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Original Article

Nvβ-actin and *NvGAPDH* as normalization factors for gene expression analysis in limb regenerates and cultured blastema cells of the adult newt, *Notophthalmus viridescens*

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ABSTRACT The red-spotted newt has the ability to fully regenerate complex structures by creating a pool of dedifferentiated cells that arise in response to tissue injury. An understanding of the mechanisms involved in the regenerative ability of the newt is limited by a lack of characterized assays. This deficiency includes the cloning and validation of housekeeping genes for normalizing gene expression data. We describe the cloning, characterization and real-time quantitative PCR evaluation of the normalization potential of the newt homologues of cytoplasmic *β-actin* and *GAPDH* during newt limb regeneration and within the blastemal B1H1 cell line. *Nvβ-actin* demonstrates a heterogeneous expression during limb regeneration and may be associated with differentiation state. The level of *Nvβ-actin* expression in B1H1 cultures under conditions of myogenesis and serum resupplementation varies with the treatment. *NvGAPDH* is ubiquitously expressed during limb regeneration and within B1H1 cultures and does not demonstrate overall variations in expression levels. Thus, *NvGAPDH* is a more appropriate normalization factor in gene expression analyses during limb regeneration and treatments of B1H1 cultures.

KEY WORDS: *regeneration, normalization factor, Notophthalmus viridescens*

Introduction

Many adult urodeles, including the red-spotted newt, *Notophthalmus viridescens*, possess the ability to regenerate entire complex appendages following amputation or serious injury. Structures such as the limbs, tail, lens, retina, craniofacial features and internal organs undergo the process of epimorphic regeneration. This involves the dedifferentiation of mature stump tissues to generate a pluripotent population of cells that proliferate and redifferentiate to restore the injured structure (Reviewed by Wallace, 1981; Mescher, 1996; Brockes, 1997; Bryant *et al.*, 2002; Nye *et al.*, 2003). Newt limb regeneration involves a series of morphological and histological stages that must be completed in order for regeneration to progress normally. These stages include wound healing or covering by a wound epithelium, wound repair, cellular dedifferentiation of tissues to form an early mesenchymal blastema structure, proliferation of these blastemal cells to produce a critical mass and the patterning and morphogenesis of the cells into the complete regenerate (Iten and Bryant, 1973). As a whole, a regenerating limb is a heterogeneous structure comprised of many different tissue and cell types undergoing a multitude of processes including dedifferentiation, proliferation and redifferentiation.

Adult newt B1H1 and A1 cell lines have been derived by the culturing of proliferating blastemal cells and satellite cells derived from intact muscle, respectively (Ferretti and Brockes, 1988). Under conditions of reduced serum concentrations, they may be induced to differentiate into myotubes that express muscle-specific markers (Ferretti and Brockes, 1988; Lo *et al.*, 1993). Upon reimplantation into a regenerating limb, A1 myotubes have the potential to dedifferentiate and transdifferentiate into cartilage (Lo *et al.*, 1993; Kumar *et al.*, 2000). Tanaka *et al.* (1997) demonstrated that serum resupplementation of A1 cultured myotubes leads to cell cycle re-entry and ultimately G2 arrest. As such, the newt blastemal and satellite cell cultures may be ideal systems to evaluate processes or pathways related to dedifferen-

Abbreviations used in this paper: GAPDH, glyseraldehyde-3-phosphate dehydrogenase; Nv, Notophthalmus viridescens; RT-qPCR, real-time quantitative polymerase chain reaction.

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tiation or myogenesis *in vitro*. A limitation of the B1H1 system is that it is heterogeneous in composition and attempts to derive clonal cultures have been largely unsuccessful (Ferretti and Brockes, 1988). Although the basic composition of this culture was characterized at its generation, little is known about the shifts in these populations in response to myotube induction and serum resupplementation.

A key aspect of quantitative comparative gene expression is the use of a suitable normalizing factor to which the levels of the gene of interest may be standardized. An ideal normalization factor demonstrates ubiquitous and stable expression over a wide variety of samples, treatments, conditions and stages of the cell cycle. Housekeeping genes are commonly used because these factors are involved in cell survival or maintenance and their expression levels should not alter dramatically between conditions or states. The most commonly used internal controls are β-*actin*, *GAPDH*, ribosomal RNA (rRNA), *histone H3* and *cyclophilin* (Bustin, 2000; Vandesompele *et al.*, 2002). Recent studies have demonstrated that a single normalizing factor is inappropriate for comparative expression analyses including Northern analysis, real-time quantitative PCR (RT-qPCR) and microarray analysis (Goldsworthy *et al.*, 1993; Thellin *et al.*, 1999; Tricarico *et al.*, 2002; Vandesompele *et al.*, 2002). None of these housekeeping genes have been characterized and validated during newt limb regeneration or in blastemal cell cultures. Previous uses of these genes as normalizing factors have depended upon the assumption that their expression profiles and role in newt studies is the same as in other organisms.

