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The 21-day postnatal rat ventricular cardiac muscle cell in culture as an experimental model to study adult cardiomyocyte gene expression

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Abstract

The purpose of this study was to develop and characterize a cardiomyocyte culture system for use as an experimental model to study the mechanism(s) by which cardiac muscle cells permanently exit the cell cycle during early neonatal life. Ventricular cardiomyocytes, isolated by retrograde perfusion of hearts from 21-day-old and adult rats, were compared through 10 days of culture. Expression patterns of genes encoding developmentally programmed proteins were determined to be similar between cardiomyocytes cultured from 21-day-old and adult rats, using the reverse transcription polymerase chain reaction. A *lacZ*-expressing reporter gene was used to test the efficiency of gene delivery in cultured cardiomyocytes. Transfections using cationic liposomes yielded 24 ± 7, 25 ± 7 and 10 ± 1% cardiomyocytes positive for β-galactosidase activity in cultured 1-day, 21-day and adult cardiomyocytes, respectively. Direct needle microinjection resulted in 48 ± 7, 35 ± 6 and 37 ± 5% cardiomyocytes positive for enzymatic activity in 1-day, 21-day and adult cardiomyocytes, respectively. Cell cycle-specific cDNA arrays were used to analyze the expression pattern of cell cycle-related genes in 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)- and non-TPA-treated cultured 21-day cardiomyocytes. Based on the similarity of cultured 21-day to adult ventricular cardiomyocytes and their high transfection efficiencies, we propose the use of cultured cardiomyocytes from 21-day-old rat ventricles as an experimental model system for the study of adult cardiomyocyte gene expression and cell cycle machinery. (Mol Cell Biochem **229**: 51–62, 2002)

Key words: adult ventricular cardiomyocytes, gene expression, transfection, microinjection, cell cycle arrays

Introduction

Growth of the mammalian heart during early development is by cell proliferation, which ceases permanently in cardiac myocytes shortly after birth. Subsequent growth of the heart is by enlargement of the cardiomyocytes. The molecular mechanism(s) controlling the permanent withdrawal of cardiomyocytes from the cell cycle are as yet undefined, but undoubtedly play a critical role in defining the anatomy, physiology and eventual pathology of the mature heart. A major objective of our laboratory is to understand the cell cycle machinery controlling cardiac muscle cell division. Unfortunately, the available experimental systems to study these phenomena remain limited.

The aim of the current study was to develop a cardiomyocyte culture system for use as a model system to investigate the mechanism(s) by which cardiomyocytes permanently exit the cell cycle during neonatal life. Ideally, these cardiomyocytes would have recently exited the cell cycle, acquired the adult phenotype, and yet have been immature enough to be manipulated genetically. After examining cultured ventricular cardiac myocytes from rat hearts of various developmental ages, cardiomyocytes cultured from 21-day-old rats (21-day cardiomyocytes) were determined to fit these criteria. This

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culture system was then characterized by microscopic examinations of cellular morphology, analyses of developmentally regulated genes using RT-PCR, transfection, and microinjection. The expression of a set of cell cycle genes was also examined in cultured 21-day cardiomyocytes treated with 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). Since it had been previously shown that TPA causes a significant increase in DNA synthesis in adult ventricular cardiomyocytes in culture [1], ventricular cardiomyocytes from 21-day-old rats were cultured in the presence of this phorbol ester, and the expression levels of cell cycle genes examined by screening a cell cycle specific cDNA array.

In light of their phenotypic similarity to cultured adult ventricular cardiac myocytes and their ability to be manipulated genetically, cultured cardiomyocytes from 21-day-old rats are proposed as an experimental model system for the study of adult gene expression and cell cycle regulation. The important advantage of this *in vitro* culture system is that it provides the researcher with a virtually pure population of ventricular cardiac muscle cells that have been isolated from the rat heart during development when they have just dropped out of the cell cycle.

Materials and methods

Animals

Two-month-old female rats (Harlan Sprague–Dawley, Indianapolis, IN, USA), approximately 200 g, represented our adult population. Timed-pregnant rats provided the postnatal hearts.

One-, 5- and 10-day-old pups were sacrificed by decapitation. Adult, 21-day-old and 17-day-old rats were injected intraperitoneally with sodium heparin (Sigma; 1000 U/g body wt.) to prevent blood clots, which could impair perfusion. One h later, the animals were anesthetized using a mixture of Ketamine (Schering-Plough Animal Health; 100 μg/g body wt.) and Xylizine (Schering-Plough Animal Health; 10 μg/g body wt.). All animal procedures were performed according to guidelines provided by the Institutional Animal Care and Use Committee of the Louisiana State University Health Sciences Center, complying with federal and state guidelines.

Isolation and culture of ventricular cardiomyocytes from neonatal 1-, 5- and 10-day-old rats

Neonatal cardiomyocytes were isolated by enzymatic treatment of ventricular tissue from 1-, 5- and 10-day-old rats. Excised ventricles were pooled, minced in ice-cold phosphate buffered saline (PBS), and digested for 15 h at 4°C with 0.125% trypsin solution (Life Technologies) in Joklik's Modified Eagle Medium (J-MEM). Following a 10-min digestion with 0.1% collagenase (Type II, Worthington Biochemical Corp.) in J-MEM at 37°C, the supernatant containing the cardiomyocytes was decanted into a centrifuge tube. An equal amount of PBS was added. After centrifugation for 3 min at low speed, the pellet was resuspended in PBS. Following a second wash in PBS and centrifugation, the pellet was resuspended in Dulbecco's Modified Eagle Medium (D-MEM), high-glucose content recipe, supplemented with 10% fetal bovine serum (FBS) from a pre-selected lot (BioWhittaker), 1 × MEM vitamin solution, 1 × MEM non-essential amino acids solution, 1 × L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml), all from Life Technologies. The isolated cells were incubated for 1 h in an uncoated flask in a humidified 37°C incubator with 95% O_2, 5% CO_2, allowing the non-myocyte population to differentially attach to the flask, thereby enriching the non-adherent cardiomyocyte population [2]. The cardiomyocytes (4×10^4 cells/cm^2) were inoculated into tissue culture grade plasticware that had been pre-coated (0.08 ml/cm^2) for 4 h with 12.5 μg fibronectin (Sigma) per ml of 0.02% Bacto-gelatin (Difco Laboratories).

Isolation and culture of 17-day-old, 21-day-old and adult cardiomyocytes

Ventricular cardiomyocytes were isolated and cultured from 17- and 21-day-old rats following a modified version of a perfusion protocol previously published for adult cardiomyocytes [3, 4]. The modified base of the perfusion apparatus was made in our laboratory from blunted 21-gauge needles to fit the smaller aortas of the 17- and 21-day hearts. While adult hearts were perfused with 1% collagenase for 30–45 min [3, 4] the 17- and 21-day hearts were perfused with 0.7% collagenase for 25–30 min. Adult cardiomyocytes were cultured at a density of 1×10^4 cells/cm^2, and 17- and 21-day-old cardiomyocytes at 1.5×10^4 cells/cm^2 on laminin-coated tissue cultureware. Proliferating non-cardiomyocytes were eliminated by the addition of 10 μM cytosine-1-β-D-arabinofuranoside (Ara-C) to the culture medium during the first 7 days of culture [4]. The cardiomyocytes were cultured in D-MEM, high glucose content recipe, supplemented with 10% pre-selected FBS, 10 μg/ml transferrin (Calbiochem), 10 μg/ml insulin, 1 × MEM vitamin mix, 1 × MEM non essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin.

