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Original Article

Distinctive expression of *Myf5* in relation to differentiation and plasticity of newt muscle cells

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ABSTRACT Regeneration in urodele amphibians such as the newt reflects the local plasticity of differentiated cells. Newt myotubes and myofibres undergo S phase re-entry and cellularisation in the limb blastema, and we have analysed the regulation of *Myf5* in relation to these events. Surprisingly, *Myf5* was expressed after fusion in cultured newt myotubes and in myofibers of the adult limb, in contrast to its familiar expression in myoblasts in other vertebrates. Its expression was markedly down regulated in cultured newt myotubes after S phase re-entry induced by serum stimulation, as well as by exposure to the trisubstituted purine called myoseverin which induces cellularisation. We have attempted to relate this striking difference from other vertebrates to the requirement for multinucleate urodele muscle cells to contribute to the regeneration blastema.

KEY WORDS: *myofiber, myogenin, myoseverin, regeneration, urodele*

Introduction

Adult urodele amphibians, such as the newt and axolotl, are capable of regenerating their limbs and tail, as well as tissues such as the lens, retina and heart (Brockes and Kumar 2002). It is not understood why regeneration on this scale is lost or drastically curtailed in other vertebrates such as mammals (Brockes *et al.,* 2001). The initiation of urodele regeneration apparently reflects the plasticity of the differentiated state in these animals (reviewed in Brockes and Kumar, 2002). For example, the regeneration of the heart and lens depends on the ability of cardiomyocytes and pigment epithelial cells of the iris to re-enter the cell cycle in the vicinity of tissue injury or removal (Eguchi and Shingai 1971, Oberpriller and Oberpriller 1974, Bader and Oberpriller 1979, Bettencourt-Dias *et al.,* 2003). One striking example of plasticity is the reversal of skeletal muscle differentiation during appendage regeneration. It has been demonstrated that labelled newt myotubes re-enter S phase and fragment into viable mononucleate cells after implantation into a limb blastema (Kumar *et al.,* 2000, Lo *et al.,* 1993). The mononucleate progeny are able to divide and contribute to the regenerate. In a recent elegant study of myofibers which were injected with a lineage tracer prior to tail regeneration in the larval axolotl, this process of cellularisation was estimated to contribute about 20% of the cells in the blastema (Echeverri *et al.,* 2001). Newt myotubes which have been blocked for S phase re-entry still undergo cellularisation (Velloso *et al.,* 2000), but the mechanism of cellularisation remains unclear.

In contrast, the regulation of myogenesis is one of the most intensively studied cases of cellular differentiation. Myogenic regulatory factors (MRFs) are muscle specific basic helix-loop-helix (bHLH) transcription factors which play essential roles in determination and differentiation of skeletal muscle cells. The property of myogenic conversion is shared by all four members of the MRF family (*MyoD, Myf5, myogenin, MRF4*), and reflects the transactivation of muscle specific promoters (Weintraub *et al.,* 1991). Notwithstanding their common ability to direct myogenic conversion, the four MRFs are thought to play distinct roles during myogenic differentiation and this view has been supported by detailed analysis of the phenotypes of a variety of single or multiple mouse null mutations (Pownall *et al.,* 2002). In both mouse and chick embryos the first MRF to be expressed is *Myf5*, and expression of either *Myf5* or *Myo D* is required for commitment of precursor cells to the myogenic lineage while expression of either *myogenin*

Abbreviations used in this paper: BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; bHLH, basic helix loop helix; HS, high serum; LS, low serum; MHC, myosin heavy chain; MRF, myogenic regulatory factor; NBT, nitroblue tetrazolium.

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or *MRF4* is required for these myogenic cells to undergo terminal differentiation (Pownall *et al.,* 2002). Myogenesis has been extensively studied in culture, and in these systems both *MyoD* and *Myf5* are expressed in proliferating myoblasts and *MyoD* expression persists after fusion while *Myf5* is down-regulated prior to myotube formation (Kitzmann *et al.,* 1998).

In order to investigate the mechanisms underlying plasticity in urodele muscle cells we have studied cultured myotubes derived from newt A1 limb cells (Ferretti and Brockes 1988). The A1 myotubes are completely refractory to growth factors which act on their mononucleate precursors, and also express markers of late myogenic differentiation. They are clearly different from their normal mammalian counterparts in that they enter and traverse S phase after serum stimulation, a process which depends on phosphorylation and inactivation of the retinoblastoma protein Rb, and hence to some extent reflects the properties of mouse myotubes missing both copies of the *Rb* gene (Schneider *et al.,* 1994, Tanaka *et al.,* 1997). One goal of these studies is to identify the precise differences between newt and mouse myotubes (Brockes and Kumar, 2002), particularly in the light of recent work indicating that expression of the *Msx1* gene (Odelberg *et al.,* 2000), or exposure to the substituted purine called myoseverin (Rosania *et al.,* 2000) or to newt blastemal extracts (McGann *et al.,* 2001), are all able to induce cellularisation in mouse myotubes(Charge and Rudnicki 2004). In the present study we have used newt *Myf5* (Simon *et al.,* 1995) to analyse the regulation of an MRF in relation to plasticity. To our surprise the expression and regulation of the gene is quite different from other vertebrates both in cultured myotubes and in myofibers of the adult limb. We suggest that the expression of *Myf5* in urodele myotubes and myofibers may play a significant part in their role as a reservoir of cells for regeneration.

