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Beeinflussung der Zellzyklusregulation von Kardiomyozyten zur Stimulation von Regenerationsprozessen des Herzmuskels

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von Dr. med. Henning Ebelt
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Herzmuskelzellen von Säugetieren verlieren in der Perinatalzeit ihre Fähigkeit zur Proliferation. Daher ist das adulte Herz unter normalen Bedingungen nicht in der Lage, untergegangene Kardiomyozyten durch neue kontraktile Zellen zu ersetzen. Im Rahmen dieser Arbeit werden in tierexperimentellen Modellen Fragen zur gezielten Induktion der Kardiomyozytenproliferation untersucht, um nach Eintritt einer Herzschädigung wieder eine Vermehrung von Kardiomyozyten zu ermöglichen.

Zellkulturexperimente können zeigen, dass in adulten murinen Knochenmarksstammzellen zwar einzelne Herzmuskel-spezifische Gene aktiviert werden können, die Induktion eines vollständigen Differenzierungsprozesses hin zu Zellen mit den funktionellen Charakteristika von Kardiomyozyten ist jedoch nicht möglich. Übereinstimmend stützen Untersuchungen im Herzinfarktmodell der Maus die Hypothese, dass die – zweifellos zu beobachtenden – günstigen Effekte von Zelltransplantationen überwiegend durch parakrine Mechanismen vermittelt werden und nicht auf der Generierung neuer kontraktiler Herzmuskelzellen beruhen.

Durch die adenovirale Expression von E2F-Transkriptionsfaktoren ist es in *in vitro*-Experimenten möglich, die Proliferation neonataler Kardiomyozyten zu induzieren, wobei der Zellzykluseintritt im Falle der Expression von E2F2 und E2F4 nicht von einer unerwünschten Steigerung der Apoptoserate begleitet ist. In der Folge wird erfolgreich eine Methode etabliert, die unter Verwendung adenoviraler Vektoren die stabile Expression von Fremdgenen in den Herzen neonataler Mäuse gestattet (Transduktion von 71±8% der kardialen Zellen). In diesem Modell führt die Expression von E2F2, nicht jedoch von E2F4, ebenfalls zur signifikanten Induktion des Zellzykluseintritts von Kardiomyozyten.

Als weiterer Angriffspunkt wird die Inhibierung von p53- und p73-abhängigen Signalwegen in die Untersuchungen einbezogen. Mittels neu generierter Adenoviren wird eine trunkierte Isoform von p73 (p73DD) mit dominant-negtiven Eigenschaften gegenüber p53 und p73 in den Herzen von Mäusen exprimiert. Dies führt entsprechend der eigenen Hypothese u.a. zur Hemmung des Proliferations-Inhibitors p21 und dem Wiedereintritt von Kardiomyozyten in den Zellzyklus. Zu erwartende Synergieeffekte, die aus der kombinierten Expression von E2F2 und p73DD resultieren, sollen im Rahmen zukünftiger Experimente analysiert werden.

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Inhaltsverzeichnis

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1 Einleitung

In Deutschland und in vielen anderen europäischen Ländern hat die veränderte Altersstruktur der Bevölkerung in Verbindung mit dem verbesserten Überleben von akuten Myokardinfarkten zu einer deutlichen Zunahme von Patienten geführt, die an chronischer Herzinsuffizienz leiden. Nach aktuellen Schätzungen beläuft sich die Inzidenz der Herzinsuffizienz aller Schweregrade in Europa auf etwa 2,3 bis 3,7 pro 1000 Einwohner und Jahr [Remme und Swedberg 2001], und die Diagnose Herzinsuffizienz stellt seit einigen Jahren den häufigsten Grund für eine Hospitalisierung in den USA dar [Ghali et al. 1990].

Im überwiegenden Teil der Fälle – schätzungsweise bei etwa 80 bis 90% aller Patienten mit Herzinsuffizienz – liegt der eingeschränkten Herzfunktion eine ventrikuläre Funktionsstörung zugrunde. Pathophysiologisch dominiert hierbei in ca. 60% der Fälle eine Beeinträchtigung der systolischen Auswurfleistung des linken Ventrikels, während bei dem geringeren Teil der Patienten die linksventrikuläre Pumpfunktion erhalten und eine diastolische Funktionsstörung als Ursache für die klinischen Zeichen der Herzinsuffizienz anzusehen ist [Gandhi et al. 2001; Senni und Redfield 2001].

