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miR-196 is an essential early-stage regulator of tail regeneration, upstream of key spinal cord patterning events.

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Short Title:
miR-196 is an essential early regulator of tail regeneration
Abstract
Salamanders have the remarkable ability to regenerate many body parts following catastrophic injuries, including a fully functional spinal cord following a tail amputation. The molecular basis for how this process is so exquisitely well-regulated, assuring a faithful replication of missing structures every time, remains poorly understood. Therefore a study of microRNA expression and function during regeneration in the axolotl, *Ambystoma mexicanum*, was undertaken. Using microarray-based profiling, it was found that 78 highly-conserved microRNAs display significant changes in expression levels during the early stages of tail regeneration, as compared to mature tissue. The role of miR-196, which was highly up-regulated in the early tail blastema and spinal cord, was then further analysed. Inhibition of miR-196 expression in this context resulted in a defect in regeneration, yielding abnormally shortened tails with spinal cord defects in formation of the terminal vesicle. A more detailed characterization of this phenotype revealed downstream components of the miR-196 pathway to include key effectors/regulators of tissue patterning within the spinal cord, including BMP4 and Pax7. As such, our dataset establishes miR-196 as an essential regulator of tail regeneration, acting upstream of key BMP4 and Pax7-based patterning events within the spinal cord.

**Key Words:**
Regeneration, microRNA, axolotl, patterning
Introduction

Regeneration is the amazing ability to reproduce precise replicas of lost structures, a phenomenon widespread among certain vertebrates like salamanders and zebrafish but all-but lost in humans (Sanchez Alvarado and Tsonis, 2006). Salamanders and zebrafish both regenerate using similar mechanisms, involving an initial, very rapid wound healing of the surface epithelium followed by the establishment of an underlying area of rapid cell division within a group of progenitor cells, referred to as a blastema. These cells then differentiate and migrate as needed to exactly replace the lost tissue. Our molecular-level understanding of how these events are initiated and so precisely calibrated remains in its infancy. Classical transplantation experiments whereby the spinal cord was rotated to invert the dorsal/ventral (D/V) pattern, resulted in an inverted positioning of the cartilage rod in the regenerate, suggesting that a faithful D/V pattern must be maintained for correct, fully faithful regeneration to occur (Holtzer, 1956). More recent work has shown that precise expression boundaries of key patterning molecules acting during embryogenesis, such as the dorsally-expressed Pax7 and Msx1/2, as well as dorsal-laterally-expressed Pax6, are maintained at strong levels in both the mature and regenerating spinal cord of the axolotl, their expression boundaries also dictating the D/V position of the regenerate (McHedlishvili L et al., 2007; Schnapp E et al., 2005). This is an interesting contrast to mammals wherein patterning molecules such as Pax7, Msx1/2 and Pax6, all of which are expressed during embryogenesis and define the dorsal and dorsal lateral domains of the neural tube, are in fact down-regulated as the neural tube matures (Liem et al., 1995).

Of course, the regeneration of an entire, complex structure such a tail or spinal cord, requires regulation of patterning not only along the D/V axis, but also along the anterior/posterior (A/P) direction. However, little is known about how precise A/P patterning is re-established during regeneration and whether its control in any way recapitulates aspects of normal development. During normal embryogenesis, the CNS is patterned along the A/P axis by the additive expression of Hox-a and Hox-b cluster genes, which progressively establishes the identities of sub-groups of neurons along the axis (Carpenter, 2002; Krumlauf et al., 1993; Maconochie et al., 1996). Results from gain- and loss-of-function experiments targeting different Hox genes in mouse have led to the theory that collinear Hox gene expression actually specifies regional identity along the main body axis (Krumlauf, 1994). Work on amphibian spinal cord and tail regeneration has shown that the number of vertebrae removed by amputation is precisely replaced during regeneration (Iten and Bryant, 1976). Classical transplantation experiments in salamanders have shown that when a tail blastema is removed and transplanted to a more proximal location on the tail
axis, the transplant retains the “memory” of its original location and regenerates the amount of tail set by its original location (Iten and Bryant, 1976). These experiments suggest that the tail blastema is a self-organizing structure and the positional identity of the cells within it is somehow defined very early on by the context at the amputation site (Carlson, 1983; Carlson et al., 2001; Nicolas et al., 2003). This also suggests that those mature tissues capable of undergoing regeneration in these species retain a positional identity along the A/P axis. At the molecular level, this invokes the possibility that the Hox genes that are used during embryogenesis to initially set up this axis, may in fact underlie this maintenance of the A/P identity in the mature tissue. Interestingly, as noted for key D/V patterning molecules, the expression of key Hox genes in salamander has been shown to be maintained in the adult tissue, thus identifying them as strong candidates for assuring the positional memory pathway as proposed above (Savard et al., 1988; Simon and Tabin, 1993). Data from the newt has shown that Hoxa9, Hoxc10, Hoxc12, and Hoxc13 are expressed at various axial levels in the mature newt spinal cord and that all four Hox genes are re-expressed during tail regeneration (Nicolas et al., 2003). Similar experiments in axolotl and Xenopus have confirmed that some Hox genes are also highly up-regulated in the regenerating spinal cord, however their exact role remains unknown (Carlson et al., 2001; Christen et al., 2003).

To date, a number of molecules and signaling pathways have been implicated to different degrees in various aspects of regeneration, including wnt signaling which was recently shown to be important for activating regeneration and regulating subsequent cell proliferation in zebrafish (Stoick-Cooper et al., 2007). Work from salamander has similarly illustrated how other, well-studied developmental pathways like BMP and Hox signaling are reused during regeneration in both tail and limb (Beck et al., 2006; Beck et al., 2003; Beck CW et al., 2003; Brown and Brockes, 1991; Gardiner et al., 1995; Savard et al., 1988; Simon and Tabin, 1993).