Results

Expression of Myf5 *in newt A1 myotubes*

A1 mononucleate cells, cultured in HS medium, were reacted with sense and antisense probes derived from the 3'untranslated region of newt Myf5, but no reaction was detected with either

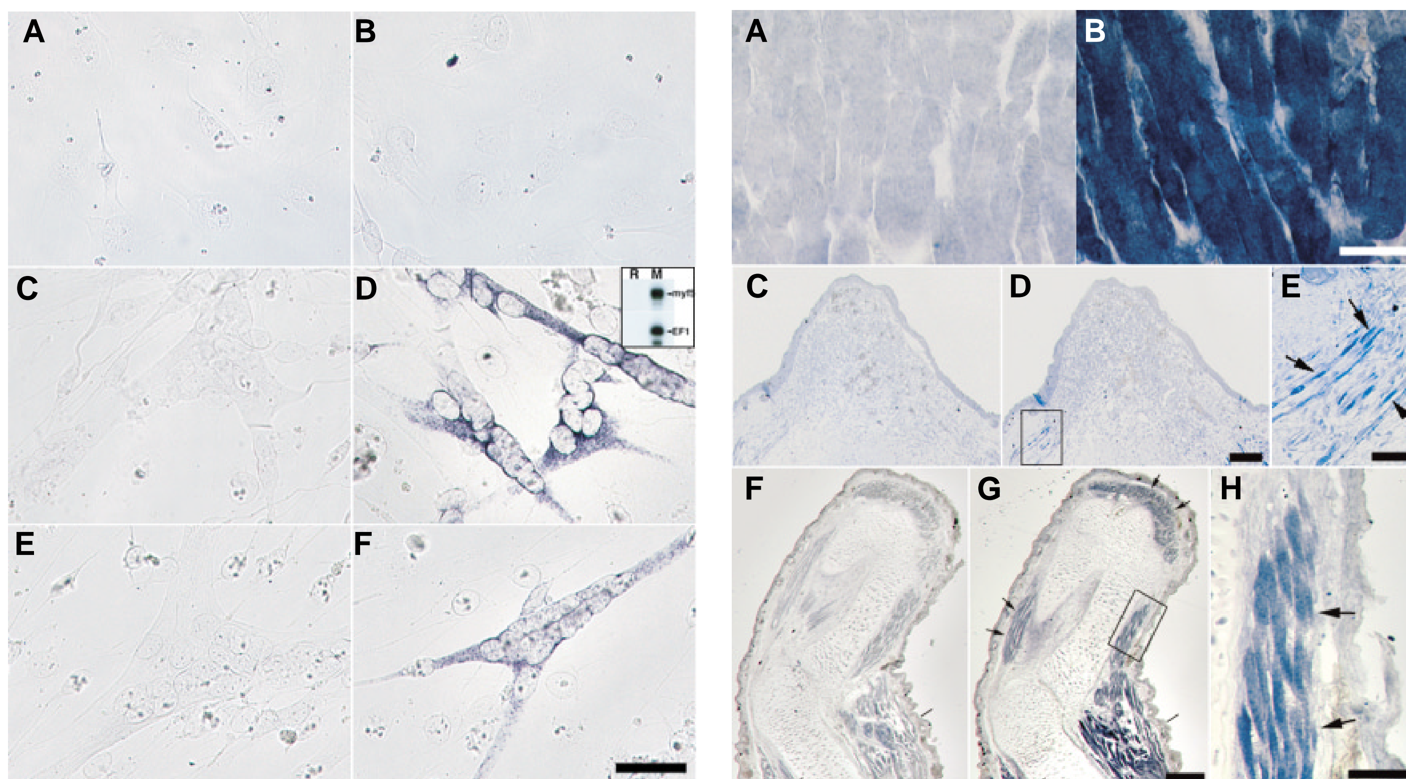

Fig. 1 (Left). Expression of newt *Myf5* and *MRF4* after fusion of A1 cells. (A,B) *Hybridisation of A1 mononucleate cells growing in HS medium to* Myf5 *sense probe (A) or antisense probe (B). Note that mononucleate A1 cells have no detectable expression.* **(C,D)** *Hybridisation of A1 myotubes and mononucleates after fusion; (C)* Myf5 *sense probe; (D) antisense probe. Note strong expression in multinucleate myotubes.* **(E,F)**, *Hybridisation of myotubes with* MRF4 *sense probe (E) and antisense probe (F). Note that MRF4 is expressed only in myotubes. Scale bar, 100 μm. The insert in (D) is an RNAse protection analysis with a* Myf5 *probe (see Materials and Methods) and an* EF-1α *control probe hybridised to RNA from an A1 myotube culture (lane M) or tRNA control (lane R). Note the bands corresponding to full length protection of* Myf5 *and* EF-1α *probes in M.*

