I) showing Cherry-positive cells associated with Col2A1 staining in head cartilage. Scale bars, 2 mm (C, E, and I) and 100 μm (J).
the micropipette holder between each round of injections. A second potential source of apparent leakiness for ERT2-Cre-ERT2 systems could be the quality of the local water because the animals are aquatically raised and constantly exposed to water. The presence of estrogen mimics in the animal water supply could confound the results, and therefore, the use of a carefully controlled water supply is recommended.

Another important technical aspect for transgenesis in this system is careful, cellular level monitoring of the germ-line transmission. Because the transgenes integrate randomly in the genome, position effects result in differences in expression of the same construct among different integrants. We therefore screen the F1 progeny of germ-line-transmitting strains by histological/immunofluorescence analysis to confirm the specificity and completeness of the expression. For example, when generating constructs driven by the CAGGs promoter, which should be expressed in every cell type, we observe some F0 founders whose progeny show no expression in specific cell types such as satellite cells or neuronal cells. We therefore always drive a fluorescent reporter gene behind the transgene and analyze the tissues of interest for appropriate expression.

We have demonstrated the ability to interrogate gene function during tail regeneration via inducible overexpression of the human p16INK4a gene. It has been proposed that the p16INK4a/ARF locus first arose in mammals and is not present in animals like the salamander, a potential basis for the restricted regeneration capacities of mammals (Pajcini et al., 2010). Here, we have tested if expression of the p16INK4a gene represses regeneration when induced ubiquitously prior to regeneration onset. Indeed, we observed a significant retardation of spinal cord regeneration in overexpressing the p16INK4a gene, consistent with an antiregenerative function for this gene. These animals could in the future be used to understand the differing downstream responses in p16INK4a-expressing or -nonexpressing cells.
These transgenic tools in combination with the growing amount of axolotl sequence data that incorporate next-generation sequencing results from our own and other labs for contig assemblies of the axolotl transcriptome and some genomic sequences (Habermann et al., 2004; Monaghan et al., 2009; Putta et al., 2004; Smith et al., 2009) open up new possibilities to parlay this classical regeneration system into a molecular genetic system to investigate the mechanistic basis of regeneration in a vertebrate and its restriction in other animals.

EXPERIMENTAL PROCEDURES

Axolotl Care and Transgenesis

Animal experiments were performed after approval by the Landesdirektion Saxony, Board of Animal Welfare. Animals were bred in Dresden, Germany, and kept in local tap water at 18°C. The water quality was controlled every day for temperature, pH, ammonia levels, and water consumption. Juveniles and adult animals were kept in continuous flow towers outfitted with particle filters, charcoal filters, and UV filters. Larvae were kept in small plastic tubs with change of fresh water every second day. Larvae were fed *Artemia* daily, whereas juvenile and adult animals were fed fish pellets. Transgenic axolotls were generated as described (Khattak et al., 2009; Sobkow et al., 2006). Briefly, one-cell-stage embryos were collected and manually dejellied and kept at 4°C until injection. Plasmids that harbored SceI meganuclease target sites flanking the expression cassette were coinjected with the SceI meganuclease enzyme (New England Biolabs). Although target sites flanking the expression cassette were coinjected with nontransgenic white siblings. Donors were amputated through the proximal half of the stylopod, and protruding bones were trimmed. Immediately, the donor’s wrist blastema was cut off and transferred in the same proximal-distal orientation onto the recipient’s stump. Animals were left asleep on a wet tissue with 0.01% benzocaine for the following 1–3 hr before placing them back into tap water to recover and regenerate lost limb structures.

**Generation of AxSox2:Cre-ERT2 Transgenic Animals**

An *Ambystoma mexicanum* (axolotl) lambda genomic library (Stratagene) was expanded in the lab using the manufacturer’s protocol. Axolotl Sox2 gene-specific primers were designed to screen the primary, secondary, and tertiary pools of the library as described by Israel (1993) with minor modifications. Briefly, the 440 pools (50,000 clones each) were screened by PCR, but no colony hybridization was done, rather the tertiary pools were further diluted down to isolate a single positive plaque. The isolated axolotl genomic DNA was recombined into a plasmid backbone, and the resulting plasmid was subjected to a second step of recombining where the Sox2 open reading frame was replaced by the Cre-ERT2-T2A-EGFP-ns gene using a liquid-recombining protocol (Sarov et al., 2006). The resulting plasmid AxSox2:Cre-ERT2-T2A-EGFP-ns was injected into one-cell-stage embryo with SceI meganuclease and allowed to develop normally. Larvae were screened for EGFP expression in brain and raised to sexual maturity.

**Generation of CAGGs:loxP-EGFP-STOP-loxP-p16Ink4A-T2A-Cherry and COL2A1:ERT2-Cre-ERT2-T2A-EGFP-ns Transgenic Animals**

To generate CAGGs:loxP-EGFP-STOP-loxP-p16Ink4A-T2A-Cherry and COL2A1:ERT2-Cre-ERT2-T2A-EGFP-ns transgenic axolotls, Tol2-CAGGs:loxP-EGFP-STOP-loxP-p16Ink4A-T2A-Cherry and Tol2-COL2A1:ERT2-Cre-ERT2-T2A-EGFP-ns constructs were co-injected with Tol2 transposase mRNA into the fertilized one-cell-stage white axolotl eggs and selected using the methods described above. Immunostaining was performed to confirm expression of human p16Ink4A using a mouse monoclonal antibody against p16 (Santa Cruz Biotechnology; #sc-56330). To detect collagen II z1-specific expression, a mouse anti-chicken Collagen type II antibody was used (Millipore; # MAB 8887).

**Distal Limb Blastema Transplantation**

Blastema donors (5–6-cm axolotls) were double transgenics of the genotype: *Car Act;EGFP; CAGGs:nucCherry*. Recipients were their nontransgenic white siblings. Donors were amputated through the forearm wrist. Nine days later, recipients were amputated through the proximal half of the stylopod, and protruding bones were trimmed. Immediately, the donor’s wrist blastema was cut off and transferred in the same proximal-distal orientation onto the recipient’s stump. Animals were left asleep on a wet tissue with 0.01% benzocaine for the following 1–3 hr before placing them back into tap water to recover and regenerate lost limb structures.

**DNA Electroporation and 4-OHT Injection**

Circular Cre expression plasmid DNA (0.5 mg/ml) was electroporated in the limb and/or spinal cord as previously described (Echeverri and Tanaka, 2003; McHedlishvili et al., 2007). For 4-OHT injections, larvae were anesthetized in 0.01% benzocaine and weighed. 4-OHT (Sigma-Aldrich; catalog # H7904 or #H6278) was diluted in DMSO to obtain a stock of 10 mg/ml. Stock aliquots were stored at –80°C until use. Fast Green (Sigma-Aldrich; F7258) was added to the thawed solution just prior to injection. A total of 50 μg/g body weight of 4-OHT was injected intraperitoneally in the ventral trunk. After injection, larvae were covered with a blanket of wet tissue for hydration for 20–30 min at room temperature until returning them to tap water.

**Immunohistochemical Analysis of Transgenic Animals**

Tails and limbs of transgenic axolotl larvae were cut and fixed, cryosectioned, and kept at –20°C until immunostained. The cryosections were stained with respective antibodies as previously described by Kragl et al. (2009).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2013.03.002.
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