A major question in regeneration is how all of these classic developmental pathways are actually regulated and reused during regeneration. In order to initiate a regenerative response and carry out to its full completion, it is clear that multiple pathways must be activated or repressed in a carefully synchronized manner, involving numerous molecular changes from rapid chromatin re-modeling to numerous transcriptional and post-translational modifications. Recently microRNAs (miRNAs) have emerged as key regulators of the differentiation status and various other aspects of cellular physiology, being capable of regulating the expression levels of many genes at the post transcriptional level (Bartel, 2004). Recent research has shown that miRNAs are not only involved in regulating differentiation but also play important regulatory roles in a fast-growing list of disease conditions (Beck et al., 2006; Choi et al., 2008; Choi PS et al., 2008; Giraldez et al., 2005; Hagen and
Lai, 2008). Certain miRNAs have also recently been implicated in regeneration in multiple species including zebrafish and newts, however the full extent of the role of these small RNAs in regeneration remains to be understood (Thatcher EJ et al., 2008; Tsonis et al., 2007; Yin et al., 2008).

Here we report our first findings from the series of studies now undertaken to advance our understanding of the molecular events underlying regeneration, starting by elucidating the role of miRNAs in this process in the axolotl tail. Microarray-based expression profiling enabled the identification of highly-conserved miRNAs that are significantly up- or down-regulated in the early tail blastema. Among these, miR-196, which has previously been implicated in normal limb development (Hornstein et al., 2005) but not in regeneration, was further characterized and identified as an essential regulator of tail regeneration, defining a novel molecular pathway acting upstream of known patterning events within the spinal cord, and thus potentially affecting both positional identity and the overall size of the regenerate.

**Materials & Methods**

**Animal handling**

All axolotls used in these experiments were bred in the axolotl facility at the MPI-CBG Dresden, Germany. For all *in vivo* experiments axolotls 2-3 cm were used, animals were kept in separate containers, fed daily with artemia and water changed daily. Animals were anesthetized in 0.01% p-amino benzocaine (Sigma) before amputations, microinjection or imaging was performed.

**Microarray analysis of miRNA expression**

Mature tail tissue and three-day regenerating blastema were collected and frozen immediately in liquid nitrogen. The tissue was ground to a powder in liquid nitrogen and then total RNA was extracted using a standard Trizol (Invitrogen) extraction protocol. Microarray analysis was outsourced to a service provider (LC Sciences). The assay started from 5 µg total RNA sample, which was size fractionated using a YM-100 Microcon centrifugal filter (from Millipore) and the isolated small RNAs (<300 nt) were 3’-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining; two different tags were used for the two RNA samples in dual-sample experiments. Hybridization was performed overnight on a µParaflo microfluidic chip using a micro-circulation pump (Atactic Technologies). On the microfluidic chip, each detection probe was and tested in triplicate consisted of a chemically modified nucleotide coding segment complementary to target microRNA (from miRBase, see http://microrna.sanger.ac.uk/sequences/) or other RNA (control) and a spacer segment of polyethylene glycol to extend the coding segment away from the
substrate. The detection probes were made by *in situ* synthesis using PGR (photogenerated reagent) chemistry. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization used 100 µL 6xSSPE buffer (0.90 M NaCl, 60 mM Na2HPO4, 6 mM EDTA, pH 6.8) containing 25% formamide at 34°C. Detection was fluorescence based using tag-specific Cy3 and Cy5 dyes. Hybridization images were collected using a laser scanner (GenePix 4000B, Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics). Data were processed by first subtracting background using regression-based mapping and then normalizing the signals using a LOWESS filter (Locally-weighted Regression). For two color experiments, the ratio of the two sets of detected signals (Log2 transformed, balanced) and p-values of the t-test were calculated. Differentially detected signal sets with p-values below 0.01 were considered statistically significant.

**Cloning and sequencing of *Ambystoma mexicanum* miRNAs**

Mixed tissue samples, including spinal cord, muscle, limbs, skin blood from 5 month old axolotls were frozen in liquid nitrogen. Small RNAs were isolated, Topo cloned as concatamers via linkers into pCR2.1 vector (Invitrogen) using the protocol developed by the Tuschl lab (Pfeffer et al., 2005). Clones were screened by PCR for inserts. Positive clones were then sequenced (MPI DNA sequencing facility). Sequences were analyzed using MiRBase to identify potential miRNA sequences, clone sequencing and analysis still in progress.

**Inhibitor injections and imaging**

miRDIAN microRNA inhibitors or mimics against miR-196 and control invertebrate inhibitors (cel-miR-67 and cel-miR-239b), supplied by Dharmacon (Thermo-Fisher, LaFayette, CO), were diluted in water to make a 40uM stock solution, and further diluted in PBS to 20nM, plus 10,000MW fluorescein dextran (Molecular Probes) and Fast Green to allow monitoring of injection. Injections were carried out using a PV820 Pneumatic Pico Pump (World Precision instruments). Directly following injection, electroporation was performed using a BTX ECM 830 electroporator. Electrodes were placed on either side of the animal, 0.5 cm away from the tissue and 5 pulses of 50V, 50 msecs each with a 300msec delay between pulses was given. Injection and electroporation was repeated two days post amputation into the cells of the blastema.

Axolotls were anaesthetized and imaged every day using an Olympus SZX16 microscope equipped with a QImaging RTV camera.

**Cryosectioning and immunostaining**

Axolotl tails were fixed overnight at 4C in freshly made 4% PFA. Tails were washed
in PBST, followed by washing in PBST + sucrose. Tails were equilibrated in 30% sucrose and then embedded in 1.5% agarose + 5% sucrose. The agarose blocks were equilibrated overnight in 30% sucrose and then embedded in Peel A-way moulds in Tissue Tec and frozen for cryosectioning. Sections of 10μm were cut and collected on SuperFrost Plus slides. Sections were allowed to air dry for 2 hours and were then rehydrated in PBS.

Sections were then permeabilized using 0.02% Triton X100, blocked using 10% goat serum in PBST for 1 hr at RT. Slides were incubated in primary antibody (anti-PAX7) (DSBH) overnight at 4C. Washed 4 x 10mins PBST and then incubated in secondary antibody (anti-mouse AP)(Invitrogen) for 1hr at RT. Washed 4 x10mins and then washed 3 x 10mins in AP buffer. Slides were then incubated in the dark in NBP+BCIP (Invitrogen) in AP buffer. The alkaline phosphatase reaction was monitored every 10mins and all slides were stopped by washing in PBST + 0.5M EDTA once the control slides showed robust staining in the expected tissues. All samples were imaged using a Zeiss upright compound microscope fitted with a QImaging camera.

