Heterogeneous proliferative potential in regenerative adult newt cardiomyocytes

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Summary
Adult newt cardiomyocytes, in contrast to their mammalian counterparts, can proliferate after injury and contribute to the functional regeneration of the heart. In order to understand the mechanisms underlying this plasticity we performed longitudinal studies on single cardiomyocytes in culture. We find that the majority of cardiomyocytes can enter S phase, a process that occurs in response to serum-activated pathways and is dependent on the phosphorylation of the retinoblastoma protein. However, more than half of these cells stably arrest at either entry to mitosis or during cytokinesis, thus resembling the behaviour observed in mammalian cardiomyocytes. Approximately a third of the cells progress through mitosis and may enter successive cell divisions. When cardiomyocytes divided more than once, the proliferative behaviour of sister cells was significantly correlated, in terms of whether they underwent a subsequent cell cycle, and if so, the duration of that cycle. These observations suggest a mechanism whereby newt heart regeneration depends on the retention of proliferative potential in a subset of cardiomyocytes. The regulation of the remaining newt cardiomyocytes is similar to that described for their mammalian counterparts, as they arrest during mitosis or cytokinesis. Understanding the nature of this block and why it arises in some but not other newt cardiomyocytes may lead to an augmentation of the regenerative potential in the mammalian heart.

Supplemental data available online

Key words: Plasticity, Cardiomyocytes, Regeneration, Heart, Newt, Cell cycle

Introduction
The newt heart is a model for adult heart regeneration as newts can functionally regenerate their heart after amputation of the apex of the ventricle. A remarkable feature of this process is that adult newt cardiomyocytes can proliferate after injury and contribute to the regenerative tissue (Oberpriller and Oberpriller, 1974; Oberpriller et al., 1995). This regenerative ability is not present in the adult mammalian heart. Cardiomyocytes in the mammalian ventricle withdraw from the cell cycle soon after birth, and subsequent growth of the heart is dependent on cellular hypertrophy and proliferation of other cell types (MacLellan and Schneider, 2000). Adult mammalian cardiomyocytes are often referred to as postmitotic: DNA synthesis is detectable at a low frequency in either normal or infarcted adult heart (Beltrami et al., 2001; Poolman et al., 1998; Soonpaa and Field, 1998), and cell cycle progression is tightly regulated. When cardiomyocytes are driven into S phase, for example by adenoviral delivery of E2F, they cannot progress to mitosis and accumulate at the G2/M boundary (Agah et al., 1997). In the adult newt ventricle the baseline proliferation of cardiomyocytes is comparably low, but after removal of ventricular tissue at the apex there is extensive DNA synthesis and mitosis in the vicinity of the wound (Oberpriller and Oberpriller, 1974; Oberpriller et al., 1995). The majority of cells undergoing DNA synthesis or mitosis have been recognised at the ultrastructural level as cardiomyocytes (Bader and Oberpriller, 1979), and no population of stem cells has been identified in the newt myocardium. These data highlight the important role of plasticity in adult cardiomyocytes for repair of the newt ventricle.

It is possible that the plasticity of the differentiated state in cardiomyocytes is related to that in other newt tissues. An adult newt is also able to regenerate its limbs and tail and ocular tissues such as the lens and retina (Brockes, 1997; Goss, 1969). In these contexts, the plasticity of the differentiated state is a key mechanism for the generation of progenitor cells (Brockes and Kumar, 2002; Brockes et al., 2001). For example, after amputation of the limb or tail, skeletal myofibres in the distal stump as well as implanted myotubes are able to re-enter the cell cycle and to fragment into viable mononucleate cells (Echeverri et al., 2001; Kumar et al., 2000; Lo et al., 1993; McGann et al., 2001; Velloso et al., 2000). In the case of the cultured newt myotube, DNA synthesis can be stimulated by mammalian serum and depends on phosphorylation of the retinoblastoma protein (Rb) (Tanaka et al., 1999; Tanaka et al., 1999). However, more than half of these cells stably arrest at either entry to mitosis or during cytokinesis, thus resembling the behaviour observed in mammalian cardiomyocytes. Approximately a third of the cells progress through mitosis and may enter successive cell divisions. When cardiomyocytes divided more than once, the proliferative behaviour of sister cells was significantly correlated, in terms of whether they underwent a subsequent cell cycle, and if so, the duration of that cycle. These observations suggest a mechanism whereby newt heart regeneration depends on the retention of proliferative potential in a subset of cardiomyocytes. The regulation of the remaining newt cardiomyocytes is similar to that described for their mammalian counterparts, as they arrest during mitosis or cytokinesis. Understanding the nature of this block and why it arises in some but not other newt cardiomyocytes may lead to an augmentation of the regenerative potential in the mammalian heart.

