# Re-programming of newt cardiomyocytes is induced by tissue regeneration

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## Summary

**Newt hearts are able to repair substantial cardiac injuries without functional impairment, whereas mammalian hearts cannot regenerate. The cellular and molecular mechanisms that control the regenerative capacity of the newt heart are unknown. Here, we show that the ability of newt cardiomyocytes to regenerate cardiac injuries correlates with their ability to transdifferentiate into different cell types. Mechanical injury of the heart led to a severe reduction of sarcomeric proteins in the myocardium, indicating a partial de-differentiation of adult newt cardiomyocytes during regeneration. Newt cardiomyocytes implanted into regenerating limbs lost their cardiac phenotype and acquired skeletal muscle or chondrocyte fates. Reprogramming of cardiomyocytes depended on contact with the limb blastema because cardiomyocytes implanted into intact, non-regenerating limbs or cultured in vitro retained their original identity. We reason that signals from the limb blastema led to de-differentiation of cardiomyocytes, cell proliferation and re-differentiation into specialized cells and propose that the ability of cardiomyocytes to transdifferentiate into different cell types reflects the cellular program that enables heart regeneration.**

Supplementary material available online at
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## Introduction

In mammals several pathogenic mechanisms might result in loss of functional myocardium, scar formation and, eventually, heart failure. The inability of mammalian hearts to rebuild lost contractile tissue is due to a restricted proliferation potential of mammalian cardiomyocytes and to the absence of effective cardiac progenitor cells, which are able to replace dead or damaged cardiomyocytes. Mammalian cardiomyocytes are terminally differentiated cells that lose their proliferation potential shortly after birth. It is commonly accepted that proliferation of adult mammalian cardiomyocytes occurs only rarely, if at all (Pasumarthi and Field, 2002; Rumyantsev, 1977). Despite recent reports about the ability of human cardiomyocytes to proliferate after cardiac infarction (Beltrami et al., 2001) the major response of mammalian cardiomyocytes to the loss of myocardial tissue is proposed to be hypertrophy (Pasumarthi and Field, 2002). Furthermore, it is not clear whether putative native cardiac precursor cells, which have been identified recently, contribute significantly to the replacement of myocardial cells in particular because they are present at very low numbers in the adult heart and show only a limited proliferation potential (Laugwitz et al., 2005).

This situation is fundamentally different in newt and zebrafish. Both organisms re-grow functional myocardial tissue after partial amputation of the heart and repair damaged areas without the generation of non-functional scar tissue. In zebrafish up to 20% of the ventricular myocardium can be replaced after resection over a two-month period (Poss et al., 2002). The repair process is associated with the proliferation of cardiomyocytes, which are characterized by a partial disassembly of sarcomeric structures – as monitored by electron microscopy (Bader and Oberpriller, 1978; Tate and Oberpriller, 1989). Although previous studies uncovered differences between the proliferative capabilities of subpopulations of cultured adult newt cardiomyocytes (Bettencourt-Dias et al., 2003), it is not clear whether different subsets of cardomyocyte or cardiomyocyte progenitor cells contribute to heart repair in vivo and whether all cardiomyocytes own an inherent plasticity that might enable them to contribute to regenerative processes. It also not known whether changes in cardiomyocyte morphology during cardiac repair reflect an obligatory de-programming and/or de-differentiation step of cardiomyocytes that is necessary for regeneration, or simply represent a feature of dividing cardiomyocytes that reduce the mechanical constraints of a dense myofibril network.

De-differentiation is a well-known phenomenon in adult urodele amphibia and has been studied mainly in regenerating limbs or tails (Brockes, 1997). During regeneration, the wound generated by amputation of the limb is rapidly covered by epidermal cells followed by the formation of a zone of proliferating progenitor cells called the blastema. Blastema cells are largely derived from the de-differentiation of muscle and dermis and are the major source for the different cell types

that are required to rebuild the lost tissue (Echeverri et al., 2001; Muneoka et al., 1986). The newt limb regeneration blastema is able to induce reversal of the differentiation of resident muscle cells and of newt A1 myotubes after implantation (Kumar et al., 2000; Lo et al., 1993). Other studies demonstrated that a tissue extract derived from the newt blastema is capable to de-differentiate newt A1 myotubes and even mouse myotubes (derived from C2C12 cells) in culture, suggesting that the ability to de-program differentiated cells is not restricted to amphibians and fish (Kumar et al., 2000; McGann et al., 2001). Although the exact molecular mechanisms that drive de-differentiation and cell proliferation in newts have remained enigmatic so far (Straube et al., 2004) several molecules were identified that seem instrumental to induce cell proliferation and de-differentiation in newts. A thrombin-activated factor that is present in the serum of several species has been proposed to serve as an effector for urodele myotubes to re-enter the cell cycle by phosphorylation of the Rb protein, thereby providing a link between the reversal of differentiation and acute events of wound healing (Tanaka and Brockes, 1998; Tanaka et al., 1999; Tanaka et al., 1997). Furthermore, Msx1 (Simon et al., 1995), Matrix metalloproteinases (Vinarsky et al., 2005), hepatocyte growth factor (Satoh et al., 2005), and the complement factors C3 and C5 (Kimura et al., 2003) have been shown to be implicated in regeneration in amphibians.

To begin to unveil the cellular processes that govern heart regeneration in newts, we decided to study the plasticity of cardiomyocytes in regenerating and non-regenerating environments. We found that regeneration of newt hearts was associated with a strong downregulation of sarcomeric proteins and cardiac-specific genes in the injured myocardium, indicating partial de-differentiation of cardiomyocytes. Transplantation of labeled cardiomyocytes into regenerating limbs did also result in a rapid downregulation of cardiomyocyte markers and in an upregulation of blastema marker molecules. By contrast, the cellular identity of cardiomyocytes was maintained after transplantation into intact limbs, indicating the requirement of a regenerative environment for cellular de-programming. De-differentiation and reprogramming were not necessarily a prerequisite for cell proliferation because cultured newt cardiomyocytes re-entered the cell cycle in vitro without the loss of sarcomeric proteins. Furthermore, we discovered that, cardiomyocytes that had lost expression of cardiomyocyte markers participated in the generation of skeletal myotubes and chondrocytes.

## Results

### Massive downregulation of sarcomeric proteins during heart regeneration in newts

In the undamaged adult newt myocardium, we observed a substantial number of nuclei that underwent karyokinesis, whereas mitotic nuclei were detected only occasionally (not shown). This picture changed dramatically upon mechanical damage to the heart. Numerous cells undergoing mitosis appeared in the compact layer and in the trabeculae, which in part seemed to be derived from differentiated cardiomyocytes, owing to the presence of residual sarcomeric structures. The appearance of mitotically active, rather immature, cardiomyocyte-like cells coincided with the loss of the expression of sarcomeric proteins in damaged areas. Immunohistochemical staining revealed a dramatic downregulation of sarcomeric myosin heavy chain (MyHC) expression (Fig. 1C) compared with intact heart (Fig. 1A) 24 hours after damage. Similar results were obtained for cardiac troponin T (cTnT) (Fig. 1D), indicating a – partial – reprogramming of cardiomyocytes. Downregulation of sarcomeric proteins was confined to damaged areas: extensive damage led to broad downregulation of sarcomeric proteins (Fig. 1C), whereas localized damage resulted in a more restricted inhibition of sarcomeric protein expression (Fig. 1D). The contractile properties of damaged hearts in situ were clearly impaired because, compared with non-injured hearts, only weak contractions were detected. This observation corresponded to the clinical appearance of operated newts, which for the first few days after ventricular damage went into a dormant-like state, were less active and predominantly rested at the bottom of the aquaria. After 1 week of recovery, however, the clinical appearance of operated newts improved, and after two weeks their behavior was almost indistinguishable from control animals. These clinical improvements did also correlate with the re-expression of sarcomeric proteins, which returned to normal levels 14 days after damage (Fig. 1E,F).