For BrdU antibody staining, slides were treated with 4N HCL for 30mins, then washed 4x 10mins in PBST, permeabilized and blocked as above. An Alexa-anti-mouse 586 secondary antibody was used. Dapi was used to visualize the nuclei. Sections were imaged using an inverted Zeiss Apotome microscope.

Haemotoxylin and Eosin (Sigma) staining was carried out on longitudinal sections. Sections were rehydrated in PBS, washed 5 mins in distilled water. Incubated in H&E solution for 10mins, washed in water and then dehydrated through an alcohol series.

Tunel analysis was carried out on longitudinal sections using the In Situ Cell Death Detection kit, TMR RED (Roche), according to manufacturer's instructions.

**miRNA in situ hybridisation**

Dioxygenin labelled Locked Nucleic Acid (LNA) probes designed to detect the mature form of the miRNA were purchased from Exqion. *In situ* hybridisation was carried out as previously described (Wienholds et al., 2005) on wholemount embryos, regenerating tails and on cryosections of regenerating tails.

**RT-PCR of Hox gene mRNA**

Total RNA was extracted as described above from mature, 3 day control regenerating blastemas and 3 day inhibitor 196 treated blastemas. Rt-PCR was performed using one step RT-PCR kit (Qiagen) using the following primers:

- **HOXA9 forward**: GTCCGACTACGGCTCCTCTC
- **HOXA9 reverse**: GGAACTCCTTCTCCAGCTCC
miRNA quantification by quantitative real-time RT-PCR (qRT-PCR)

Total RNA from homogenized axolotl tail tissue samples by grinding frozen tissue in liquid nitrogen and then extracting total RNA using Trizol. cDNA was synthesized from total RNA, using RT reagents that were specific for each of the miRNAs (hsa-miR-196, and snoRNA135 TaqMan MicroRNA Assays from Applied Biosystems). Real-time qPCR was performed using TaqMan Universal PCR mastermix (Applied Biosystems), and reactions were run on an ABI7900HT machine. qRT-PCR protocols were exactly as recommended in the manufacturer's manual. For higher robustness, four qRT-PCR replicates were performed per sample, for each of the miRNA targets, with the error bars indicating the variance of the four replicates. Per sample, miRNA target levels were normalized against the average of four replicate snoRNA135 amplifications.

Cloning of axolotl Pax7 3' UTR

For cloning of the axolotl 3' UTR, a 3'RACE kit from Invitrogen was used. The following primers were designed based on the partial cDNA sequence of axolotl Pax7, Accession Nr: AY523019

CGACATTCCCTGGGTGGAATC  
TGAAAGCTGCCAGTTGATTG

Total RNA was used from mature tissue and 3 day regenerating tail blastemas for subsequent PCR reactions.

Western Blotting

Tissues samples from normal, control or inhibitor-196 tail samples was amputated and placed directly into RIPA lysis buffer containing a protease inhibitor cocktail (Roche). Samples were homogenized using a tissue disruptor (Qiagen), spun down and the supernatant removed. Total protein was measured using Bradford reagent (Sigma) and samples were further diluted with sample buffer (Sigma). Western blots were carried out using standard protocol.

Antibody against the axolotl shh protein was generated using the peptides
CGPGRGIGKRRQPKKL and CDIIFKDEENTGADRL (Eurogentech). Serum was purified against the peptides.

Antibody used: rabbit anti-BMP4 (Abcam ab38342), anti-Meis 1/2/3 (Upstate), anti-msx1/2 (DSHB) and anti-tubulin (Sigma).
Results

Expression profiling of 1,550 vertebrate microRNAs reveals extensive regulation during early axolotl tail regeneration

In order to characterize the expression of evolutionarily-conserved microRNAs during axolotl tail regeneration, and in particular, to identify those, if any, that display differential regulation during the early stages of this process, we conducted miRNA profiling experiments using a microarrayed probe set covering 1,550 vertebrate miRNAs from human, mouse, zebrafish and rat. Extracted RNA samples from control non-regenerating tail tissue were compared to equivalent extracts from 3-day tail blastemas, both prepared from young adult animals. The resulting differential hybridization datasets were processed for background subtraction, normalized, and subjected to statistical analyses, yielding the results shown as volcano plots in Figure 1, and the data listed in Supplementary Table 1. It is worth noting that these analyses were significantly aided by the strong built-in redundancy of most miRNA-specific probes found in this array, resulting itself from the high cross-species conservation of many miRNAs. Applying a threshold for statistical significance of \( p < 0.01 \), probe sets for 78 of the tested miRNA families were found to reveal significant changes in expression levels during early tail blastema formation, with 32 showing down-regulation and 46 showing up-regulation, as compared to mature non-regenerating tissue. As seen in the volcano plot of Fig 1A, the levels of miRNA modulation observed in the blastema ranged widely, from an ~89-fold decrease up to a 62-fold increase, with several miRNA families emerging from the rest based on statistical significance or amplitude of regulation, or both (Figure 1B).

The miRNA family showing the most consistent and statistically significant up-regulation in the 3-day tail blastema was miR-196 (Figure 1A, B), with an average expression level 37.2-fold higher than in mature tail, as detected by 2 distinct probes (from \( \text{Pan paniscus} \) and \( \text{Xenopus tropicalis} \)) yielding 6 datapoints, all of which gave \( p \) values below 1.2x10E-9. Other notably up-regulated miRNAs included miR-671 (Figure 1B, with a mean 40.3-fold increase), miR-489 (Figure 1B, mean 39.4-fold increase), miR-681 (Figure 1B, mean 32.5-fold increase), miR-497 (Figure 1B, mean 16.3-fold increase), and miR-183 (Figure 1B, mean 7.4-fold increase). Conversely, several miRNA families were found to be notably down-regulated in the 3-day blastema, including miR-338 (Figure 1B, mean 25.4-fold decrease), miR-101 (Figure 1B, mean 22.1-fold decrease), miR-141 (Figure 1b, mean 14.7-fold decrease), and miR-124 (Figure 1B, mean 4.3-fold decrease).
As further discussed below, several of these miRNAs have been implicated previously in specific cellular functions and/or developmental processes. Particularly notable among these was miR-196, both in view of the dramatic amplitude of its observed regulation here, and because it has previously been implicated in the regulation of \textit{Hoxb8} gene expression during normal limb development (Mansfield et al., 2004; Yekta et al., 2004; Hornstein et al., 2005). In our ongoing effort to comprehensively isolate and sequence small RNA species from axolotl, our growing dataset of cloned axolotl miRNAs has revealed the very high level of conservation of axolotl miR-196 with its vertebrate homologues, including a perfect match to human and mouse miR-196b (Fig 1C). Based on these observations, it was chosen to start with miR-196 in our further analysis of the role of miRNA molecules in axolotl tail regeneration.

