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TECHNIQUES

Quantitative Evaluation of Morpholino-Mediated Protein Knockdown of GFP, MSX1, and PAX7 During Tail Regeneration in *Ambystoma mexicanum*

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Vertebrate regeneration is a fascinating but poorly understood biological phenomena. Urodele amphibians such as *Ambystoma mexicanum* (the axolotl) can functionally regenerate complex body structures such as the limb and tail, including the spinal cord, throughout life. So far, molecular studies on regeneration have been limited due to the paucity of tools for knocking-down gene and protein function. In this article, we quantitatively assessed the ability of morpholinos to specifically down-regulate protein expression in both cultured urodele cells and in vivo. We focused on the down-regulation of green fluorescent protein and two axolotl proteins, MSX1 and PAX7. Our data show that the expression of these proteins can be efficiently reduced by morpholinos. MSX1 has been hypothesized to be involved in muscle dedifferentiation based on experiments using cultured myotubes. Our studies in in vivo muscle fibers so far have shown no influence of overexpressing or down-regulating MSX1 on the dedifferentiation process. *Developmental Dynamics 232: 162–170, 2005.* © 2004 Wiley-Liss, Inc.

Key words: morpholino; axolotl; amphibian; regeneration; blastema; MSX1; PAX7

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INTRODUCTION

The ability of urodeles to regenerate complete body structures has fascinated generations of scientists since Spallanzani's initial description in 1768. Surprisingly, there is still relatively little known about the underlying molecular mechanisms. The difficulty of manipulating gene and protein function and analyzing phenotypes in complex adult tissues compared with embryonic tissues is a primary reason for the limitations in molecular analysis so far.

A common method used to knockdown protein function during vertebrate development is the application of morpholino antisense oligonucleotides against the 5′ region of a target mRNA. Morpholinos are short (usually 25 bp), antisense oligoribonucleotides where an additional amine has been introduced into the ribose rings. This modification renders the oligoribonucleotides nuclease-resistant while maintaining their binding affinity and specificity. Morpholino oligoribonucleotides complementary to sequences spanning the start codon of the target mRNA interfere with ribosomal binding and prevent translational initiation. This technique has been implemented successfully in various organisms, including *Xenopus* (Heasman et al., 2000), zebrafish (Nasevicius and Ekker, 2000), chick (Kos et al., 2001), and mouse (Coonrod et al., 2001). In zebrafish and mouse morpholinos against specific proteins produced phenocopies of the known genetic mutants of these proteins (Nasevicius and Ekker, 2000; Coonrod et al., 2001; Lele et al., 2001). The application of morpholinos has been limited so far to embryogenesis, where it is possible to inject the morpholinos into the cleaving egg (Heasman et al., 2000; Nasevicius and Ekker, 2000) or to electroporate the morpholinos into the relatively soft, simple embryonic tissue structures (Mellitzer et al., 2002; Kos et al., 2003).

We endeavored to assess if morpholino knockdown of protein translation is possible in the context of regeneration. One question was whether mor-

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pholinos function at all in urodele cells. A second major issue was whether sufficient morpholinos could be delivered into cells at the amputation plane to elicit efficient protein knockdown. Particularly interesting would be the ability to deliver morpholinos into the cells that will contribute to the regeneration blastema. This strategy would allow for the testing of proteins involved in the conversion of mature cell types into regeneration progenitor cells. Recently, we demonstrated that cells in the axolotl tail could be efficiently transformed with DNA plasmids by means of in vivo electroporation (Echeverri and Tanaka, 2003). This technique has been used to trace cell fate by the transfection of plasmids encoding fluorescent proteins such as green fluorescent protein (GFP). A question is whether this method can be used to perturb gene and protein function during regeneration by morpholino-mediated protein knockdown.

Two major tissue types of interest during axolotl tail regeneration are spinal cord and muscle. After tail amputation the ependymal cells of the mature spinal cord migrate and proliferate to form an elongating ependymal tube, which subsequently differentiates into the regenerated spinal cord. How the ependymal cells are activated to form the regenerating neural progenitor cells is not yet understood. We wished to address whether it is possible to knockdown protein expression in the ependymal cells that undergo spinal cord regeneration. PAX7 is a transcription factor that is expressed in the dorsal progenitor cells of the developing neural tube, and we have found that PAX7 is expressed in the dorsal ependymal cells that will contribute to the regenerate (Mchedlishvili, manuscript in preparation). The role of PAX7 during spinal cord regeneration was not known. We, therefore, chose PAX7 as one target in our present morpholino studies.

Muscle is a particularly important target for morpholino-mediate knockdown studies. The dedifferentiation of multinucleate fibers into proliferating mononucleate cells is a signature feature of regeneration, and understanding the molecular basis of this phenomenon is a major goal in regeneration research. In this regard, MSX1 represents a particularly interesting protein to study. *Msx1* gene expression is induced in the urodele limb blastema and in the regenerating mouse digit tip (Koshiba et al., 1998; Han et al., 2003). It has been suggested that MSX1 plays a role in muscle fiber dedifferentiation, because overexpression of *msx1* in cultured mouse myotubes caused a small percentage of cells to fragment into proliferating mononucleate cells (Odelberg et al., 2000), a feature not normally associated with these mammalian cells. However, the in vivo role of MSX1 in muscle dedifferentiation has not yet been tested.

Here, we demonstrate that exogenously expressed proteins such as GFP, as well as endogenous proteins such as PAX7 can be down-regulated by the electroporation of morpholino antisense oligoribonucleotides into regenerating tissues. We have developed the methods to target both dedifferentiating muscle fibers and spinal cord ependymal cells. These experiments indicate that the morpholino technique provides a means to test gene function in specific cell types and during specific cellular processes such as dedifferentiation during regeneration.

