REVERSIBILITY OF THE MONONUCLEATE-TO-MULTINUCLEATE MYOGENIC TRANSITION DURING AMPHIBIAN LIMB REGENERATION

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SUMMARY

Muscle differentiation involves the fusion of mononucleate myoblasts to form multinucleate syncytial myotubes. In order to assess reversibility of the mononucleate-to-multinucleate transition in urodele limb regeneration, myotube formation was induced in cultured newt limb blastemal cells. Myotubes were purified, replated at low density and injected with a cytoplasmic lineage tracer. In some cases nuclei of myotubes were labelled by incorporation of tritiated thymidine. Labelled myotubes were stable in culture for 6–8 weeks and no transfer to mononucleate cells was observed. The myotubes were implanted under the wound epidermis of a hindlimb blastema. Labelled mononucleate cells were observed 1 week after implantation and such cells could derive both the cytoplasmic lineage tracer and the nuclear marker. The number of such cells increased by 2–3 weeks after implantation. These results provide strong support for the reversibility of muscle differentiation during urodele limb regeneration, and raise questions about the mechanism of such a reversal.

INTRODUCTION

Cellular differentiation is often viewed as irreversible, but recent work has suggested that maintenance of the differentiated state requires continuous regulation and hence that the process is reversible[1,2]. Much of this work has been concerned with the transcriptional regulation of differentiation, and the requirement for active suppression of genes that are inappropriate for a particular cell type. De-differentiation, the reversal of differentiation, and trans-differentiation, a switch to another cell type, are of particular interest in this context[3]. Much of the literature about these processes is concerned with phenomena originally observed in regeneration, for example the transition between retinal pigment epithelium and lens in Wolffian regeneration. The present account is concerned with reversal of muscle differentiation. The fusion of myoblasts to give multinucleate myotubes is one of the most striking examples of differentiation. It is also a key system for studying molecular mechanisms since the discovery of the myogenic genes, transcription factors of the helix-loop-helix family which are able to impose a myogenic phenotype after transfection into non-muscle cells[4,5].

One context where muscle de-differentiation may occur is limb regeneration in urodele amphibians such as the newt and axolotl. After amputation of a limb the wound surface is healed by migrating epithelial cells which form the wound epidermis. The blastemal cells, the progenitors of the regenerate, arise from the mesenchymal tissue underneath the wound epidermis. One possible source of blastemal cells is by de-differentiation of multinucleate myofibres to yield mononucleate blastemal cells which then proliferate and contribute to the regenerate[6–8]. Such reversal of the mononucleate-to-multinucleate transition has been difficult to establish unequivocally and remains controversial[9,10]. In this paper we have exploited the availability of cultured newt blastemal cells by inducing myotube formation, injecting the myotubes with a lineage tracer, and implanting them under the wound epidermis of a limb blastema[11]. Our results do not speak directly to the contribution of resident myofibres to the blastema, but they do establish the reversibility of the mononucleate-to-multinucleate transition of vertebrate myogenesis.

RESULTS AND DISCUSSION

The design of the experiment is illustrated in Fig. 1 and full details are given in Lo *et al.*[11] Cultured newt myotubes were enriched relative to mononucleate cells by sieving through Nylon mesh. The resulting population was plated at low density to allow unambiguous identification of myotubes, and subsequent injection of rhodamine-conjugated lysinated dextran as lineage tracer. In some experiments the nuclei of myotubes were labelled by prior incubation in medium containing [^{3}H]thymidine. Examples of labelled myotubes are shown in Fig. 3.

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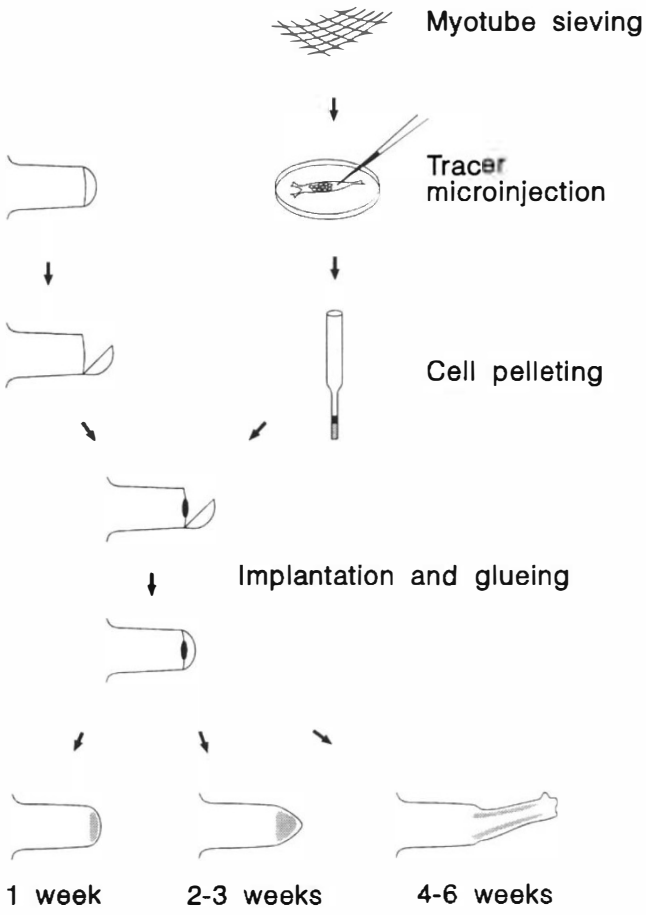