**Expression of miR-196 is up-regulated in blastema and spinal cord early during axolotl tail regeneration**

As a first step towards characterizing in more detail the expression patterns of mir-196 during regeneration, locked nucleic acid (LNA) probes were used for \textit{in situ} hybridization analyses in whole-mounts and cross-sections of axolotl tails (Fig. 2). The low levels of normal expression noted for miR-196 in mature, non-regenerating tissue from the above microarray datasets (Supp, Table 1), were confirmed by corresponding \textit{in situ} data from these tissues, consistently falling below the detection limits of the LNA-based methodology used here (Fig. 2A). Nonetheless, as early as 24 hours post amputation, elevated levels of miR-196 were readily detectable in the cells immediately adjacent to the wound epidermis and in those slightly anterior to the plane of amputation (Fig. 2B). Between 2 and 4 days post-amputation, miR-196 is present in the regenerating tissue, within the cytoplasm of essentially all blastema cells and in the dorsal cells of the regenerating ependymal tube (Fig. 2 C, D, H). During this period, miR-196 is also detected in a zone of mature tissue up to ~500µm immediately anterior to the plane of amputation (Fig. 2 C, D), within the cytoplasm of a population of cells localizing to the dorsal and lateral domains of the spinal cord (visible in cross-sections from this zone, Fig. 2 G, H). By 7 days post-amputation the elevated expression within the regenerate is highest in the anterior region of the blastema (Fig. 2 E) and at 14 days of regeneration, when the first morphological signs of differentiation are visible as a rod cartilage, miR-196 is no longer detected in the regenerate by the present \textit{in situ} hybridization methodology.

Using real-time RT-PCR to generate more quantitative characterization, we confirmed and extended the patterns observed by microarray and \textit{in situ} analyses, finding miR-196 levels to be very low in mature tail, but increasing by 3 days post amputation, the highest level seen at 7 days post amputation (Fig. 2J). By 30 days
post amputation when all tissues types are normally differentiated in the regenerate.

**miR-196 is an essential early stage regulator of tail regeneration**

To examine the function of miR-196 during tail regeneration, the formation of the mature miRNA was blocked by injection of an inhibitor against miR-196 into amputated tails. The first injection was directed into the spinal cord and surrounding cells shortly after amputation, and then repeated at two days post-amputation, to maximize the penetration and distribution of the inhibitor within the tail region. Inhibitor-treated animals were then compared to animals treated identically with a negative invertebrate control inhibitor, and these were found in these studies to behave indistinguishably from wild type, untreated animals. Inhibition of miR-196 was thereby found to cause severe defects in tail outgrowth visible from day 6, when the blastema is consistently smaller than in the control injected animals (Fig. 3 B, E). By 14 days post amputation, when a rod of differentiated cartilage is visible in the regenerate, the inhibitor injected animals had consistently shorter tails than the control animal (Fig. 3 C, F). Detailed measurements of the regenerating blastema and final tail length over time (Fig 3 G) indicated that the growth defect occurred from the very beginning of regeneration, causing an overall slower growth rate, and reaching a clear plateau after which growth was stopped altogether, far short of the controls (Fig. 3 E).

It should be noted that, despite best efforts in optimizing the injection protocols, it is likely that the present phenotype reflects only partial penetrance of the inhibitor to a subset of cells within the tail area. Indeed, although qRT-PCR analysis from blastema and tissue adjacent to the plane of amputation confirmed the near-complete knock-down of miR-196 levels in inhibitor-injected compared to control tails (Fig 2 J), the measured residual levels of miR-196 detected by qRT-PCR may come from such “escaper” cells. Thus, the possibility that if all cells could be reached uniformly, the present phenotype of shortened re-growth might progress to an even earlier growth arrest yielding an even shorter tail, remains an open question beyond the reach of currently available techniques. Nonetheless, the fact that regeneration can still occur at all in animals where miR-196 has been down-regulated, and that the observed re-growth always reaches a clear plateau at a shortened tail length, strongly suggests that miR-196 may in fact be regulating genes involved in specifying exactly how much regeneration is needed, rather than merely triggering the overall process at all.

The morphological composition of main tissues within the regenerating tail was therefore examined in more detail to determine whether miR-196 inhibition also
impacts how faithfully individual structures are reformed. As seen in Fig. 3F, an apparently normal, though shortened, rod of cartilage clearly re-forms in the inhibitor treated animals, as further confirmed by alcian blue staining (Supp. Figure 1). Using an antibody against myosin heavy chain, apparently normal muscle fibers were observed in the regenerate of miR-196-inhibited animals, though far fewer than in the control animals (data not shown). However, when the spinal cord was more closely examined, it was found that the inhibitor treated animals display a morphologically aberrant terminal vesicle, compared to the control animals, as seen initially by H&E staining (Fig. 4 A-D). A marked accumulation of cells was noted within the lumen of the spinal cord, at its posterior end nearest to the plane of amputation, where the terminal vesicle is normally observed (Fig. 4,B, D). This was not seen in control animals. Whole-mount GFAP staining of the injected animals, which stains the glial cells of the spinal cord allowed a more detailed observation of the terminal vesicle’s geometry, revealing a “bulb”-like structure with an apparently open-ended lumen (Fig. 4 E). By contrast, the end of the spinal cord in inhibitor-treated animals showed no bulb-like terminal vesicle, but instead, a more irregular, blunt end (Fig. 4F).