RESULTS

Morpholinos Lead to Down-Regulation of Ectopically Expressed GFP Both in Cultured Cells and In Vivo

In this study, we characterized the function of morpholinos in two situations: a myogenic cell line (A1) derived from *Notophthalmus viridescens* that is widely used for regeneration research, as well as in vivo during tail regeneration in *Ambystoma mexicanum* (axolotl). We initially assessed the ability of morpholinos to block GFP expression in the newt A1 cell line, a nontransformed cell line derived from newt limb tissue explants (Ferretti and Brockes, 1988). To test the morpholinos, A1 cells were electroporated with *egfp* and *dsred* expression plasmids, together with either the antisense morpholino against *gfp*, or with the inverted control morpholino (for precise conditions see Experimental Procedures section). At 24 and 48 hr postelectroporation, the cells cotransfected with the *gfp* and *dsred* plasmids and the inverted morpholino expressed both fluorescent proteins robustly (Fig. 1A). In contrast, cells transfected with the morpholino against *gfp* displayed undetectable or extremely dim GFP signal (Fig. 1B). Upon quantifying the GFP and DsRed fluorescence in the transfected cells the GFP/DsRed ratio was 10-fold reduced in the cells transfected with the anti-*gfp* morpholino (GFP/DsRed ratio: inverted morpholino, 1 ± 0.27; anti-*gfp* morpholino, 0.1 ± 0.12 Fig. 1C). This finding indicated that morpholinos exert a specific function in newt cells. It should be noted that, although morpholinos are reported to be nonionic oligonucleotides they efficiently entered cells, presumably by diffusion through holes in the cell membrane created by the electroporation.

We previously have developed the methods to electroporate cells in vivo in the axolotl tail, including muscle cells, blastema cells, and spinal cord cells (Echeverri and Tanaka, 2003). To test the efficacy of morpholinos in vivo, we again overexpressed *gfp* together with *dsred* plasmids in axolotl tail muscle fibers by single cell electroporation, together with either anti-*gfp* morpholinos or inverted control morpholinos. Similar to our in vitro results, GFP expression was strongly inhibited when anti-*gfp* morpholino was included in the electroporation (Fig. 2A,B). In this case, we also observe a 10-fold decrease in GFP expression in vivo (GFP/DsRed ratio: inverted morpholino, 0.72 ± 0.38; anti-*gfp* morpholino, 0.078 ± 0.076; Fig. 2C). These results indicate that morpholinos are an effective tool for inhibiting protein expression from transfected plasmids in the urodele.

Identification of *pax7* and *msx1* 5′ Sequences by RACE

To address whether morpholinos can interfere with endogenous axolotl protein expression, we initially chose to test protein knockdown of two axolotl genes, *pax7* and *msx1*. We chose these genes for several reasons. First, the availability of antibodies that recognize the respective proteins allowed us to assess whether protein knock-

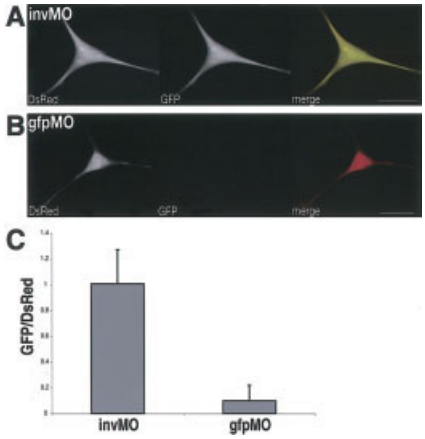

Fig. 1. Morpholinos specifically down-regulate ectopically expressed green fluorescent protein (GFP) in cultured newt A1 cells. **A,B:** Newt A1 cells were electroporated with two plasmids, encoding the GFP and DsRed proteins and either the inverted control morpholino (invMO, A), or the morpholino against *gfp* (gfpMO, B). Pictures in the green and red channels were taken with the same exposure time. **C:** Graph of the GFP to DsRed fluorescence ratio as measured in single cells (n = 8). The two ratios are different to a *P* value of 5.29e-06. Scale bars = 100 μm in A,B.

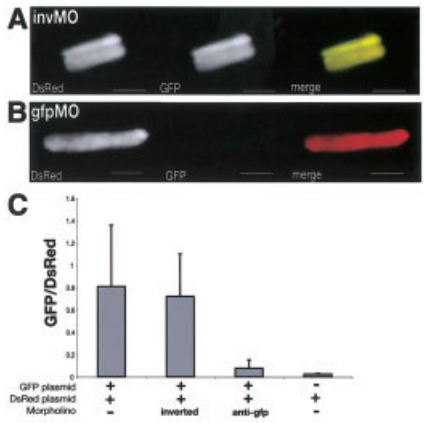

Fig. 2. Morpholinos specifically disrupt green fluorescent protein (GFP) expression in axolotl muscle fibers in vivo. **A,B:** Single muscle cells of the axolotl tail were electroporated in vivo with two plasmids encoding eGFP and DsRed proteins, together with either the control morpholino (invMO, A) or the morpholino against *egfp* (gfpMO, B). Pictures were taken of live animals and with the same exposure time in green and red channels. **C:** Fluorescence intensities of GFP and DsRed were measured in single cells (n > 14), and the ratio of GFP/DsRed fluorescence was calculated. Because fluorescence also produces a weak signal in the GFP channel of the microscope, cells electroporated with only DsRed were also quantitated to determine the "background" ratio of DsRed only. The ratios for inverted morpholino and anti-*gfp* morpholino are different to a *P* value of 1.81e-08. Scale bars = 100 μm in A,B.

down had been successful. Second, PAX7 protein is expressed in the dorsal domain of the mature and regenerated spinal cord similar to the expression found during development of the neural tube (Mchedlishvili et al., manuscript in preparation). By focusing on this gene, we could determine whether it was possible to deliver morpholinos into the spinal cord and whether such morpholinos would be retained in the neural progenitor cells that give rise to the regenerating spinal cord. We were interested to know if the PAX7 knockdown would have a phenotype on spinal cord regeneration, although *pax7*$^{-/-}$ mice have a minimal phenotype due to redundancy in gene expression with *pax3* (Mansouri and Gruss, 1998). The *msx1* gene was of particular interest, since Odelberg et al. proposed that MSX1 expression can induce muscle cell dedifferentiation in culture mouse myotubes (Odelberg et al., 2000). We believed that it would be interesting to test the protein's in vivo function in a setting where muscle dedifferentiation normally takes place.