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differentiated cells, or are there defined sub-populations of cells that have this ability? Do newt cardiomyocytes progress through several cycles, or do they show the complex regulation of cell cycle progression of the mammalian cardiomyocyte? Finally, is cell cycle re-entry triggered by mammalian serum and is it dependent on Rb phosphorylation? To answer these questions we have exploited a culture system where S phase entry and cell cycle progression can be analysed in single cells.

Materials and Methods
Preparation and labelling of cardiomyocytes
Adult newt (Notophthalmus viridescens) ventricles were removed and stored overnight at 25°C in 70% L-15 medium (AL15). They were digested in 2 ml phosphate-buffered saline with 0.5% Bactotrypsin (Difco), 380 U/ml collagenase (Sigma), 0.15% bovine serum albumin and 0.3% glucose for 8 hours in a shaking water bath at 27°C, with a change of the enzyme solution every 2 hours. The resulting cell suspension was added to 4 ml amphibian MEM [AMEM; 70% MEM (Ferretti and Brookes, 1988; Tanaka et al., 1999)] with 10% FBS, passed through a 100 μm microsieve, and centrifuged to collect the cells. The pellet was resuspended in AMEM (10%FBS) and preplated in 6 cm culture dishes for 3 days at 25°C in a humidified CO₂ incubator. Blood cells, connective tissue cells and some pigmented cells attached to the culture dish during this step, while myocytes remained in the non-adherent fraction, in suspension. The myocytes were plated onto laminin-coated dishes at a density of 4000 cells/cm² in AMEM (10% FBS). For studies on the dependence of serum concentration for re-entry, the medium was washed and changed to AMEM 0.5% FBS 2 days after plating, and cells were stimulated with different concentrations of FBS 4 days later.

Cells were labelled with 3-bromo-2-deoxyuridine (BrDU; 10 μg/ml) or with [³⁵S]thymidine (0.01 μCi/ml). For cumulative labelling with [³⁴C]thymidine, fresh medium with label was added every 3 days until day 15, and then medium without label was added for 3 days prior to fixation and autoradiography with Ifford K5 emulsion. The concentration of [³⁴C]thymidine chosen for these experiments did not affect cell cycle progression (results not shown). For pulse labelling, BrDU was added to the medium and cells were fixed 8, 9 or 18 hours afterwards depending on experimental convenience, as described in the figure legend.