To further prove the loss of cardiomyocyte-specific gene expression in regenerating cardiomyocytes, we performed

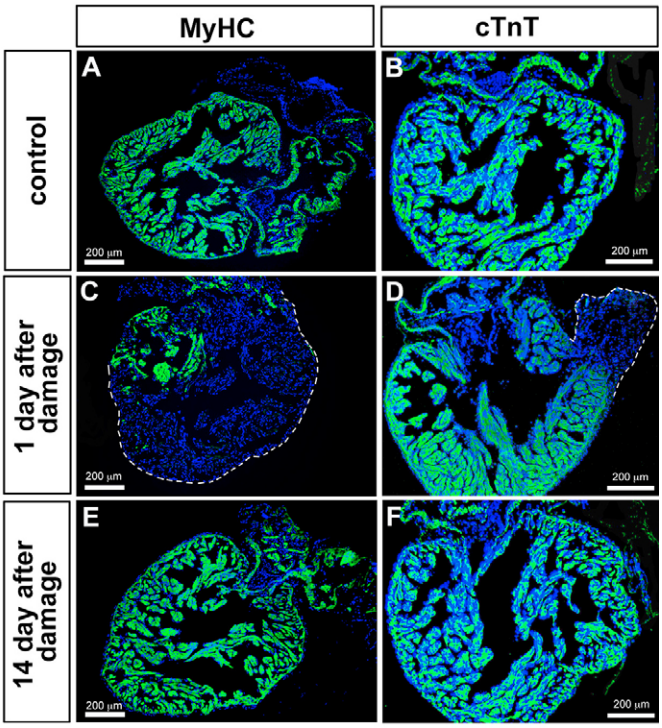


**Fig. 1.** Cardiac injury in newts results in a transient downregulation of contractile proteins. Immunofluorescence staining (green) of sham operated (A,B) and damaged newt hearts (C-F) for MyHC (A,C,E) and cTnT (B,D,F) 1 (C,D) and 14 days (E,F) after mechanical injury. The white dashed lines in C and D indicate the margins of the hearts. Note the massive and widespread downregulation of MyHC and cTnT, which corresponded to the degree of damage exerted to the heart. 14 days after the injury cardiomyocytes in the injured areas re-expressed MyHC and cTnT (E,F). A Hoechst 33342 counterstaining was used to label all nuclei on the sections.

semi-quantitative RT-PCR experiments using RNA from hearts that had been damaged to different extents. Primers were selected from newt cDNA sequences, which represented homologues to genes known to be expressed specifically in the heart. A detailed description of the newt EST database from which the cDNA sequences were derived (our unpublished results). We found a strong downregulation of the expression of MyHC, cTnT, cTnI, cTnC and cardiac actin 7 days after the injury, whereas the expression of the ribosomal protein S21, which was used as a control, remained unchanged (Fig. 2). The extent of the downregulation corresponded approximately to the degree of the heart injury: the heart with the most damage (Fig. 2, lane 4) showed a more pronounced loss of cardiomyocyte markers than the heart that encountered a less severe injury (Fig. 2, lane 5). Between 14 and 21 days the expression of most markers returned to normal levels and, thereafter, was indistinguishable from sham-operated animals (Fig. 2).

### Newt cardiomyocytes retain their differentiated phenotype in culture and upon transplantation into intact limbs

Using the preplating technique, we were able to obtained highly purified (>95%) cardiomyocyte cultures. Isolated newt cardiomyocytes expressed cTnT and sarcomeric MyHC for an extended period of time (Fig. 3C,F,I). Furthermore, most cardiomyocytes plated on laminin started to beat after 6-8 days in culture, clearly indicating stability of the differentiated phenotype in culture.

To further challenge the differentiated phenotype of newt cardiomyocytes, we labeled isolated cardiomyocytes with CM-DiI, Hoechst 33342 or with an adenovirus-expressed enhanced green fluorescent protein (EGFP), and transplanted the labeled cells into an intact non-regenerating limb (Fig. 3 and supplementary material Fig. S2). In our hands DiI labeling was superior compared with other labeling techniques, yielding signals that were stronger and more stable during the course of the experiments than EGFP- and Hoechst-derived signals. Since we essentially obtained identical results using either labeling technique, we employed DiI labeling for the majority of our experiments. Transplanted cardiomyocytes were readily located at various time points after implantation and continued to express cTnT (Fig. 3) and MyHC (data not shown) when implanted into a non-regenerating limb. After 8 days the bulk of implanted cardiomyocytes were found in close proximity near the original injection site (Fig. 3A,D,G). After 15 days, the transplanted, DiI-labeled cardiomyocytes had spread and covered a larger area but maintained a robust expression of cTnT (Fig. 3B,E,H). Taken together, these results clearly demonstrated that cardiomyocytes maintained their original phenotype even after extended time periods in a non-regenerating environment both in vitro and in vivo after transplantation.

### Transplanted newt cardiomyocytes undergo a rapid de-differentiation in regenerating limbs and hearts

To investigate whether cardiomyocytes lose their differentiation status under regenerative conditions we implanted labeled cardiomyocytes into regenerating limbs close to the blastema that had formed 5 days after limb amputation. We observed that cardiomyocytes underwent a rapid downregulation of heart muscle proteins in regenerating limbs. Already 24 hours after cell implantation myocytes lost most of the expression of MyHC (Fig. 4), cTnT and troponin I (data not shown). Similar results were obtained 48 hours and 7 days after implantation (Figs 4, 7, and data not shown). A quantitative assessment revealed that 95% of the labeled cardiomyocytes had lost expression of cardiomyocyte markers 1 day after transplantation, whereas not a single DiI-labeled cell was detected that stained positive for a heart muscle

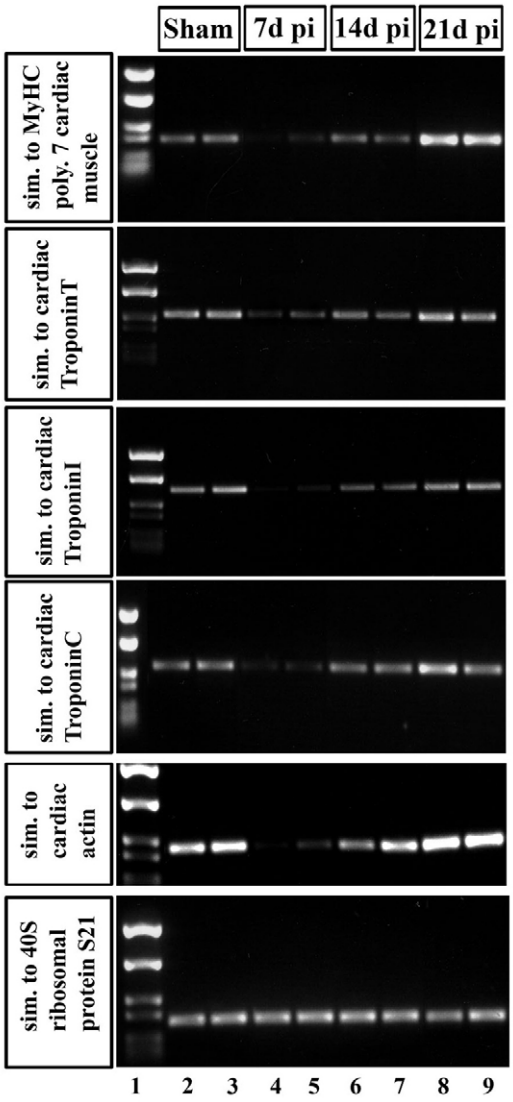


**Fig. 2.** Transient downregulation of numerous cardiac-specific genes after cardiac injury in newts. RT-PCR of RNA isolated from sham-operated animals (sham), and 7, 14 and 21 days after injury (7d pi, 14d pi, 21d pi). Two separate hearts were analyzed for each time point. Note that hearts shown in lanes 4, 6, and 8 received a more extensive damage than those shown in lanes 5, 7 and 9. The expression of MyHC, cTnT, cTnI, cTnC and cardiac actin was downregulated 1 week after heart injury but returned to levels of sham-operated hearts within three weeks. 40S ribosomal protein S21 was expressed at constant levels during regeneration and served as an internal control. Lane 1, molecular mass marker; lanes 2 and 3, sham operated hearts; lanes 4 and 5, 7 days post injury (pi); lanes 6 and 7, 14 days pi; lanes 8 and 9, 21 days pi.