Fig. 2 (Right). *Myf5* expression in sections of normal and regenerating newt limbs. *Sections of normal limb musculature were reacted with* Myf5 *sense* **(A)** *or antisense* **(B)** *probes. Sections of a mid-bud stage limb blastema were reacted with sense* **(C)** *or antisense* **(D)** Myf5 *probes. Note that no specific reactivity is associated with the mesenchymal or epidermal compartments of the blastema but* Myf5 *was expressed in some muscle fibres between the stump and blastema arrowed in* **(E)**. *Scale bar, 300 μm. (E) is a higher magnification micrograph of the square in (D). Scale bar, 100 μm.* **(F,G)** *Sections of a palette stage blastema were reacted with sense (F) and antisense (G)* Myf5 *probes. Scale bar, 300 μm.* **(H)** *shows a higher magnification micrograph of the square in (G). Note that* Myf5 *is expressed in newly formed myofibres (arrows) of the regenerate. The two dashs indicate the amputation planes in (F) and (G). Scale bar, 100 μm.*

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                                    :    :  :                                 *
newtMyf5            RAPSG----HHQAGHCLLWACKACKRKSSTMDRRKAATMRERRRLKKVNSAFETLKRCTT 111
XenopusMyf5         RAPIG----HHQAGNCLMWACKACKRKSSTTDRRKAATMRERRRLKKVNQAFETLKRCTT 111
mouseMyf5           RAPTG----HHQAGHCLMWACKACKRKSTTMDRRKAATMRERRRLKKVNQAFETLKRCTT 111
mouseMyogenin       LGTP-----EHCPGQCLPWACKVCKRKSVSVDRRRAATLREKRRLKKVNEAFEALKRSTL 109
XenopusMyogenin2    SPHPNVTQQEHCPGQCLPWACKVCKRKTVSMDRRKAATLREKRRLKKVNEAFEALKRSTL 120

                                                               *         .
newtMyf5            ANPNQRLPKVEILRNAISYIESLQELLREQVENYYTLPG--QRCSEPGSPLSNCSDGMAE 169
XenopusMyf5         TNPNQRLPKVEILRNAIQYIESLQDLLREQVENYYSLPG--QSCTEPGSPMSSCSDGMSD 169
mouseMyf5           TNPNQRLPKVEILRNAIRYIESLQELLREQVENYYSLPG--QSCSEPTSPTSNCSDGMPE 169
mouseMyogenin       LNPNQRLPKVEILRSAIQYIERLQALLSSLNQEERDLR--YRGGGPQPMVPSECNSHSA 167
XenopusMyogenin2    LNPNQRLPKVEILRSAIQYIERLQTLLASLNQQERDQRDLLFISNGSQRVVSSECGSSSS 180

                    .                *                   *
newtMyf5            CNSPAWSRRNGSFDSAYCSDIPTMYPTDKLSTLSSLDCLSSIVDRISSPEEPALPQQDNL 229
XenopusMyf5         CSSPQWSGRNSSFDNVYCSDLQTSFSSTKL-TLSSLDCLSSIVDRISSPQQCSLPIPDSI 228
mouseMyf5           CNSPVWSRKNSSFDSIYCPDVSNACAADKS-SVSSLDCLSSIVDRITSTEPSELALQDTA 228
mouseMyogenin       SCSPEWGNAL-----EFGPNPGDHLLAADPTDAHNLHSLTSIVDSITVEDMSVAFPDETM 222
XenopusMyogenin2    SCSPEWNDS------DFSGSQSDHLLSDDSSEQRDINSLSSIVNSITSGEVSITYPEQHI 234
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Fig. 3. Alignment of amino acid sequences of various Myf5 and myogenin family members. *The residues in red are conserved between all the MRFs shown; those in green are Myf5 specific while those in orange are myogenin specific. The black residues are not specific to either MRF. The asterisks above the alignment identify Myf5 specific cys residues, and the single dot identifies those Myf5 specific cys residues that immediately precede myogenin specific cys residues. The double dots identify cys residues conserved between the MRFs. Note the cys residue at position 109 which is a Myf5 specific residue in the centre of a highly conserved region for both MRFs. Significant differences between Myf5 and myogenin can also be found after the highly conserved region.*

probe in most cells (Fig. 1 A,B). There were some examples of bipolar cells that reacted with the antisense probe and which could be about to fuse. In contrast, cultures which had been switched to fusion medium showed strong reaction of the antisense probe with multinucleate myotubes and this was not observed with the sense probe (Fig. 1 C,D). The myotubes were analysed in randomly selected fields, and 97% (n = 2005) were clearly positive for reaction with the antisense probe. The expression of Myf5 in the myotube cultures was further confirmed by RNase protection analysis. It is noteworthy that RNA from such cultures was able to protect a full length antisense probe from digestion (Fig. 1D insert).

In view of the striking difference with respect to expression of Myf5 during myogenesis in other vertebrates, we investigated the expression of newt MRF4 in the A1 myotube cultures. This member of the MRF family was not detected in mononucleate A1 cells but was expressed after fusion (Fig. 1 E,F), and this corresponds to findings in other vertebrates, thus underlining the surprising result with Myf5.