Microinjection of cells
Injection of plasmid DNA or fluorescently labelled dextrans was performed with a Narishige manipulator connected to a pneumatic micropipet. Cells were microinjected (Barrie et al., 2003) for more details on the antibody]. PKH-labelled cells were fixed with 4% PFA for 5 minutes followed by permeabilisation with TBS-0.1% Tween 20 for 10 minutes. Phosphatase treatment was performed by incubating 100 U/coverslip of lambda phosphatase (NEB) at 30°C for 5 minutes. Controls were performed using phosphatase inhibitors (NAF: 10 mM; B-glycerolphosphate: 10 mM). Controls for all antibodies and cell staining
Mouse monoclonal antibodies were used against BrdU (BU-20; Amersham), sarcomeric myosin heavy chain (A4.1025; Dr Simon Hughes, Randall Institute, King’s College, London), p16 (DCS-50.2, used at 10 μg/ml; Dr Gordon Peters, Imperial Cancer Research Fund, London), titin and troponin T (clones 9D10 and CT3, Dr Elisabeth Ehler, ETH, Zurich), Rb (51B7, used at 13 μg/ml; Dr Sybille Mittnacht, ICR, London, available from Serotec (MCA2104), see Barrie et al. (Barrie et al., 2003) for more details on the antibody]. 51B7 is specific for Rb as it stained SF 295 Rb-positive cells (NIC); but not the matching SF 539 cells (NCI; Rb deletion). The other antibody was polyclonal rabbit: anti-phospho histone H3 (Upstate Biotechnology, New York, USA). For BrdU, anti-phospho histone H3, p16 and sarcomeric proteins staining, cultured cells were fixed, after rinsing with PBS, with 100% methanol at –20°C for 5 minutes. Cells were processed for BrdU and MyHC staining as previously described (Tanaka et al., 1999). PKH-labelled cells were fixed with 4% paraformaldehyde (10 minutes), permeabilised with 0.5% saponin (BDH) in PBS for 30 minutes and stained for MyHC. Cells labelled with dextran were fixed in 4% paraformaldehyde in PBS containing 0.2% Triton X-100 pH 7.4 for 10 minutes and stained for BrdU and MyHC. Cells containing alkaline phosphatase were fixed with acid alcohol (5% glacial acetic acid in ethanol) at –20°C for 5 minutes. Endogenous alkaline phosphatase activity was destroyed by incubation in PBS at 65°C for 15 minutes, and cells were developed using ELF-97 (Molecular Probes). Cells for Rb staining were fixed in 4% PFA for 5 minutes followed by permeabilisation with TBS-0.1% Tween 20 for 10 minutes. Phosphatase treatment was performed by incubating 100 U/coverslip of lambda phosphatase (NEB) at 30°C for 30 minutes. Controls were performed using phosphatase inhibitors (NAF: 10 mM; B-glycerolphosphate: 10 mM). Controls for all antibodies were performed by omitting primary antibody incubation or by using mouse IgG as a primary. DNA was stained with Hoechst 33258 (1 μg/ml).

Results
Newt cardiomyocytes undergo DNA synthesis and mitosis in dissociated culture
Adult newt cardiomyocytes were dissociated by proteolysis. Routinely, 40-60% of the cells obtained were cardiomyocytes,
as evidenced by the distinct elongated shape and the presence of striations. The resulting suspension was enriched by differential adhesion, as cardiomyocytes do not adhere to the plastic of the culture dish. Newt cardiomyocytes have a branched morphology which is different from the rod shape of their mammalian counterparts, and striated myofibrils were clearly visible (Fig. 1A-C). After plating the suspension of cells onto laminin more than 98% of the cells were mononucleate, and more than 90% were cardiomyocytes as evidenced by staining for expression of troponin T and sarcomeric myosin heavy chain (MyHC; Fig. 1E,F). The cells spread slowly and after 1 week in culture many had formed junctions and started to beat synchronously (Fig. 1D). Myocytes continued to be the majority of the population of cells even after long periods of culture (24 days).

In an initial analysis of cell cycle progression, we determined if cardiomyocytes could incorporate BrdU and undergo mitosis as assessed by staining for phosphorylated histone H3. The cells entered S phase (Fig. 2A,B and D) with a peak at 10 days after plating (Fig. 2D) when 25.8% were strongly labelled with a pulse of BrdU. A peak of mitotic activity was also observed at 10 days (Fig. 2C,D), and mitotic cells were readily detected under phase contrast optics with their prominent chromosomes (Fig. 2E1,E2). They often remained flat and attached to neighbouring cells, and during mitosis most of the myofibrils seem to have disassembled and the remaining were seen in the cell periphery (Fig. 2E1-E4). Occasionally, myofibrils extended into the cleavage furrow at cytokinesis. The daughter cells often resumed beating after division.

The time course and extent of DNA synthesis (Fig. 2D) were comparable to those reported for adult newt ventricular cells after injury in situ (Bader and Oberpriller, 1979). In Bader and Oberpriller’s experiment, the tip of the ventricle was cut, minced and grafted back into the ventricle in order to increase the number of myocytes near the wound surface. Animals were sacrificed 1 hour after being injected intraperitoneally with tritiated thymidine. The percentage of labelled cells in the minced graft (morphological criterion used to distinguish cardiomyocytes from other cells) was determined. They reported a peak of DNA synthesis at 16 days after injury where 24% of the cells in the graft incorporated the label. These similarities suggest that this culture system is appropriate to study the mechanisms regulating plasticity.