Screening parameters of ventricular cardiomyocytes

To estimate a developmental age at which to develop the culture system, three conditions were initially examined in a screening of cardiomyocytes cultured from 1-, 5-, 10-, 17-,

21-day-old and adult (60-day-old) rats. Conditions examined were the protocol used to isolate the cells, the extracellular matrix attachment factor that was optimal for cell attachment, and the morphology of the freshly isolated cardiomyocyte.

Photomicroscopy

Cultured 21-day cardiomyocytes were examined using a Nikon inverted optics phase contrast microscope with a 20 × objective, and photographed using Kodak TMC100 black and white film on the 1st, 3rd, 5th and 8th day of culture. Digital fluorescent images of MF-20/Hoechst stained cardiomyocytes, as well as TPA and control 21-day-old cardiac muscle cells were acquired with a Micromax cooled CCD digital camera system (Advanced Imaging Corporation), and analyzed using MetaMorph software, version 4.5 (Advanced Scientific).

Immunofluorescence

Cultured 1-day, 21-day and adult cardiomyocytes were examined with an antibody to sarcomeric myosin (MF-20; Developmental Studies Hybridoma Bank, Dept of Biological Sciences, Iowa City, IA, USA) conjugated to FITC, and counterstained using Hoechst 33342 for simultaneous examination of their cellular morphology and nucleation. Differentiation at the morphological level was evaluated by examining cellular size, structural organization of the myofibrils, and proportion of binucleated cells in the cultures.

Ventricular cardiomyocytes cultured on coverslips were fixed for 30 min at room temperature in ethanol:acetic acid: H_2O (18:1:1) and permeabilized with 0.1% Triton X-100 for 3 min at room temperature. The mouse MF20 monoclonal antibody to sarcomeric myosin heavy chain was used as the primary antibody for indirect immunofluorescent staining. A FITC-conjugated goat anti-mouse IgG (Sigma) was used as the secondary antibody. The cardiomyocytes were counterstained with Hoechst 33342. Digital fluorescent images of MF-20/Hoechst stained cardiomyocytes were acquired with a Micromax cooled CCD digital camera system (Advanced Imaging Corporation), and analyzed using MetaMorph software, version 4.5 (Advanced Scientific).

RNA isolation and RT-PCR

Expressions of the developmentally regulated genes for myosin heavy chain (MHC), α-actin, connexin43 (Cx43) and atrial-natriuretic factor (ANF) were used in the present studies to compare the gene expression of the 21-day and adult cardiomyocytes. Rat tissues used in these studies included ventricular tissue from 17-day-old embryos (e17), adult atria (Atria), and adult skeletal muscle (SkM). Freshly isolated and cultured (day 10 of culture) 21-day-old and adult ventricular cardiomyocytes represented our cellular population. Total RNA was isolated from tissues and cells with TriZOL Reagent (Life Technologies), according to the manufacturer's protocol. The RNA was treated with RNase-free DNase I (Life Technologies) according to the manufacturer's protocol, and reverse transcribed using 1 μM of an oligo$(dT)_{12–18}$ primer (Life Technologies), 20 μM dNTP, 1 × reverse transcription buffer, 200 units of MMLV-reverse transcriptase (Promega) and 0.2 μg DNase I-treated RNA in a 20 μl reaction.

Each 50 μl PCR reaction contained 1 μl of reverse transcribed RNA, 0.2 μM each of the appropriate sense primer and corresponding antisense primer, 1.5 mM $MgCl_2$, 0.2 μM dNTPs, 2.5 units of Taq DNA polymerase (Promega) and 1 × manufacturer-supplied thermophilic DNA buffer. Primer pairs to amplify the transcripts for α-MHC, β-MHC, α-cardiac actin, α-skeletal actin, connexin43 (Cx43) and atrial natriuretic factor (ANF), as well as the amplification protocol were previously published [5].

In the triiodothyronine (T_3) experiment, the medium was supplemented on day 7 of culture to a final concentration of 1.0 μM 3,3′, 5-triiodothyronine (Sigma), after the removal of Ara-C from the culture medium. The cardiomyocytes were harvested 48 h later.

Transfection

Transient transfections of 1-day-old, 21-day-old and adult cardiomyocytes were performed on cells cultured in six-well plates. One-day cardiomyocytes were cultured at a density of 2×10^5 cells/well, 21-day cardiomyocytes at 7.5×10^4 cells/well, and adult cardiomyocytes at 5×10^4 cells/well. The exogenous gene used in these studies was a pUC19-based plasmid capable of expressing *LacZ* under the control of the cytomegalovirus promoter. On day 10 of culture, 2 μg of the exogenous *LacZ* plasmid was added to 0.4 ml of Opti MEM I (Life Technologies), and 40 μl of LipofectAMINE (Life Technologies) was added to a different tube containing 0.4 ml of Opti MEM I. The two solutions were combined, and allowed to incubate for 45 min at room temperature. To this DNA/LipofectAMINE solution, 1.2 ml of Opti MEM I was added to create the transfection mixture, which contained no antibiotics. After the cells were rinsed once with Opti MEM I, they were overlaid with 2 ml/well of transfection mixture and allowed to incubate for 8 h in a humidified 37°C incubator in 95% O_2, 5% CO_2. The transfection mixture was then replaced with standard culture medium. The cells were assayed for β-galactosidase activity 24 h after the start of transfection.

Microinjection

The microinjection protocol is modified from a previously published report [6], with changes in the culture conditions during microinjection to minimize cellular damage and death. For microinjection, cardiomyocytes were cultured on pre-etched glass coverslips (Cellocate, Eppendorf, Hamburg, Germany). One surface of these special coverslips was pre-etched by the manufacturer with an alphanumeric microgrid to allow the exact localization of each injected cell. To remove dead cells, the culture medium was changed on day 1 of culture, after which it was changed every other day.

One h prior to the microinjection, the cardiomyocytes were placed into injection medium. For the 21-day-old and adult cardiomyocytes, this medium was composed of J-MEM, 5% FBS, 10 μg/ml transferrin, 10 μg/ml insulin, 1 × MEM vitamin mix, 1 × non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 μM of the slow-channel calcium blocker verapamil (Sigma) to prevent contraction and cell death during the microinjection. For the neonatal cardiomyocytes, the medium was composed of J-MEM, 10 mM Hepes, 5% FBS, 1% L-glutamine, 1 × MEM vitamin mix, 1 × MEM non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 μM verapamil. In each case, the medium was immediately replaced with fresh microinjection medium after the injection, and the serum concentration was increased from 5% to 20%. Two h later, the medium was replaced with our standard culture medium. The DNA samples for microinjection (1 mg/ml) were kept in injectant buffer (100 mM potassium chloride, 5 mM potassium phosphate, pH 7.4). Just prior to loading into the microcapillaries, these samples were centrifuged at 16,000 × g for 15 min, in order to pellet any insoluble material that might potentially plug the micropipette. The microinjection efficiency was calculated as:

$$\frac{\text{number of cells positive for β-galactosidase activity.}}{\text{total number of cells injected}}$$

β-Galactosidase assay

The cardiomyocytes were fixed for 5 min at 4°C in a fixative composed of 2% paraformaldehyde, 0.2% glutaraldehyde and phosphate buffer. After fixation, the cells were washed 3 times with PBS and overlaid with a solution of 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$, 1.3 mM X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in PBS. Following an 8-h incubation at 37°C, the cells were viewed in a phase contrast microscope, and photographs taken using Kodak Gold ASA200 color film.

