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 regenerated 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., 1997).

Here we address several questions regarding the cellular and molecular mechanisms that underlie the plasticity of the cardiomyocyte. First, is this ability to re-enter the cell cycle in response to injury a general property of the differentiated state in newt cardiomyocytes, as described for other newt

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 Brockes, 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 CO_2 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/cm^2 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 [^{14}C]thymidine (0.01 μCi/ml). For cumulative labelling with [^{14}C]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 Ilford K5 emulsion. The concentration of [^{14}C]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 picopump PV820 mounted on a Zeiss Axiovert microscope, 5 days after plating. At 1 hour before microinjection of the cells, the medium was changed to serum free AL15 with 2,3-butanedione monoxime (BDH, 4 mM) to inhibit myofibril contraction. DNA was injected into the nucleus and dextrans (Texas Red or fluorescein-conjugated 70 kDa dextrans, Molecular Probes; used as an additional control for the effect of microinjection) into the cytoplasm. After microinjection, the medium was changed to AMEM with 10% FBS for 7 days prior to addition of BrdU for 18 hours. In plasmid pTL1-p16 the coding sequence for human p16^{INK4} is expressed under control of the SV40 promoter in the expression vector pTL1, as described previously for the retinoic acid receptor (Ragsdale et al., 1989). In plasmid pCAP the coding sequence for human placental alkaline phosphatase is expressed under control of the SV40 promoter in the expression vector pSG5 as described earlier (Schilthuis et al., 1993).

Lineage tracing with tracker dye

Cardiomyocytes were labelled in suspension with the PKH-26 fluorescent tracker dye (Sigma) according to the manufacturer's instructions. The PKH-labelled cells were mixed with unlabelled cells (1:24) and 4×10^4 cells were seeded onto a 35 mm dish scored with a grid. After 4-5 days in culture the cell numbers were adjusted to 1 labelled mononucleate cell per grid square by removing supernumeraries with a microinjection pipette attached to a Narishige micromanipulator. The cells were fixed and examined 24 days after plating.

Imaging

Live cardiomyocytes were viewed under phase contrast objectives and images were captured with a monochrome CCD (SONY) camera and image contrast was enhanced with Image Pro Plus software. For continuous time lapse analysis, cells were maintained at room temperature in AL15 with 10% FBS overlaid with mineral oil to prevent evaporation. For observation once or twice a day, cells were plated in a dish scored with an oriented grid and kept in the incubator in AMEM with 10% FBS. For sequential observations cells could readily be recognised by their position in the grid. Images for immunofluorescence were collected on a CV-12 (Photonic Sciences) cooled monochrome camera. Z-series stacks were acquired under a Leica TCS SP confocal system and projections generated using Leica TCS imaging software.

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 [51β7, 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]. 51β7 is specific for Rb as it stained SF 295 *Rb*-positive cells (NCI) 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 dextrans 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,