Expression in the normal and regenerating newt limb

Striated myofibres of the normal adult limb were strongly positive for reaction with the antisense Myf5 probe in comparison to the sense probe control (Fig. 2 A,B). After amputation of the forelimb at mid-humerus level, the resulting blastema was analysed at the mid-bud stage at 18-20 days post amputation. The mesenchymal and epithelial cells of the blastema were only weakly reactive with both sense and antisense probes (Fig. 2 C,D) and hence no expression was detectable in this tissue at this stage. In contrast, the myofibres at the base of the stump expressed Myf5 (Fig. 2E). In palette stage blastemas at 25 days post amputation the myofibres in the regenerate, and those at the junction of stump and regenerate, were strongly positive for the Myf5 antisense probe (Fig. 2 F-H). These data on expression in the animal are consistent with the findings on cultured A1 cells, demonstrating the absence of Myf5 in mononucleate A1 and blastemal cells, while Myf5 expression was readily detected in multinucleate myotubes and myofibers.

Alignment of newt Myf5 sequence

The onset of expression of newt Myf5 resembles data on the expression of myogenin in other vertebrates, and it is therefore critical to confirm the identity of the newt sequence. Figure 3 shows an alignment with Xenopus and mouse Myf5 and myogenin. The amino acid residues conserved between the different MRFs are in red while Myf5 specific residues are highlighted in green, and myogenin specific residues are in orange. The detailed analysis of newt Myf5 with its family members from other species leaves no doubt that the identity of this sequence is newt Myf5 (see Fig. 3 and legend). In view of the clear difference in regulation of Myf5 in the newt and other vertebrates whose regenerative ability is limited, it was of significant interest to investigate Myf5 expression in relation to the two aspects of plasticity that are characteristic of urodele myotubes and myofibres (Brockes and Kumar, 2002).

Regulation after S phase re-entry

Parallel cultures of A1 cells were maintained in fusion medium for five days, and either maintained in low serum medium or switched to HS medium for a further four days. When analysed for Myf5 expression in LS cultures, the majority of cells expressed Myf5 and only 3.6% of the myotubes were negative (n = 2623). In contrast, in HS medium, significant numbers of myotubes reacted weakly or not at all with the probe, approximately 24.1% were completely negative (n=2633) (Fig. 4 A,B).

In order to investigate the correlation of Myf5 down regulation with S phase re-entry, at the end of the culture period the LS or HS cultures were labelled with ^{3}H-thymidine (TdR). In order to allow for detection of TdR uptake after *in situ* hybridisation the cultures were processed for autoradiography. Figure 4C, taken from a LS culture, illustrates a myotube which is not labelled by TdR, while Fig. 4D, from a HS culture, shows a Myf5 negative/TdR positive myotube next to a Myf5 positive/TdR negative cell. Overall we observed an approximately 5.7 fold increase of TdR labelled myotubes: 5.7% of myotubes (n = 618) were Myf5 negative/TdR positive in LS cultures in comparison to 32.5% (n = 1011) in HS cultures.

As a control probe, we analysed expression of EF-1α and TdR uptake in LS versus HS cultures and observed that EF-1α

expression was not affected by S phase re-entry (Fig. 4 E,F). In conclusion it appears that Myf5 expression is correlated with cell cycle re-entry in the post-mitotic cell. The putative link between re-entry and repression of Myf5 expression could play an important role in the reversal of muscle differentiation and the creation of blastemal cells.

Regulation during cellularisation

In order to induce cellularisation, A1 myotube cultures were treated with the trisubstituted purine myoseverin, or with YC41, an inactive control compound differing by a single methyl substituent (Rosania *et al.,* 2000). The action of myoseverin was originally identified on C2C12 mouse myotubes; however the compound was found to be fully active on A1 myotubes as well. In cultures treated with myoseverin for 24 hours we observed a 5 fold increase in the number of myosin positive mononucleate cells over parallel YC41 treated control cultures, and approximately 50% of these were Myf5 positive (data not shown).

After overnight exposure to myoseverin the newt myotubes showed a marked flattening which was not observed with YC41 (Fig. 5 A,B), and has not been previously reported for the smaller mouse myotubes. Interestingly the flattened multinucleate cells revealed a decreased Myf5 expression (Fig. 5C) which was not observed in parallel cultures treated with YC41 (Fig. 5D). It is possible that the lower signal is an artifact of 'spreading' the mRNA over a larger area, but expression of the control EF1α mRNA was not altered in either culture (Fig. 5 E,F). These results suggest that myoseverin may act to down regulate the expression of myogenic factors before cellularisation has occurred. It is noteworthy that flattened myotubes were often associated with Myf5 positive mononucleate cells which may arise in some cases prior to flattening (Fig. 5C, arrowhead).