**Progression and arrest in single cells; S phase and mitosis**

In several mammalian species, cardiomyocytes become polyploid after birth as a result of a G2 arrest or inability to finish cytokinesis (Brodsky, 1991; MacLellan and Schneider, 2000; Soopna et al., 1996). In order to assess the proliferative potential of newt cardiomyocytes, we determined the proportion of these cells that enter S phase and the subsequent progression of each cell through the cycle. We analysed the progression of single cells by following them for 18 days by time lapse microscopy. Cardiomyocytes were plated onto a dish scored with a numbered grid and pictures of the grid squares were taken once or twice a day. Since cells did not move between different squares, we could follow every cell division in each square for 18 days. To avoid any effect of density-dependent inhibition of mitosis, the squares selected for analysis had on average only 2-3 cells. In order to identify which cells were entering the cycle and synthesising DNA, cells were continuously labelled in three experiments with [14C]thymidine for 15 days, followed by a further 3 days in unlabelled medium. This incubation in unlabelled medium was chosen to allow labelled cells to finish mitosis before the end of the experiment. The 3 day period was chosen based on the average duration of (G2+M) phases in blastemal cells in regenerating limbs, which is 43 hours (Wallace and Maden, 1976). After analysis by autoradiography, we observed that 75% of the cells initially chosen for analysis entered S phase, and that 76% of those subsequently entered mitosis. These results show that the majority of adult newt cardiomyocytes can be activated to enter S phase, and although a portion of these cells appear to undergo a subsequent block, 60% have the ability to progress into mitosis.

**Progression and arrest in single cells; mitosis and cytokinesis**

Approximately 29% of the initial cells (n=195) progressed...
through one or more complete rounds of cell division (including karyokinesis and cytokinesis) giving rise to beating mononucleate progeny (Table 1, Fig. 3A,B). Some cells gave rise to clones that showed weaker and more disorganised staining for MyHC, compared to cells that did not divide (Fig. 3A, final panel). A detailed description of the variety of lineages produced by dividing clones is illustrated in Fig. 3B. Although there was great variation of the proliferative potential between different clones, there was a tendency for sister cells to be similar in two important respects (Fig. 3B). First, in 52 divisions that gave rise to mononucleate cells and occurred at least 5 days before the end of the experiment, there was a significant propensity for symmetric divisions. Second, there was a correspondence in cell cycle time between sibling cells that was also significant. The detailed analysis of both parameters is given in the supplementary information (http://jcs.biologists.org/supplemental).

Approximately 31% of the initial cardiomyocytes gave rise to a binucleate cell in their first mitosis (Table 1, Fig. 3C). We observed a small number of candidate fusion events between non-sister cells (less than 7%) but most multinucleate cells (2 or more nuclei) clearly resulted from incomplete mitosis (Fig. 3C). This was apparently due to a problem in resolving the cleavage furrow, as mitosis often resulted in two partially separated cells with distant nuclei (Fig. 3C, at 12 days), which eventually became closer (Fig. 3C, 13 days). Interestingly, 19% of the multinucleate cells subsequently entered S phase and completed mitosis and cytokinesis, giving rise to a variety of outcomes (Fig. 3D); this shows that the formation of a multinucleate cell does not preclude further proliferation. In the population that divides more than once (Fig. 3B,D) most of the cells that go through one complete cycle also finish cycles in subsequent divisions.

The finding that more than half of the cardiomyocytes can undergo karyokinesis and that half of these can successfully complete cell division was confirmed using another technique. Cells were labelled with a fluorescent tracker dye PKH-26 (Sigma), seeded onto a gridded surface and adjusted so there was one labelled cell per square (see Materials and Methods). This fluorescent tracker dye is coupled to long aliphatic tails that incorporate into lipid regions of the cell membrane. PKH-26 has been characterised in a wide variety of applications, particularly in the study of cell biology and cell behavior. The results shown are from time-lapse microscopy analysis of the progeny of 195 cells pooled from five independent experiments.