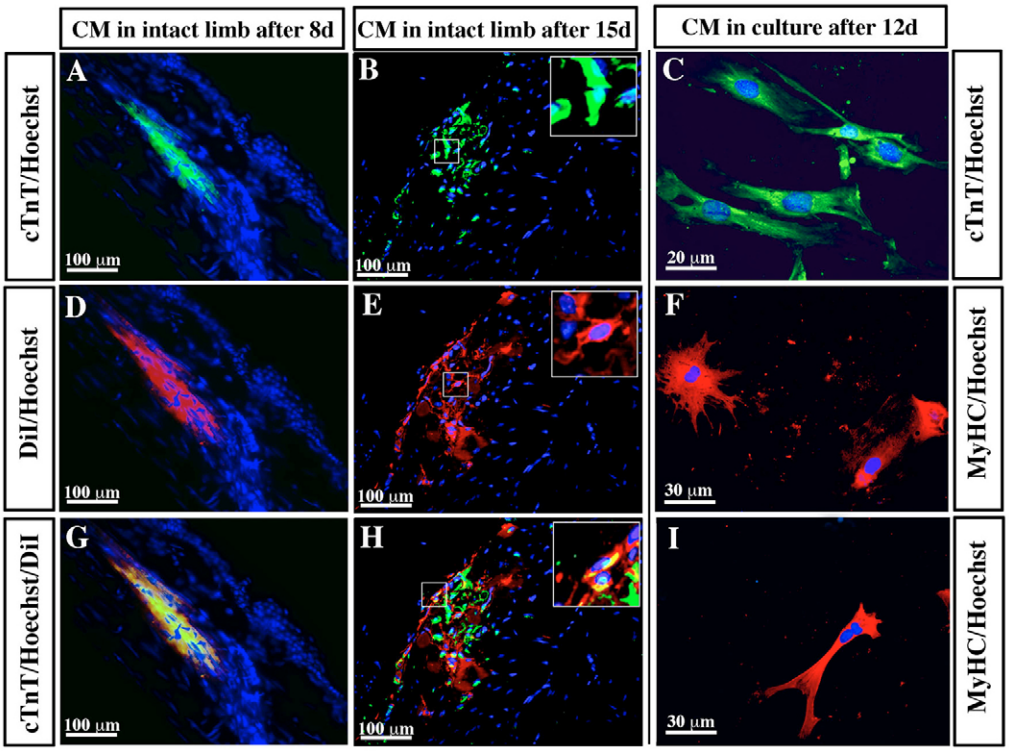


**Fig. 3.** Cardiomyocytes maintain the expression of heart-muscle-specific genes after transplantation into intact limbs and under culture conditions. (A,B,D,E,G,H) Stained cryosections. DiI-labeled cardiomyocytes (red) transplanted into intact limb maintained the expression of the cardiac-muscle-specific cTnT (A,B,G,H; green). (C,F,I) Newt cardiomyocytes in culture expressed (C) cTnT and (F,I) MyHC (MF20-staining). Sections and cultures were stained with Hoechst 33342 (blue) to visualize all nuclei.

specific marker after 15 and 30 days (Table 1). In contrast to the disappearance of cardiac-specific sarcomeric muscle proteins, the intermediate filament protein desmin, which is expressed in striated and smooth muscle cells, was present at all stages investigated. Interestingly, we found a re-expression of MyHC at late stages of limb regeneration 15 and 28 days after implantation of cardiomyocytes (Fig. 4F,L,R and supplementary material Fig. S3). Quadruple staining for DiI, Hoechst 33342 dye, Desmin and MyHC confirmed these findings: transplanted DiI-labeled cells expressed desmin but not MyHC 1 day after transplantation; transplanted cells that had maintained desmin expression started to re-express MyHC 15 days after transplantation (supplementary material Fig. S3). It should be noted, however, that the MF20 antibody detects both cardiac and skeletal MyHC, and thus does not allow discrimination between re-expression of cardiac MyHC and de

**Table 1. Fate of cardiomyocytes transplanted into regenerating limb stumps or intact limbs**

| | Labeled, implanted cells (in %) after | | |
|---|---|---|---|
| | 1 day | 15 days | 30 days |
| Transplantation into the non-injured limb (control) | | | |
| Apoptosis | 6±1 | 1±1 | 0±0 |
| Transplantation into regenerating limb stump | | | |
| Apoptosis | 3.5±1.5 | 0±0 | 0±0 |
| Loss of cardiomyocyte markers | 96.5±1.5 | 100±0 | 100±0 |
| Presence of blastemal markers | 35±5 | 0±0 | 0±0 |
| Expression of skeletal muscle markers | 0±0 | 40±5 | 65±5 |

The numbers represent approximate calculations from at least five transplantation experiments expressed as deviation of the means. Since transplanted cardiomyocytes often formed clusters it was not always possible to count individual cells.

novo expression of skeletal muscle MyHC (see below). To further prove the downregulation of cardiac-specific proteins in cardiomyocytes transplanted into regeneration limbs we performed western blot analysis. As expected, we observed expression of the skeletal-muscle-specific sTnI in isolated muscles, in the injured and in the intact limb that had received injections of cardiomyocytes, but not in the heart and in isolated cardiomyoctes (Fig. 5). Expression of the cardiac-muscle-specific cTnT was detected in the heart and in isolated cardiomyocytes but not in isolated skeletal muscle. In accordance with our immunofluorescence data, we detected cTnT protein in intact but not in regenerating limbs that had received injections of cardiomyocytes. Cardiomyocytes located in intact limbs continued to express cTnT for as long as 28 days, whereas transplantation into regenerating limbs efficiently erased cTnT expression shortly after implantation (Fig. 5). No re-expression of cTnT was monitored in regenerating limbs even 28 days after implantation of cardiomyocytes. Although attempts to implant labeled cardiomyocytes orthotopically into regenerating hearts varied considerably owing to technical difficulties caused by the thinness of the ventricular myocardium, we were able to locate clusters of DiI-labeled cells. Interestingly, all DiI-labeled cardiomyocytes had lost expression of the cardiomyocyte marker cTnT 1 day after implantation and were indistinguishable from the surrounding regenerating myocardium, which had also lost its cTnT expression (supplementary material Fig. S4). Apparently, both orthotopic and heterotopic regenerative environments induced de-differentiation of transplanted cardiomyocytes.