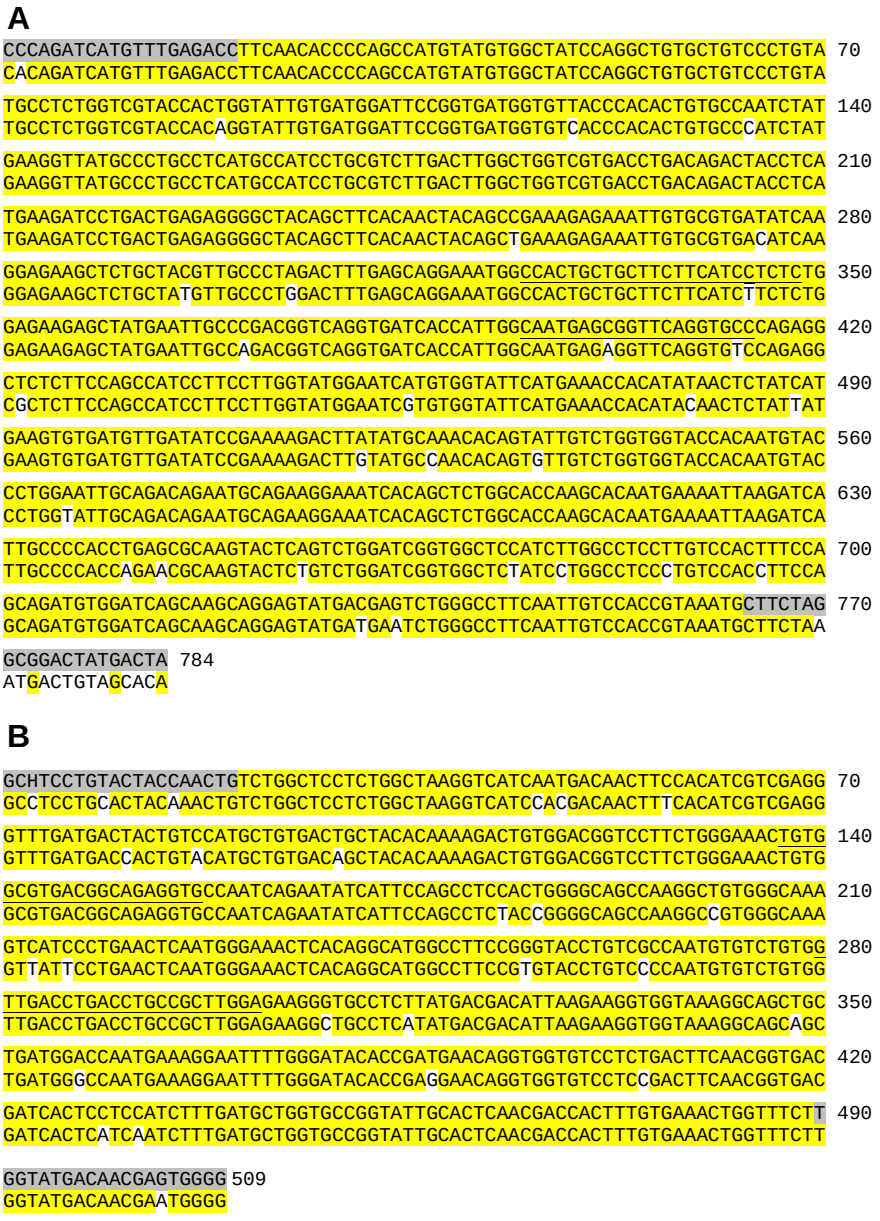

Fig. 1. Nucleotide sequences of *Notophthalmus viridescens* β-*actin (Nvβ-actin)* and *GAPDH (NvGAPDH)* indicating primer locations. (A) *Comparison of* Nvβ-actin *(upper) to* Cynops ensicauda β-actin *(AB117093) (lower) shows 96% identity at the nucleotide level. Shaded areas denote synthetic oligonucleotides used to isolate* Nvβ-actin. **(B)** *Alignment of* NvGAPDH *(upper) to* Pleurodeles waltl GAPDH *(AF343978) (lower) shows 95% nucleotide identity. Shaded areas indicate hybridization site for synthetic degenerate oligonucleotides for* GAPDH. *For both (A,B), underlined regions indicate primer sequence used in RT-qPCR.*

The actins are a class of structural proteins involved in cell structure, mobility and filopodia extension. Isoforms of *actin* have demonstrated differential expression during different stages in the regeneration process. For example, during tail regeneration, *striated muscle actin* is downregulated during dedifferentiation and subsequently upregulated during muscle differentiation (Khrestchatisky *et al.*, 1988). Yang and Zalik (1994) demonstrated heterogeneous expression of α and γ muscle actins during lens regeneration, while filamentous actin proteins appeared ubiquitous. Characterization of *actin* expression in *Fugu rubripes* suggests that, of all types examined, cytoplasmic β-*actin* demonstrates the broadest expression profile and this form is frequently employed as a normalization factor in other systems (Venkatesh *et al.*, 1996). Therefore, the newt homologue of cytoplasmic β-*actin* (*Nvβ-actin*) may be an appropriate candidate to pursue for normalization purposes.

GAPDH is an essential glycolytic enzyme that results in phosphorylation of glyceraldehyde-3-phosphate into 1,3-biphosphoglycerate and the reduction of NAD+ to NADH. It is one of the most commonly used genes for normalization because its expression has been either presumed or demonstrated to be stable in a wide variety of tissues and experimental conditions. GAPDH is generated in a relatively low copy number, has a low turnover rate, has few pseudogenes and demonstrates sufficient homology across genera to make it a relatively easy target for cloning (Sturzenbaum and Kille, 2001). Despite its wide usage, however, for some systems *GAPDH* is considered to be a poor normalizing factor because of its involve-

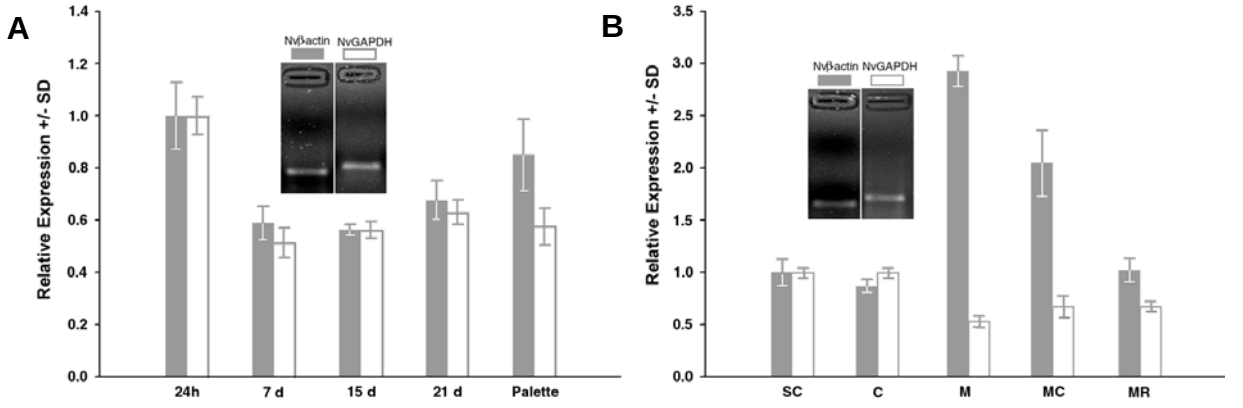

Fig. 2. Real-time RT-qPCR analysis of *Nvβ-actin* and *NvGAPDH* during limb regeneration and B1H1 culture. (A) *RT-qPCR of regenerating limbs reveals that* Nvβ-actin *and* NvGAPDH *expression levels are similar from 24 hours until 21days after amputation. In the regenerating palette, however,* Nvβ-actin *expression is upregulated.* **(B)** *RT-qPCR analysis of* Nvβ-actin *and* NvGAPDH *expression in subconfluent (SC), confluent (C), myogenic (M), extended myogenic cultures (MC) and FBS-resupplemented myogenic (MR) cultures demonstrates stable* NvGAPDH *expression throughout (within a 1.5 fold variation).* Nvβ-actin *expression is elevated in myogenic and extended myogenic cultures, but decreases when the myotubes are resupplemented with FBS. Insets in both (A,B) show an ethidium-bromide stained agarose gel obtained during the log phase of PCR amplification, indicating the specificity of the primers.*