TPA treatment of cultured 21-day ventricular cardiomyocytes

Ventricular cardiomyocytes were isolated from 21-day-old female Sprague–Dawley rats and cultured in tissue culture flasks, as described. On day 7, Ara-C-supplemented culture medium was replaced with culture medium containing no added Ara-C. On days 9, 11 and 13, TPA (Sigma; 50 ng/ml final concentration in culture medium) was added with fresh medium to the experimental set of cardiomyocytes. DMSO (Sigma; 0.02%), the vehicle for TPA, was added to the control set of cardiomyocytes.

Cell cycle-specific expression array protocol

RNA isolated from 21-day ventricular cardiac muscle cells cultured in the presence or absence of TPA was dissolved in RNase-free water. This RNA was used as a template for [^{32}P]-cDNA probe synthesis according to the protocol supplied by the manufacturer of the cell cycle arrays (SuperArray, Bethesda, MD, USA). Briefly, cDNA probes were synthesized using gene-specific primers provided by the manufacturer and [α-^{32}P]-dCTP (Amersham). The cDNA probes were denatured by heating at 94°C for 5 min, and quickly chilled on ice. Each probe was added to a pre-blocked array membrane, and incubated for 15 h at 68°C. Following the hybridization, each membrane was washed twice with a 68°C solution of 2 × SSC, 1% sodium dodecyl sulfate for 20 min at 68°C with agitation. The damp membranes were immediately wrapped with plastic wrap, and exposed to a PhosphorImager screen (Molecular Dynamics) for 12 h. The screen was scanned with the STORM PhosphorImager and ImageQuant software, version 4.0 (Molecular Dynamics) was used to quantify the signals.

Each array is a nylon membrane on which cDNA fragments from 22 cell cycle-related genes have been immobilized in duplicate. Each membrane contained pUC18 cDNA as a negative control, as well as GAPDH and β-actin cDNA, which served as internal controls for the normalization of the signals. Each signal was normalized to GAPDH. The mean values ± S.E.M. obtained from the non-TPA-treated 21-day cardiomyocytes were compared to the corresponding values obtained from TPA-treated 21-day cardiomyocyte cultures, using the paired Student's *t*-test. Differences were considered significant at a p value < 0.05.

Results

The small size of the early neonatal hearts precluded use of the perfusion technique for the isolation of cardiomyocytes. Instead, ventricular cardiomyocytes were isolated from 1-,

Table 1. Examination of cardiomyocyte isolation and culture conditions

Parameters examined	Days of postnatal development					
	1d	**5d**	**10d**	**17d**	**21d**	**Adult**
(A) Isolation protocol	*Neonatal*	*Neonatal*	*Neonatal*	**Adult**	**Adult**	**Adult**
(B) Extracellular attachment protein	*Fibronectin*	*Fibronectin*	*Fibronectin*	*Fibronectin*	**Laminin**	**Laminin**
(C) Cell shape	*Round*	*Round*	*Round*	*Round*/**Rod**	**Rod**	**Rod**

Ventricular myocytes were isolated and cultured from 1-day- (1d), 5-day- (5d), 10-day- (10d), 17-day- (17d), 21-day- (21d) old and adult rat ventricles. (A) Isolation protocol refers to the technique used to isolate ventricular myocytes from heart tissue. Dissected ventricles were immersed in collagenase to disperse individual cardiomyocytes in the neonatal isolation protocol. Whole hearts were perfused with a collagenase solution prior to immersion in collagenase in the adult isolation protocol. (B) Cells were cultured in either fibronectin- or laminin-coated tissue culture flasks. (C) Cardiomyocytes were examined under a light microscope, and the shape of the cells recorded. Neonate-like characteristics are in italic font, and adult-like characteristics are in bold font.

5- and 10-day rat hearts by incubation in trypsin/collagenase (Table 1). The spherically shaped cardiomyocytes isolated from these age groups attached readily to fibronectin-coated flasks within 4 h of isolation. The cells were spread out within 1 day, and contracted spontaneously by day 2 in culture. Adjacent cells that were in physical contact were observed to contract synchronously.

Attempts were made to isolate ventricular cardiomyocytes from 17-day-old, 21-day-old and adult hearts using the trypsin/collagenase enzymatic incubation method described above. These cardiomyocytes were rounded, with irregularly shaped plasma membranes. They did not survive in culture, whether in fibronectin- or laminin-coated flasks. In contrast, using the retrograde perfusion technique, both round- and rod-shaped cardiomyocytes were isolated from the 17-day-old rat heart. These cardiomyocytes attached to fibronectin-coated flasks within 1 day of isolation, and were spread out by day 5 in culture.

While the cardiomyocytes isolated from 17-day-old hearts were successfully cultured in fibronectin-coated flasks, 21-day-old cardiomyocytes showed a definite preference for laminin. When the 21-day cardiomyocytes were inoculated into fibronectin-coated flasks, the cells attached, but did not survive in culture. These cardiomyocytes remained rod-shaped, did not spread out, and did not contract.

Ventricular cardiomyocytes were isolated from the adult rat heart using the retrograde perfusion protocol. Adult cardiac muscle cells, like the 21-day cardiomyocytes, did not spread out on fibronectin-coated flasks. When cultured in laminin-coated flasks, however, these cells attached within 3 days, and spread out within 7 days of isolation.

Light microscopy

Cardiomyocytes freshly isolated from the 21-day rat ventricles consisted of two distinct morphologies. Immediately following the isolation procedure, 84 ± 11% of these cells were cylindrically shaped, with cross striations. The remainder were spherical. Both types of cells contracted spontaneously. Under our culture conditions, the cardiomyocytes became progressively rounded (Fig. 1B), and usually non-contracting 24 h after isolation. At this point, the majority of the cells were either unattached or loosely attached to the surface of the laminin-coated flask. By day 3 of culture, the majority of the cardiomyocytes had made focal attachments to the substratum (Fig. 1C). Pseudopod-like processes projected out as spreading and flattening of the cells progressed. About 30% of the cardiomyocytes resumed spontaneous contractions at this time. By day 5 in culture (Fig. 1D), the cardiomyocytes contracted in synchrony in areas where they had made contact with one another. These cardiac myocytes were flattened and spread out by day 8 in culture (Fig. 1E).