## Activation of blastema cell markers in cardiomyocytes transplanted into regenerating limbs

We next wanted to know whether the apparent de-

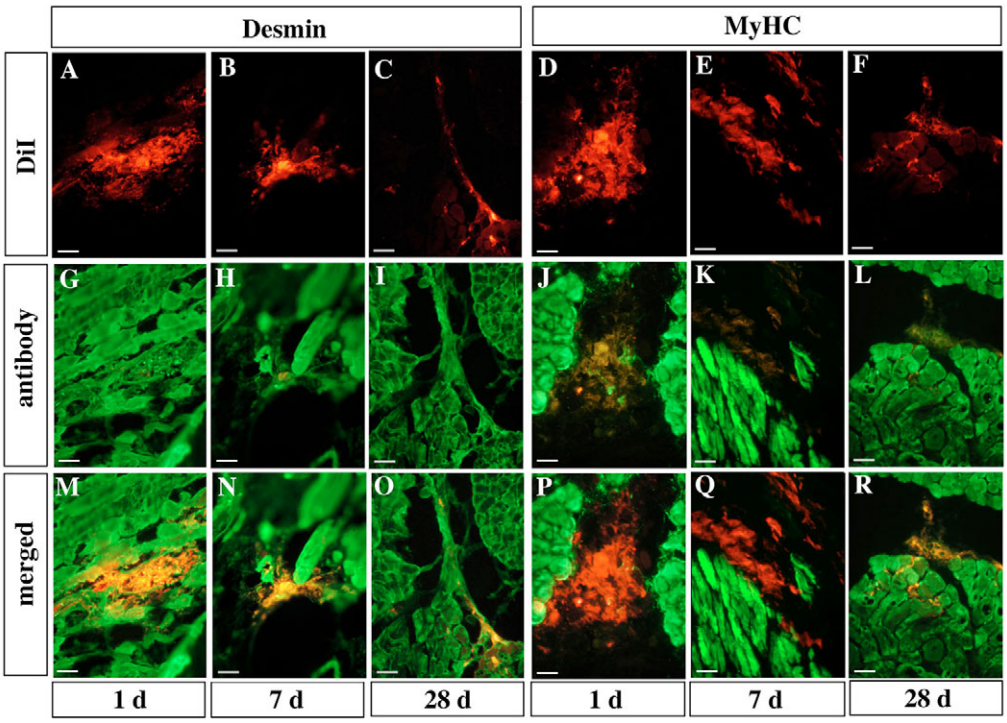


**Fig. 4.** Transplantation of newt cardiomyocytes into regenerating limbs results in a rapid downregulation of heart-muscle-specific genes. (A-L) DiI-labeled cardiomyocytes (A-F, red) were transplanted into a regenerating limb 5 days after amputation and staining for desmin (G-I, green) or MyHC (J-L, green) after 1, 7 and 28 days. Newt cardiomyocytes maintained the expression of the intermediary filament protein desmin in the majority of transplanted cells but rapidly lost the expression of MyHC (MF20 staining; note that MF20 detects both skeletal and cardiac MyHC isoforms). After 28 days, transplanted cells re-expressed MyHC. (M-O,P-R) Mergend images. Bars, 100 μm.

differentiation and the loss of cardiac-specific markers of cardiomyocytes implanted into regenerating limbs was associated with the expression of molecules characteristic for undifferentiated blastema cells. Staining with antibodies against vimentin and the blastemal cell marker 22/18 (Fekete and Brockes, 1987) revealed a strong expression of both antigens in approximately 30% of DiI-labeled cardiomyocytes 24 hours after implantation (Table 1). This expression decreased during the course of regeneration and was absent 15 and 30 days after implantation (Fig. 6 and Table 1). Like 22/18, expression of vimentin indicates an early stage of cell development. Vimentin is not only expressed in cardiomyocytes transplanted into regenerating limbs but also in the damaged areas of the regenerating heart during early stages of regeneration (data not shown). It is interesting that the expression of blastema cell markers (Fig. 6) was inversely correlated with the re-expression of differentiation markers 15 and 30 days after transplantation, respectively (Fig. 7).

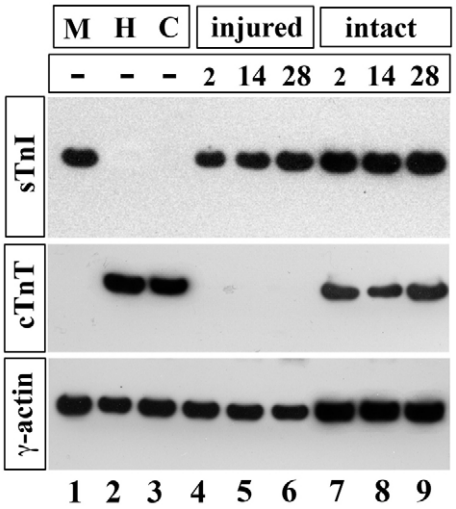


**Fig. 5.** Newt cardiomyocytes rapidly loose the expression of cardiac specific proteins after transplantation in regenerating but not in intact limbs. Western blot analysis of protein extracts prepared from skeletal muscle (M, lane 1), heart (H, lane 2), and cultured cardiomyocytes (C, lane 3) as well as injured and intact limbs (lanes 4-6 and 7-9, respectively) at different time points (2, 14 and 28 days) after transplantation of cardiomyocytes. Note the absence of cTnT after transplantation into injured but not intact limbs.

The presence of regeneration-associated cytoskeletal molecules, originally identified in limb blastema cells, within transplanted cardiomyocytes suggested that similar cellular events lead to the establishment of an actively proliferating population of progenitor cells in different organs. De-differentiation of cardiomyocytes and successful integration of transplanted cells into host tissue presumably depends on the processing of signals received from neighboring cells. A major signaling complex that relays extracellular signals into the cell is comprised by the focal adhesion kinase (FAK) at sites of focal adhesion. We used an antibody against FAK phosphorylated at Tyr397 to monitor phosphorylation of FAK. As shown in Fig. 6E,J,P transplanted cardiomyocytes, like most of the surrounding cells, stained strongly positive for FAK-Tyr397-*P*, indicating the activation of an intracellular pathway that promotes cell survival. Furthermore, the simultaneous appearance of phosphorylated FAK in transplanted cells and surrounding cells between 1 and 30 days post implantation demonstrated a successful integration into the regenerating tissue and a synchronization of signaling pathways.

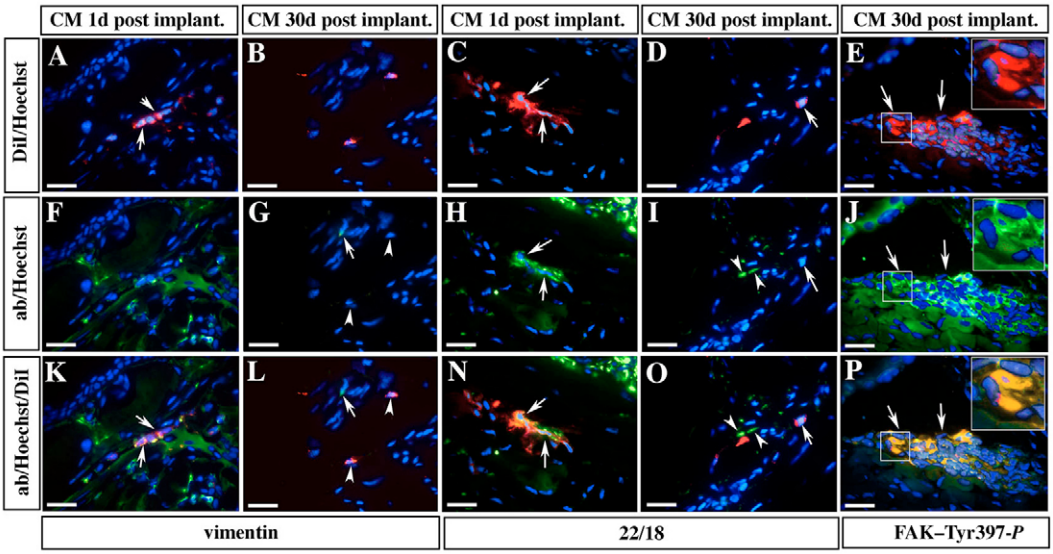


**Fig. 6.** Cardiomyocytes that undergo blastema-induced re-programming transiently express blastema cell markers and activate focal adhesion kinase at later stages. (A-L,N-P) Immunofluorescence staining of DiI-labeled cardiomyocytes (red) with vimentin (A,B,F,G,K,L), the blastema cell marker 22/18 (C,D,H,I,N,O) and FAK-Tyr397-*P* (E,J,P) 1 day and 30 days after transplantation. Bound antibodies on cryosections were detected with FITC-conjugated secondary antibody (green). Hoechst 33342 counterstaining was used to label all nuclei on the sections. Notice that, vimentin and 22/18 are expressed 1 day but not 30 days after transplantation. Activation of FAK occured simultaneously in transplanted and neighboring cells indicating successful integration into the recipient tissue. Bars, 50 μm.