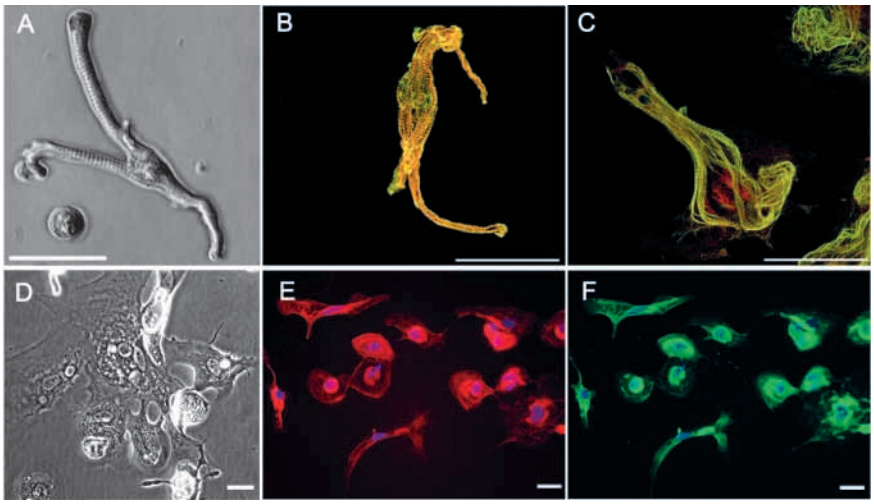

Fig. 1. Adult ventricular cardiomyocytes in culture. (A) Micrograph of an isolated cardiomyocyte in suspension after dissociation. Note the branched morphology and striated myofibrils. (B,C) Isolated cardiomyocytes after plating. Note the cross striations and expression of the myofibril markers MyHC (green, B and C) and troponin (red, B) or titin (red, C), as visualised by double label indirect immunofluorescence. (D) An interconnected group of cells, at 8 days after plating onto laminin, that beat in synchrony. (E,F) More than 90% of the cells in the culture are cardiomyocytes; expression of the myofibril markers troponin (E) and MyHC (F) at 8 days after plating (DNA stained with Hoechst 33258 in blue). Scale bar: 50 μm.

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 ventricles 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; Soonpaa 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 [^{14}C]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