ment in other cellular activities (Sirover, 1996; Tatton *et al.*, 2000; Mazzola and Sirover, 2002). Many studies that invalidate *GAPDH* as an internal standard have focused on single cell masses such as tumours, structures such as developing collateral arteries, microdissected tissues containing contaminating cells, or individual cells such as erythrocytes (Thellin *et al.*, 1999; Bustin, 2000; Schmittgen and Zakrajsek, 2000; Goidin *et al.*, 2001; Hamalainen *et al.*, 2001; Deindl *et al.*, 2002; Tricarico *et al.*, 2002). Recently, the *GAPDH* homologues for the axolotl (*Ambystoma mexicanum*) and *Pleurodeles waltl* were cloned and used for semiquantitative RT-PCR expression studies (Christensen *et al.*, 2001; Ferretti *et al.*, 2001). Although not specifically evaluated, the apparent stable expression lends credibility for isolating the newt *GAPDH* homologue (*NvGAPDH*) as another normalization factor for gene expression analyses.

We describe the cloning and characterization of the expression profile of *Nvβ-actin* and *NvGAPDH* during adult newt forelimb regeneration and in B1H1 cultures under conditions of proliferation, myogenesis and serum resupplementation. While *NvGAPDH* expression is expressed in most cell types during regeneration and in B1H1 cultures, *Nvβ-actin* expression is heterogeneous and seems to be correlated to cells representing a less differentiated phenotype. Our current results suggest that *NvGAPDH* may be an appropriate candidate for use as a normalizing factor for characterizing gene expression during limb regeneration or in the heterogeneous B1H1 cell cultures and that caution must be exercised in relying upon the stable expression of newt cytoplasmic β-*actin*.

Results

Identification of newt β-actin *and* GAPDH *cDNA fragments*

The PCR amplification of *Nvβ-actin* using human derived primers yielded a 784 base pair (bp) fragment that contains 2/3rds of the 3' ORF of β-*actin* (Fig. 1A). All BLAST analyses identified this fragment primarily as cytoplasmic β-*actin* and secondarily as cytoskeletal γ-*actin*. *Nvβ-actin* has 96% nucleotide identity to *Cynops ensicauda* β-*actin* and 100% amino acid identity to *Xenopus laevis* cytoplasmic β-*actin* (data not shown).

Amplification with degenerate *GAPDH* primers isolated a 509 bp sequence that was identified as GAPDH by BLAST searches (Fig. 1B). The fragment shows 96% nucleotide identity and 97% amino acid identity to *Pleurodeles waltl GAPDH*. As well, NvGAPDH has high similarity to axolotl (85%), *Oncorhynchus mykiss* (90%) and *Gallus gallus* (90%) polypeptides (data not shown). Overall, *NvGAPDH* is a highly conserved gene at both the nucleotide and amino acid levels. The nucleotide mismatch at the 5' end may not be real and may result from degeneracy in the oligonucleotides. The resulting codon, nevertheless, specifies the same amino acid.

RT-PCR analysis of β-actin and GAPDH expression during limb regeneration

Both *NvGAPDH* and *Nvβ-actin* primers are ideal for real-time PCR analysis. The primers amplified a single product and demonstrated no primer-dimer formation (Fig. 2). The PCR efficiencies were above 90%, with *NvGAPDH* and *Nvβ-actin* at 91.8% (R^2 = 0.996) and 95.5% (R^2 = 0.992), respectively (data not shown). Comparison between both normalizing genes was valid because the absolute slope of ΔCT is 0.094 (p = 0.162, R^2 = 0.102), below an arbitrary cut-off value of 0.1 indicating that the relative PCR efficiencies are equal (data not shown).

Comparison of the ΔCT values revealed that expression values for both normalizing factors was similar up to 21d post-amputation (Fig. 2A), at which point *Nvβ-Actin* levels significantly increased while *NvGAPDH* remained the same (ANOVA, p = 0.03; Tukey Test, p < 0.05). Replication with different RNA sources yielded similar profiles.

Spatial expression of newt β-actin and GAPDH during newt regeneration

In situ hybridization analysis of *Nvβ-actin* expression during limb regeneration demonstrates a spatially heterogeneous distribution of transcription (Figure 3). During dedifferentiation, there is

strong *Nvβ-actin* expression in the cells of the dedifferentiating distal margin (Figure 3A). During the blastemal stages, there is strong staining within the dedifferentiated and proliferating blastemal cells as well as throughout all layers of the thickening apical epithelial cap (Figure 3 A-C). There is strong expression throughout the wound epithelium up to and including the 21d regenerate. During the palette stage there is staining throughout the condensing cartilage, differentiating myotubes and blastemal cells (Figure 3 D-F). In the palette, there appears to be a greater proportion of the cells stained positively for *Nvβ-actin* versus either the 10 or 21d regenerates due to a larger component of the section being composed of the intensely staining differentiating paddle. At this point there is a distinct lack of expression in the upper layers of the newly differentiated epidermis. Within intact tissues *Nvβ-actin* is localized in satellite cells adjacent to muscle fibers (Figure 3G) and dispersed cells of the periosteum and connective tissues. Within intact skin, *Nvβ-actin* expression is localized to basal dermal cells but is not expressed in the epidermis (Figure 3H). The majority of the glands, pigmented cells, cells of the stratum spongiosum, muscle myotubes, mature keratinized epidermis and red blood cells were not stained (Figure 3H and data not shown).

NvGAPDH is almost ubiquitously expressed throughout the regeneration process, although the expression intensity varies by tissue (Figure 4). Extensive staining is observed in the muscle, connective tissues, epithelial cells and distal blastemal cells of the 7d regenerate (Figure 4 A,F,G). Noted is the occasional staining of the small subcutaneous glands, little staining of the stratum spongiosum and stratum compactum layers and no staining of the stratum serosa layer. While the 15d regenerates demonstrate similar staining patterns as the 5d (data not shown) and 7d regenerates, they also show greater staining of blastemal cells (Figure 4B). The 21d regenerate demonstrates heavier and more uniform expression throughout the blastema and notably less staining in dedifferentiated cells at the base of the regenerate (Figure 4 C,H). The palette stage sections demonstrate extensive staining in the condensing chondrocytes and differentiating myotubes and uniform expression in the distal blastemal cells (Figure 4 D,I).