### Newt cardiomyocytes transdifferentiate mainly into skeletal muscle cells in regenerating newt limbs and contribute to muscle fiber formation

As shown in Fig. 4 some transplanted cells began to express sarcomeric MyHC at 30 days after implantation. To analyze whether this expression was due to the re-expression of cardiac-specific markers or the transdifferentiation of (de-programmed) cardiomyocytes into skeletal muscle cells, we used antibodies specific for either cardiac or skeletal muscle proteins. It became apparent that transplanted cells began to express skeletal-muscle-specific sTnI at approximately day 15 after implantation (Fig. 7A,E,I). Fifteen days later, transplanted cells formed small muscle fibers that stained positive for sTnI. These fibers were associated with each other and with endogenous myofibers (Fig. 7B,F,J), indicating that the transplanted, cardiomyocyte-derived cells participated in the regeneration of skeletal muscle in a similar manner as blastema cells originating from the limb. Fifteen days after transplantation approximately 35% of DiI-labeled cardiomyocyte-derived cells stained positive for skeletal muscle markers. This ratio increased further to 65% 30 days after transplantation (Table 1).

In contrast to the expression of skeletal-muscle-specific proteins cardiomyocyte-derived cells seemed to have lost their ability to express cardiac specific genes. Two days after implantation the expression of the cardiac-muscle-specific cTnT was lost, corresponding to the upregulation of blastema cell markers that clearly indicated de-differentiation of transplanted cardiomyocytes. Cardiomyocyte-derived cells did not regain any expression of cTnT. Although DiI-labeled cells were easily identified 30 days after implantation, they did not resume expression of cTnT (Fig. 7D,H,L and Table 1). Cardiomyocyte-derived cells did also contribute to the generation of chondrocytes albeit at a much lower rate than to skeletal muscle cells. Thirty-five days after amputation, single DiI-labeled chondrocytes, which were identified by staining with LE-Lectin coupled to a fluorochrome, were present at different planes in regenerating limbs both in a proximal position close to the amputation site and more distally in the developing toe buds (supplementary material Fig. S5).

### Induction of cell-cycle entry and de-differentiation are not necessarily coupled

The observed downregulation of sarcomeric proteins in cardiomyocytes in regenerating hearts and after transplantation into regenerating limbs might imply that de-differentiation is a prerequisite for cell-cycle re-entry. We tested this hypothesis by cultivating newt cardiomyocytes in vitro for different periods of time. Interestingly, newt cardiomyocytes maintained expression of MyHC despite the onset of histone H3 phosphorylation. Although only a small number of MyHC-positive cells were in G2 after 24 hours (Fig. 8A,F,K) this ratio increased significantly after 72 hours rendering most cardiomyocytes in culture positive for phosphorylated histone H3 (Fig. 8B,G,L), clearly indicating that loss of the differentiated phenotype and cell-cycle entry are not necessarily coupled. Cardiomyocytes that were transplanted into regenerating limbs and that had lost their expression of MyHC and cTnT (Figs 1, 7) did also show a robust expression of phosphorylated histone H3, which exceeded mitotic activity of neighboring cells (Fig. 8C,H,M). Surprisingly, the mitotic index of regenerating limbs was comparatively low once the blastema was established. Fig. 8E,J,O shows cells positive for phosphorylated histone H3 in the blastema and in the adjacent skeletal musculature 5 days after amputation.

In mammals, transplantation of cells often leads to a loss of transplanted cells due to programmed cell death. To analyze whether the high rate of cells in G2 phase can in part be

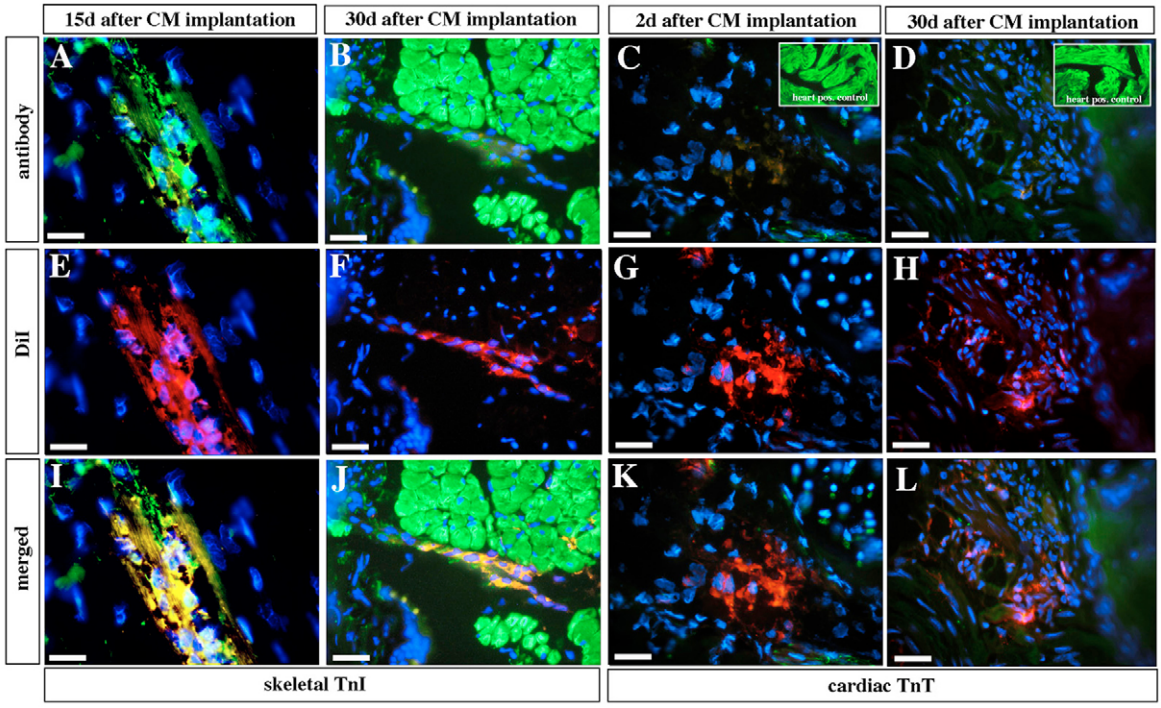


**Fig. 7.** Newt cardiomyocytes transdifferentiate into skeletal muscle cells in regenerating newt limbs. Immunofluorescence staining of DiI-labeled cardiomyocytes (red) with anti the skeletal-muscle-specific anti-sTnI antibody (A,B,I,J; green) and with the cardiac-muscle-specific anti-cTnT antibody (C,D,K,L; green). Hoechst 33342 counterstaining was used to localize all nuclei on the cryosections. Inlets in C and D, are positive controls for cTNT staining using sections derived from the heart. Note the rapid downregulation of cTnT in transplanted cardiomyocytes and the upregulation of skeletal-muscle-specific proteins in DiI-labeled cells 15 days after transplantation. The formation of cardiomyocyte-derived skeletal myotubes are clearly visible 30 days after transplantation. Bars, 100 μm.

explained by a selective loss of non-cycling cells, we performed terminal deoxynucleotide transferase (TdT)-mediated nick-end labeling (TUNEL) analysis. Clearly, transplanted cardiomyocytes did not undergo massive programmed cell death. TUNEL-positive cells were found only rarely within the transplanted cell populations and were most often found when isolated cells were not accompanied by other transplanted cells (Fig. 8D,I,N). Apoptotic cells were only detected during the first 2 days after cell transplantation. Later stages were virtually devoid of apoptotic cells (Table 1). We also did not find signs for oncosis or other modes of cell death in transplanted cells (data not shown), suggesting that most of the transplanted cardiomyocytes were successfully incorporated into the regenerating limb blastema.