Together, these data indicate significant inhibitor-196-induced defects in spinal cord structure in the immediately anterior vicinity of the amputation plane, exactly where and when miR-196 is normally up-regulated. They are also suggestive of a possible defect in the putative posterior migration of spinal cord cells within the lumen of the ependymal tube, that might normally be exiting the spinal cord at this site.

**Down-regulation of miR-196 decreases cell proliferation in the blastema but does not increase apoptosis**

To investigate the potential molecular and cellular mechanisms underlying the shortened regenerates and spinal cord defects of the miR-196 inhibition phenotype, the hypothesis of a basic, early defect in cell proliferation was first assessed. This analysis was initially focused on the same region of mature spinal cord tissue within 500μm anterior to the plane of amputation, in which miR-196 was found to be up-regulated early during regeneration (Fig 2). This area has also been reported previously to be the source of cells giving rise to the new ependymal tube during regeneration (Echeverri and Tanaka, 2002, 2003; McHedlishvili L et al., 2007). Using BrDU incorporation to detect proliferating cells undergoing S phase, inhibitor-196-treated animals showed numerous actively-cycling, BrDU-labeled cells (Fig. 5 and Supp. Fig. 2), however from detailed quantifications from serial cross-sections through this part of the spinal cord, it was found that in inhibitor-196 injected animals there are more cells undergoing S-phase than in control animals (Fig. 5 and Suppl. Fig. 2D). It was also noted that the total number of cells in this 500μm zone is higher in the inhibitor treated animals. However this is probably because cells are
dividing but are unable to migrate into the regenerate and accumulate in the lumen of the spinal cord as observed in histological sections (Fig. 4 B, D). Also, no apparent gross differences were noted in the number of cells undergoing mitosis, as revealed by condensed chromosomes. The same analysis of cell proliferation was then focused on the blastema itself, where a statistically significant decrease in the incidence of BrDU-labeled cells was detected, which then results in an overall smaller size of the blastema in miR-196-inhibited animals, suggesting that miR-196 may play a role in regulating the amount of cell division taking place in the early stages of regeneration.

To ascertain if decreased cell proliferation alone was responsible for the inhibitor-induced shortened regenerates the levels of apoptosis was next examined. Using an acridine orange assay for loss of membrane integrity and Tunel staining as markers for apoptotic cells, the same regions, i.e. the spinal cord just anterior to the amputation plane and the blastema, were examined as described above, using wholemounts and serial cross-sections. These analyses revealed no detectable differences between control and inhibitor-196-treated animals (Suppl. Fig 3), thus suggesting that the observed “short tail” regeneration phenotype is not due to increased apoptosis.

**miR-196 pathway regulates several key components of tissue patterning in blastema and spinal cord**

As the present data so far suggested that miR-196’s role in controlling the amount of tail regeneration is neither directly inhibiting cell proliferation or increasing rates of apoptosis, the possibility that it may be exerting its functions by regulating tissue patterning events was investigated. As this would be consistent with previous reports that miR-196 is a key regulator of *hoxb8* expression during limb development (Hornstein et al., 2005; Mansfield et al., 2004; Yekta S et al., 2004), this analysis was begun by looking at levels of Hox gene transcripts by RT-PCR in tail regenerates, i.e. focusing first on blastema tissues, at 3 days post-amputation (Supp. Figure 4). While the axolotl orthologue of HoxB8 has not yet been cloned and PCR probes have so far failed to yield interpretable signals for it in axolotl extracts, other Hox genes of apparent relevance to the present context were found to be readily detectable. These included HoxA9 and HoxA5, which are also predicted in mouse and human (miRBASE) to have seed sequences for miR-196 in their 3’ UTRs. Also of interest, HoxC10 and HoxB13 have been shown to be up-regulated within 48hrs of tail amputation in axolotl and are down-regulated as the cells in the blastema begin to differentiate (Carlson et al., 2001). Despite the fact that such elevated expression levels at a time when miR-196 is also up-regulated in the tail, combined with the absence of seed sequences from their 3’UTRs, makes these particular Hox genes
unlikely to be direct targets of miR-196, the possibility that these and other Hox genes may be active downstream components of the miR-196 pathway made all of them worthy of further analysis in the present context.

This analysis (Supp. Fig 4) first revealed most of these Hox transcripts to already exhibit strong levels in mature tail tissue, while also confirming the previously reported up-regulation of HoxB13, in the 3-day tail regenerate (Carlson et al., 2001). A similarly marked down-regulation in HoxA5 was also apparent. While this result appeared to suggest that axolotl HoxA5 may be a direct target of miR-196 regulation, as suggested by the 3'UTR sequences of its human and mouse orthologues, the results from inhibitor-196-treated animals showed a barely detectable increase in HoxA5, which thereby failed to strongly confirm this hypothesis. In fact, the most notable results from inhibitor-196-treated axolotls were slight but reproducible reductions in HoxA9 and HoxC10 transcripts, as compared to controls (Supp. Fig 4). Although this data argues against these genes being direct targets of miR-196, however the possibility remains that both genes could act as potential downstream components of the present miR-196 pathway.