Gegenwärtig stehen im klinischen Alltag vor allem pharmakologische Ansätze als etablierte Verfahren zur Behandlung der Herzinsuffizienz zur Verfügung. Durch die kombinierte Gabe von β-Rezeptorblockern, Inhibitoren des Renin-Angiotensin-Aldosteron-Systems, Diuretika und Digitalis-Glykosiden ist es möglich, sowohl die klinischen Beschwerden herzinsuffizienter Patienten als auch deren Prognose bezüglich Morbidität und Mortalität zu verbessern [Swedberg et al. 2005]. Das Spektrum der verfügbaren Therapie-Möglichkeiten ist darüber hinaus in den vergangenen Jahren durch die Weiterentwicklung von mechanischen Kreislaufunterstützungssystemen (*assist devices*) sowie von Geräten zur kardialen Resynchronisations-Therapie erweitert worden. Die Herztransplantation stellt bislang die einzige etablierte chirurgische Therapieoption zur Behandlung von Patienten mit refraktärer Herzinsuffizienz dar, wobei jedoch insbesondere die nur begrenzte Verfügbarkeit von Spenderorganen eine deutliche Limitation dieses Verfahrens begründet.

Das adulte Herz ist unter normalen Bedingungen nicht in der Lage, die nach einer Schädigung untergegangenen Kardiomyozyten durch neue kontraktile Zellen zu ersetzen, da Kardiomyozyten von Säugetieren in der Perinatalzeit ihre Fähigkeit zur Proliferation verlieren [Soonpaa et al. 1996; Soonpaa und Field 1997]. Da sich eine Herzinsuffizienz in vielen Fällen als Folge des Verlustes von kontraktilem Myokard entwickelt, erscheint es als sinnvoller Therapieansatz, die Menge an Herzmuskelzellen wieder durch therapeutische Eingriffe zu

vermehren, um so der Entwicklung bzw. dem Voranschreiten des kardialen Funktionsverlustes zu entgegnen.

Prinzipiell sind verschiedene Ansätze – sowie deren Kombination – als denkbar beschrieben, die zu einer Vermehrung der Kardiomyozyten beitragen können (siehe auch [von Harsdorf et al. 2004]): i) die gezielte Anreicherung von körpereigenen Stammzellen im Herzen [Orlic et al. 2001]; ii) die Transplantation von Kardiomyozyten oder Kardiomyozyten-Vorläuferzellen bzw. geeigneten Stammzellen (Übersicht in [Dimmeler et al. 2005]); iii) die Induzierung der Proliferation von Kardiomyozyten des verbliebenen Rest-Myokards [Pasumarthi et al. 2005]; iv) die Transdifferenzierung von im Herz lokalisierten Nicht-Muskelzellen zu Kardiomyozyten [Etzion et al. 2002].

Im Rahmen der eigenen Untersuchungen wurden von diesen Möglichkeiten schwerpunktmäßig zwei Ansätze verfolgt. Zunächst wurden verschiedene Fragen zu dem Einsatz von Stammzell-Transplantationen im Rahmen der ‚zellulären Kardiomyoplastie' untersucht. Hierbei war es jedoch nicht möglich, adulte murine Stammzellen gezielt in Kardiomyozyten zu differenzieren. Deshalb wurde im folgenden stattdessen analysiert, inwiefern es möglich ist, durch gezielte Interventionen den Wiedereintritt von Herzmuskelzellen in den Zellzyklus auszulösen, um so die verfügbare Anzahl von Kardiomyozyten wieder zu vermehren.

2 Ergebnisse und Diskussion

2.1 Homogenität und Differenzierungspotential von adulten murinen Knochenmarksstammzellen *in vitro*

Bereits in den 1990er Jahren konnte gezeigt werden, dass es möglich ist, isolierte fetale oder neonatale Kardiomyozyten erfolgreich in die Herzen von Empfängertieren zu transplantieren [Soonpaa et al. 1994; Klug et al. 1996]. Die Zellen waren in der Lage, über mehrere Monate nachweisbare Transplantate zu bilden, wenngleich spätere Untersuchungen zu dem Ergebnis kamen, dass letztendlich nur etwa 20% der transplantierten Zellen in den Empfängerherzen überleben [Muller-Ehmsen et al. 2002]. Ergänzende Studien konnten darüber hinaus belegen, dass die Transplantation von Kardiomyozyten tatsächlich auch zu einer partiellen Wiederherstellung der gestörten linksventrikulären Funktion im Tiermodell beiträgt [Reinecke et al. 1999; Etzion et al. 2001; Muller-Ehmsen et al. 2002].

Eine therapeutische Anwendung von humanen embryonalen oder fetalen Kardiomyozyten ist aus nahe liegenden Gründen undenkbar, so dass in der Folge eine intensive Suche nach alternativen Zelltypen, die zur Zellersatztherapie geeignet sein könnten, begonnen wurde. Embryonale Stammzellen wurden aufgrund ihres Potentials zur multilineären Differenzierung und der damit verbundenen Möglichkeit der gleichzeitigen Bildung von Blutgefäßen und Myokard von verschiedenen Arbeitsgruppen intensiv untersucht [Boheler et al. 2002; Gepstein 2002; Min et al. 2002; Mummery et al. 2002; Sachinidis et al. 2002; Trounson 2002]. Zusätzlich rückten auch adulte Stammzellen zunehmend in den Blickpunkt der Forschung, da diese Zellen für therapeutische Zwecke beim Menschen mit den geringsten ethischen Bedenken behaftet sein sollten und erste tierexperimentelle Arbeiten auch diesen Zellen ein geeignetes Differenzierungs- und Regenerationspotential zuschrieben [Gussoni et al. 1999; Orlic et al. 2001; Jiang et al. 2002]. Vor diesem Hintergrund wurden bereits frühzeitig adulte humane Stammzellen auch in klinischen Studien bei Patienten mit Myokardinfarkt untersucht [Assmus et al. 2002; Strauer et al. 2002; Wollert et al. 2004] – wenngleich zu diesem Zeitpunkt eine Vielzahl von Fragen bezüglich der Biologie der überwiegend aus dem Knochenmark isolierten Zellen noch ungeklärt war.