## Discussion

A better understanding of the mechanisms leading to the regeneration of the heart might be a first step to evoke similar processes in mammals. In this study, we employed the newt model to analyze the extent of plasticity of cardiomyocytes and its dependence on regenerative environments. Newt cardiomyocytes implanted into regenerating limbs showed a remarkable plasticity and rapidly acquired properties that resembled blastema cells. At later stages of regeneration cardiomyocyte-derived cells exhibited expression of skeletal-muscle-specific genes and of a cartilage marker, and participated in the re-growth of the limb and the generation of skeletal muscle.

In damaged or partially amputated hearts neither did typical blastema form nor did fully differentiated cardiomyocytes start to proliferate. In contrast to a blastema-based regeneration process, heart repair in newts seems to rely on cells that own properties of both blastema cells and cardiomyocytes. In fact, bona fide blastema cells that are able to form various cell lineages might not be required in the heart because the myocardium contains only a limited number of different cell types and a limited need for positional information. Yet, cardiomyocytes clearly own the ability to form blastema-like cells and can differentiate into different cell types when placed in regenerating limbs – even if they do not need this function during heart regeneration. It seems an attractive idea that all newt cells share a similar ‘core’ program that enables them to respond to regenerative cues. Under certain circumstances, when only a limited number of different cell types are needed – such as in the heart – this program might be executed only partially. In this respect, it is interesting that most cardiomyocyte-derived cells maintained the expression of the intermediate filament desmin, which is found in all striated muscle cells, smooth muscle cells and muscle precursor cells. Cells earmarked for skeletal muscle formation most likely maintained expression of desmin and/or expressed vimentin (and the blastema cell marker 22/18, which is often expressed together with other intermediate filament proteins during the early stages of development). Unlike desmin, the expression of vimentin and 22/18 disappeared when differentiation proceeded. Fifteen days after implantation cells never showed a positive response for vimentin and 22/18, indicating a re-differentiation of the transplanted cells. The ability of newt

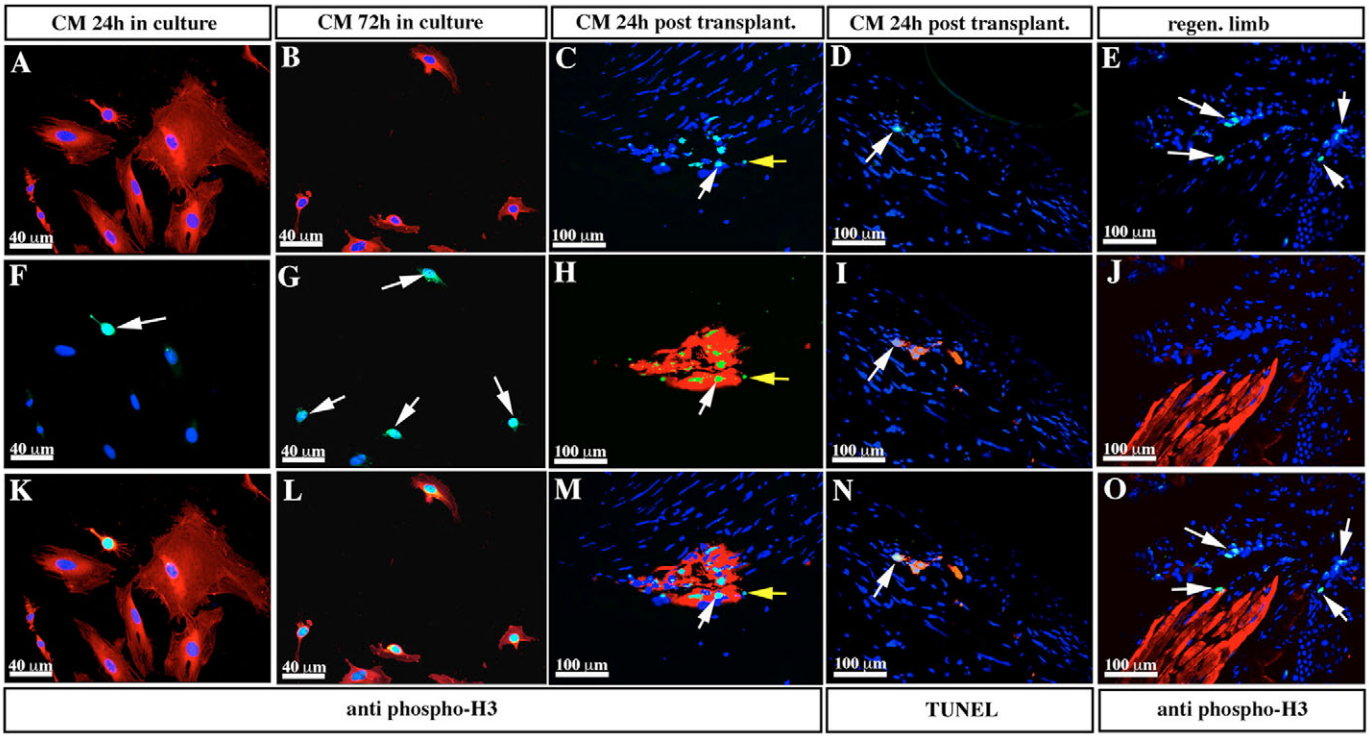


**Fig. 8.** Newt cardiomyocytes re-enter the cell-cycle in vitro and in vivo and rarely undergo programmed cell death after transplantation. Immunofluorescence staining of phosphorylated histone H3 in cultured cardiomyocytes 24 hours (A,F,K) and 72 hours (B,G,L) after isolation. Cells were labeled with MyHC (red, MF20 staining), anti-phosphorylated histone H3 (green, appears cyan due to color overlay) and Hoechst 33342 to indicate all nuclei. White arrows indicate cells in G2. Immunofluorescent detection of phosphorylated histone H3 in cardiomyocytes 24 hours after transplantation (C,H,M). Cardiomyocytes were DiI-labeled before transplantation and stained with anti-phosphorylated histone H3 (green fluorescence, appears cyan due to color overlay) and Hoechst 33342 to indicate all nuclei. White arrows in (C,H,M) indicate cardiomyocyte-derived cells in G2, yellow arrow indicates a limb-derived cell in G2. TUNEL-labeling of DiI-labeled cardiomyocytes 24 hours after transplantation (D,I,N). Note that transplanted cardiomyocytes only rarely underwent programmed cell death (white arrow). (E,J,O) Cycling cells in regenerating limbs; cells were labeled with MyHC (red, MF20 staining), anti anti-phosphorylated histone H3 (green, appears cyan due to color overlay) and Hoechst 33342 to visualize all nuclei. Note that cell-cycle progression in the regenerating limb occurred both in the skeletal myotubes (red) and in the blastema.

cells to undergo a major reprogramming is also emphasized by the fact that urodele amphibians are able to reprogram spinal cord cells to skeletal muscle and cartilage upon tail regeneration, which indicates an ectoderm-to-mesoderm switch (Echeverri and Tanaka, 2002).