As miR-196 is also up-regulated during early tail regeneration specifically in cells of the mature spinal cord within ~500μm anterior to the plane of amputation, genes involved in patterning the spinal cord were examined next. During normal regeneration, cells within this region of the spinal cord show a slight down-regulation in the levels of Pax7 protein, a homeobox domain protein that is a marker of dorsal cells in the spinal cord and is also expressed in muscle satellite cells (Schnapp E et al., 2005). The cells in this zone lose their mature structure and begin to form a simple single cell layer ependymal tube. At the same time, Pax7 remains restricted to the dorsal-most cells of the spinal cord and this dorsal identity is also seen in the newly regenerating ependymal tube (Fig. 6 A-D). In clear contrast to this, however, inhibitor-196-injected animals show markedly higher levels of Pax7 protein, though still restricted to the dorsal-most cells, within exactly the same region of the mature spinal cord where the inhibited miR-196 would normally be up-regulated at this time, i.e. post-amputation day 3 (compare Fig. 6E to 6A). This more intense anti-Pax7 staining is also visible in dorsal cells of the spinal cord further posterior, both at the amputation plane and throughout its full length within the early blastema of inhibitor-196-treated axolotls. This was also different from control axolotls, in which Pax7 levels in the regenerating ependymal tube and in cells adjacent to the plane of amputation are much lower than in the more anterior mature zone noted above (Fig 6B-D). Finally, another notable difference in inhibitor-196-treated animals was the clearly higher level of Pax7 protein in putative satellite cells interspersed among the outer layers of muscle fibers both in mature tissues anterior to the amputation plane, and within the regenerate (asterisks in Fig 6A-C and E-G).
As these results clearly indicate that the miR-196 pathway somehow regulates Pax7-based spinal cord patterning events during early axolotl tail regeneration, other known markers of dorsal/ventral patterning were also examined (Fig. 6I). Protein levels for Msx1, another homeobox domain-containing transcription factor thought to act upstream of Pax7 and whose expression is also restricted to the dorsal spinal cord (Schnapp E et al., 2005), were also found to be highly increased in inhibitor-196-treated axolotls. Similarly, protein levels for BMP4, which itself acts upstream of both Msx1 and Pax7, all genes are known to be involved in maintaining dorsal cell identity (Timmer JR et al., 2002), were also higher in inhibitor-196-treated axolotls. Conversely, these animals showed no detectable change in levels of sonic hedgehog protein, a known regulator of spinal cord ventral identity (Schnapp E et al., 2005).

Perhaps most interestingly, however, levels for Meis2, an atypical homeobox domain protein previously described as playing a role in defining proximo-distal identity during limb regeneration (Mercader et al., 2005), were found to be significantly lowered in inhibitor-196-treated axolotls. This is particularly relevant in the present context, as defects in Meis gene function might be predicted, based on observations from axolotl limb regeneration that Meis is expressed higher proximal than distal in the blastema (Mercader et al 2005), to lead to a failure to correctly translate the amount of tissue to be re-formed, resulting in an aberrant calibration of tail re-growth along the antero-posterior axis.

To further strengthen the hypothesis that miR-196 regulates Pax7, we first examined the 3'UTR of of Pax7 from other species due to lack of sequence data in the axolotl. Using currently available prediction programs (miRBase, TargetScan, Pictar), no regulatory element for miR-196 was identified in the 3'UTR of human or mouse Pax7 genes. 3' RACE was then used to amplify the axolotl 3'UTR, interestingly here we identified a 7-mer miR-196 regulatory element (Supp. Fig. 5). To further test the functional role of miR-196, axolotls were injected with a synthetic form of miR-196 (Mimic) to address the issue of how increased levels of miR-196 affect regeneration and levels of Pax7 protein. Interestingly it was found that the overall length of the regenerate increased significantly in the presence of high levels of miR-196 (Fig.7, A-C). This increase in overall tail length correlates with an observed increase in cell proliferation in the early blastema (Supp. Fig. 5). How the levels of increased miR-196 affects the expression of Pax7 protein was addressed by co-injecting the control mimic or mimic miR-196, with a plasmid encoding a GFP to allow transfected cells to be identified. Increased levels of miR-196 were found to downregulate Pax7 expression in a cell autonomous manner, when spinal cord cells are transfected with miR-196 Pax7 protein is no longer detectable in these cells, however the satellite cells which did not receive the mimic still express normal levels of Pax7 protein (Fig. 7D –K).
Discussion

Axolotl tail regeneration involves extensive regulation of miRNA levels

The present study offers the first comprehensive profiling of miRNA expression during early stages of regeneration in axolotl tail. The high level of differential miRNA regulation observed in the early tail blastema suggests that miRNAs are playing key roles in reprogramming cells to initiate a regenerative response. A similar study has recently identified miRNAs involved in zebrafish caudal fin regeneration (Yin et al. 08). Comparing the two datasets, multiple similarities emerge in the dynamic behavior of several miRNAs during early stages in these two types of regeneration, including, for example, miR-101 and, more subtly in the axolotl, miR-133, both of which are down-regulated in the regenerating tissue. Another example is miR-141, which was recently reported to be down-regulated in cells undergoing an epithelial to mesenchymal transition (Gregory et al., 2008), and is also down-regulated in the early phases of both tail and fin regeneration. These common patterns suggest that these miRNAs may regulate those processes that are common to the early events in both regenerative contexts. Interestingly, miR-196 shows the opposite result: the zebrafish study showed miR-196 to be down-regulated in fin and potentially negatively regulated by fibroblast growth factors, in contrast to the present data showing its strong up-regulation to be essential for faithful axolotl tail regeneration to occur. Furthermore, miR-196 is in fact down-regulated in early stages of axolotl limb regeneration (Sehm & Echeverri, unpublished observation), similar to the zebrafish, supporting the view that fin and limb are more closely similar to each other than to tail. This likely also reflects the direct involvement of the spinal cord in tail, but not fin nor limb regeneration, consistent with the present findings of miR-196 regulating key patterning events within the spinal cord itself. Whether mir-196 is also regulated by fgf signaling during axolotl limb regeneration remains to investigated.

miR-196 emerges as key regulator of early tail regeneration

During mouse development, miR-196 has been shown to act upstream of Hoxb8 and shh to ensure faithful expression domains of patterning genes during limb development (Hornstein et al., 2005). In this study, it is clearly shown that up-regulation of miR-196 in the early blastema is needed to ensure the correct amount of tail is regenerated. miR-196 does not inhibit cell division or increase cell death in the early blastema, however it does cause a decrease in rates of cell proliferation in the early blastema. However in contrast to mouse limb development no evidence was found to suggest that miR-196 is acting directly on Hox genes during regeneration. When miR-196 is inhibited HoxA9 and HoxC10 are both slightly down-
regulated, various Hox genes have been described in different species including newt, xenopus and axolotl to be maintained in mature tissue and to be up-regulated during regeneration, however a direct function has never been shown.