To design morpholinos against these genes, we isolated the 5′ sequences of the axolotl *pax7* and *msx1* genes by 5′ rapid amplification of cDNA ends (RACE). We initially isolated the axolotl *pax7* sequence using degenerate primers (see Experimental Procedures section). The 5′ RACE primers were then designed from this sequence, and subsequent RACE reactions led to the isolation of the 5′ end of the gene (Fig. 3A).

Although Koshiba et al. had reported the full-length axolotl *msx1* sequence (Koshiba et al., 1998), this published sequence is approximately 50 amino acids shorter at the N-terminus when compared with full-length *msx1* sequences from any other species. The reported N-terminus of the axolotl gene was positioned at a conserved internal methionine found in other species. To determine the 5′ end of the *msx1* gene, we designed a reverse primer at the 5′ end of the published axolotl sequence and performed 5′ RACE. We indeed found an additional 164 nucleotides of in-frame sequence at the 5′ end that placed a start codon in a similar position to the other vertebrate *msx1* genes (Figs. 3B, 4 for MSX1 amino acid alignment). During this work, we also identified that the Koshiba sequence contained four sequencing errors marked at nucleotides 48, 294, 295, and 366 in our new sequence (see arrow and blue nucleotides Fig. 3). These errors had resulted in a frameshift at amino acid positions 1 to 55 and 47 to 66 according to our new amino acid sequence (Fig. 4).

Morpholinos Reduce PAX7 Levels in the Differentiated and Regenerating Spinal Cord

PAX7 protein is expressed in dorsal progenitor cells of the developing spinal cord (Kawakami et al., 1997; Mansouri and Gruss, 1998; Yamamoto et al., 2001). The monoclonal antibody against chick PAX7 recognizes the endogenous axolotl PAX7 protein, marking a dorsal domain in the mature and regenerating spinal cord (see Fig. 5). To reduce PAX7 levels in the spinal cord, the anti-*pax7* morpholino together with a fluorescently tagged standard control morpholino was electroporated into spinal cord cells by inserting a morpholino-filled glass microelectrode into the lumen of the spinal cord immediately after tail amputation as previously described (Echeverri and Tanaka, 2003). The fluorescent standard control morpholino was included to locate the successfully electroporated cells. In parallel control experiments, the inverted anti-*pax7* morpholino was electroporated together with the fluorescent control morpholino. Axolotl tails were fixed at 2, 4, 6, and 8 days after electroporation, and immunohistochemistry of PAX7 was performed on cryosections. Figure 5A depicts typical PAX7 staining of the differentiated axolotl spinal cord where the dorsal cells in and adjacent to the spinal cord ependymal layer are positive for PAX7. By previous cell fate tracing studies, these ependymal cells have been shown to be the cells that will form the regenerating spinal cord after tail amputation (Echeverri and Tanaka, 2003). PAX7 staining is reduced in the axolotl cells that took up the anti-*pax7* morpholino (Fig. 5B, the arrow points to cells that down-regulated PAX7). Down-regulation of