Table 1. The proliferative potential of cardiomyocytes

<table>
<thead>
<tr>
<th>Category of cells</th>
<th>% cardiomyocytes in each category</th>
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</thead>
<tbody>
<tr>
<td>Did not undergo mitosis</td>
<td>40</td>
</tr>
<tr>
<td>Divided once</td>
<td>16</td>
</tr>
<tr>
<td>Divided twice</td>
<td>9</td>
</tr>
<tr>
<td>Divided 3 or more times</td>
<td>4</td>
</tr>
<tr>
<td>Became multinucleate</td>
<td>25</td>
</tr>
<tr>
<td>Became multinucleate and then underwent one or more cycles with completion of cytokinesis</td>
<td>6</td>
</tr>
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The results shown are from time-lapse microscopy analysis of the progeny of 195 cells pooled from five independent experiments.
of systems for in vitro and in vivo cell tracking applications and has been used previously on newt cells (Kumar et al., 2000). After 24 days in culture we observed that 35% of the initial cardiomyocytes (myosin-positive cells) had divided at least once (Fig. 4A) and that 38% of the initial cells had become multinucleate (Fig. 4B). The fact that more cells in this experiment became multinucleate, as compared to those in the time lapse experiment described above, could be because of the longer incubation time or the unavoidable inclusion of putative fusion events (Przybylski and Chlebowski, 1972) or membrane overlapping events in this category.

Our results clearly indicate that the ability to enter S phase is a general property of the differentiated state in adult cardiomyocytes. This is possibly related to the similar property in other newt differentiated cells, such as the cultured skeletal myotube, where at least 75% of the cells may enter S phase (Tanaka et al., 1997). This similarity suggests that the same mechanism may underlie entry into S phase in these cells.

S phase re-entry is enhanced by mammalian serum and dependent on Rb regulation

To determine whether DNA synthesis in newt cardiomyocytes was stimulated by mammalian serum, we performed a dose response assay for FBS. We found that newt cardiomyocytes respond to serum by S phase entry, a response that is maximal at about 10% FBS (Fig. 5A).

In order to analyse the phosphorylation state of the retinoblastoma protein in the newt cardiomyocyte, we used a monoclonal antibody that specifically recognises an epitope that includes phosphoserine 608 in human Rb and is conserved in the newt protein (Barrie et al., 2003; Tanaka et al., 1997). This residue is hypophosphorylated in cells in G0/G1, becomes phosphorylated prior to entry into S phase, and remains phosphorylated throughout the cell cycle (Zarkowska et al., 1997). This antibody stained the nucleus of 59% of the cardiomyocytes at 9 days after plating in medium containing 10% FBS (Fig. 5B,C), and the staining was significantly diminished by phosphatase digestion of the fixed cells (see Materials and Methods). This result indicates that cell-cycle
associated Rb phosphorylation arises in these cells. The presence of Rb phosphorylation in the overall population was confirmed using immunoprecipitation followed by western blotting using a pan specific Rb antibody (data not shown).

In order to evaluate the functional role of Rb in S phase entry, we injected adult cardiomyocytes with a plasmid encoding human p16INK4, a CDK inhibitor that specifically inhibits CDK4/6 (Ruas and Peters, 1998). The regulation of Rb activity, and possibly one other member of the pocket protein family, is absolutely required for p16-mediated cell cycle arrest (Bruce et al., 2000; Lukas et al., 1995; Medema et al., 1995). After exposure to a pulse of BrdU, the cells were stained with antibodies to BrdU, human p16INK4 and MyHC. Expression of the p16INK4 protein produced an approximately 13-fold inhibition of S phase entry relative to uninjected cells, whereas cells injected with a control plasmid were only inhibited 1.3 fold (Table 2). We conclude that a serum-activated pathway leading to phosphorylation of Rb is a strong candidate to mediate re-entry to the cell cycle by the adult cardiomyocyte.

### Discussion

This study has for the first time examined the proliferative potential of single adult newt cardiomyocytes. Two major findings emerge from this study. First, the majority of adult newt cardiomyocytes are able to re-enter S phase. Second, only a third of these cells are able to undergo one or more complete cycles of cell division, as the remaining cells do not undergo mitosis or fail to complete cytokinesis (a model summarising the results of the different experiments is presented in Fig. 6). These results suggest that the differentiated state of adult newt cardiomyocytes is compatible with complete cycles of division and that newt heart regeneration depends on the retention of proliferative potential in a subset of cardiomyocytes.