During axolotl tail regeneration it was found that Pax7, BMP4 and Msx1 are all up-regulated in inhibitor 196 treated animals suggesting that this miRNA is acting directly on or upstream of one or more of these genes. We identified a 7mer binding site for miR-196 in the 3'UTR of axolotl Pax7, suggesting that it is acting directly to regulate Pax7 expression, this is further supported by the data showing that by increasing or decreasing the levels of miR-196, Pax7 protein levels are accordingly affected, which in turn affects cell division during regeneration, giving rise to the short tail phenotype in inhibitor treated axolotls. This data suggests that Pax7 acts in a feedback loop with BMP4 and Msx1 to regulate both patterning and proliferation during regeneration. The role of BMP signaling in controlling cell proliferation in the spinal cord has previously been described in chick and is thought to be mediated via the Wnt signaling pathway (Chesnutt C et al., 2004), whether this is also the case in axolotl remains to be determined. Interestingly in mammalian digit tip regeneration Msx1 has been shown to directly regulate regulate BMP4 during digit tip regeneration (Han M et al., 2003).

In axolotl limb regeneration, Meis genes are expressed in proximal blastema and if over-expressed in distal cells cause these to move to a more proximal location (Mercader et al., 2005). This suggests that high levels of Meis proteins play a role in distinguishing proximal from distal. The fact that Meis is down-regulated when miR-196 is down-regulated and that a shorter tail is regenerated in these animals correlates with the idea that high levels of these genes may play a role in determining positional identity and translating the information of how much must be regenerated, which in turn dictates levels of proliferation necessary to facilitate that amount of regrowth.

Our data suggests that miR-196 acts directly on Pax7 to downregulate Pax7 protein levels in cells in a 500µm zone anterior to the plane of amputation. This acts as a signal to the cells to increase their proliferation and to migrate out to form a new ependymal tube. When miR-196 in inhibited, Pax7 protein levels remain high, leading to defects in transmitting the information of how much must be regenerated as indicated by downregulation of Meis and therefore levels of proliferation are decreased probably due to the secondary effect of alterations in the levels of BMP4 and Msx1 protein. Although BMP's are needed for cell proliferation, too high a level of protein can also act to inhibit proliferation. These results show that levels of gene expression must be precisely calibrated to ensure faithful regeneration occurs and
that microRNAs act as key regulators of this complex process
Previous studies in xenopus have shown that regeneration can be promoted at a
non-regenerative competent stage in larval xenopus by reactivation of the BMP
signaling pathways, and if the BMP pathway is inhibited at a regeneration permissive
stage then regeneration can be inhibited (Beck CW et al., 2003). These studies from
axolotl suggest that if too much Pax7 protein is produced then regeneration is also
perturbed, suggesting that the genes reused during regeneration must be carefully
calibrated to ensure faithful regeneration and that microRNAs are potentially acting to
fine tune gene expression levels during regeneration.

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Figure Legends

Figure 1. Expression profiling of 1,550 vertebrate miRNAs in early axolotl tail regenerate.

(A) Volcano plot showing results of microarray-based differential hybridization comparing RNA extracts from 3-day tail blastema versus mature tail. Background-subtracted and normalized data for each miRNA probe were used to plot log2(3dReg/Mature) on the x axis and (–Log10(p value)) on the y axis. Probes showing value differentials considered statistically significant by p values below 0.01 (-Log10 value > 2) are shown as dark blue triangles, and those with p values above 0.01 (-Log10 value < 2) appear as light blue triangles. Probe datapoints showing statistically significant values for miR-196 are highlighted as green circles.

(B) Bar chart showing amplitude of regulation observed by microarray analysis for selected miRNA families, as indicated. Each bar shows single data point value for differential between 3-day tail blastema and mature tail from one individual miRNA probe. Each probe, having been tested in triplicate, yields 3 datapoints or bars. See Supplementary Table 1 for list of probes and associated data.

(C) Alignment of mature forms for cloned axolotl miR-196 (ame, highlighted in pink) against known vertebrate miR-196 sequences from human (hsa), mouse (mmu), chicken (gga), zebrafish (dre), Xenopus tropicalis (xtr), and pigmy chimpanzee (ppa). Areas of sequence identity are shaded.

Figure 2. Localization and quantification of miR-196 expression during regeneration

(A) Using LNA probes miR-196 cannot be detected in non-regenerating tissue.

(B,C) By 24hrs post amputation miR-196 can be seen in cells at the plane of amputation. Dashed line indicates the plane of amputation.

(D,E) miR-196 remains expressed in cells of the blastema and slightly anterior to the level of amputation.

(F) 14 days post amputation as a rod of cartilage is visible in the regenerate, miR-196 is no longer detected by in situ hybridization.
Cross sections indicate that miR-196 is specifically expressed in the cytoplasm of dorsal and lateral cells of the spinal cord and in all cells of the blastema.

qRT-PCR of miR-196 supports the *in situ* data, miR-196 expression increases during the early stages of regeneration and decreases at later stages. Injection of an inhibitor against miR-196 specifically reduces its expression level *in vivo*.

**Figure 3. Inhibitor-based down-regulation of miR-196 inhibits tail regeneration**

(A,B,C) Control inhibitor injected animals, regeneration occurs via healing of the wound (A), formation of a blastema (B) and eventual differentiation of lost tissue (C) (*n*= 30)

(D,E,F) Inhibitor-196 injected axolotls, two days post amputation defect already visible. (E) Blastema is significantly smaller than in the control animals. (F) 10 days post amputation differentiated cells can be seen but overall size of regenerate is significantly smaller (*n*= 35).

(G) Measurement of regenerating tails, blue line control animals, red inhibitor 196 treated animals. Down-regulation of miR-196 inhibits regeneration resulting in an inaccurately sized regenerate

**Figure 4. Terminal vesicle formation is disrupted in inhibitor-196-treated animals**

(A, B) H&E staining on longitudinal sections of 3 day regenerating tails. Control injected animals have a “bulb” like structure at the end of the spinal cord called the terminal vesicle. (B) is a higher magnification of the terminal vesicle.

(C,D) Longitudinal sections of inhibitor-196 injected animals 3 days post amputation. (D) higher magnification of the end of the spinal cord shown in (C), no bulb like structure visible and an accumulation of cells can be seen in the lumen adjacent to the plane of amputation.