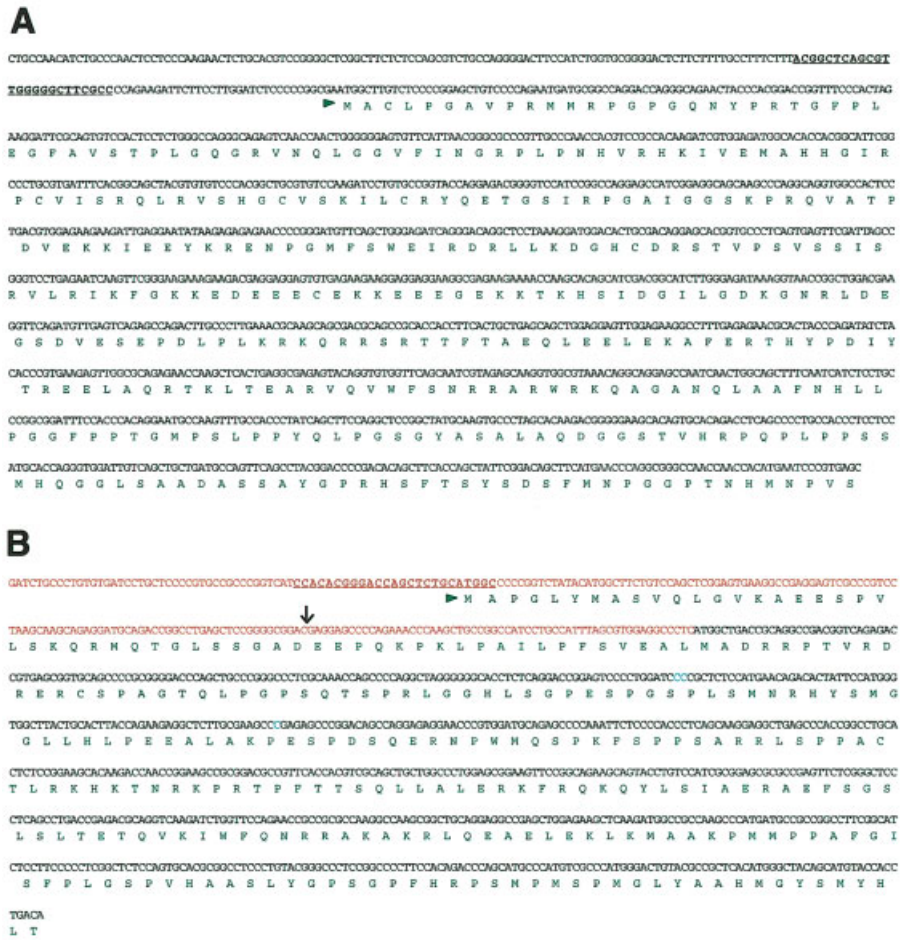

Fig. 3. A,B: Sequences of the axolotl *pax7* (partial, A) and *msx1* (B) genes. The binding site of the antisense morpholino to the 5′ end of the genes is in bold and underlined. The red nucleotides in the *msx1* sequence represent the additional 5′ sequence that was isolated in the present work compared with the published axolotl *msx1* gene (Koshiba et al., 1998). The black arrow and the three blue nucleotides in the central region of the *msx1* sequence mark sequence differences between our and Koshiba's result.

Fig. 4. Alignment of the MSX1 amino acid sequences from different species. The numbers are the NCBI accession numbers. The axolotl sequences are in red; mouse and chick sequences are in black. The axolotl sequence identified by Koshiba (D82577) is 55 amino acids shorter than the axolotl sequence identified by us (AY525844, sequence is marked with asterisks). Differences in amino acid sequence between the old and the new axolotl MSX1 sequence are highlighted in blue.

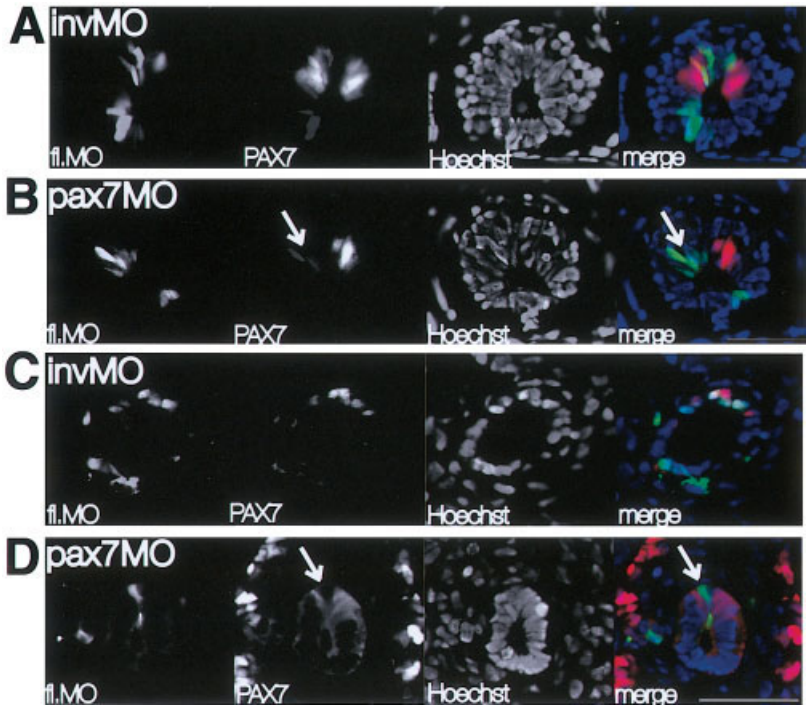

Fig. 5. Morpholinos down-regulate axolotl PAX7 expression in the mature and regenerating spinal cord. **A,B:** Inverted control *pax7* morpholino (invMO, A) or anti-*pax7* morpholino (pax7MO, B) was coelectroporated with fluorescein isothiocyanate (FITC) -labeled standard control morpholino into spinal cord cells. Cross-sections of the differentiated axolotl spinal cord 2 days postelectroporation show FITC-labeled standard control morpholino (fl.MO) in green and PAX7 antibody staining in red and nuclear Hoechst staining in blue. The arrows point to the electroporated region of the dorsal spinal cord in which the PAX7 protein is specifically down-regulated by the morpholino against axolotl *pax7*. In the mature spinal cord, PAX7 is expressed in the dorsal spinal cord cells but not or only weakly in the dorsal midline. This finding is also the case in unelectroporated samples and likely represents the roofplate cells. **C,D:** Cross-sections of 6-day regenerating spinal cords that were electroporated with morpholino on day 0 postamputation. PAX7 staining is present even in the distal tip of the 6-day control blastema (C) but not in the regenerating spinal cord cells that were treated with *pax7* antisense morpholino (D, see arrows). The same results were obtained with a *pax7* morpholino that is directly coupled to fluorescein. In all pictures, dorsal is at the top and ventral is at the bottom. Scale bars = 100 μm in B (applies to A,B), in D (applies to C,D).

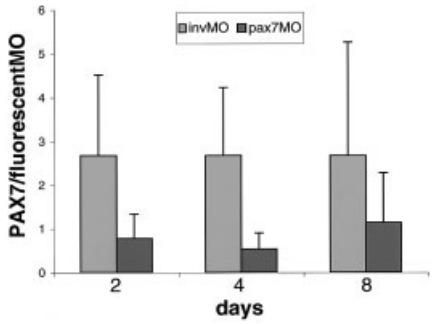

Fig. 6. Quantification of endogenous axolotl PAX7 protein after morpholino treatment. Axolotl spinal cord cells were electroporated with a mixture of a fluorescently labeled standard control morpholino and either the anti-*pax7* (pax7MO) or the inverted control morpholino (invMO). Tails were fixed at the indicated time points after electroporation and stained for the PAX7 protein. Fluorescence intensities from PAX7 antibody staining and from the fluorescent morpholino were measured in single cells ($n > 22$). Each column in the graph represents the average ratio of PAX7 antibody fluorescence to standard control morpholino fluorescence per cell normalized to the value for the inverted morpholino on day 2.