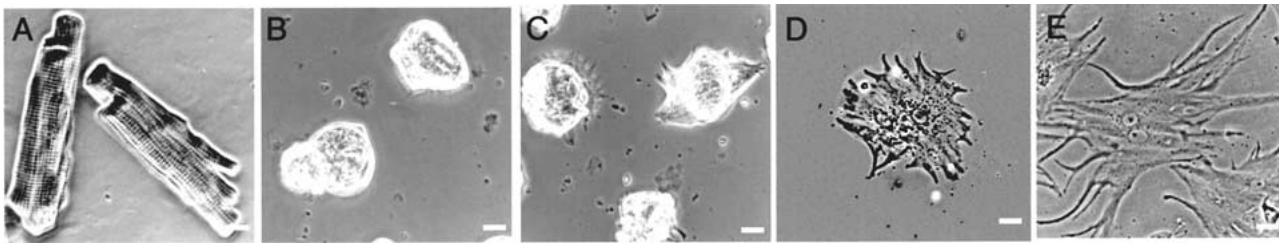

Fig. 1. Phase contrast light photomicrographs of freshly isolated and cultured ventricular cardiomyocytes from 21-day-old rat hearts. Cardiomyocytes were grown on laminin-coated glass coverslips, and photographed on successive days of culture. Panel A represents cardiomyocytes approximately 4 h after isolation. Panels B, C, D and E represent cardiomyocytes on days 1, 3, 5 and 8 of culture, respectively (magnification 400 ×. Bar 20 μm).

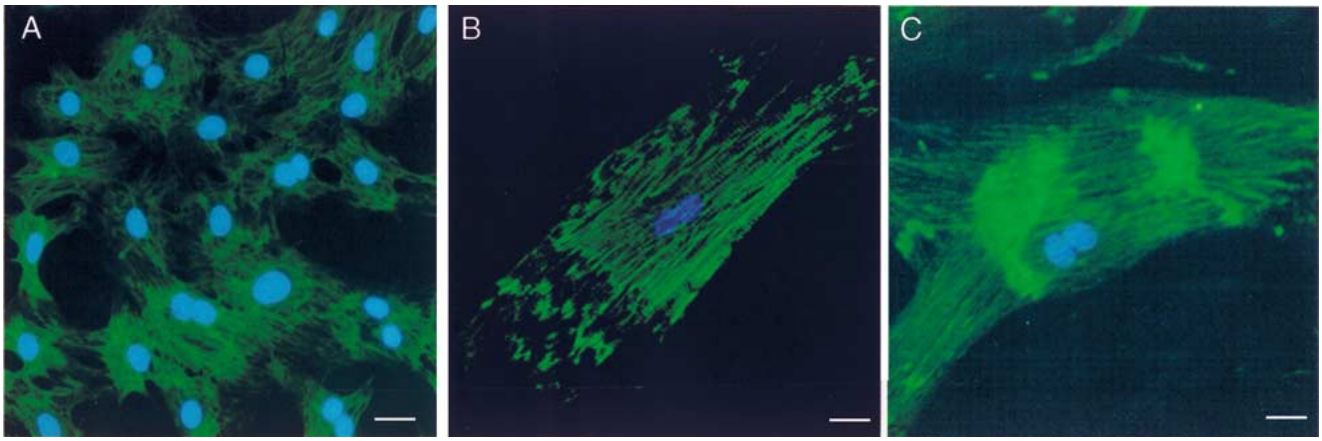

Fig. 2. Photomicrographs of immunohistochemcially-stained cultures of cardiomyocytes isolated from the ventricles of rats of different ages. Panels A, B and C represent ventricular cardiomyocytes isolated from 1-day-old, 21-day-old and adult rat hearts, respectively (magnification 200 ×). Cells were stained with both the MF20 anti-sarcomeric antibody and Hoechst 33342. Panel A illustrates ventricular cardiomyocytes isolated from 1-day-old rat hearts cultured on fibronectin-coated glass coverslips. These cultures from 1-day-old rat ventricles typically contained a small percentage of fibroblasts and other cell types, and were fixed and stained on day 2 of culture, prior to becoming overgrown with the highly proliferative non-cardiac muscle cells. Ventricular cardiomyocytes isolated from 21-day-old (Panel B) and adult (Panel C) rats were cultured in the presence of cytosine-1-β-D-arabinofuranoside (Ara-C) for the initial 7 days of culture. Ara-C was removed from the culture medium after day 7, and the pure populations of cardiomyocytes were fixed and stained on day 10 of culture (Bar 20 μm).

Immunofluorescence

Cellular size, structural organization of the myofibrils, and proportion of binucleated cells were evaluated in cardiomyocytes cultured from 1-day, 21-day and adult rats. On day 10 of culture, cardiomyocytes cultured from 21-day ventricles (Fig. 2B) were similar in size to the adult cardiomyocyte (Fig. 2C). The mouse anti-sarcomeric MF-20 antibody was used to verify that each cell was a myocyte, to identify the presence of myofibrils, and also to examine the density of these contractile elements. Ventricular cardiomyocytes cultured from 1-day-old rat hearts (Fig. 2A) were 18 ± 5% (n = 400) binucleated, whereas 21-day and adult cardiomyocytes were 84 ± 11% (n = 425) and 87 ± 8% (n = 400) binucleated, respectively.

Comparisons of gene expressions in 21-day-old vs. adult cardiomyocytes

Expressions of the developmentally regulated genes for MHC, α-actin, Cx43 and ANF were used in the present study to characterize the biochemical phenotype of the cells as either embryonic or adult. As expected, ventricular tissue isolated from embryonic day 17 (e17) hearts expressed the β- (Fig. 3A, lane 1) but not the α-MHC mRNA (Fig. 3A, lane 6). Freshly isolated samples of both 21-day-old ($21d_i$; Fig. 3A, lane 9) and adult ($Adult_i$; Fig. 3A, lane 10) cardiomyocytes expressed the adult (α) transcript of MHC. After 10 days in culture, however, both 21-day ($21d_c$; Fig. 3A, lane 2) and adult ($Adult_c$; Fig. 3A, lane 3) cardiomyocytes expressed the embryonic (β-MHC) mRNA, and no longer expressed the α-MHC transcript ($21d_c$; Fig. 3A, lane 7 and $Adult_c$; Fig. 3, Lane 8). These results suggest that both the 21-day and adult cardiomyocytes in culture undergo a reversion to a more embryonic state. The similarity in expression patterns exhibited by the 21-day and adult cardiomyocytes is noteworthy. Both freshly isolated 21-day and adult cardiomyocytes express α-MHC almost exclusively, whereas both cultured 21-day and adult cardiomyocytes express mainly β-MHC mRNA.

Expression of α-skeletal actin mRNA was undetectable in freshly isolated cardiomyocytes from 21-day ($21d_i$; Fig. 3B, lane 4) and adult ($Adult_i$; Fig. 3B, lane 5) animals. Following 10 days of culture, both 21-day ($21d_c$; Fig. 3B, lane 2) and adult ($Adult_c$; Fig. 3B, lane 3) cardiomyocytes expressed the α-skeletal actin transcript. A significant observation is the similarity in expression patterns between freshly isolated 21-day ($21d_i$; Fig. 3B, lane 4) and adult ($Adult_i$; Fig. 3B, lane 5) cardiomyocytes, and between cultured 21-day ($21d_c$; Fig. 3B, lane 2) and adult ($Adult_c$; Fig. 3B, lane 3) cardiomyocytes. A similar expression pattern is seen for α-cardiac actin. Both freshly isolated 21-day ($21d_i$; Fig. 3B, lane 10) and adult ($Adult_i$; Fig. 3B, lane 11) cardiomyocytes expressed α-cardiac actin. After 10 days of culture, α-cardiac actin mRNA is expressed by both 21-day ($21d_c$; Fig. 3B, lane 8) and adult ($Adult_c$; Fig. 3B, lane 9) cardiomyocytes at a reduced level. Again, the similarity in expression patterns between the 21-day and adult cardiomyocytes is noteworthy.