(E,F) Wholemount GFAP of 5 day regenerating tails. GFAP stains the radial glial cells of the spinal cord. (E) Bulb like terminal vesicle at the end of the regenerating spinal cord in control injected animals. (F) Inhibitor 196 injected animals no terminal vesicle is observed.
Figure 5. Cell division in inhibitor-196-treated animals

(A) Quantification of BrdU incorporating cells within a 500μm zone spanning the plane of amputation and in the early blastema. Columns each represent the mean percentage of BrDU positive cells 4 days post amputation. ***: p-value <0.001, Student’s t-test.

Figure 6: miR-196 pathway regulates several key components of spinal cord patterning, including Pax7

(A) Pax7 expression in the dorsal cells of the mature spinal cord, approximately 500μm anterior to the plane of amputation in control injected animal

(B) Expression adjacent to the plane of amputation, asterisk marks putative satellite cells.

(C,D) Pax7 is still expressed in the dorsal most cells of the immature regenerating ependymal tube

(E) High levels of Pax7 protein in inhibitor 196 injected in spinal cord cells approximately 500μm anterior to the plane of amputation. Expression domains appear expanded in comparison to control (A)

(F-G) Pax7 protein remains high in regenerating spinal cord in comparison to control (B,C,D). Protein level also appears elevated in potential satellite cells (asterisk) adjacent to the muscle fibers (F)

(I) Protein levels of key patterning genes are affected in inhibitor treated animals. Levels of BMP4 and Msx1 protein are increased in inhibitor-196 treated animals in comparison to control animals. Meis2 protein levels are downregulated in treated animals, while levels of sonic hedgehog protein are unaffected.

Figure 7: Increased levels of miR-196 affect the length of the regenerate and downregulate Pax7 protein

(A) Control mimic injected animals regenerate normally and approximately the same length of tail that was originally amputated. Dashed line marks plane of amputation

(B) Mimic 196 injected animals regenerate normal tail structures, cartilage
(blue staining), but the length of the regenerate is significantly longer than the control (A).

(C) Measurement of the regenerated tail, length is measured from the plane of amputation (interface between the notochord and the cartilage, dashed line in A, B). *** p: value < 0.0001, Students t-test.

(D-K) Cross-sections of axolotl tail transfected with control or Mimic 196 plus a plasmid encoding a GFP. (D, H) Dapi staining, (E, I) GFP expressing cells, (F, J) Pax7 antibody staining and (G, K) Overlay. Normally Pax7 protein is expressed in the dorsal cells of the spinal cord and in the satellite cells adjacent to muscle fibers (F, G). Mimic 196 decreases Pax7 expression, GFP marks cells that have received the plasmid and the mimic 196, the majority of cells of the spinal cord but not the satellite cells are GFP+ (I, K). Pax7 protein is not detected in the GFP positive cells of the spinal cord (*) but normal expression is detected in the GFP negative satellite cells, indicated by arrows (J, K).

Supplementary Material

Supplementary Table 1: Microarray-derived differential expression analysis of vertebrate miRNAs in axolotl tail, comparing 3-day regenerate versus mature tissue.

Supplementary Figure 1: Small cartilage rod is regenerated in inhibitor-196 treated axolotls

Supplementary Figure 1

(A) Alcian Blue staining of regenerated tail, blue histological stain is specifically taken up by the cartilage cells in the regenerated tissue. Interface between the notochord and cartilage marks the plane of amputation as indicated by the dashed line. (n=10)

(B) Differentiation of cartilage occurs normally in inhibitor miR-196 treated animals, however the amount is smaller than the control animals (A). (n=12)

Supplementary Figure 2

(A-D) Cross sections of regenerating spinal cord, cells incorporate BrdU in cells in all positions within the spinal cord and in surrounding tissues (n=15)
(E-H) Inhibitor-196 injected axolotls dividing cells can be seen within the spinal cord and in adjacent cells (n=10).

Supplementary Figure 3: Tunel Staining of Inhibitor treated animals

(A-C) Positive Control, cross sections of regenerating tail are treated with DNAse to induce DNA damage, prior to carrying out Tunel staining.

(D-F) Control injected animals, low level of apoptosis apparent in tissue sections.

(G-I) Inhibitor miR-196 treated animals, low level of apoptosis, comparable to the control animals is seen.

(J) Quantification of Tunel staining at 3 and 7 days post amputation. Three days after amputation approximately 6-7% cells are undergoing apoptosis in both control and inhibitor treated animals, by seven days post-amputation this level has reduced to approximately 1% of the total cells in the animal (n=10). Student t-test showed no significant difference between samples.

Supplementary Figure 4. miR-196 pathway regulates expression levels of several Hox genes

(A) mRNA levels of Hox genes during regeneration. Lane 1: mature tail tissue, Lane 2: 3 day control inhibitor and Lane 3: 3 day inhibitor-196 treated tissue.

(B) Semi-quantification of Hox gene levels. HoxA9 decreases slightly in inhibitor-196 treated animals. HoxB13 is upregulated during regeneration but is unaffected by downregulation of miR-196. HoxA13 and HoxC10 are both mildly downregulated in treated animals. hoxA5 is downregulated in regenerating tissue but is also unaffected by downregulation of HoxA5. Sec61 levels are equivalent in all RNA samples.

Supplementary Figure 5. Higher levels of miR-196 increase cell proliferation

(A) Levels of miR-196 were increased by injecting a chemically synthesized form of the miRNA (Mimic). The number of BrdU positive cells were assessed 4
days post-amputation. Augmented levels of miR-196 increased the % of BrdU positive cells in the blastema. *** : p-value < 0.0001, Students t-test.

(B) 3’UTR of axolotl Pax7 contains a 7-mer binding site for miR-196b, complimentary binding site highlighted in red.
Figure 1C
Figure 3

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G

Time Course of Axolotl Tail Regeneration

- Control
- Inhibitor mR-196
Figure 4

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Figure 5

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Figure 7

Control

+ Inhibitor miR-196

4 day reg + control inhibitor + Inhibitor miR-196

Bmp4

Msx1

Meis2

Shh

Tubulin