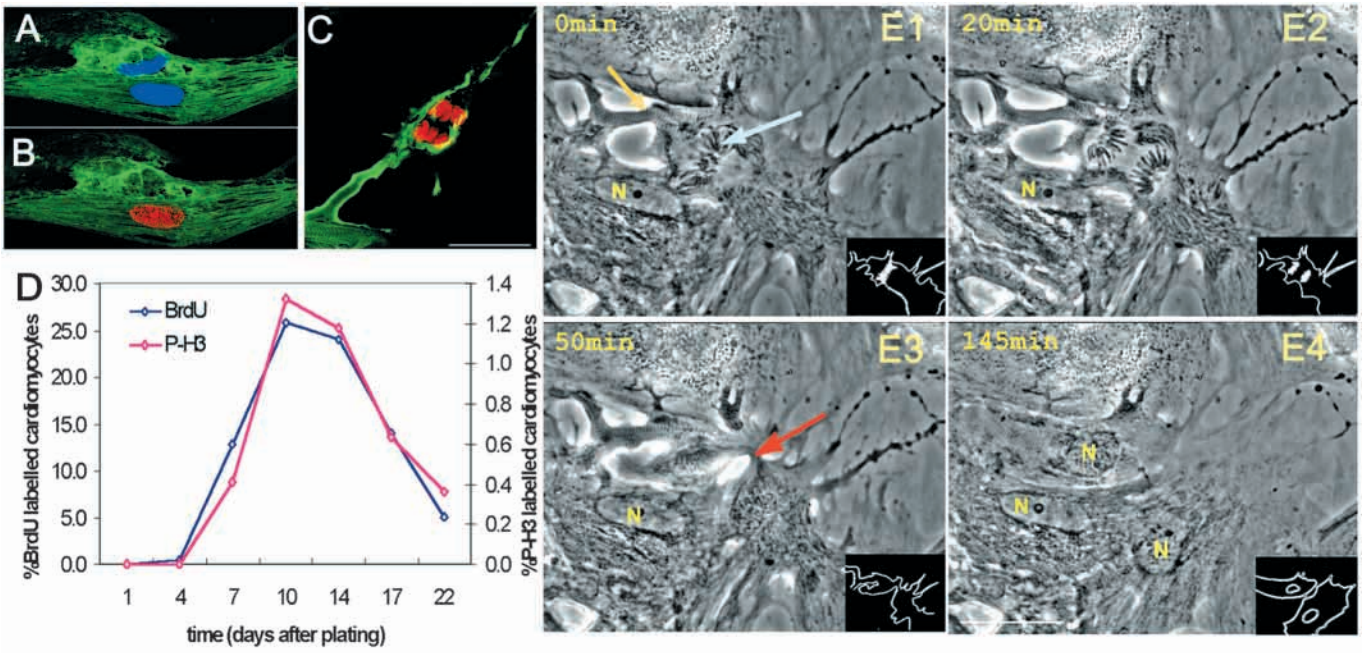

Fig. 2. Cardiomyocytes undergo DNA synthesis and mitosis. Cells were pulsed with BrdU for 9 hours at various times after plating and then fixed and stained for BrdU, MyHC and DNA. Parallel wells were stained for phosphorylated histone H3 (P-H3), MyHC and DNA. (A,B) Labelling of cardiomyocytes with BrdU. Confocal micrograph of two cells stained for BrdU (red), DNA (Hoechst 33258, blue) and MyHC (green). Note that the lower cardiomyocyte has incorporated BrdU. (C) Labelling of cardiomyocytes with an antibody against phosphorylated histone H3 (red) and MyHC (green). Myofibrils are excluded from the central region where mitosis is occurring. (D) Time course of entry into S phase and mitosis. Note that there is a peak of cells undergoing S phase and mitosis at 10 days. The results are the average of duplicate dishes. (E1-E4) Time lapse of a cardiomyocyte undergoing mitosis. Both daughter cells were contracting at 145 minutes (E4). An outline of the cell is seen in the right, lower side of each picture. The time elapsed is shown in the left top side. Note the myofibrils (yellow arrow) and the chromosomes (blue arrow). Red arrow points to the cleavage furrow; N, nuclei. Scale bar: 50 μm (in C and E4).

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).

Table 1. The proliferative potential of cardiomyocytes

Category of cells	% cardiomyocytes in each category
Did not undergo mitosis	40
Divided once	16
Divided twice	9
Divided 3 or more times	4
Became multinucleate	25
Became multinucleate and then underwent one or more cycles with completion of cytokinesis	6

The results shown are from time-lapse microscopy analysis of the progeny of 195 cells pooled from five independent experiments.