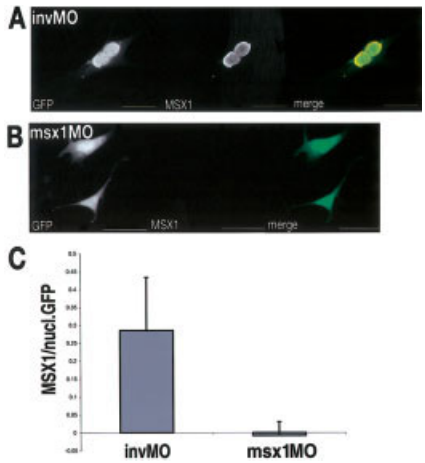

Fig. 7. Morpholinos knock down protein expression of ectopically expressed axolotl MSX1. **A,B:** Newt A1 cells were electroporated with two plasmids, encoding *egfp* and *msx1*, and either the inverted control morpholino (invMO, A) or the morpholino against *msx1* (msx1MO, B). Green fluorescent protein (GFP) is expressed in the cytoplasm and in the nucleus, whereas MSX1 is exclusively found in the nucleus, consistent with its role as a transcription factor. Cells receiving anti-*msx1* morpholino show weak or undetectable MSX1 levels. **C:** Fluorescence intensities from the MSX1 antibody staining and from nuclear eGFP expression were measured in single cells ($n > 13$), and the ratio of MSX1/nucGFP fluorescence was calculated.

PAX7 protein expression was quantified in single cells and in relation to the fluorescent standard control morpholino. Cells that had taken up the fluorescent control morpholino and that were located in the PAX7 expression domain were chosen randomly for quantifications. The comparison of PAX7 expression after electroporation of antisense or control morpholinos is shown in Figure 6. We observe up to fivefold reduction in PAX7 protein levels in vivo and continue to observe repression 8 days postamputation (Student's *t*-test values for 2, 4, and 8 days: 3.88e-05, 3.067e-09, and 0.0077, respectively).

The cells electroporated with the morpholinos represent the progenitors for the regenerating spinal cord, so an important question was whether protein down-regulation continued as these cells contributed to the regenerating tissue. Figure 5C,D indeed shows that fluorescent morpholinos are still easily detectable in the regenerating spinal cord. Furthermore, PAX7 protein expression continues to be inhibited in the ependymal cells of the regenerating spinal cord (Figs. 5D, 6). When we observed the tails containing anti-*pax7* morpholinos, no obvious phenotype on regeneration could be detected.

MSX1 Protein Knockdown or Overexpression Has No Effect on Muscle Dedifferentiation

MSX1 is up-regulated during urodele limb regeneration and mouse digit tip regeneration (Koshiba et al., 1998; Han et al., 2003). Odelberg and Keating proposed a role for MSX1 in muscle dedifferentiation. They conditionally expressed *msx1* by means of

TABLE 1. *Msx1* Morpholino Does Not Inhibit *In Vivo* Muscle Fiber Dedifferentiation

	Fiber stable	Fiber fragments	Total	% Fragmented
Experiment 1				
DsRed + msx1MO	6	16	22	72.7
DsRed + invMO	6	14	20	70
Experiment 2				
DsRed + msx1MO	7	11	18	61
DsRed only	6	11	17	65

retroviral transduction in cultured mouse myotubes and found that a small percentage of the myotubes produced proliferative, mononucleated cells (Odelberg et al., 2000). The role, however, of MSX1 in dedifferentiation has not been tested in vivo. Here, we have assayed the role of MSX1 in axolotl muscle fiber dedifferentiation during tail regeneration in vivo. Our goal was to determine whether blocking MSX1 expression or overexpressing MSX1 in muscle fibers at the plane of amputation would inhibit or enhance the percentage of muscle fibers that underwent dedifferentiation. In vivo immunohistochemistry of MSX1 resulted in very faint signals that were difficult to quantitate, so we first assessed the effectiveness of the anti-*msx1* morpholino to block protein expression by overexpressing the protein in A1 cells. When we transfected the full-length axolotl *msx1* sequence into A1 cells, we could easily detect the protein with a monoclonal antibody against chick MSX1+2. Axolotl MSX1 was correctly located in the nuclei of the cells (Fig. 7A), according to its role as a transcription factor. The morpholino designed against our *msx1* sequence worked well in inhibiting protein expression, because cells positive for GFP and the anti-*msx1* morpholino showed no detectable staining of the MSX1 protein (Fig. 7B). MSX1 down-regulation was quantified by measuring the ratio of fluorescence from MSX1 antibody staining to nuclear GFP fluorescence as depicted in Figure 7C.

Having established the effectiveness of the anti-*msx1* morpholino, we tested the role of MSX1 in muscle dedifferentiation in vivo. Anti-*msx1* morpholino or the inverted control morpholino was electroporated into single muscle fibers of the axolotl tail, together with a *dsred* expression plasmid to monitor successful electroporation. Three days postelectroporation, the tail was amputated directly behind the DsRed-expressing fibers. The muscle cells were followed up once per day for 8 days under the fluorescence microscope, and dedifferentiating fibers were scored as described previously in (Echeverri et al., 2001). There was no significant difference in the number of dedifferentiating fibers containing either *msx1* antisense or control morpholino (Table 1). A typical example of a morpholino-containing muscle fiber that dedifferentiated is depicted in Figure 8A,B. This muscle fiber was electroporated in vivo with the *dsred* expression plasmid plus the *msx1* antisense morpholino. The fibers containing the anti-*msx1* morpholinos dedifferentiated 3 to 5 days after amputation, indistinguishable from control fibers.