In order to provide additional evidence for these events, we combined fluorescent *in situ* detection of Myf5 mRNA with immunostaining for MHC. As illustrated in Fig. 6 A-C for YC41 treated control cultures, Myf5 is co-expressed with MHC in myotubes; neither of the markers, however, were expressed in mononucleate cells. After myoseverin treatment, Myf5 mRNA was down regulated in flattened MHC positive myotubes (Fig. 6 D,E) but as observed above, in some cases MHC and Myf5 double positive mononucleate cells were present – always in close contact with myotubes (Fig. 6 D-F arrowed). This finding

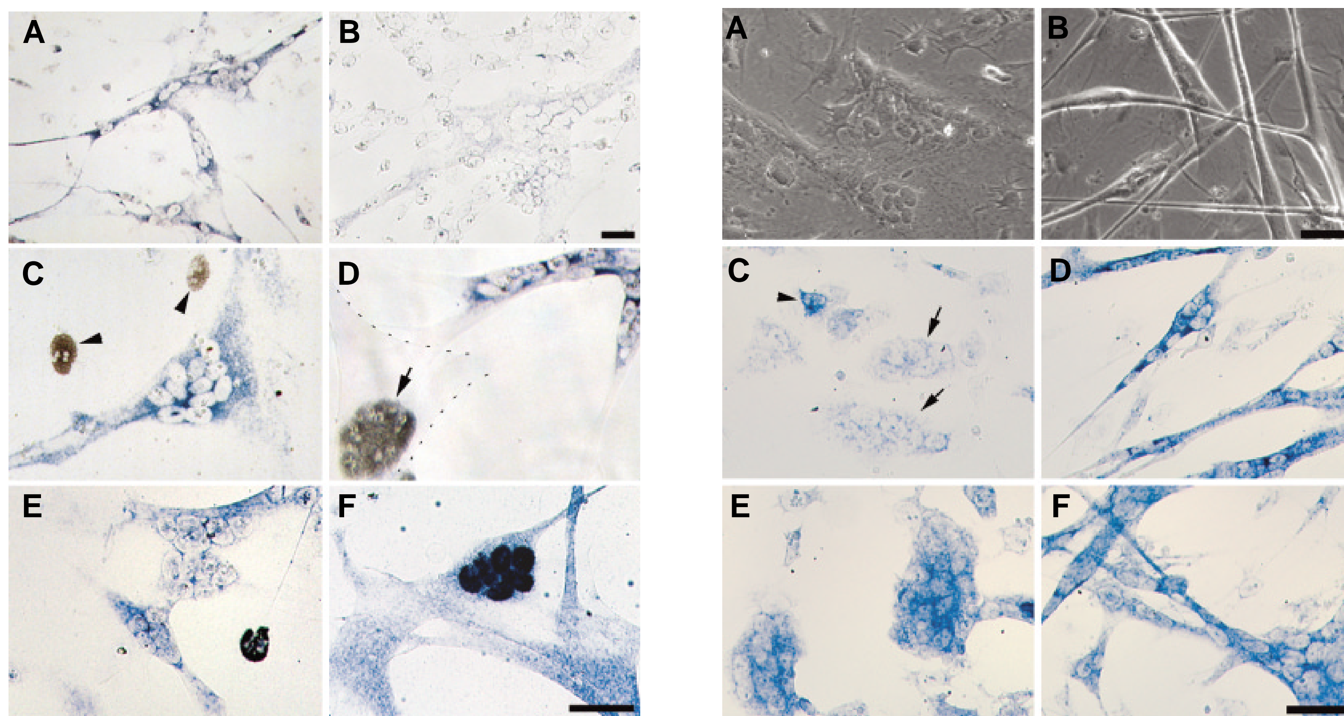

Fig. 4 (Left). Down regulation of *Myf5* in A1 myotubes is correlated with S phase re-entry in high serum medium. (A) Myf5 *expression after hybridisation of antisense probe to myotubes after 9 days culture in LS medium.* **(B)** Myf5 *expression in myotubes after 5 days culture in LS medium and 4 days in HS medium. Note the low expression relative to (A).* **(C,D)** *Correlation of* Myf5 *down regulation in myotubes with* ^{3}*H-thymidine labelling.* **(C)** Myf5 *expression and* ^{3}*H-thymidine labelling of partially purified myotubes in LS medium. Arrowheads show thymidine labelled nuclei of mononucleate cells while the* Myf5 *positive myotube has unlabelled nuclei.* **(D)** Myf5 *expression and* ^{3}*H-thymidine labelling of myotubes in HS medium. Note the absence of* Myf5 *expression in the left myotube with labelled nuclei (arrowed), and the* Myf5 *positive right myotube with unlabelled nuclei.* **(E,F)** *Expression of* EF1-α *is not regulated by S phase re-entry of A1 myotubes.* **(E)** EF1-α *expression and* ^{3}*H-thymidine labelling in LS medium and* **(F)** *HS medium, showing labelled nuclei in one of the myotubes. Scale bar, 100 μm.*

Fig. 5 (Right). Regulation of *Myf5* by myoseverin. *A1 myotube cultures were treated with either myoseverin (A,C,E) or YC41 control compound (B,D,F).* **(A,B)** *Appearance of cultures under phase contrast optics; note the flattening induced by myoseverin.* **(C,D)** *Expression of* Myf5*; note the down regulation in flattened myotubes (arrowed), and the presence of a positive mononucleate cell (arrowhead).* **(E,F)** *Expression of* EF1-α*; note the absence of downnregulation by myoseverin. Scale bar, 100 μm.*