While ANF mRNA was expressed in e17 ventricular tissue (e17; Fig. 3C, lane 1), it was undetectable in freshly isolated 21-day ($21d_i$; Fig. 3C, lane 4) and adult ($Adult_i$; Fig. 3C, lane 5) cardiomyocytes. In contrast, cultured 21-day ($21d_c$; Fig. 3C, lane 2) and adult ($Adult_c$; Fig. 3C, lane 3) cardio-

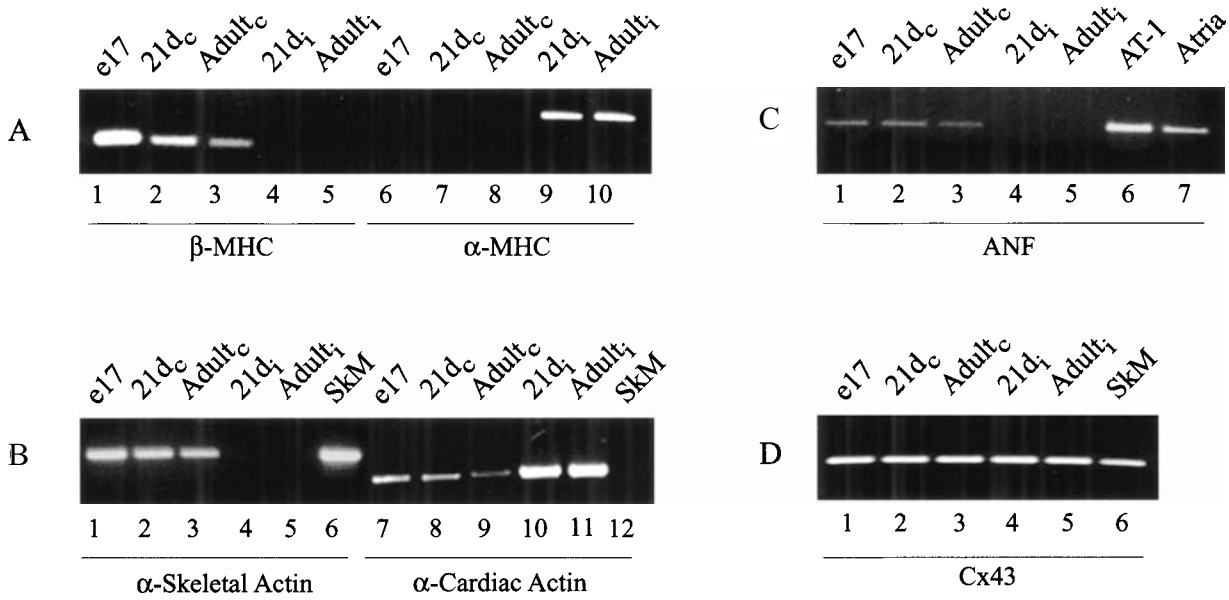

Fig. 3. RT-PCR-based analyses of β-MHC, α-MHC, α-skeletal actin, α-cardiac actin, ANF and Cx43 gene expression in cardiomyocytes and tissues. (A) β-MHC and α-MHC, (B) α-skeletal and α-cardiac actin, (C) ANF and (D) Cx43 expression in cardiomyocytes and tissues. Sources of the total RNA used in the RT-PCR and subsequent reactions were: embryonic day 17 ventricular cardiac tissue (e17), postnatal day 21 cultured ventricular cardiomyocytes (21d$_c$: day 10 in culture), freshly isolated 21-day ventricular cardiomyocytes (21d$_i$), cultured adult ventricular cardiomyocytes (Adult$_c$: day 10 in culture), freshly isolated adult ventricular cardiomyocytes (Adult$_i$), AT-1 cardiomyocytes (AT-1), adult atrial tissue (Atria) and adult skeletal muscle (SkM) tissue.

myocytes expressed mRNA transcripts coding for ANF. The resulting PCR product was confirmed by comparison with ANF-specific transcripts from atrial tissue (Atria; Fig. 3C, lane 7) and the AT-1 cardiomyocytes, in which expression of the SV40 large T antigen has been targeted via the ANF promoter (AT-1 [7, 8]; Fig. 3C, lane 6).

Primer pairs used to detect Cx43 were used to further characterize cultured 21-day cardiomyocytes. Freshly isolated (Fig. 3D, lanes 4 and 5) and cultured (Fig. 3D, lanes 2 and 3) 21-day and adult cardiomyocytes, as well as e17 ventricular tissue (Fig. 3D, lane 1) expressed transcripts for Cx43.

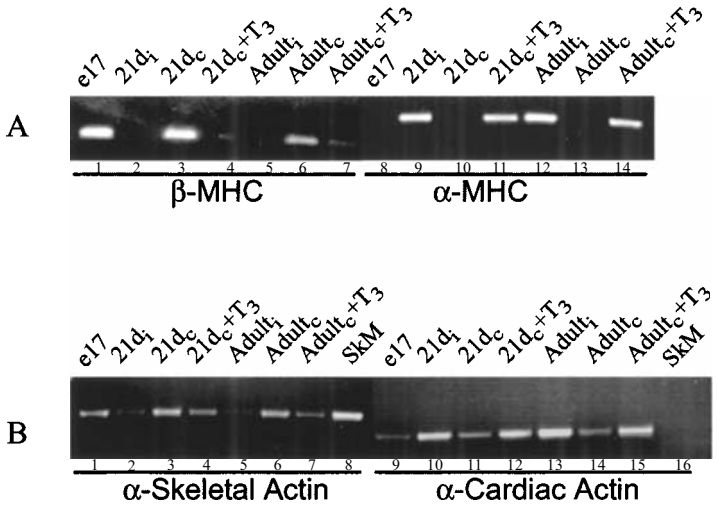

Fig. 4. RT-PCR-based analyses of β-MHC, α-MHC, α-skeletal actin and α-cardiac actin gene expression in T$_3$-treated and non-treated cardiac myocytes, and in embryonic day 17 and adult skeletal muscle tissues. Sources of the total RNA used in the RT-PCR reactions included: embryonic day 17 ventricular cardiac tissue (e17), postnatal day 21 cultured ventricular cardiomyocytes (21d$_c$: day 10 in culture), freshly isolated 21-day ventricular cardiomyocytes (21d$_i$), cultured adult ventricular cardiomyocytes (Adult$_c$: day 10 in culture), freshly isolated adult ventricular cardiomyocytes (Adult$_i$), T$_3$-treated 21-day cultured ventricular cardiomyocytes (21$_c$+ T$_3$) and T$_3$-treated cultured adult cardiomyocytes (Adult$_c$+ T$_3$). Primer pairs used in the RT-PCR reactions were designed to amplify products specific for α-MHC, β-MHC, α-skeletal actin and α-cardiac actin mRNA.