We also tested the effect of overexpressing MSX1 in muscle fibers. Single tail muscle fibers were electroporated with a plasmid encoding the axolotl *msx1* gene driven by the cytomegalovirus (CMV) promoter together with the *dsred* plasmid to monitor positively electroporated fibers. Between 5 and 10% of muscle fibers undergo dedifferentiation in the absence of tail amputation, likely triggered by the small injury that is done to the tail during the electroporation process (Table 2). Such a condition of a small but detectable muscle dedifferentiation frequency was ideal when looking for enhancement of dedifferentiation by MSX1 expression. As can be seen from the results in Table 2, the overexpression of MSX1 did not stimulate dedifferentiation significantly above the level of the controls transfected with *dsred* and empty vector. Figure 8C,D shows a muscle fiber that was cotransfected in vivo with the *dsred* and the *msx1* expression plasmid. The fiber did not dedifferentiate but remained stable even 6 days after transfection.

The expression of MSX1 in the electroporated muscle fibers was confirmed by staining electroporated tails with the monoclonal anti-MSX1 antibody. As expected, the ectopically expressed MSX1 was easily detectable in the nuclei of the electroporated fibers (Fig. 9).

DISCUSSION

Here, we have described the use of morpholinos to knockdown protein expression during regeneration. This approach represents, to date, the only available method beyond expression of dominant negative plasmid constructs or the use of pharmacological inhibitors to negatively interfere with gene function during urodele regeneration. Attempts to implement RNA interference have been unsuccessful so far in our hands. Electroporation of long dsRNA or small interfering RNA (siRNA) against GFP in the newt A1 cells or in vivo in the axolotl did not interfere with GFP expression (Schnapp and Tanaka, unpublished observations). The conditions we used in our RNA interference experiments effectively interfere with expression in mouse embryos (Calegari et al., 2002).

Morpholino electroporation can be used to target individual cells and study their behavior in the context of normal regeneration as shown here for muscle fibers. The advantage of knocking down proteins only in a few cells of a given tissue is that their behavior can be studied on the background of a wild-type organism and observations can be made down to the single cell level. Genetic knockouts can have the disadvantage of either being lethal or having such a strong phenotype that interpretations are

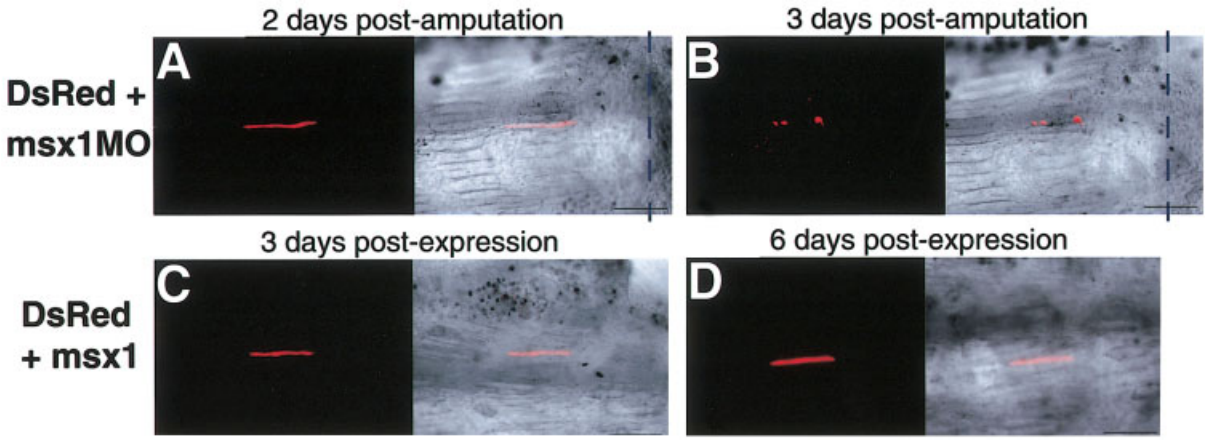

Fig. 8. Muscle fibers electroporated in vivo with either the *msx1* antisense morpholino or the *msx1* expression plasmid display normal muscle dedifferentiation. **A,B:** Tail muscle fiber electroporated with *dsred* and anti-*msx1* morpholino. Up to 2 days postamputation, the fiber is stable (A) and 3 days postamputation it dedifferentiates into mononucleate cells (B). The dashed lines mark the amputation plane. **C,D:** Tail muscle fiber electroporated with *dsred* and *msx1* expression plasmids. The fiber remains stable 3 days (C) and 6 days (D) after expression of the plasmids. The left panel of A–D shows the red fluorescence from the DsRed protein, and the right panel is the overlay of the fluorescence and the differential interference contrast microscopy picture. Scale bars = 100 μm in A–D.

TABLE 2. *Msx1* Overexpression Does Not Stimulate *In Vivo* Muscle Fiber Dedifferentiation

Overexpression of	Fiber stable	Fiber fragments	Total	% Fragmented
DsRed + msx1	29	2	31	6.4
DsRed + empty vector	37	4	41	9.7

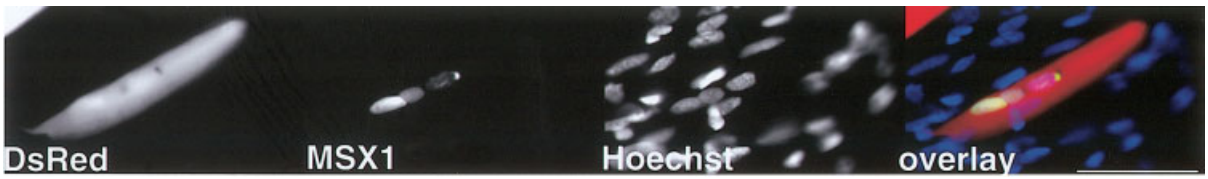

Fig. 9. Overexpressed MSX1 is present in the nuclei of in vivo electroporated muscle fibers. Muscle fibers in the axolotl tail were coelectroporated with the *dsred* and the *msx1* expression plasmid. Six days after electroporation, the tails were fixed and 20-μm-thick cryosections were stained with the monoclonal antibody against MSX1. DsRed fluorescence is in red, MSX1 antibody staining in green, and nuclear Hoechst stain is in blue. Scale bar = 100 μm.

very difficult. In our studies, we were able to electroporate cells in the mature tissue that ultimately contribute to the regenerating tissue. Furthermore, we continued to observe depression of protein expression for 8 days postamputation. This finding indicates that morpholino knockdown is valuable for studying genes involved in the early steps of blastema formation. Unlike zebrafish, we have so far not observed any nonspecific, deleterious effects of morpholinos on newt and axolotl cells.