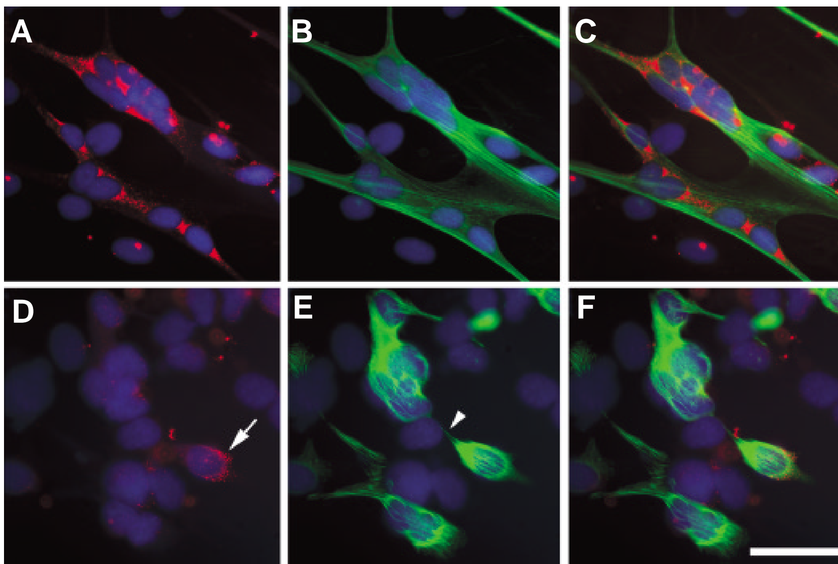

Fig. 6. Double label analysis of budding of mononucleate cells from myotubes. *A1 myotube cultures were treated with either YC41 (A-C) or myoseverin (D-F).* **(A,D)** *Expression of* Myf5 *as detected by fluorescence* in situ *hybridisation (red). Note the down regulation by myoseverin.* **(B,E)** *Expression of MHC protein by immunocytochemistry (green). Note the junction (E, arrowhead) between the mononucleate cell expressing* Myf5 *(D, arrow) and the flattened myotube.* **(C,F)** *Merged image of A,B and D,E respectively. The co-expression of* Myf5 *and MHC appears as yellow/orange. Note difference in myotubes between C and F as a consequence of* Myf5 *down regulation. Scale bar, 100 μm.*

indicates that these mononucleate cells may indeed be derived by budding from the myotube.

Discussion

Our results have disclosed a striking difference between the regulation of Myf5 in urodeles and other vertebrates. Newt Myf5 has an expression pattern analogous to myogenin in other vertebrates in that its mRNA is expressed after fusion of myoblasts into myotubes, a result obtained with two non-overlapping RNA probes including one from the 3' untranslated region. There is only limited information about the expression of Myf5 in anurans at post-embryonic stages; however, there is presently no reason to believe that Xenopus differs from other vertebrates in this regard (Rescan 2001). We suggest that newt Myf5 may be acting in place of or might be substituting for myogenin during myogenesis. It is important to note that the results are not confined to *in vitro* cultures of newt myotubes but also extend to myofibers of the limb *in vivo*. In this regard it is noteworthy that MRF4, another urodele MRF for which a probe is currently available (Simon *et al.,* 1995), has an expression pattern comparable to that in other vertebrates. These findings raise a number of interesting issues for which there are no clearcut answers at present. One relates to the identity of the putative MRF that is expressed in urodele myoblasts in place of Myf5. This clearly requires an analysis of the expression of urodele MyoD and myogenin, as well as candidate upstream myogenic regulators such as Pax3 and Pax7, which have been shown to play a role in muscle lineage specification (Pownall *et al.,* 2002).

A second set of issues arises from the relationships between Myf5 expression in the urodele and the plasticity of myotubes and myofibres. Myf5 is markedly down regulated in cultured A1 myotubes stimulated for re-entry to S phase, and this aspect may parallel the finding that this gene is down regulated in cycling mouse myoblasts (Kitzmann *et al.,* 1998). Nonetheless our data have only established a correlation and it is unclear if S phase re-entry induces down regulation of Myf5, or vice versa, or if the two pathways are independent. We have observed TdR negative, Myf5 negative cells in the cultures but these were so infrequent that no firm conclusion can be drawn. In the context of regeneration it may be important that some of the progeny mononucleate cells derived by cellularisation do not express MRFs, as is apparently the case for the early limb blastema. In a previous study Myf5 expression has been demonstrated in mid bud blastemas (Simon *et al.,* 1995). In that investigation, however, RNA of a blastema pool was used and hence the appropriate time window or subpopulation of non-expressing blastema cells may have been missed. The existence of MRF negative blastema cells may permit an additional flexibility with respect to lineage commitment in the regenerate; for example there is evidence for some degree of transdifferentiation from muscle to cartilage (Brockes and Kumar 2002, Lo *et al.,* 1993). It is also possible that MRF expression might interfere with key properties of the early blastemal cell phenotype.