Although culture conditions may enhance dedifferentiation in cardiomyocytes (Claycomb, 1991; Eppenberger et al., 1988) and could in principle induce an artifactual response, several lines of evidence suggest that the results described in this work reflect the properties of the population of newt cardiomyocytes after injury and account for them at the single cell level. First, the cells in long term culture show a time dependence and extent of entry into S phase (Fig. 2D) which is comparable to

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**Table 2. Expression of human p16INK4 in newt cardiomyocytes inhibits DNA synthesis**

<table>
<thead>
<tr>
<th>Injected substance</th>
<th>% BrdU labelled cardiomyocytes</th>
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<tr>
<td></td>
<td>Injected</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>Plasmid coding for p16</td>
<td>2 (223)</td>
</tr>
<tr>
<td>Plasmid coding for alkaline phosphatase</td>
<td>24 (220)</td>
</tr>
<tr>
<td>Texas Red-dextran</td>
<td>22 (348)</td>
</tr>
</tbody>
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Separate plates of cardiomyocytes were microinjected with an expression plasmid encoding either the CDK inhibitor p16 or, as a control, human placental alkaline phosphatase at a concentration of 50 μg/ml. Micronjection of Texas Red-dextran (70 kDa) was also used as a control. Cells were incubated for 18 hours with BrdU prior to fixation, 7 days after injection. The results presented are pooled from three or four independent experiments. A block to entry into S phase is evident upon expression of p16. Details of the plasmids can be found in Materials and Methods.

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**Fig. 4.** Fate of single cardiomyocytes. One cell per grid square was labelled with the red fluorescent tracker dye, PKH-26 (see Materials and Methods), and cultured for 24 days prior to fixation and analysis. There were a total of 238 initial cells from three independent experiments. (A,B) Confocal micrographs of squares where two daughter cells (A) or a binucleate cell (B) were observed. Staining for MyHC is in green and DNA (Hoechst 33258) in blue. Scale bar: 50 μm.

**Fig. 5.** Regulation of S phase entry in newt cardiomyocytes. (A) Dependence of entry on serum concentration. Cells were exposed to various concentrations of FBS, as described in Materials and Methods, and pulsed with BrdU 4 days later for 8 hours. Each point is from a separate dish of the same culture, and the peak at approximately 10% FBS was observed in three other comparable experiments. (B,C) Detection of Rb in cardiomyocytes by indirect immunofluorescence with an antibody recognising phosphoserine 608. The cells were stained with anti-MyHC and Hoechst 33258 (DNA; B), anti-phosphoserine 608 (in Rb; C). In control experiments the intensity of nuclear staining was significantly diminished by digestion of fixed cells with lambda phosphatase prior to reaction with the antibody (not shown). Note that most of the nuclei in B are positive for the Rb phosphorylated epitope in C. Scale bar: 50 μm.
An adult newt has an extraordinary regenerative ability, being able to regenerate not only large sections of its heart, but also its jaws, lens, retina, limbs and tail in response to tissue damage or removal. The regenerative ability of adult urodeles is associated with high plasticity of the differentiated state (Brockes and Kumar, 2002). This is manifest in different ways depending on tissue type. Iris pigmented epithelial cells transdifferentiate and proliferate during lens regeneration (Eguchi et al., 1974). Multinucleate newt myotubes and myofibrils re-enter the cell cycle and undergo conversion to mononucleate cells during limb and tail regeneration (Echeverri et al., 2001; Kumar et al., 2000; Lo et al., 1993; Velloso et al., 2000). We have shown that the majority of newt cardiomyocytes can enter into S phase and the differentiated state is compatible with complete cycles of division in 29% of newt cardiomyocytes. In each of these cases of plasticity, there is entry into the cell cycle and it is restricted to the zone adjacent to the wound (Brockes and Kumar, 2002; Oberpriller et al., 1989). Our results suggest that the same pathway drives newt cardiomyocytes and skeletal myotubes into S phase (Tanaka et al., 1997), since in both cell types this is enhanced by mammalian serum and is dependent on Rb inactivation, as evidenced by the strong inhibitory activity of p16INK4. Work of Sadoshima and Izumo (Sadoshima et al., 1997) suggests that serum leads to Rb phosphorylation but not DNA synthesis in cultured neonatal rat cardiomyocytes. To explain that difference it will be necessary to investigate how factors in mammalian serum stimulate entry into S phase in newt cardiomyocytes, namely whether Rb phosphorylation is regulated differently.