We initially chose the *pax7* and *msx1* genes because of their putative function in spinal cord and muscle dedifferentiation and because of the availability of antibodies to assess protein knockdown. Although we could reduce PAX7 expression in spinal cord neural progenitor cells, we did not see an overall phenotype. This finding may either be due to redundancy in gene function and/or due to insufficient knockdown. We favor the former, redundancy in gene function, because the *pax7* knockout mouse does not show a phenotype in the central nervous system (Mansouri et al., 1996).

MSX1 has been suggested to induce muscle dedifferentiation, based on experiments on cultured mouse myotubes (Odelberg et al., 2000). We would like to note, however, that Koshiba et al. (1998) found that the onset of *msx1* expression during limb regeneration occurred after 10 days in the blastema, which is a time after dedifferentiation has already occurred. In our experience, in situ hybridization of *msx1* shows robust staining in the mature and regenerating spinal cord, but expression in dedifferentiating muscle fibers is not detectable at similar levels (data not shown). Such observations also question the role of MSX1 in muscle dedifferentiation.

We have functionally tested here the role of MSX1 in axolotl muscle fiber dedifferentiation in vivo, with the result that there is no stimulation or block of dedifferentiation by overexpressing or down-regulating MSX1 in the muscle fibers at the amputation plane. Considering the results from

Odelberg et al., it is surprising that neither overexpression nor protein knockdown produced a detectable phenotype in vivo. These results may indicate that MSX1 has no role in in vivo muscle dedifferentiation. Further work will be required to resolve the true role of MSX1 in muscle dedifferentiation, for example whether other factors influence its ability to induce muscle dedifferentiation.

Taken together, we have shown successful protein knockdown during vertebrate regeneration. Such a tool will be important in the future to move from descriptive studies towards a more detailed understanding of the molecular aspects of regeneration.

EXPERIMENTAL PROCEDURES

Axolotl Care

Ambystoma mexicanum (axolotls) were bred in our facility and kept at 18°C in Dresden tap water and fed daily with artemia. For all surgery, animals were anesthetized in 0.01% ethyl-p-aminobenzoate (Sigma). The experiments described here were performed on 3- to 5-cm-long axolotl.

Cloning of Axolotl *msx1* and *pax7* Genes

The 5′ ends of both the axolotl *msx1* and *pax7* genes were cloned by 5′ RACE (BD Bioscience). The 5′RACE cDNA was prepared from 6 day axolotl tail blastema total RNA. The reverse primer used for *msx1* 5′ RACE was 5′CTCTGCATCCACGGGTTCCTCTCCT3′.

Part of the sequence of the axolotl *pax7* gene was amplified from cDNA by using degenerate primers. The forward and reverse primers were 5′GGNATCCTNGGCGACAAAG3′ and 5′GCNGAGNTGGAGTTGGTGGT3′, respectively. Several subsequent polymerase chain reactions (PCRs) were necessary to clone the 5′ end of the *pax7* gene, because each PCR only yielded a fraction of the total upstream gene sequence. Primers used were (in experimental order) 5′AGTGAGCTTGGTTCTCTGCGCCAAC3′,5′CTGGGTAGTGCGTTCTCTCAAAGGC3′, 5′CATCCTTTAGGAGCCTGTCCCTGATC3′, 5′CCTCAATCTTCTTCTCCACGTCAGGAGT3′.

Msx1 and *pax7* cDNA sequences were verified by comparison to the homologous sequences from other species. The axolotl *msx1* gene was cloned into the eGFP-N2 expression vector (Clontech, map available at http://www.clontech.com) behind the CMV promoter (replacing the *egfp* gene). The *egfp* sequence was cut out of the vector with *Hin*dIII and *Not*I restriction enzymes. The full-length *msx1* sequence was amplified by using the forward primer from the 5′ RACE kit and the reverse primer 5′CTTATGTCAGGTGGTACATGC3′, including the *msx1* stop codon. The PCR product was cloned into the pCRII vector (TA Cloning Kit, Invitrogen) and cut out with the *Hin*dIII and *Not*I restriction enzymes to ligate it into the respective restriction sites of the N2-backbone.

Morpholinos

All morpholinos used in this study were designed by Gene Tools (Gene Tools LLC, Philomath, OR, www.gene-tools.com) based on the cDNA sequences of the respective genes. The morpholino sequence against the *msx1* mRNA is 5′GCCATGCAGAGCTGGTCCCGTGTGG3′, against the *pax7* mRNA 5′GGCGAAGCCCCCAACGCTGAGCCGT3′ and against the *egfp* mRNA 5′ACAGCTCCTCGCCCTTGCTCACCAT3′. The binding sites of the morpholinos against the *msx1* and the *pax7* mRNA are depicted in Figure 3. All control morpholinos were the 3′ to 5′ inverted sequences of the respective antisense morpholinos. The standard control fluorescein-tagged morpholino was also purchased from Gene Tools.