Fig. 1. *Schematic representation of an implantation experiment. After injection of lineage tracer into individual myotubes in culture, myotubes were implanted under the wound epidermis of a hind limb blastema. After regeneration, limbs were collected at three different times and analysed by fluorescence microscopy. (Reprinted from Lo* et al.[11] *with permission.)*

The substituted dextran is not taken up by cultured newt cells, nor is it transferred to mononucleate cells in high-density culture. Its properties as a lineage tracer have been established by experiments in a variety of different systems. The cultured cells were trypsinised, pelleted and implanted under the wound epidermis of a hind limb blastema.

The blastemas were analysed by sectioning and fluorescence microscopy to determine the fate of implanted myotubes. At 1 week after implantation labelled mononucleate cells were observed in the vicinity of the implant (Fig. 2*a*). These cells were strongly positive for the lineage tracer, and in cases where the nuclei had been labelled with [^{3}H]thymidine, we observed mononucleate cells with both a labelled nucleus (after autoradiography) and with cytoplasmic lineage tracer (Fig. 2*b,c*). The incidence of labelled mononucleate cells was consistent with de-differentiation of at least 15–20% of the input nuclei in myotubes, although it should be noted that this is a lower estimate.

By 2–3 weeks after implantation the number of labelled cells had increased, indicating that the products of de-differentiation were capable of proliferation. Numerous