The effects of thyroid hormone on cardiac gene expression

Since our goal was to develop a model system for use in the study of adult cardiomyocyte gene expression, the transition back to an embryonic phenotype as exemplified by the re-expression of β-MHC, α-skeletal actin and ANF in cultured 21-day ventricular cardiomyocytes, presented a dilemma. In contrast to the absence of α-MHC mRNA in cultured 21-day ($21d_c$; Fig. 4A, lane 10) and adult ($Adult_c$; Fig. 4A, lane 13) cardiomyocytes, the addition of T_3 to the culture medium resulted in an up-regulation of α-MHC in both 21-day ($21d_c + T_3$; Fig. 4A, lane 11) and adult ($Adult_c + T_3$; Fig. 4A, lane 14) cultured cardiomyocytes. Similarly, presence of this hormone led to a down-regulation of the embryonic (β) form of MHC in both cultured 21-day and adult cardiomyocytes, as evidenced in Fig. 4A, lanes 3, 4, 6 and 7.

The expression pattern of the α-skeletal and α-cardiac actin genes was also examined in the T_3-supplemented cultures. As shown in Fig. 4B, both 21-day ($21d_c$, lane 3) and adult ($Adult_c$, lane 6) cultured cardiomyocytes expressed the fetal (α-skeletal) actin isoforms. The expression level of this fetal isoform was down-regulated in 21-day ($21d_c + T_3$; Fig. 4B, lane 4) and adult ($Adult_c + T_3$, lane 7) cardiomyocytes cultured in T_3. Cultured cardiomyocytes, both 21-day ($21d_c$; Fig. 4B, lane 11) and adult ($Adult_c$; Fig. 4B, lane 14), expressed low levels of α-cardiac actin mRNA transcript. The α-cardiac mRNA, however, was up-regulated in cultures grown in the presence of T_3 (Fig. 4B, lanes 12 and 15).

Microinjection and transfection

As seen in Fig. 5A, 27 ± 7% (n = 3) of the transfected 21-day cardiomyocytes expressed β-galactosidase. This is similar to the transfection efficiency obtained with cultured 1d cardiomyocytes (24 ± 7%; n = 3), and significantly higher ($p < 0.05$) than that obtained with adult cardiomyocytes (10 ± 3%; n = 3). The 35 ± 6% (n = 3) microinjection efficiency obtained with 21-day-cardiomyocytes was similar to that obtained with adult cardiomyocytes (37 ± 6%; n = 3). Representative photomicrographs of microinjected (Fig. 5B) and transfected (Fig. 5C) cultured 21-day-cardiomyocytes are presented.

Cell cycle gene expression in TPA-treated vs. control 21-day cardiomyocytes

Adult ventricular cardiomyocytes cultured in the presence of TPA have been shown to increase the amount of ^{3}H-thymidine incorporated into their DNA by approximately 250% [1]. These cells were very much enlarged, and more spread out than the untreated control cardiomyocytes. Compared

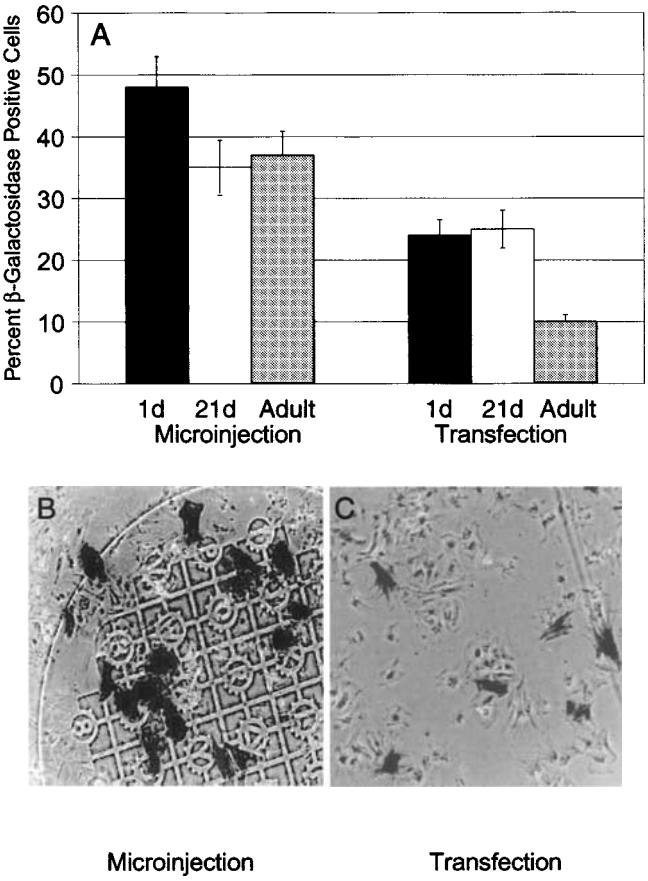

Fig. 5. A comparison of the efficiency of transfection vs. microinjection in cultured ventricular cardiomyocytes. The reporter gene, *LacZ*, driven by the CMV promoter, was either transfected using LipofectAMINE (n = 3), or microinjected (n = 3) into cultured 1-day (day 3 in culture), 21-day (day 10 in culture) and adult (day 10 in culture) cardiomyocytes. Following transfection, both the total number of cardiomyocytes and the number of cardiomyocytes expressing β-galactosidase were counted in each of 12 randomly selected microscopic fields.

The percent β-galactosidase expression per field of transfected cardiomyocytes equals:

$$\frac{\text{The number of cardiomyocytes positive for β-galactosidase activity} \times 100.}{\text{Total number of cardiomyocytes}}$$

For microinjection, the cardiomyocytes were cultured on sterile CELLocate (Eppendorf) coverslips, whose pre-etched microgrid design allowed for the quick location of microinjected cells. During microinjection, the location of each microinjected cardiomyocyte was marked on a CELLocate log sheet with a grid design corresponding to the coverslip. The percent β-galactosidase expression in microinjected cardiomyocytes equals:

$$\frac{\text{The number of cardiomyocytes positive for β-galactosidase activity} \times 100.}{\text{Total number of cardiomyocytes microinjected}}$$

Photomicrographs of microinjected (Panel B) and transfected (Panel C) cardiomyocytes cultured from 21-day rat hearts are shown.