Recently, it has been proposed that skeletal muscle de-differentiation involves satellite-cell activation indicating that limb regeneration and mammalian tissue repair share common cellular and molecular programs (Morrison et al., 2006). This is in contrast to the established view that de-differentiation of mature cells is the major source for blastema formation (Kumar et al., 2000; Kumar et al., 2004). If reversal of the differentiated state of mature cells and activation of stem cells in regenerating tissues co-exist, the quantitative aspects of their relative contribution in vivo remain to be determined. At present, it cannot be excluded that de-differentiating skeletal muscle will contribute to the generation of muscle stem cells, which are Pax7-positive (Morrison et al., 2006). Since Pax7 is required for the renewal and maintenance of muscle satellite cells and is strongly upregulated during muscle regeneration in muscle progenitor cells (Oustanina et al., 2004), it seems likely that any pathway leading to muscle regeneration will result in an increase of Pax7-positive cells, irrespective of their origin.

Although adult newt cardiomyocytes seem to posses a heterogeneous proliferation potential in vitro, the fact that the majority of cultured cardiomyocytes can enter S phase (Bettencourt-Dias et al., 2003) and contain the G2 marker phosphorylated histone H3 (this study), argues against a stem-cell-based mode of heart regeneration. It seems extremely unlikely that we have transplanted cardiac stem cells into the regenerating limbs, in particular because the cardiomyocyte cultures used for transplantation were highly homogenous (>95% MyHC-positive). Thus, we conclude that the contribution of cardiomyocytes to limb regeneration depends on reversal of the differentiated state of mature cells, re-emphasizing the original idea of de-differentiation of mature cells as a major mode for organ regeneration (Brockes, 1997). It should be pointed out, however, that we cannot completely rule out the possibility that the contribution of cardiomyocyte-derived cells to limb regeneration depends on cell fusion rather than on cell-autonomous de-differentiation and/or re-differentiation processes. Although technical limitations of the newt system prevented to solve the problem of cell fusion unambiguously, we assume that we have described a bona fide de-differentiation and/or re-differentiation process for two reasons: (1) usually cell fusion of non-muscle cells is a rare

event (Schulze et al., 2005), whereas in our experiments the majority of transplanted cells acquired skeletal-muscle-specific markers; (2) we found expression of skeletal muscle markers in cardiomyocyte-derived cells, which had no direct contact to neighboring skeletal muscle cells, making cell fusion unlikely.

The ability to reprogram differentiated cells is certainly not an unique feature of newts within the vertebrate phyla. It has been shown that differentiated cells can be reprogrammed by cell fusion (Blau et al., 1985; Schulze et al., 2005), by nuclear transfer in cloning experiments (Hochedlinger and Jaenisch, 2002), and as a part of a developmental program (Patapoutian et al., 1995). It is surprising that reprogramming of mammalian cells, despite its feasibility, is rarely observed in physiological settings. Evolution might have selected against de-differentiation and transdifferentiation to accomplish regeneration because of a number of reasons. First, de-differentiation within damaged tissue results in an additional, transient decrease in the number of functional cells that might further deteriorate tissue function. Second, de-programming of cells is an additional step, which will give rise to unwanted dysfunctional cells that might be detrimental for the function of certain organs. A nice example is the switch from smooth to skeletal muscle, which involves the appearance of individual cells, which express a mixed phenotype (Patapoutian et al., 1995). Although it might be more common that a differentiation status is erased before a new phenotype is established, intermediate situations apparently exist in which de-differentiation and re-differentiation occur more or less simultaneously, as demonstrated by the continued expression of desmin in transplanted cardiomyocytes. Last, – and probably most importantly – de-differentiation leads to the mobilization of cells that have already encountered many potentially hazardous conditions. Unlike stem cells, which divide very slowly and are usually kept aside in well-protected niches, differentiated cells might have collected mutations both in the nuclear and mitochondrial DNA, and carry otherwise dangerous cargo. Any future approach to utilize de-differentiation for therapeutic purposes must take this into account and aim on a careful selection of appropriate cells.

The finding that cardiomyocytes in culture enter the cell cycle and proliferate without a major downregulation of cardiac-muscle-specific genes contrasts to the situation in regenerating hearts and in cardiomyocytes transplanted into regenerating limbs and demonstrates that de-differentiation and cell-cycle re-entry are not necessarily coupled. Yet, cardiomyocytes in culture do not proliferate efficiently (Bettencourt-Dias et al., 2003), whereas cardiomyocyte-derived cardiac progenitor cells in the heart and cardiomyocytes that had been transplanted in regenerating limbs show proficient proliferation. This might indicate that de-programming of cells greatly improves the ability of cardiomyocyte-derived cells to re-populate damaged tissue areas or re-grow complete organs.

The fact that cultured cardiomyocytes continue to express cardiac-specific genes corresponds to our finding that cardiomyocytes maintained the differentiated state when transplanted into non-injured limbs. Only the presence of regenerating tissue, such as in damaged hearts and amputated limbs, evoked de-programming of cardiomyocytes. The presence of an activity within the blastema that can induce de-dedifferentiation of cells to become proliferative progenitors is well documented (McGann et al., 2001). It remains to be seen whether the same factors are present in regenerating hearts and contribute to the (partial) de-differentiation of cardiomyocytes in situ and in cardiomyocytes transplanted into regenerating limbs (Straube et al., 2004). The exact identity of signals that link tissue injury to tissue regeneration needs to be elucidated, as it may reveal key signaling pathways involved in the initiation of cellular de-programming and progenitor-cell formation. Our results have unveiled a previously unknown plasticity of newt cardiomyocytes. Although we cannot exclude that heart regeneration in urodeles involves activation of cardiac stem cells, our transplantation experiments have clearly established that a uniform population of cultured MyHC-positive cardiomyocytes can change its identity. We reason that cardiac regeneration in newts is based on de-differentation of mature cardiomyocytes, which enables them to respond efficiently to the induction of DNA synthesis and to proliferate efficiently. Since mammalian cardiomyocytes still own a certain ability to re-enter the cell cycle (Ebelt et al., 2005) and to proliferate (Engel et al., 2005), a better understanding of the molecular signals that control heart regeneration in newts might open the way for heart regeneration in mammals.

## Materials and Methods

### Animal maintenance and manipulation

Adult newts, *Notophthalmus viridescens* were obtained from Charles Sullivan & Co. (Nashville, TN) and were maintained in aquaria with water at 18-20°C. Newly arriving animals were treated with a disinfection solution (0.5% sulfamerazine) for 24 hours. Animals were fed with gnat larvae and artemia two to three times each week. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All animal experiments were permitted and approved by the local authorities. All operations were performed under anaesthesia [0.1% tricaine (ethyl 3-aminobenzoate methanesulfonate salt; Sigma) for 5-10 minutes]. Hindlimbs were amputated below the elbow. Heart injury was achieved by application of massive mechanical damage (15 times squeezing of the heart with a forceps), which resulted in intracardial hemorrhage and blood clot formation. After the operation newts were placed on ice for 45 minutes, transferred to a 0.5% sulfamerazine solution overnight, and then into normal aquarium water. Cell transplantations into the limb stumps were performed 5 days after amputation by multiple injections (three to four) of labeled cardiomyocytes suspended in amphibian PBS (aPBS; 70% of normal PBS in water).