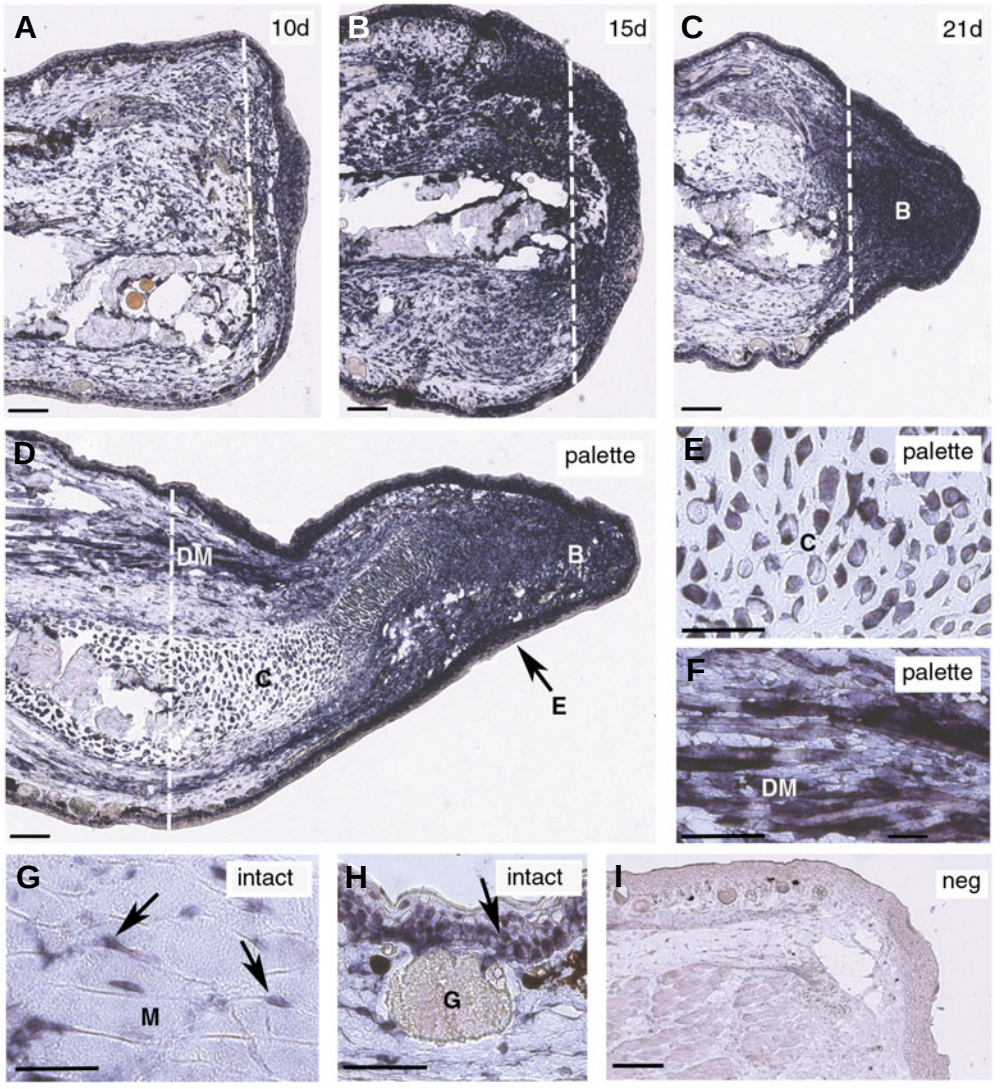

Fig. 3. *Nvβ-actin* expression in adult newt forelimb regenerates. *Intense* Nvβ-actin *staining is apparent in the blastema cells and the overlying wound epithelium in 10 day* **(A)**, *15 day* **(B)** *and 21 day* **(C)** *regenerates. In the regenerating palette* **(D-F)**, Nvβ-actin *expression is present in the condensing cartilage (C), differentiating myotubes (DM) and blastema cells (B). However, there is no expression in the upper layers of the newly differentiated epidermis (E). In the intact stump, staining is seen in the satellite cells of muscles (arrows in* **(G)** *) and in the basal cell layer of the epidermis (arrow in* **(H)***). Dotted line indicates amputation plane. (M) intact muscle, (G) integument gland. Scale bar, 100 μm.*

RT-PCR analysis of newt β-actin and GAPDH expression in B1H1 culture

Expression levels for both genes were examined for subconfluent and confluent B1H1 cells, for 4d and 8d myotube cultures and for myotubes resupplemented with fetal bovine serum (FBS). *NvGAPDH* and *Nvβ-actin* expression levels are equal in subconfluent and confluent cultures. However, while *NvGAPDH* demonstrates uniform expression levels for all B1H1 cell culture conditions, *Nvβ-actin* expression is elevated during myogenesis (Figure 2B). *Nvβ-actin* is significantly upregulated upon serum withdrawal and returns to subconfluent levels with serum resupplementation (ANOVA, $p < 0.05$; Tukey Test, $p < 0.05$). This was replicated three times with three different RNA sources and showed consistent results. In order to determine whether these profiles are affected by minute variations in starting material or pipetting errors, the differences in threshold cycle (ΔCT) be-

tween *NvGAPDH* and *Nvβ-actin* were evaluated. These differences should not be significant if the expression levels of the genes did not vary since both samples are amplified from the same master mix. Under myogenic conditions there are large variations in ΔCT between *NvGAPDH* and *Nvβ-actin*, suggesting that *Nvβ-actin* levels are dramatically changing between different culture conditions (data not shown).

Changes in cellular populations in B1H1 cells in response to different culture conditions

B1H1 cultures are heterogeneous. In order to determine whether the changes in *Nvβ-actin* expression seen with the qRT-PCR result from differential expression in specific cell types, we examined changes in cell types or morphologies under different culture conditions. The results of the morphology counts are summarized in Figure 5. Stable subconfluent B1H1 cultures are comprised of approximately 80% pleiomorphic, 10% bipolar, 10% signet and less than 1% giant cells. Notable features include the distinct lack of myotube structures in these cultures.

The presence of myotubes is first identified in confluent cultures even prior to the reduction in serum. Eight days after decreasing the serum concentration, myotubes make up approximately 25% of the total culture. In the long-term myotube culture, although the number of multinucleated myotubes is high, the majority would not be classified as mature as described by Ferretti and Brockes (1988). Coincident with myogenesis is a significant increase in the proportion of bipolar cells ($p < 0.05$). There is a significant decrease in the proportion of signet cells with an increase in confluency and differentiation, as well as a significant increase in the proportion of giant cells (ANOVA, $p < 0.05$).

With serum resupplementation, the proportion of bipolar cells, which had increased with myogenesis, returns to basal levels. As well, there is a concomitant increase in the proportion of pleiomorphic cells. The percentage of myotubes declines from 25% of the culture to less than 10%. Myotubes that are maintained are large, mature and contain definite sarcomere striations of skeletal muscle, which are only observed to a limited degree in short term myotube cultures.