Although these are plausible rationalisations, it remains unclear why Myf5 should be playing such a role in multinucleate cells rather than say myogenin, assuming that this is present in urodeles. It is interesting that myoseverin causes down regulation of Myf5 prior to cellularisation as well as the formation of Myf5 positive mononucleate progeny. The mechanism

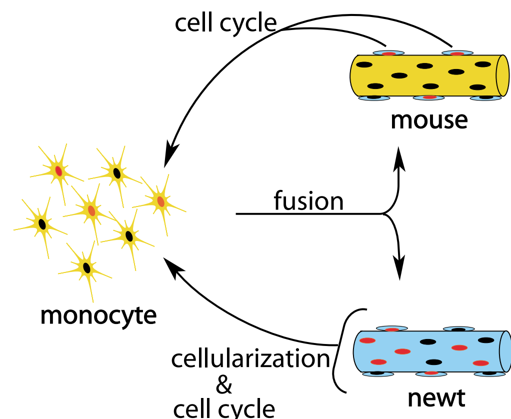

Fig. 7. Schematic diagram illustrating the reservoir function of Myf5 positive cells in mouse and newt. *In the mouse, Myf5 is expressed in mononucleate myoblasts before fusion, and in the reserve satellite cell population (red nuclei) within the basal lamina. In the newt, Myf5 is expressed in multinucleate myofibers and possibly in reserve cells located outside the basal lamina (red nuclei). Myf5 positive myofibers are an important source of mononucleate precursors during appendage regeneration. The blue colour in the cytoplasm of either mouse or newt cells indicates Myf5 expression, and red nuclei denotes the ability to re-enter the cell cycle.*

of action of myoseverin is not understood at present but it clearly has regulatory effects on MRF expression that occur before fragmentation rather than as a secondary consequence of it.

Finally we note that Myf5 expression is an important feature of satellite cells associated with muscle fibers, the mononucleate reserve population that is believed to facilitate regeneration of vertebrate muscle (Beauchamp *et al.,* 2000, Charge and Rudnicki 2004). During epimorphic regeneration in urodeles the myofibers themselves form a comparable resevoir of quiescent cells as illustrated in Fig. 7. Studies on urodeles have identified a potential satellite cell equivalent which is located outside the basal lamina, but its origin and the contribution of this cell to epimorphic regeneration is unclear (Cameron *et al.,* 1986, Popiela 1976). The work of Echeverri *et al.* (2001) has demonstrated that a significant proportion of the cells in the tail blastema derives from cellularisation of striated myofibers. It is also known that epimorphic regeneration can extend over an indefinite number of cycles of amputation without apparent exhaustion of a limited pool of reserve cells. Myf5 expression may in some sense be linked to this extensive proliferative potential and its distinctive expression in urodele myotubes and myofibers certainly warrants further study to understand its functional role in regeneration.

Materials and Methods

Animals

Adult Notophthalmus viridescens were obtained from Charles Sullivan and Co. (Tennessee) and maintained as described (Ferretti and Brockes 1988). After bilateral forelimb amputation at mid-humerus level, newts were allowed to regenerate at about 22°C. Blastema stages were staged according to Iten and Bryant (Iten and Bryant 1973).

Cell culture and myotube purification

Newt A1 cells were cultured essentially as described (Lo *et al.,* 1993; Kumar *et al.,* 2000). Cells were grown on gelatine-coated plastic dishes in 65% Eagles MEM, 10% heat-inactivated foetal bovine serum, 25% H_2O, 10 μg/ml insulin and penicillin/ streptomycin. Myogenesis was induced in confluent plates of A1 cells by lowering the serum concentration from 10% (HS) to 0.5% (LS). After 5 days, > 90% of cells fused into multinucleate myotubes. For myotube purification, the myotubes were trypsinised, neutralised in 0.5% serum-containing medium, sieved through a 100μm nylon mesh (Cell MicroSieve, BioDesing Inc.) to remove clumps, followed by passage through a 35 μm mesh to remove mononucleate cells. The myotubes retained on the 35 μm meshes were washed into LS medium and plated into a fibronectin-coated 35 mm dishes (Sigma, 10-20 μg/ml, 1 hour, room temperature) and left to adhere overnight.

[^{3}H]-thymidine labelling

For thymidine labelling, partially purified myotubes were stimulated in HS medium and exposed to medium containing 1 μCi/ml [^{3}H]-thymidine (Amersham). The plates were fixed, processed for *in situ* hybridisation, dried and coated with Ilford K5 emulsion.

In situ *hybridisation to cultured A1 cells and blastemal tissue*

A 0.35kb fragment (3' untranslated region) of newt Myf5 (Simon *et al.,* 1995) was cloned into Bluescript vector (Stratagene) and the resulting construct pMyf»5 was linearised either with Eco109I or with XbaI. A 0.1kb fragment (3' untranslated region) of newt EF1α was cloned into Bluescript vector (Stratagene) and the resulting construct pNVEF1α was linearised either with XbaI or with XhoI. A 1.25kb fragment of newt MRF4 (Simon *et al.,* 1995) was cloned into Bluescript vector (Stratagene) and the resulting construct pMRF4 was linearised either with NotI or with EcoRI. The respective linearized templates were transcribed with T3 or T7 RNA polymerase to generate digoxigenin-UTP labelled antisense or sense riboprobes following the manufacturer's protocol (Roche).