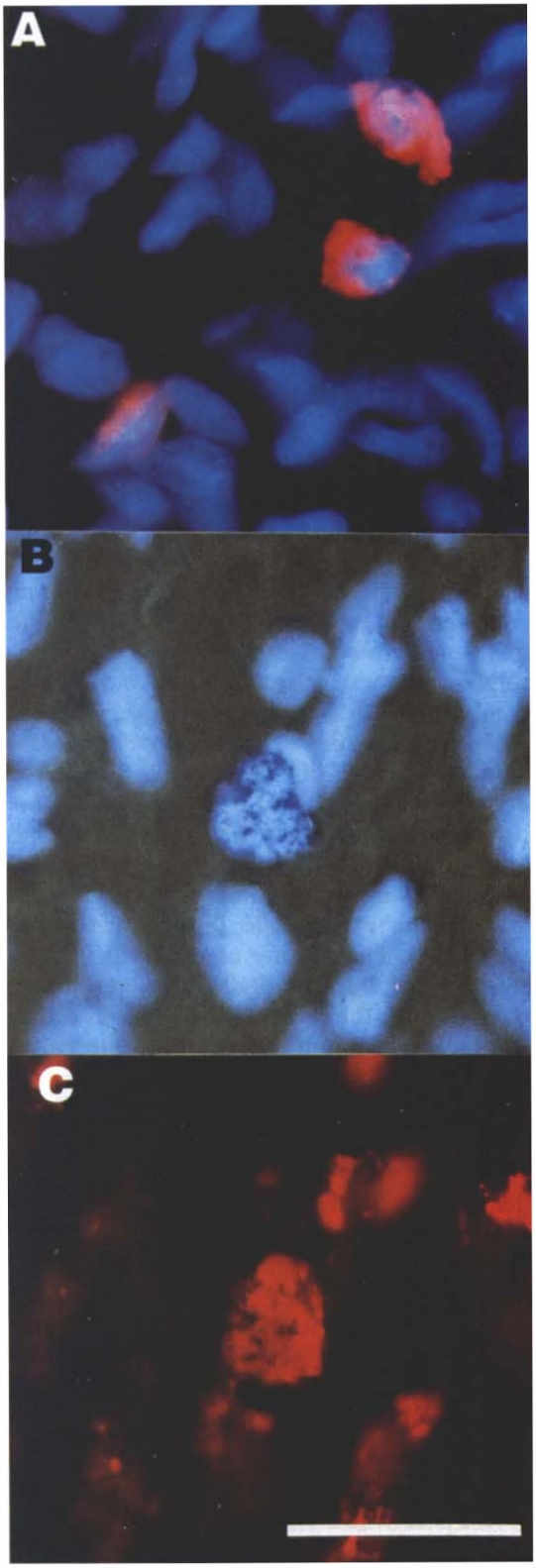

Fig. 2. *Labelled mononucleate cells in sections of regenerating limbs 9–10 days after implantation of labelled myotubes. (a) Three mononucleate cells with rhodamine-labelled cyoplasm from the lineage tracer. (b) Mononucleate cell labelled with [^{3}H]thymidine and visualised after autoradiography. (c) Same cell as in (b) labelled by fluorescence to show the lineage tracer. Scale bar represents 50 μm. (Reprinted from Lo* et al.[11] *with permission.)*

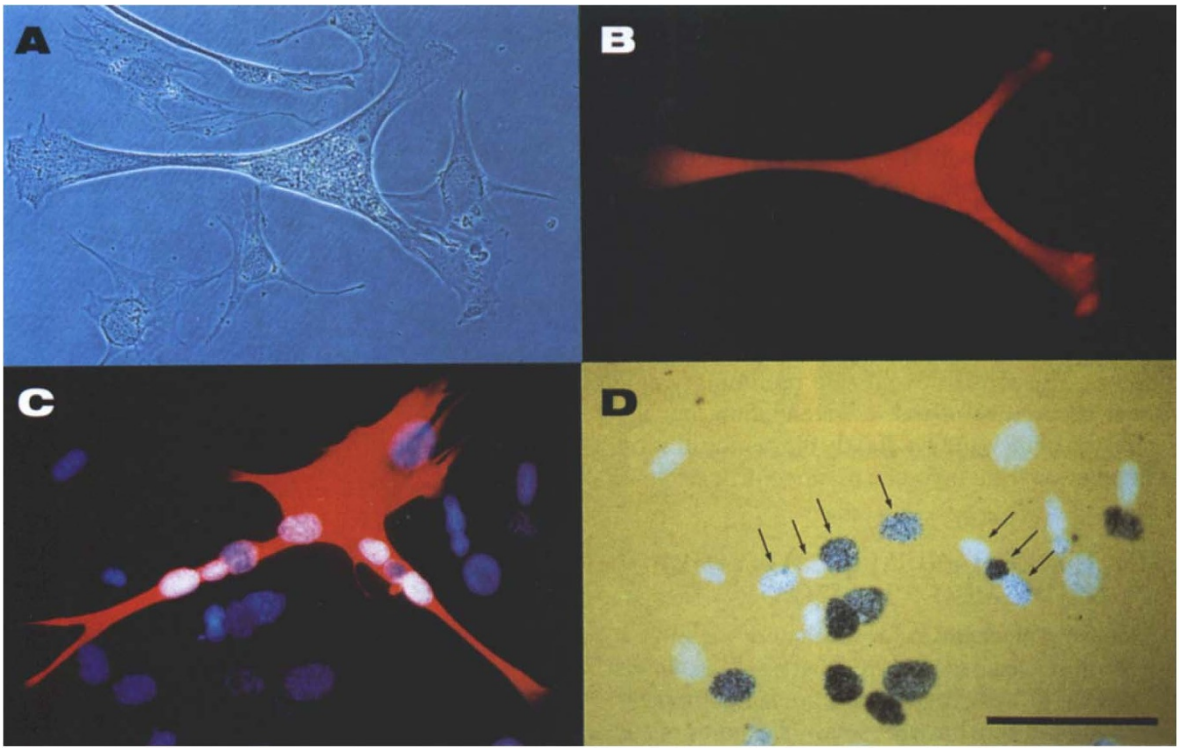

Fig. 3. *Cultured newt myotubes labelled by intracellular injection. A labelled myotube is shown with (a) phase-contrast microscopy and (b) fluorescence optics. A myotube previously labelled with [^{3}H]thymidine and microinjected, is shown with Hoechst stained nuclei by (c) fluorescence or (d) bright-field and fluorescence to show nuclei with silver grains. Scale bar represents 200 μm. (Reprinted from Lo* et al.[11] *with permission.)*