Spatial expression of newt β-actin and GAPDH in B1H1 culture

In situ expression analysis of *Nvβ-actin* and *NvGAPDH* in confluent B1H1 cultures produced specific intense cytoplasmic staining in all cell types of the heterogeneous cultures (data not shown). This includes those bearing myotube, pleiomorphic, osteoclast-like and signet-like cell morphologies as previously described (Ferretti and Brockes, 1988; Maier and Miller, 1992; Washabaugh and Tsonis, 1994; Eddinger, 1998; Corcoran and Ferretti, 1999). Within the limits of detection of the *in situ* hybridization assay, the changes in *Nvβ-actin* shown by the RT-qPCR analysis could not be attributed to changes in expression in any particular cell type, although the *relative* levels of expression *within* specific cell types was difficult to assess.

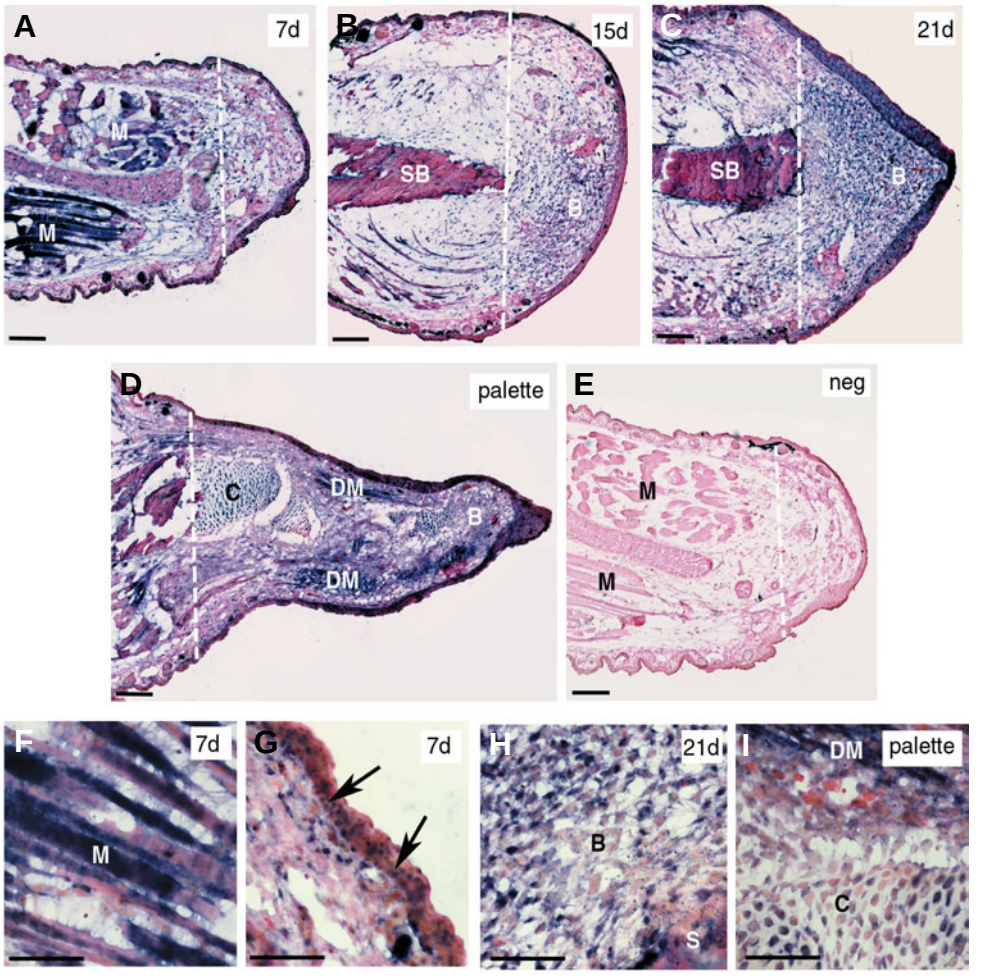

Fig. 4. *GAPDH* expression in adult newt forelimb regenerates. *Intense staining is apparent in the muscle (M), connective tissue, basal epithelial cells and blastema cells of the 7 day* **(A,F,G)**, *15 day* **(B)** *and 21 day* **(C,H)** *regenerates. In the regenerating palette* **(D,I)**, *intense staining is present in the differentiating muscles (DM), chondrocytes (C) and distal blastema cells (B). Dotted line indicates amputation plane. (SB) bone of intact stump, (M) myotubes. Scale bar, 100 μm.*

Discussion

Conservation of cytoplasmic β-actin and GAPDH in urodeles

The structural actins are a class of proteins represented by multiple subtypes with relatively distinct distributions (Khrestchatisky *et al.*, 1988; Yang and Zalik, 1994; Venkatesh *et al.*, 1996). They have demonstrated high conservation across genera and are frequently used as standards for expression studies at either the RNA or protein levels (Vandesompele *et al.*,

2002). The successful amplification of *Nvβ-actin* using primers derived from human cytoplasmic β-*Actin* confirms nucleotide conservation across species. Due to over 90% amino acid identity between actins both across species and between isoforms within a species (Venkatesh *et al.*, 1996), we used RT-PCR to isolate newt β-*actin*. The primers chosen for the amplification are in a region of the gene which demonstrates lower conservation between actin isoforms within a species. Consequently, the amplification product derived from this region may demonstrate higher specificity for β-*actin* over other members of the family (Venkatesh *et al.*, 1996).

GAPDH demonstrates a high degree of conservation from bacteria to eukaryotes, clearly showing the importance of this gene in metabolism. Degenerate oligonucleotides were used to amplify a 509 bp fragment by RT-PCR. *NvGAPDH* demonstrates almost 96% nucleotide identity to *GAPDH* in the urodele *P. waltl.*

Nvβ-actin expression is not ubiquitous during regeneration

In situ hybridization analysis on sections of intact and regenerating adult newt forelimbs revealed a heterogeneous pattern of *Nvβ-actin* expression. In the limb regenerates, *Nvβ-actin* was strongly expressed in the dermis, blastema, satellite cells between myotubes and a sub-population of cells throughout the connective tissue. This suggests that newt cytoplasmic β-*actin* is not ubiquitous in the context of an intact limb and regenerate but may only be expressed in a subpopulation of cells. Expression of *Nvβ-actin* is unlikely to be a marker specific to actively cycling cells, since it is expressed in many cell types proximal to the amputation plane and also satellite cells that are quiescent until triggered to re-enter the cell cycle. *Nvβ-actin* may also be associated with cells that have the potential to contribute to regeneration. Connective tissues, satellite cells and dermis all contribute to the regeneration blastema (Chalkley, 1954; Hay and Fischman, 1961; Lo *et al.*, 1993).