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

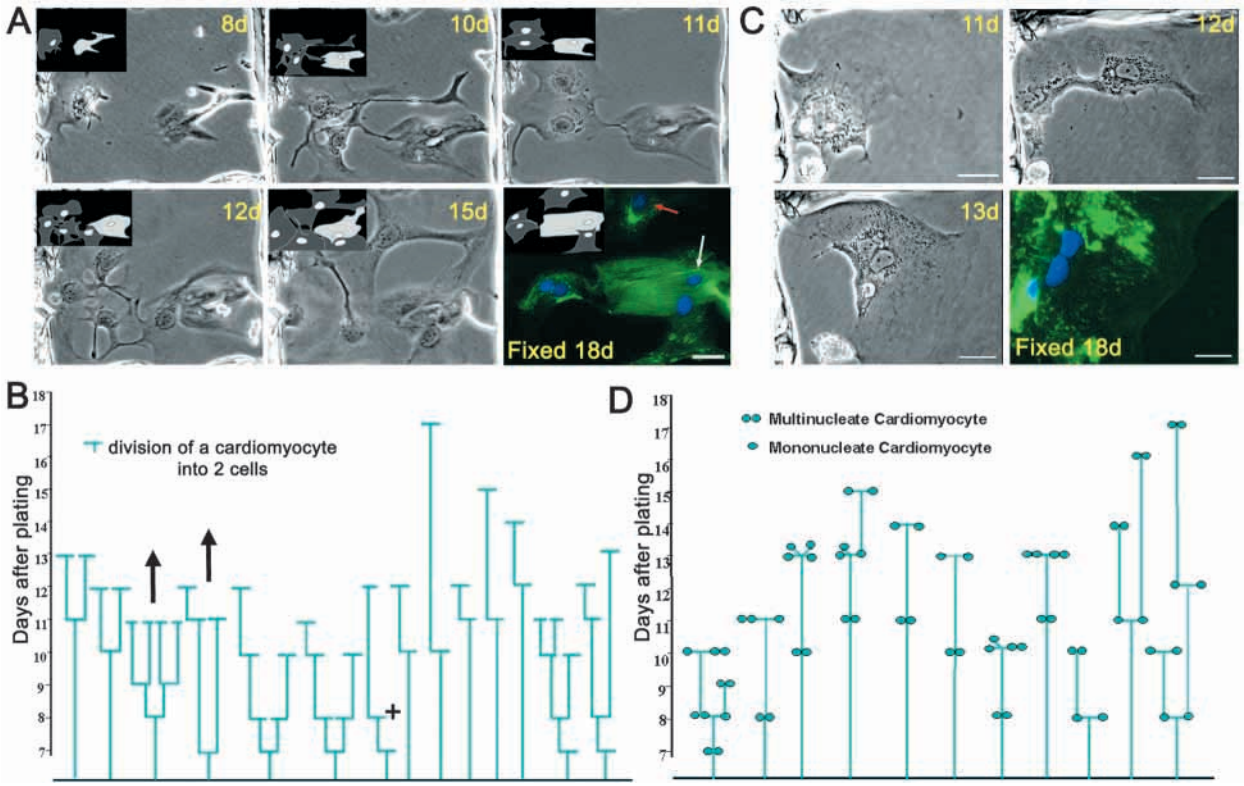

Fig. 3. Analysis of cycle progression by time lapse microscopy. Cells were plated out in a grid, photographed as described in Materials and Methods, then fixed and stained at 18 days. (A,B**)** Cardiomyocytes may divide more than once. (A) A field where a cardiomyocyte divided twice and another did not divide. The number at the top right of each picture shows the number of days elapsed since the cells were plated. A schematic drawing of the field is shown on the top left part of the figure. Note that the cell on the left at 8 days gives rise to 2 daughter cells at 10 days. Each of the daughter cells divides once more at 12 days. All the progeny cells stain positive for MyHC (lower right), but the staining is less strong and organised (red arrow) than in the non-dividing cell (white arrow). (B) Tree diagrams (one per starting cell) for the proliferation of cardiomyocytes which divided more than once giving rise to mononucleate progeny. Cells that divided only once (n=17) are not represented. Pedigrees were constructed from the time-lapse analysis of three independent cultures and they illustrate the diversity observed. All the cells were followed for 18 days (to simplify the scheme, lines for each cell stop in the last division observed during the 18 days). The arrows indicate clones which continued to divide; using this experimental set-up it was impossible to trace those divisions as accurately as all the others represented. The cross indicates that a cell died. (C) Example of a field where a cardiomyocyte underwent acytokinetic mitosis, thus becoming multinucleate. (D) Same experiment as in B. Pedigrees of cardiomyocytes that gave rise to multinucleate progeny. Cells that underwent only one cycle are not represented. Note that the mitotic division of a multinucleate cell can have several different outcomes. Scale bars: 50 μm.

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

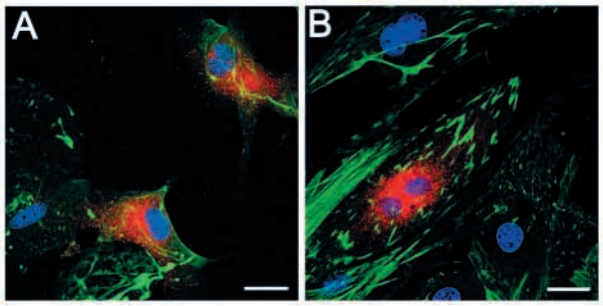

Fig. 4. Fate of single cardiomyocytes. One cell per grid square was labelled with the red fluorescent tracker dye, PKH-26 (see Materials and Methods), and cultured for 24 days prior to fixation and analysis. There were a total of 238 initial cells from three independent experiments. (A,B) Confocal micrographs 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.

Table 2. Expression of human p16^{INK4} in newt cardiomyocytes inhibits DNA synthesis

	% BrdU labelled cardiomyocytes	
Injected substance	Injected cardiomyocytes (*n*)	Non-injected cardiomyocytes (*n*)
Plasmid coding for p16	2 (223)	26 (685)
Plasmid coding for alkaline phosphatase	24 (220)	31 (598)
Texas Red-dextran	22 (348)	37 (712)

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. Microinjection 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.