A surprising result from our work is that newt cardiomyocytes have a heterogeneous proliferative potential. Although all cells are exposed to serum-containing medium, only a small subset seem to be responsible for the increase in cell number observed upon regeneration. The behaviour of the remaining cells resembles that observed for their mammalian counterparts at several stages of development. The G2/M boundary and the ability to undergo cytokinesis have long been recognised as critical checkpoints to the proliferation of mammalian cardiomyocytes. Thus cardiomyocytes in several mammalian species become polyploid and/or multinucleate after birth (Brodsky, 1991; MacLellan and Schneider, 2000; Poolman et al., 1998; Soonpaa et al., 1996). In cases where neonatal or adult mammalian mouse and rat cardiomyocytes traverse S phase, mitotic figures are rarely seen and cytokinesis is not observed (Claycomb and Bradshaw, 1983; MacLellan and Schneider, 2000; Soonpaa and Field, 1998). Finally, the presence of striated myofibrils in the equatorial region of the cell has been noted as a possible factor in the formation of mononucleate cardiomyocytes in neonatal mammals (Li et al., 1997), and this was also observed here for the newt cells.

Why do newt cardiomyocytes show this heterogeneity in proliferative potential? The cells do not show any apparent distinction in terms of their differentiated state as they have very homogeneous morphology, they all express muscle markers and contract (our observations in culture) and they are very homogeneous in terms of their differentiated state as they have very homogeneous morphology, they all express muscle markers and contract (our observations in culture) and they are all quiescent in the adult newt (Oberpriller et al., 1989). However, a clue to this variable behaviour is the fact that these cardiomyocyte clones show a similar pattern of cell division to the one previously described for embryonic cardiomyocytes (Burton et al., 1999). We found that sister-cell cardiomyocytes are significantly correlated, both in terms of undergoing a subsequent cell cycle and also in respect of their cell cycle time. It may be that this subgroup of cells has not undergone the complete programme of terminal differentiation, and the absence of signals keeps these cells quiescent in a non-injured animal. A molecular comparison of the cells in this culture should help us to analyse the regulation of the differentiated state and cell cycle progression in an adult cardiomyocyte.

The recent finding that zebrafish can regenerate the heart through cardiomyocyte division (Poss et al., 2002) shows that the potential for cardiomyocyte division is more widespread than previously thought. The similarities between the regulation of the cell cycle of newt and mammalian cardiomyocytes suggest that the large difference in

![Fig. 6. Model of cell cycle progression by adult newt cardiomyocytes in culture. This incorporates, into a diagram, the various results described in the text. This figure should be seen only as a model based on the data, since it incorporates data from different experiments (time lapse analysis; dose response assay for FBS; Rb phosphorylation and inhibition of re-entry by p16INK4). Note that all percentages are given with reference to the starting population of cardiomyocytes. Although our data does not rule out the possibility that another member of the pocket-protein family may also mediate the regulation of cell cycle entry, to date no other members of this family have been cloned in amphibia.](image-url)
regenerative ability may reflect differences in regulation of the same pathways. Consequently, one might expect that such differences could be subject to genetic variability. It is noteworthy that cardiac repair has recently been described after cryogenic infarction of the right ventricle in the MRL strain of mice (Leferovich et al., 2001). This strain has an enhanced capacity to heal surgical wounds, a complex trait that maps to at least seven genetic loci, and significant re-entry to S phase was noted after injury to the heart (Leferovich et al., 2001). Additionally, it is possible that mammalian cardiomyocytes may also display a heterogeneous proliferative potential, as telomere shortening has been shown in a small percentage of adult rat cardiomyocytes (Anversa and Nadal-Ginard, 2002; Kajstura et al., 2000). The results presented here raise the possibility that heart regeneration through cardiomyocyte proliferation, while not normally a significant occurrence, might become possible in mammals. It is worthwhile to explore further the possibility that mammalian cardiomyocytes may also show a heterogeneous proliferative potential and to investigate whether there may exist populations more susceptible to stimulation to proliferate. Additionally, the newt cardiomyocyte culture system offers an opportunity to further analyse the molecular regulation of the differentiated state and cell cycle progression in an adult cardiomyocyte by directly comparing cells with different abilities to proliferate. These efforts might complement the current approaches to heart regeneration that are based on implantation of cells (Grounds et al., 2002; Kessler and Byrne, 1999; Orlic et al., 2001; Reinlib and Field, 2000).

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