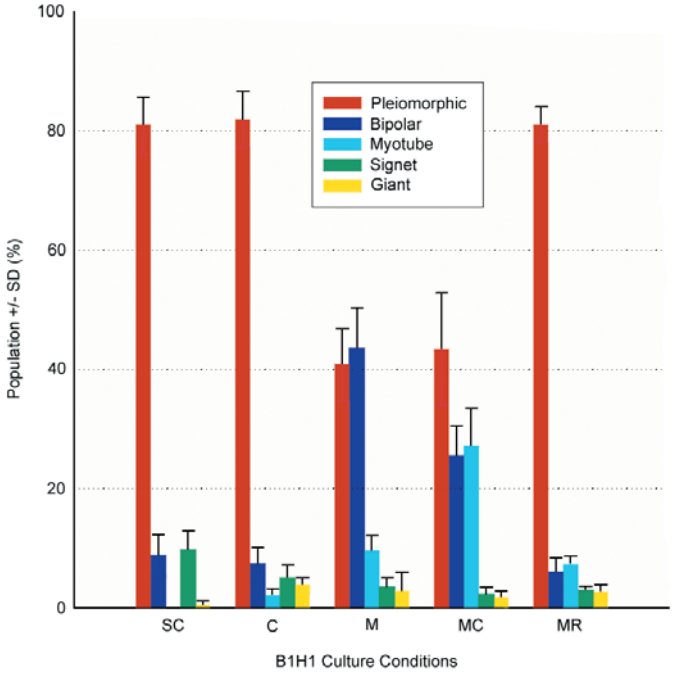

Fig. 5. Cell composition analysis of the heterogeneous B1H1 cultures *during subconfluent (SC), confluent (C), 4-day myotube induction (MI), 8-day myotube induction (MC) and 4-day myotube cultures resupplemented with serum (MR). Counts indicate a change in morphological frequency with the onset and continuation of myogenic conditions and restoration to near original frequencies with serum resupplementation. See text for description of individual cell types.*

NvGAPDH is ubiquitously expressed during limb regeneration and throughout B1H1 culture conditions

NvGAPDH displayed a heterogeneous expression pattern throughout various regenerating tissues. Transcripts were detected in the epidermis, dermal layers, mesenchymal cells, periosteum, muscle cells and differentiating cells. Tissues with elevated rates of metabolism (e.g., muscle), as well as actively proliferating cells appeared to have stronger staining. Furthermore, *NvGAPDH* was equally expressed in all cell morphologies in B1H1 cultures undergoing proliferation, myogenesis (serum withdrawal) and serum resupplementation.

B1H1 cell morphologies change with different culture conditions

Maier and Miller (1992) performed an in depth analysis of primary cell culture derived from the blastema. From 14d blastemas, they identified four main cell morphologies. The population was composed of 60-90% large, stellate transparent pleiomorphic cells, 10-40% non-proliferating signet cells bearing autofluorescent granules, less than 1% bipolar cells and less than 1% round multinucleate giant cells (Maier and Miller, 1992; Washabaugh and Tsonis, 1994). Based on their fluorescent properties, morphology and ability to interact with each other *in vitro* the signet cells were hypothesized to represent leukocytes of hematopoietic origin and the giant cells were thought to represent osteoclasts (Maier and Miller, 1992; Washabaugh and Tsonis, 1994).

A number of stable cell lines have been derived from newt and axolotl tissues and blastemata. These include the clonal primary cultures derived from the ependyma during tail regeneration in *P. waltl*, an axolotl limb connective tissue cell line and the blastema cell (B1H1) and muscle satellite cell lines (A1) derived from *N. viridescens* (Ferretti and Brockes, 1988; Benraiss *et al.*, 1996; Roy *et al.*, 2000). The A1 cell line and to a lesser degree the blastema derived B1H1 cell line, have been used to address issues of pluripotency, myogenesis, myotube cell cycle reentry and complete dedifferentiation (Lo *et al.*, 1993; Tanaka *et al.*, 1997; Kumar *et al.*, 2000; McGann *et al.*, 2001).

Overall, both the A1 and B1H1 cultures are heterogeneous in morphology. We examined whether the differential expression patterns obtained for *Nvβ-actin* under different culture conditions resulted from changes in cellular morphology. A number of observations were noted. Firstly, the cellular composition of the B1H1 line is different in subconfluent cultures compared with primary blastema cultures. Maier and Miller (1992) suggest that bipolar cells and signet cells represent <1% and 10-40%, respectively in the primary cultures. We identified bipolar and signet cell morphologies as each representing approximately 10% of the culture. In no case did the signet cell composition increase to become a major component and as the cell density increased, the signet cell composition significantly declined. Coincidental with

the decrease in signet cells, there was an increase in the proportion of giant cells. This finding supports the hypothesis of Washabaugh and Tsonis (1994) that signet cells may fuse to produce giant cells. Another interesting result is the significant increase in the proportion of myotubes and bipolar cells with myotube induction. This is in agreement with the suggestion that bipolar cells may represent immature myotubes (Ferretti and Brockes, 1988; Maier and Miller, 1992). In fact, prior to fusion into myotubes, pleiomorphic cells align and seem to acquire "bipolar" morphologies and are nonproliferative (Ferretti and Brockes, 1988). The increase in proportion of pleiomorphic cells upon serum resupplementation along with the decrease in myotubes and bipolar cells may be due to proliferation of remaining undifferentiated pleiomorphic cells, or a decrease in myotubes and bipolars due to cell death, or fusion of myotubes and bipolar cells into larger and more mature myotubes. While Lo *et al.* (1993) indicate that A1 myotubes contain up to several hundred nuclei, Kumar *et al.* (2000) suggest that A1 myotubes tend to contain between 3 and 6 nuclei. The current study found that most B1H1 myotubes tend to be disordered, clumped and contain between 2 and 3 nuclei, in agreement with Corcoran and Ferretti (1999).