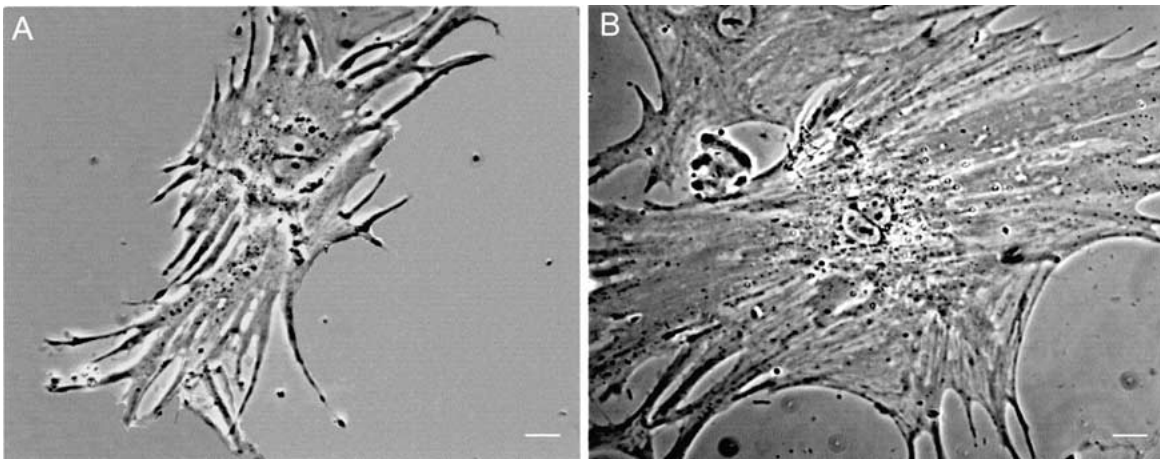

Fig. 6. Phase contrast photomicrograph of control and TPA-treated ventricular cardiomyocytes cultured from 21-day-old rat heart. Control ventricular cardiomyocytes cultured from 21-day-old rats (Panel A) received DMSO, the vehicle for TPA, in the culture medium. Panel B represents a single TPA-treated postnatal day 21 cultured ventricular cardiomyocyte. DMSO or TPA was added to the culture medium on days 10 and 12 of culture, and the cells were photographed on day 14 (magnification 200 ×; Bar, 15 μm).

to controls (Fig. 6A), 21-day cardiomyocytes cultured in the presence of TPA were also greatly enlarged (Fig. 6B).

An array of cell cycle-specific cDNAs was chosen to study cell cycle gene expression in the 21-day culture system. TPA treatment of these cardiomyocytes resulted in a significant increase in the levels of cyclin D1, cyclin E, cyclin A and Cdc2 mRNA (Fig. 7A). These are accompanied by a down-regulation of $p15^{Ink4b}$ (Fig. 7B). For each set of TPA vs. control comparisons, p is less than 0.05 (Figs. 7A and B).

Discussion

The rationale of this work was to develop a cell culture model system using terminally differentiated ventricular cardiomyocytes that had recently exited the cell cycle. Our hypothesis was that younger cardiomyocytes, with a less fully developed ultrastructure, would more readily accept genetic manipulation.

After culturing ventricular cardiomyocytes from 1-, 5-, 10-, 17- and 21-day rat hearts, and comparing their char-

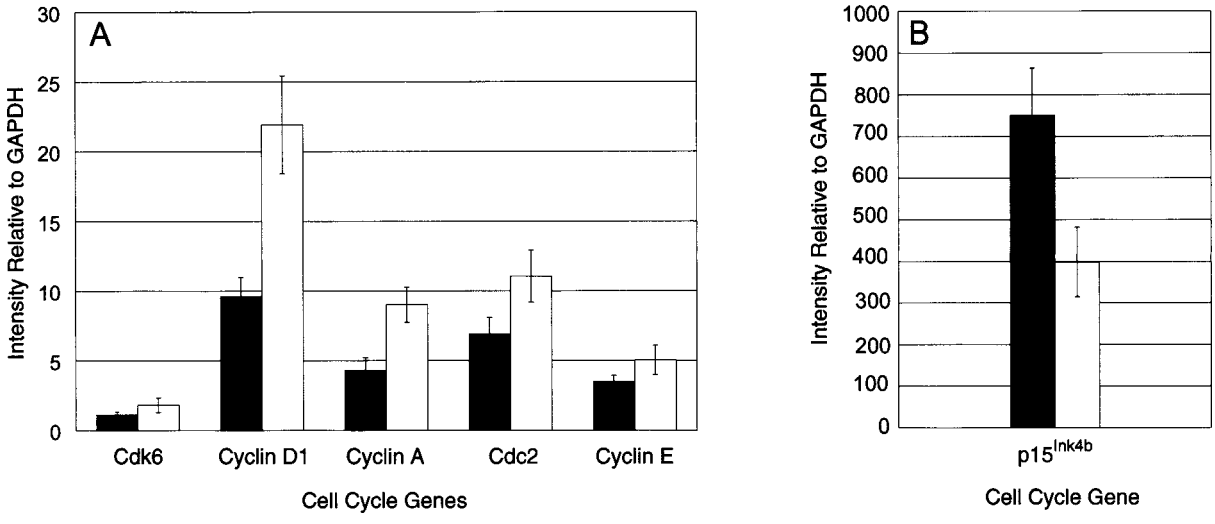

Fig. 7. Expression of mRNA for Cdk6, cyclin D1, cyclin A, Cdc2, cyclin E and $p15^{Ink4b}$ in control and TPA-treated ventricular cardiomyocytes cultured from 21-day-old rats. On day 10 of culture, ventricular cardiomyocytes from 21-day-old rat hearts were treated with TPA for 5 days. Total RNA isolated from 3 untreated and 3 TPA-treated cardiomyocyte cultures was reverse-transcribed, then this cDNA was used as a template for [α-^{32}P]-cDNA synthesis. After denaturation, this [α-^{32}P]-cDNA was hybridized to each membrane. The image of the blot, captured by the optical system of a PhosphorImager, was converted into digital information. The intensity of each signal is directly proportional to the amount of radioactivity present within each area. The signal from each cDNA spot was normalized to the mean GAPDH. Data was analyzed using the Student's paired *t*-test. (A) Cdk6, cyclin D1, cyclin A, Cdc2 and cyclin E; (B) $p15^{Ink4b}$. For cyclin D1 cyclin A, Cdc2, Cycin E and $p15^{Ink4b}$.

acteristics with those of the cultured adult cardiomyocyte, the 21-day postnatal ventricular cardiac myocyte was found to meet these criteria. Collagen accumulates in the extracellular matrix of the heart throughout the life of the mammal [9, 10]. The degree of accumulation of the collagenous network is a developmentally regulated determinant of the maturity of the heart. In the current study, the isolation of cardiomyocytes from 17-day and 21-day-old hearts was found to require collagenase perfusion, followed by incubation in a collagenase solution. Due to the smaller size of the organs and the underdeveloped collagenous network in the hearts of the 1-day-, 5-day and 10-day-old rats, ventricular cardiomyocytes were isolated from these early neonatal hearts by an overnight trypsin incubation at 4°C, followed by a short collagenase incubation. These observations are in agreement with the data reported by Lundgren *et al.* [11], showing that the connective tissue network in the 20-day-old rat heart is morphologically indistinguishable from that found in the adult heart.

The preferential attachment of cardiomyocytes of different developmental age groups to fibronectin- or laminin-coated flasks from rats was used as another indicator of the maturity of the hearts. Interaction of the cardiomyocyte with the components of the extracellular matrix (ECM) depends on the specificity of membrane receptors for its ligand. The α-chains of the integrins confer ligand specificity to these interactions with ECM substrates [12]. The $\alpha_1\beta_1$, $\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrins expressed in fetal and neonatal cardiomyocytes mediate the attachment of these cells to fibronectin, laminin and type IV collagen, respectively [12, 13]. Adult cardiomyocytes, on the other hand, which express no detectable levels of $\alpha_1\beta_1$ and $\alpha_5\beta_1$ integrins, adhere only to laminin and type IV collagen, and weakly to fibronectin [13]. The preferential attachment of 21-day cardiomyocytes to laminin observed in the current experiment, which is in agreement with the data presented by Borg *et al.* [13], suggests that these cells are adult-like by this parameter. The morphology of the cardiomyocyte immediately upon isolation, an indicator of the complexity of its ultrastructural organization *in vivo*, was one parameter used in the present studies to screen for 'adult-like' features in 1-, 5- 10- 17- and 21-day cardiomyocytes.