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 p16^{INK4}, 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 p16^{INK4} and MyHC. Expression of the p16^{INK4} 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

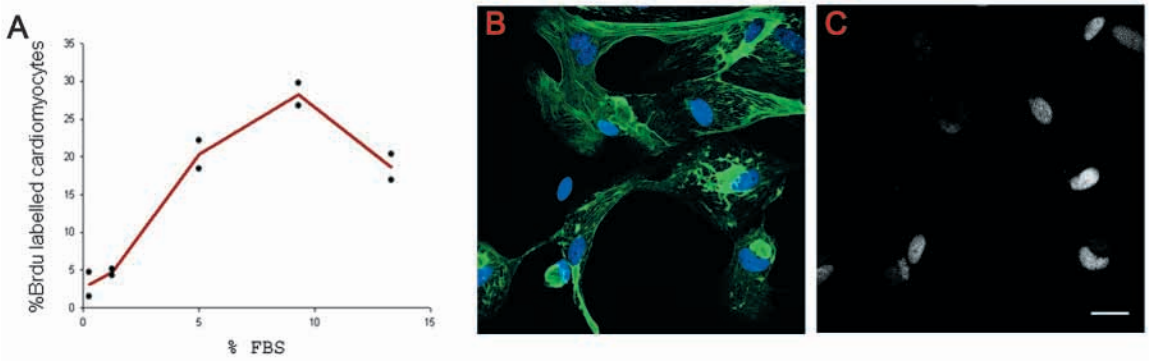

Fig. 5. Regulation of S phase entry in newt cardiomyocytes. (A) Dependence of entry on serum concentration. Cells were exposed to various concentrations of FBS, as described in Materials and Methods, and pulsed with BrdU 4 days later for 8 hours. Each point is from a separate dish of the same culture, 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.

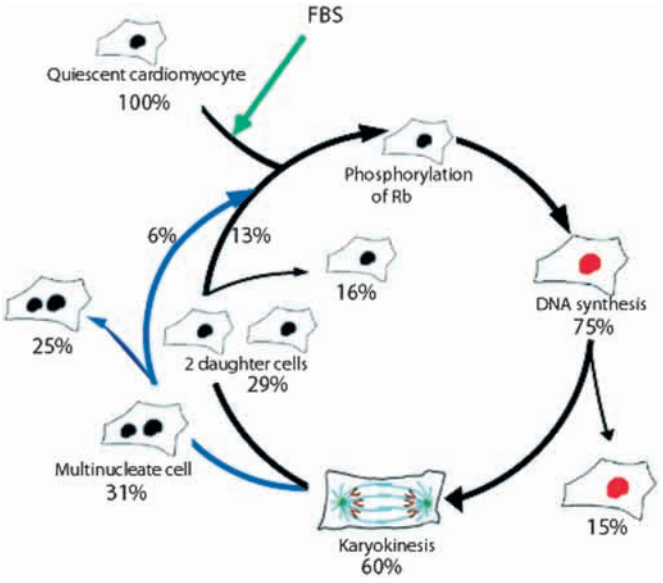

Fig. 6. Model of cell cycle progression by adult newt cardiomyocytes in culture. This incorporates, into a diagram, the various results described in the text. This figure should be seen only as a model based on the data, since it incorporates data from different experiments (time lapse analysis; dose response assay for FBS; Rb phosphorylation and inhibition of re-entry by $p16^{INK4}$). 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.

that observed after injury to the newt ventricle (Bader and Oberpriller, 1979; Bader and Oberpriller, 1978). Second, 19% of the cells go through more than one round of cell division in culture which may account for the 2.5 fold increase in the number of cardiomyocytes described in vivo, after injury (Bader and Oberpriller, 1979). Finally, the blocks to cell cycle progression described here are in agreement with the observations that 45 days after mincing the tip of the newt ventricle and grafting it back to the heart, 6% of the cells in the graft are binucleate and 7% are mononucleate with a polyploid nucleus (Oberpriller et al., 1989; Oberpriller et al., 1995). In control animals, only 1% of the cardiomyocytes have a polyploid nucleus and less than 1% are binucleate (Oberpriller et al., 1989).

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 myofibres 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 $p16^{INK4}$. 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 binucleate 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 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 then previously thought. The similarities between the regulation of the cell cycle of newt and mammalian cardiomyocytes suggest that the large difference in

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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