A surprising difference between the B1H1 cell line and the published A1 culture data may be the existence of signet and giant cells in the B1H1 cultures. Although these cell types were identified in primary cell cultures derived from the blastema (Ferretti and Brockes, 1988; Washabaugh and Tsonis, 1994), they were not previously described in the B1H1 long-term cultures. Washabaugh and Tsonis (1994) concluded that these cell types were non-dividing leukocytes derived from the circulating blood. The maintenance of these cells in the B1H1 cultures suggests that they may be spontaneous differentiation products of blastema cells. Observations of differing ratios of signet to pleiomorphic morphologies between populations are consistent with this latter interpretation (Maier and Miller, 1992). Since previous observations on primary blastema cultures suggest that bipolar morphs, giant cells and particularly signet cells do not appear to proliferate *in vitro* (unpublished observations, Maier and Miller, 1992; Washabaugh and Tsonis, 1994), their existence should have been selectively lost since the inception of the B1H1 line. In addition, the heterogeneous morphologies of B1H1 cells reappear in cultures derived from single cells (data not shown). Overall, our results suggest that all the different cell morphologies can be derived from a single cell type and that the morphological composition of B1H1 cultures is variable under different conditions and distinct from the published A1 line. This variation should be considered when evaluating the impact of a treatment upon the B1H1 line.

NvGAPDH is a better normalization factor for expression studies in vivo *and* in vitro *than Nvβ-actin*

NvGAPDH is an acceptable normalizing factor for analysis of gene expression during newt limb regeneration and B1H1 blastema cell cultures. During regeneration, this gene is ubiquitously expressed and demonstrates a stable expression profile as identified by qRT-PCR. In addition, *NvGAPDH* appears to be stably expressed in B1H1 cultures undergoing proliferation, myogenesis and serum resupplementation. Although GAPDH has been demonstrated in other systems to fluctuate within differentiation (Steele *et al.*, 2002), during experimental treatments (Zhong and Simons, 1999; Schmittgen and Zakrajsek, 2000) and between some cancer cells and lines (Gong *et al.*, 1996; Kim *et al.*, 1998; Wu and Rees, 2000), these differences tend to be cell-specific (Gorzelniak *et al.*, 2001). Although there are likely subtle differences in expression levels in specific areas undergoing regeneration in adult newt structures, these differences are likely buffered by the adjacent intact tissues. In the polyclonal B1H1 cell line, *NvGAPDH* does not appear to fluctuate greater than a factor of 1.5 under various culture conditions, even though GAPDH has been implicated in nonglycolytic activities such as membrane transport and fusion, microtubule assembly, nuclear RNA export, translational control, phosphotransferase/kinase reactions, apoptosis, proliferation and DNA replication and repair (Epner *et al.*, 1999; Sirover, 1999). Although these changes may be occurring within B1H1 cultures and/or during limb regeneration in the adult newt, the degree of change is not large enough to produce a significant change in the overall expression level as detected by qRT-PCR.

By qRT-PCR, *Nvβ-actin* and *NvGAPDH* show similar profiles in the regenerating forelimb up to 21 days post-amputation. In the palette stage regenerate, *Nvβ-actin* expression increases, but this may be somewhat expected due to differentiation events. The spatial expression analysis demonstrates the heterogeneity in the distribution of *Nvβ-actin* positive cells. Studies of cytoplasmic β-*actin* in mouse suggest the potential for differential expression in mouse tissues (Tokunaga *et al.*, 1988). Surprisingly, *Nvβ-actin* expression was upregulated in our B1H1 cells under conditions of myogenesis, but downregulated in longer-term myogenic cultures and with serum resupplementation. Although myogenic cultures contain a sub-population of pleiomorphic cells which increases in the longer-term cultures, the proportion of these cells is not as high as within subconfluent cultures. As such, one might expect a decrease in cytoplasmic β-*actin* expression. *In situ* analysis suggests that the myotubes within myogenic cultures still express *Nvβ-actin*. As this analysis did not evaluate the relative levels of expression between the different cell types, it is possible that specific cell types may be expressing *actin* at a higher level in myogenic cultures. Alternatively, there is evidence to suggest that β-*actin* may be associated with moving cytoplasm (Hoock *et al.*, 1991) and the B1H1 cell migration, alignment and fusion during myogenesis could explain the increase in expression. This is supported by the high expression of *Nvβ-actin* during *in vivo* chondrogenesis and within early myotubes and further supports the assertion that β-*actin* should not be used when connective tissue physiology is under states of active flux (Sturzenbaum and Kille, 2001). Other studies of β-*actin* expression have found it to increase during keratinocyte differentiation and to vary significantly during experimental conditions in certain cell lines (Zhong and Simons, 1999; Schmittgen and Zakrajsek, 2000; Steele *et al.*, 2002). Although expression increases during myogenesis, *Nvβ-actin* can be used as a normalizing factor during proliferative states since expression appears to be relatively stable.

Conclusions

The current studies identify the sequences of housekeeping genes *Nvβ-actin* and *NvGAPDH* which can be used as normalizing factors for adult newt gene expression analyses. Expression profiles for both genes suggest that *NvGAPDH* is a more suitable

normalizing agent for both *in vitro* and *in vivo* regeneration studies in the adult newt.

Materials and Methods

Animals

Adult red spotted newts (*N. viridescens*) were obtained from Charles Sullivan (Tennessee) and maintained under standard conditions (Cameron *et al.*, 2004). Forelimb amputations were performed at the mid-stylopodium under MS-222 anesthesia (Cadinouche *et al.*, 1999). Samples were harvested after 1, 5, 7, 10, 15, 21 days post-amputation and at the palette stage for *in situ* hybridization expression analysis and qRT-PCR.

B1H1 Cell Culture

B1H1 cells were maintained on 1% gelatin coated plates (Ferretti and Brockes, 1988). Myotube induction and serum resupplementation were performed based on the methods of Lo *et al.* (1993). Subconfluent cultures (SC) were harvested at ~70% confluency under normal culture conditions in A.MEM with 10% FBS. Myogenic samples (M) were harvested following 4 days of culture in low serum medium and long-term myogenic samples (MC) were harvested following 8 days in this medium. For serum resupplemented myogenic cultures (MR), cells were maintained in low serum medium for 4 days and in 10% serum medium for an additional 4 days prior to harvesting.

For morphological composition analyses, cultured cells incubated under the above conditions were counted in 6 random fields of view in a 10 cm diameter culture dish using an Olympus inverted microscope at 40X. Cultured newt cells are relatively large with visible nuclei, allowing them to be easily observed. The classification of cell morphology was as follows: Pleiomorphic cells were those that were flat, large, stellate and transparent (Ferretti and Brockes, 1988; Maier and Miller, 1992). Bipolar cells were defined as long, thin, mononucleate cells with apparent adhesion points at both poles (Ferretti and Brockes, 1988; Maier and Miller, 1992). Signet cells were relatively small and round with no extensions, had a small centrally located nucleus and could contain visible cytoplasmic granules (Maier and Miller, 1992; Washabaugh and Tsonis, 1994). Giant cells were much larger than pleiomorphic cells, contained one or more centrally located large nuclei and bore visible "filaments" radiating from the nucleus towards the cell (Ferretti and Brockes, 1988; Washabaugh and Tsonis, 1994). Myotubes were much larger than pleiomorphic cells, contained multiple nuclei and demonstrated cytoplasmic longitudinal striation patterns (Ferretti and Brockes, 1988; Eddinger, 1998; Corcoran and Ferretti, 1999). The count for each morphological cell type was normalized to the total number of counts per field of view and the results evaluated by ANOVA.