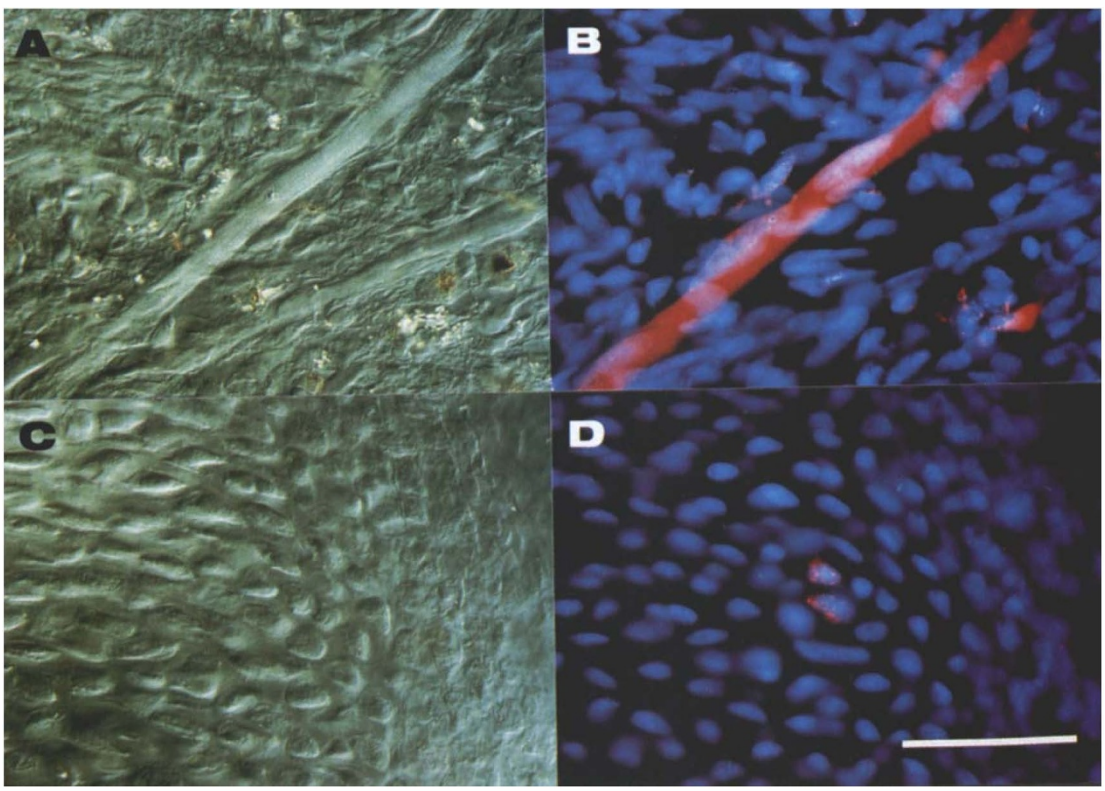

Fig. 4. *Muscle fibre and cartilage cells in sections of regenerating limbs after implantation of labelled myotubes. (a) and (b) show a labelled muscle fibre 9 days after implantation under differential interference optics or fluorescence. (c) and (d) show differentiating cartilage 26 days after implantation also under interference or fluorescence. Note the two rhodamine-labelled nuclei within the cartilage matrix. Scale bar represents 100 μm. (Reprinted from Lo* et al.[11] *with permission.)*

examples of strongly labelled muscle fibres were observed at this stage but it is unclear whether these were derived from fusion with myotubes or with mononucleate cells. At 4–6 weeks after implantation there was significant cartilage differentiation in the regenerate. By this time the number of cells labelled with the dextran had decreased, presumably because of dilution due to cell division. Labelled cells were nonetheless occasionally observed in cartilage, generally in clusters of two to four (Fig. 4*c,d*). While cartilage can be identified reliably in the regenerating limb at this stage with light microscopic criteria, it will be necessary to confirm these observations by use of molecular markers. The present results are preliminary evidence for the occurrence of transdifferentiation, but the major conclusion of this study is clearly the demonstration of de-differentiation from myotube to mononucleate cells in the blastema.

POSSIBLE MECHANISM OF MYOTUBE DE-DIFFERENTIATION

The most plausible mechanism for reversal of the mononucleate-to-multinucleate transition is that nuclei enter the cell cycle, and the process of cytokinesis eventually fragments the myotube. There have been several studies of the effects of viral oncogenes, in particular SV40 large T antigen, on inducing DNA synthesis in the nuclei of cultured rodent myotubes. For example, Iujvidin *et al.*[12] have shown that after introducing large T into primary myoblasts and inducing fusion, nuclei in multinucleate fibres synthesise DNA and enter mitosis. Such myotubes contain late prophase and metaphase nuclei, but these events lead to destruction of the fibres, possibly as a result of programmed cell death. The availability of cultured newt myotubes should allow us to investigate the cellular and molecular mechanisms of de-differentiation *in vitro*. Thus it will be interesting to introduce T into these cells and to determine whether they follow the same course as rodent myotubes or a different one, such as successful completion of cytokinesis. The ability to make these cellular transitions may be a fundamental aspect of regeneration.

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Key words: Limb regeneration, Lineage tracer, Muscle, Urodele amphibian.

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