Cell division is irreversibly halted in the rat heart by day 4 of postnatal development [14], and DNA synthesis continues until postnatal day 17 [15, 16]. The difference in binucleation between 21-day cardiomyocytes (84 ± 11%) and adult (87 ± 8%) was statistically non-significant. Binucleation in cardiomyocytes cultures from 1-day-old rats (18 ± 4%), however, was considerably lower. During early neonatal development, both DNA synthesis and cytokinesis are ongoing events. Although cytokinesis ceases by day 4 of postnatal development in the rat, DNA synthesis continues until the third week of development. Thus, in the 1-day-old rat, ventricular cardiomyocytes undergo cytokinesis following each DNA replication, resulting in mainly mononucleated cells. The binucleation observed in cardiomyocytes from 21-day and adult rat hearts can be attributed to the 3-week period of postnatal development during which DNA replication occurred without the corresponding cytokinesis. The transition from hyperplastic to hypertrophic cardiac myocyte growth is complete in the 21-day rat heart. Based on this, the 21-day rat heart can be considered to possess many adult-like characteristics. Unlike the adult cardiac ventricular cardiomyocyte, however, the 21-day cardiac muscle cell has not yet completed the full hypertrophy program that results in the adult heart. Its less complex ultrastructure may allow it to be successfully manipulated on a genetic level. Based on this rationale and our data, the 21-day culture system was selected as the optimal experimental model of choice.

Immediately following the isolation protocol, 84 ± 11% of the 21-day cardiomyocytes were cylindrical in shape, and the remainder spherical. Both cyclindrical and spherical cells underwent spontaneous contractions. Over the next 10 days in culture, the 21-day cardiomyocytes followed a sequence of events that resembled those reported for adult ventricular cardiomyocytes isolated and cultured under similar conditions [4]. The sequential morphological changes included rounding up, attachment to the substratum, and spreading out. This sequence of events resembles those reported for adult ventricular cardiomyocytes isolated and cultured under similar conditions.

Simultaneous staining with the anti-sarcomeric myosin antibody, MF-20, and the Hoechst 33342 nuclear dye, showed that 84 ± 11% of the 21-day cardiomyocytes were binucleated, compared to 87 ± 8% and 18 ± 4% of binucleated cardiomyocytes in adult and 1-day ventricular cardiomyocytes, respectively. MF-20 was used to identify the presence of myofibrils. Thus, on a morphological level, data from immmunohistochemistry as well as phase contrast microscopy indicate that the 21-day cardiac muscle cells were adult-like.

To characterize the gene expression of these cultured cardiomyocytes, RT-PCR was used to examine their expression of a number of developmentally regulated genes. The expressions of α- and β-MHC, α-cardiac and α-skeletal actin, ANF and Cx43 were used to assess the maturity of cardiomyocytes. In all cases, freshly isolated 21-day and adult cardiac myocytes expressed the adult isoforms. After 10 days in culture, however, neither 21-day nor adult cardiomyocytes expressed the adult phenotype. This is interpreted to indicate that, in some respects, 21-day and adult cells in culture reverted to an embryonic-like phenotype. This return to a less differentiated or more embryonic state in culture, which has also been documented by others using α-smooth muscle actin as a phenotypic marker [17], could be problematic in studies of terminal differentiation or gene expression in the adult heart. The addition of T_3 to the culture medium of the 21-day cardiomyocytes prevented this switch. Apparently the FBS in the culture medium contained an inadequate amount of T_3 to

maintain the adult-like phenotype of these cardiomyocytes in culture.

Because the 21-day culture system was developed to study the machinery of cardiomyocyte terminal differentiation, it was particularly important to demonstrate that these cells were capable of accepting exogenous DNA. Two different gene delivery techniques, transfection and microinjection, were chosen to introduce the *LacZ* reporter gene. A transfection efficiency of 25 ± 7% was obtained with 21-day cardiomyocytes. A significantly lower ($p < 0.05$) transfection efficiency (10 ± 1%) was obtained with cultured adult cardiomyocytes. Although the percentage of positively staining cardiomyocytes was higher by microinjection (35 ± 6% for 21-day and 37 ± 5% for adult), the total number of microinjected cardiomyocytes expressing β-galactosidase was lower when compared with transfection. These results suggest that, while transfection and microinjection can both be used successfully to introduce exogeneous DNA into the terminally differentiated cardiomyocyte, the higher number of transfected cardiomyocytes makes the less labor-intensive transfection technique the method of choice. The significantly higher transfection efficiency obtained with the 21-day cardiac muscle cells, compared to that obtained with adult cardiomyocytes, indicates that these less mature cardiomyocytes present an excellent experimental model system for the study of adult cardiomyocyte gene expression and cell cycle machinery. It is also proposed as a system for investigating the molecular mechanism(s) that suppress the cardiomyocyte cell cycle irreversibly during early neonatal development.

One aspect of cell cycle regulation is the control of DNA synthesis. Relying on previously documented increases in DNA synthesis resulting from TPA treatment of cultured adult ventricular cardiomyocytes [1], the effect of TPA on the 21-day culture system was used to study cell cycle regulation. At the light microscope level, the most obvious difference between TPA-treated and control 21-day ventricular cardiac myocytes was a substantial increase in the size of the treated cells (Fig. 6).

The TPA-treated 21-day cardiomyocytes demonstrated a significant increase in the mRNA levels for cyclins D1 ($p = 0.003$), E ($p = 0.040$) and A ($p = 0.001$), and for Cdc2 ($p = 0.020$), accompanied by a significant decrease in $p15^{Ink4b}$ ($p = 0.0004$). This increase in mRNA expression of the positive regulators of the cell cycle (cyclins D1, E and A, and Cdc2), with a concomitant decrease in mRNA expression of the $p15^{Ink4b}$ cell cycle inhibitor in TPA-treated 21-day ventricular cardiomyocytes suggests that these cells retain some capacity to re-enter the cell cycle.

Although the TPA-treated cardiomyocytes re-initiated DNA synthesis, they did not appear to undergo cytokinesis. However, multinucleated cardiomyocytes could be observed in the TPA-treated cultures. This suggests that a checkpoint in mitosis may be preventing cytokinesis in these TPA-treated cells. Further detailed studies utilizing this 21-day postnatal culture system will assess whether these cells can be induced to fully re-enter the cell cycle and possibly undergo mitosis.

In light of their phenotypic similarity to cultured adult ventricular cardiac myocytes and their ability to be manipulated genetically, cultured ventricular cardiomyocytes from 21-day-old rats are proposed as an experimental model system for the study of adult gene expression and cell cycle regulation. This system would be useful in the investigation of the molecular mechanism(s) leading to cell cycle withdrawal in ventricular cardiomyocytes during early neonatal development.

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