Isolating newt cytoplasmic β-actin and GAPDH cDNA fragments

Low stringency RT-PCR was performed using human cytoplasmic β-*actin* specific primers on the first strand product generated from newt B1H1 total RNA. Specifically, total RNA was isolated from subconfluent B1H1 cells using Trizol (Invitrogen). First strand cDNA was generated from 1 μg of total RNA using M-MLV reverse transcriptase (Invitrogen) and the specific reverse primer (Figure 1A). The reverse primer is at the 3' end of the human β-*actin* gene and includes the ORF termination sequence and part of the 3' UTR. RT-PCR using 5 μl of first strand cDNA and the forward and reverse primers (Figure 1A), was performed in a final reaction mixture of 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 125 ng of each primer and 0.2 U *Taq* DNA Polymerase (Invitrogen). The PCR condition involved an initial denaturation at 94ºC for 3 minutes followed by 30 cycles of 94ºC for 60 seconds, 55ºC for 90 seconds and 72ºC for 120 seconds on a Perkin Elmer 480 Thermocycler. The product was analyzed by agarose gel electrophoresis and cloned using the pGEM-T Easy Cloning System II (Promega) as per manufacturer's instructions. Clones were sequenced on ABI automated sequencers using the M13 forward and reverse primers.

Degenerate *GAPDH*-specific oligonucleotides were designed after aligning nucleotide sequences of *GAPDH* from *Bos taurus* (U85042), *Columba livia* (AF036934), *Gallus gallus* (AF047874), *Homo sapiens* (NM_002046), *Mus musculus* (AK002273), *Oryctolagus cuniculus* (L23961), *Oncorhynchus mykiss* (AF027130), *Pleurodeles waltl* (AF343978), *Sus scrofa* (AF017079) and *Xenopus laevis* (U41753). The resulting forward and reverse primers (Figure 1B) yielded an expected amplicon of ~510 bp corresponding to the *gapC* fragment (Mounaji *et al.*, 2002). cDNA synthesis was generated from 2 μg liver total RNA as above. PCR was performed using 100 ng of the forward and reverse primers and a 1:25 dilution of the first strand cDNA under the reaction conditions: initial denaturation of 95°C for 2 minutes, 30 cycles of 94ºC for 1 min, 49ºC for 1 min and 72ºC for 50 s on an Eppendorf Mastercycler Gradient Thermocycler. Following analysis by electrophoresis, the PCR product was cloned into pGEM-T Easy vector, recombinants were screened and clones were sequenced, as above.

Newt β-*actin* and *GAPDH* were amplified, cloned and sequenced independently at least twice to obtain a consensus nucleotide sequence.

In situ *hybridization analysis*

Preparation of tissues for *in situ* analysis was the same regardless of source. Briefly, tissues were fixed overnight at 4°C in 4% paraformaldehyde in PBS and then equilibrated in 30% sucrose in PBS overnight. Following a one hour incubation in 1:1 OCT and 30% sucrose in PBS, tissues were flash frozen in the OCT:sucrose solution. Embedded tissues were sectioned at 16 um and mounted on Superfrost Plus (Fisher) slides.

Digoxygenin-UTP labeled RNA probe was generated as per Cadinouche *et al.*(1999). The 784 bp *Nvβ-actin* probe was generated from the cloned fragment following linearization with *SpeI* and *NcoI* to generate sense and anti-sense probes, respectively. Antisense and sense *NvGAPDH* probes were generated by digestion of the recombinant *NvGAPDH* clone with *SpeI* and *NcoI*, respectively. Sectioned *in situ* expression analyses were performed as per Myat *et al.* (1996). The reaction was stopped by washing with 1X PBS and sections were either mounted in 50% glycerol in PBS or counterstained with Eosin-Y and mounted with Permount. *In situ* expression analysis on B1H1 tissue cultures was performed as per Cash *et al.* (1998).

Real-time quantitative RT-PCR analysis

To evaluate the relative PCR efficiencies of both *NvGAPDH* and *Nvβ-actin* primers, first strand cDNA was generated from 2 μg of DNaseI-treated total RNA from confluent B1H1 cells primed with 500 ng each of oligo(dT) and random hexamers (Promega) in a 30 μL reaction using the M-MLV reverse transcriptase (Invitrogen). Serial dilutions of first strand product of 1:2, 1:10, 1:20, 1:100, 1:200, 1:1000 and 1:2000 were used for RT-qPCR. The 25 μL PCR reaction included 1X PCR Buffer, 2 mM $MgCl_2$, 200 μM of each dNTP, 200 nM of each primer (Figure 1), 0.8 U *Taq* DNA polymerase, 3:125000 SYBR Green I Dye (Molecular Probes) and 8 nM calibration dye (Bio-Rad). RT-qPCR was performed on a iCycler iQ real-time thermocycler (Bio-Rad) using the following parameters: an initial denaturation step of 3 min at 95°C, 40 cycles of 94°C and 63°C of 25 sec each. This was followed by a 10 min extension at 72°C with a dissociation-curve analysis of a 60 sec denaturation step at 95°C, annealing step for 60 sec at 55°C and 80 cycles of 0.5°C increments every 10 sec. Each reaction condition was performed in triplicate and the products were subjected to 2.5% agarose gel electrophoresis to ascertain specific PCR amplification. ΔCT and standard deviation values were calculated as per Livak and Schmittgen (2001).

To profile *Nvβ-actin* and *NvGAPDH* expression during regeneration, RT-qPCR was performed on first strand cDNA derived from 2 μg of total RNA from intact newt limb and regenerate samples harvested at 1, 7, 15, 21 days post-amputation and at the palette stage. First strand cDNA was also generated, as above, from subconfluent B1H1 cells, B1H1 cultures

following myotube induction with low serum and myotube cultures following resupplementation of medium with 10% FBS. RT-qPCR was performed independently for *Nvβ-actin* and *NvGAPDH* using the methodology described above. Relative fold overexpression and standard deviation values were calculated as per Livak and Schmittgen (2001). Results were analysed by ANOVA and Tukey statistical tests.

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