Electroporation of Newt A1 Cells

Newt A1 cells (kind gift from Jeremy Brockes) were grown in 10% fetal calf serum–modified MEM at 25°C and 2% CO_2 as previously described (Ferretti and Brockes, 1988). For electroporation, the cells were grown to confluency in a 160-cm^2 tissue culture flask and electroporated in 300-μl Steinberg's solution with 10 μg each of eGFP-N2 and pDsRed2-N1 (Clontech) expression plasmid, plus 150 μg of either anti-*gfp* or control morpholino (inverted sequence of anti-*gfp* morpholino). Cells were electroporated in 4-mm electroporation cuvettes (Eurogentec SA, Seraing, Belgium, www.eurogentec.com). Electroporation conditions for A1 cells were 100 V, 5 pulses, 35-msec pulse length on an Electro Square Porator ECM 830. For electroporation of A1 cells with the *msx1* gene, 5 μg of the *msx1* expression plasmid, 5 μg of *egfp* (eGFP-N2 plasmid), or *egfp-nls* (kind gift from Wulf Haubensak, Max Planck Institute, Dresden, Germany), plus 100 μg of anti-*msx1* or inverted control morpholino were used.

In Vivo Electroporation of Single Muscle Fibers and Image Acquisition

The set up for axolotl electroporation was identical to that described by Echeverri and Tanaka (2003). For electroporation of single muscle fibers, the skin from one side of the tail was removed so that the electroporation needle could make direct contact with the surface of the muscle fiber. DNA and morpholino concentrations used were 0.2 μg/μl for cytoplasmic eGFP-N2 and pDsRed2-N1 and 7 μg/ml of either anti-*gfp* or control morpholino. For MSX1 down-regulation experiments, 0.2 μg/μl of pDsRed2-N1 and either 4 or 7 μg/μl of *msx1* morpholino were electroporated. For MSX1 overexpression, the same concentration of pDsRed2-N1 plus 0.4 μg/μl of the *msx1* plasmid were used. Several nanoliters of liquid were injected (using a World Precision Instrument pressure injector) close to the muscle cell and 5 pulses of 50 V, 200 Hz, and pulse length of 20 msec were applied using a SD9 Stimulator (Grass Telefactor).

Dedifferentiating muscle cells were followed by light microscopy as previously described (Echeverri and Tanaka, 2003). Briefly, we used the white strain of axolotls that contain negligible skin pigment and imaged the fluorescent muscle fibers through the skin that is only several cell layers thick. Animals were anesthetized as described above and imaged by using a 10× plan-neofluar objective on a Zeiss Axiovert 200 system with a Spot digital camera, controlled by MetaMorph image acquisition software.

In Vivo Electroporation of Axolotl Spinal Cord Cells

For bulk electroporation of the axolotl spinal cord, the animal was mounted on a tilted stage, to allow visualization of the spinal cord lumen. The tail was amputated and then the spinal cord lumen was injected with morpholino solution by insertion of a needle into the central canal of the spinal cord (for more details, see Echeverri and Tanaka, 2003). Concentrations of morpholinos used were 4–8 μg/ml of standard control morpholino and 8–14 μg/ml of either anti-*pax7* or inverted *pax7* control morpholino. After injection, the needle was removed from the spinal cord and two electrodes were placed dorsolaterally on the sides of the tail. The homemade electrodes consisted of rounded platinum plates of 0.5 cm diameter at the end of Plexiglas homemade tweezers. Pulse conditions were 25–40 V (depending on the animal size) given in five repeats with pulse length 50 msec by using the BTX Electro Square Porator ECM 830.

Immunofluorescence of A1 Cells and Axolotl Tail Cryosections

A1 cells were fixed for 15 min in 4% paraformaldehyde, washed in TBS, and incubated overnight at 4°C with 100 μg/ml mAB against MSX1+2 (concentrated supernatant of 4G1 cell line from Developmental Studies Hybridoma Bank, IA, www.uiowa.edu/~dshbwww/) in TBS plus 20% goat serum. The secondary antibody was tetrarhodamine isothiocyanate–labeled rabbit anti-mouse (DAKO, Glostrup, Denmark, www.dako.com) diluted to 1:200.

Electroporated axolotl tails were fixed in 4% fresh paraformaldehyde overnight at 4°C, washed in phospate buffered saline, equilibrated in 30% sucrose, and frozen in Tissuetek (OCT compound, Sakura). Twenty-micrometer-thick cross-sections of the tail were processed for immunohistochemistry with the anti-PAX7 mAB (PAX7, Developmental Studies Hybridoma Bank). A Cy5-labeled secondary antibody (Dianova, Hamburg, Germany, www.dianova.com) was used in a 1:200 dilution. Nuclear stainings were done with 1 μg/ml of Hoechst.

Microscopy and Fluorescence Quantification

Quantifications of GFP and DsRed fluorescence in A1 cells and in axolotl muscle fibers were done from live cells and animals, whereas quantifications of fluorescence from MSX1 and PAX7 antibody staining and from nuclear GFP were done on fixed samples. Digital images of axolotl spinal cord cross-sections were taken on an upright Olympus BX61 microscope, and all pictures of A1 cells and live muscle fibers on the inverted Zeiss Axiovert 200M microscope, using a Spot CCD camera. For A1 cells and live muscle fibers, the same pictures were used for fluorescence quantifications. For fluorescence quantifications of PAX7-stained spinal cord cells, pictures were taken on a confocal ZEISS Axiovert 200M microscope.

Average fluorescence intensities of one cell or one nucleus were measured in the MetaMorph program, and background fluorescence was subtracted for each field separately. The ratios of GFP/DsRed fluorescence (for the morpholino against *gfp*), MSX1/nuclearGFP fluorescence (for the morpholino against *msx1*), and PAX7/fluorescent morpholino fluorescence (for the morpholino against *pax7*) were calculated. NCBI GenBank accession numbers for axolotl *msx1* and *pax7* are AY525844 and AY523019, respectively.

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