Cultured newt A1 cells were reacted with one of the digoxigenin-UTP labelled riboprobes. In brief, A1 mononucleate cells or A1 multinucleate myotubes were fixed in 4% paraformaldehyde (PFA) for 10 mins, washed in PBS containing 0.1% Tween 20 (PBT), and stored in 50% ethanol at –20ºC until use. Mononucleate cells or multinucleate myotubes were rehydrated in PBT and hybridised with the Myf5 probe in buffer containing 50% formamide, 5x SSC at 55 ºC overnight. After hybridisation, these A1 cells and myotubes were washed first with 50% formamide, 5x SSC, 1% SDS for 20 mins at 50ºC, followed by a second wash in 50% formamide, 2x SSC, 1% SDS under the same conditions. The cells were reacted with affinity purified alkaline phosphatase-labelled sheep anti-digoxigenin antibody (Roche) and finally developed with BCIP/NBT substrate (Promega) containing 10% polyvinyl alcohol (MW 31,000-50,000; Aldrich), until optimal colour development. *In situ* hybridisation on sections was performed essentially as described (Cash *et al.,* 1998). In brief, animals were anaesthetised with 0.1% tricaine (3-aminobenzoic acid ethyl ester, Sigma) and blastemas were fixed in 4% PFA for at least 4 hours at 4°C and rinsed in PBS followed by dehydration in graded ethanol (25%, 50%, 75%, 90%, 100%), then xylene and paraffin, and embedded. The paraffin blocks were sectioned on a rotary automatic microtome (Leica RM 2155) at 10-13 μm, floated on superfrost plus microscope slides (BDH), dried at 37 °C, and kept at –70°C until use. The sections were rehydrated in graded ethanol (100%, 90%, 75%, 50%, 25%), and successively treated with proteinase K (1 μg/ml), 4% PFA and 50% deionised formamide, and then reacted with the hybridisation solution containing riboprobe. Hybridisation was performed at 50°C for 21 hours and the sections were washed twice with 50% formamide, 2x SSC for 30 mins at 50ºC, RNase A (20 μg/ml) at 37ºC for 5 mins, followed by a wash in 50% formamide, 1x SSC for 30 mins at 50ºC. The sections were reacted with affinity purified alkaline phosphatase-labelled sheep anti-digoxigenin antibody (Roche) and finally developed with BCIP/NBT substrate (Promega) containing 10% polyvinyl alcohol (MW 31,000-50,000; Aldrich), until optimal colour development.

RNase protection assays

RNase protection analysis was performed as described (Cash *et al.,* 1998) with a Myf5 fragment generated by PCR between oligonucleotides CGGAATTCCGGTTCTCGAGGGGCTTTAT and CGGGATCCCACACTTAACTCACTAACAA, cloned into the SmaI site within the polylinker of Bluescribe (Stratagene). A 0.1kb fragment of newt EF1α was used as a normalising control.

Myoseverin treatment

Myoseverin, a 2,6,9-trisubstituted purine (Rosania *et al.,* 2000), was added to A1 myotubes in LS medium at 30-40 μM. DMSO or YC41 were used as vehicle control or as an inactive trisubstituted purine control (Rosania *et al.,* 2000). The myotubes were fixed with 4% PFA at 48-72 h and processed for *in situ* hybridisation with either Myf5 or EF1α riboprobes. Cells were viewed with an Axioskop microscope (Zeiss) for *in situ* hybridisation, with an Axiovert 135 for observation of morphological changes. Phase contrast micrographs were taken by Sony CCD camera (model SSC-M370CE) on the Axiovert 135. Images of *in situ* hybridisation were acquired using a JVC digital camera and Image Pro Plus software (Media Cybernetics, USA). The images were exported to Adobe Photoshop 6.0 (Adobe Systems, USA) for processing and printing.

Double staining by in situ *hybridisation and immunocytochemistry on cultured cells*

In situ hybridisation with fluorescence detection was performed up to the point of the hybridisation washes as described above. The cells were subsequently treated in PBS containing 2% hydrogen peroxide for 20 mins and were then washed in PBS, blocked in 2% blocking reagent (Roche) in buffer 1 (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.1% Tween 20),

and incubated in mouse mAb against myosin heavy chain (MHC, 1:250) as well as HRP-conjugated rabbit anti digoxigenin antibody (Roche, 1:100), followed by TSA Cyanine 3 system (PerkinElmer Life Science, Inc.) to detect Myf5 expression. The cells were incubated for 30 mins at room temperature with FITC-conjugated rabbit anti mouse secondary antibody (DAKO, 1:40), and mounted in 0.1% p-phenylenediamine (Fluka) prior to observation under an Axioplan 2 microscope.

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