### Isolation and labeling of cardiomyocytes

For the isolation of cardiomyocytes 30-35 newt hearts were dissected under aseptic conditions and collected in 70% Leibovitz-15 (L-15) medium supplemented with penicillin-streptomycin. Newt ventricles were dissociated as described (Bettencourt-Dias et al., 2003) with several modifications. The enzyme solution was prepared in aPBS containing gentamicin (50 μg/ml). After 2 hours of dissociation the cell suspension was discarded. Depending on the activity of trypsin and collagenase batches the dissociation solution was changed every 1-2 hours. Cell suspensions were neutralized with complete L-15 medium (70% L-15 with 10% FBS and penicillin-streptomycin), centrifuged in a table-top centrifuge 10 minutes at 500 rpm, and resuspended in complete L-15 medium. Cells were pre-plated in 6-cm uncoated culture dishes for 2 days at 25°C before the culture supernatant containing the myocytes was transferred onto laminin-coated dishes (15 μg/ml laminin in serum-free medium, coating for 60 minutes). Culture dishes were left undisturbed for 3 days before the first medium change. Cardiomyocytes were used for labeling and implantation experiments 12-14 days after plating on laminin-coated dishes.

Cardiomyocytes were labeled with CM-DiI (C-7001, Molecular Probes) according to the manufacturer's instructions. In brief, the medium (complete L-15) was removed and the cells were washed with aPBS. Attached cells were incubated with CM-DiI (2 μg/ml) in aPBS (2 ml labeling solution/dish) for 5 minutes at 25°C, and then for additional 15 minutes at 4°C. Cells were washed with aPBS and harvested by trypsinization (0.05% trypsin/0.02% EDTA for 20 minutes). Trypsin was neutralized by adding complete L-15 medium. Cells (approximately 100,000 cardiomyocytes) were resuspended in 150-200 μl aPBS after centrifugation and transplanted into limb stumps by 3-4 successive injections. Although the efficiency of the transplantation procedure varied between individual experiments, we were usually able to retrieve between 40 and 60% of injected cells. These numbers did

not change dramatically during the first two weeks after transplantation, although the DiI-labeled cells were more dispersed at later stages. Since most labeled cells were located within tightly packed clusters it was not possible to count individual cells within the first 24-48 hours after transplantation. For double or triple labeling of cells, the CM-DiI solution was removed and myocytes were incubated with Hoechst 33342 (1 μg/ml) in aPBS for 5 minutes at 24°C and/or with an adenovirus encoding EGFP as described previously (Ebelt and Braun, 2003).

### Immunohistochemistry

Regenerating and intact organs were dissected at various time points as indicated and fixed in 4% paraformaldehyde (in aPBS) for 2 hours, washed in aPBS, treated with aPBS containing 30% Saccharose at 4°C overnight and embedded in Polyfreeze tissue freezing medium (Polysciences, Inc.) on dry ice. Tissue blocks were sectioned on a cryostat at 10 μm. For immunohistochemistry the freezing medium was washed off and the sections were blocked with 0.5% BSA and 3% horse serum in aPBS. After 2 hours the blocking solution was removed and the sections were incubated overnight with the appropriate antibody at 4°C, followed by three washes with aPBS. Sections were exposed to the secondary antibody labeled with FITC, DTAF or another fluorochrome for 2 hours at room temperature to visualize bound antibody. Finally, sections were washed, incubated with Hoechst 33258 (1 μg/ml) to detect nuclei, washed again twice and mounted in Mowiol. Detection of nuclear antigens was achieved by treatment of the sections with 0.1% Triton X-100 in PBS for 10 minutes followed by an additional washing step before blocking. Semithin sections and histochemical staining procedures were performed using established protocols (Kostin et al., 2003).

Cardiomyocytes grown in culture dishes were washed twice with aPBS and fixed with 70% methanol/30% aceton (v/v) at –20°C. Fixation was repeated with fresh methanol-aceton for 20 minutes at –20°C. After aspiration of the fixation agent the dishes were dried. Cells were treated three times with 0.1% Triton X-100 in aPBS, blocked with 0.5% BSA, 0.1% Triton X-100, 3% horse serum in aPBS and washed before incubation with primary antibodies overnight at 4°C (or 2 hours at room temperature). Cells were washed three times with PBS and incubated with the secondary antibody for 2 hours at room temperature. Cells were washed, incubated with Hoechst 33342 as described above and washed twice. Mowiol was used for mounting cells. Pictures were taken on an Axioplan 2/Axiophot 2 microscope (Zeiss) and assembled using Adobe Photoshop 7.0.

### RT-PCR, TUNEL assay, western blot analysis and antibodies

mRNA was isolated from regenerating newt hearts using published procedures (Schulze et al., 2005). Reverse transcription PCR (RT-PCR) was performed as described previously (Schulze et al., 2005). cDNA sequences for newt genes encoding various sarcomeric proteins were selected from a newly created newt EST database, which will be published elsewhere. Reverse transcribed RNAs were amplified in 32 cycles with annealing temperatures at 55°C and an elongation time of 25 seconds. Oligonucleotide sequences used for RT-PCR were: myosin-heavy-chain polypeptide 7 (1242s 5′-TTCACCAGCTAAAGGCAGAC-3′, 1496as 5′-GGTATCCCTGCAGTGTCTTG-3′); cardiac troponin T (360s 5′-AAGTTGTT-TATGCCAAACCTG-3′, 721as 5′-TATCCGCCAAAATGTAAAGAG); cardiac troponin I (292s 5′-GCCTCAGGTTAAGAGAAAACC-3′, 621as 5′-TTATCCA-CATCGTCCTTCTTC-3′); cardiac troponin C (103s 5′-AACTCTCCAGGG-ACTCTGAAC-3′, 449as 5′-TATCATCTCCTGCAACTCCTC); cardiac actin (54s 5′-ATGTACCCTGGTATTGCTGAC-3′, 335as 5′-AACTCCTTGGAGATGATAA-AGG-3′); 40S ribosomal protein S21 (35s 5′-AAGTAACCATGCAGAACGATG-3′, 261as 5′-GGCTAACCGAAGGATAGAGTC-3′). TUNEL assays (Roche) were performed according to manufacturer's instructions. To detect the presence of proteins by western blot analysis tissue samples were prepared by ultraturax homogenization and boiled for 1 minute; 20 μg of protein samples were resolved on 4-12% SDS polyacrylamide gradient gels (InVitrogen) and blotted onto nitrocellulose membranes. After incubation with primary antibodies overnight at 4°C, membranes were washed and incubated with the appropriate horseradish-peroxydase-coupled secondary antibodies (Dianova) for 45 minutes at room temperature. Bound antibodies were visualized using the Femto detection kit (Perbio Science) according to instructions of the manufacturer.

The following antibodies were used in the course of this study: anti-skeletal troponin I (A9), anti-cardiac troponin T (1F2); anti-troponin I (cardiac and skeletal, C5; Acris/DPC Biermann), anti-vimentin (XL-VIM-14.13; Progen Biotechnik), anti-blastemal marker (22/18) and anti-skeletal muscle marker (12/101) were both from DSHB – University Iowa, IA. Anti-skeletal myosin (MY-32) and anti-phosphorylated histone H3 (HTA28) were both from Sigma. Anti-myosin (MyHC, MF-20, hybridoma cell culture supernatant) and anti-FAK-Tyr397-*P* (rabbit polyclonal) were from Calbiochem. Anti-desmin (rabbit antiserum) was from Sigma. Usually, primary antibodies were used in a concentration of about 10 μg/ml. The specificity of primary antibodies was analyzed in control sections of skeletal and heart muscle (supplementary material Fig. S1). Secondary antibodies against mouse (FITC-conjugated immunoaffinity purified $F(ab')_2$ fragment goat anti-mouse IgG) and rabbit (DTAF-conjugated immunoaffinity purified goat anti-rabbit IgG) primary antibodies were obtained from Dianova. When necessary, additional fluorochrome-conjugated secondary antibodies were used as indicated.

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