Im Rahmen von Experimenten der eigenen Arbeitsgruppe wurden zunächst Fragen zu dem Verhalten von adulten Knochenmarksstammzellen der Maus in Zellkulturexperimenten untersucht. Es sollte analysiert werden, inwiefern die Isolation von Knochenmarksstammzellen mittels üblicher Präparationsprotokolle (*differential adhearance*, *differential survival*) zu Zelllinien mit identischen Eigenschaften führen würde oder ob bei diesem Vorgehen Linien mit differenten Phänotypen entstehen würden. Außerdem sollte

untersucht werden, inwiefern sich bei den neu zu etablierenden Stammzelllinien tatsächlich *in vitro* eine Differenzierung zu reifen Kardiomyozyten induzieren lässt [Belema Bedada et al. 2005].

Im Rahmen der Untersuchungen war es möglich, zwei klonale Stammzelllinien aus dem Knochenmark adulter Mäuse zu etablieren (*murine bone marrow-derived multipotent adult stem cell populations*, mBM-MASCs). Beide Zelllinien (mBM-MASCs1 und -2) zeigen hinsichtlich Morphologie und Verdopplungszeit in Kultur ein weitgehend identisches Verhalten. Bei der durchflusszytometrischen Analyse konnte festgestellt werden, dass mBM-MASC1 und -2 eine nahezu identische Expression der Marker c-Kit, CD45, Ter119, Flk-1, H-2Dd, CD13, SSEA-1 und CD133/prominin aufweisen, während jedoch signifikante Unterschiede hinsichtlich der Expression von CD34 und Sca-1 bestehen (mBM-MASCs1: $CD34^{-}/Sca\text{-}1^{high}$; mBM-MASCs2: $CD34^{+}/Sca\text{-}1^{moderate}$).

In der Folge wurde unter anderem untersucht, inwiefern es möglich ist, eine gezielte Differenzierung der beiden Stammzelllinien zu Herzmuskelzellen auszulösen. Hierfür wurden zum einen verschiedene Mitglieder der Wnt-Familie verwendet, die bekanntermaßen potente Induktoren der myogenen Differenzierung während der Embryonalentwicklung darstellen (Übersicht in [Cohen et al. 2008]). Mittels retroviraler Vektoren, die die cDNAs zur Expression von Wnt4, Wnt7a, Wnt7b bzw. Wnt11 codierten, wurden zunächst Fibroblasten *in vitro* infiziert. Anschließend wurden die Stammzellininen mit den transduzierten Fibroblasten co-kultiviert, so dass eine permanente Anreicherung des Kulturmediums durch die sezernierten Wnt-Proteine sichergestellt werden konnte. Es zeigte sich, dass nach einigen Tagen alle getesteten Wnt-Faktoren die mRNA-Expression der kardiomyozyten-typischen Gene Nkx-2.5, GATA-4, Hand 2 (dHand) und TEF1 sowie von kardialem Tropomyosin induzieren, jedoch führt nur die Behandlung der Zellen mit Wnt11 auch zur Expression von β-*myosin heavy chain* (β-MHC) und *brain natriuretic protein* (BNP). Darüber hinaus war es allerdings nicht möglich, die Aktivierung anderer kardiomyozytärer Gene wie α-MHC oder *atrial natriuretic protein* (ANP) nachzuweisen. Mittels Immunfluoreszenz-Färbungen konnte belegt werden, dass die Stimulation der Stammzellen mit Wnt11 zwar die Expression von kardialem Troponin T induziert, es fanden sich jedoch keine Zeichen für die Etablierung eines organisierten Kontraktionsapparates oder einer mikroskopischen Querstreifung der Zellen, wie sie bei reifen Kardiomyozyten typischerweise zu beobachten ist. Die immunhistologische Doppelanfärbung der Wnt11-behandelten mBM-MASCs mit gegen GATA-4 und Nkx-2.5 gerichteten Antikörpern zeigt ein großes Maß an Heterogenität innerhalb des Zellverbandes: während einige Zellen nur jeweils einen der beiden Marker exprimieren, finden sich

gleichzeitig auch Zellen, die entweder beide Proteine oder aber keines davon aufweisen. Zu keinem Zeitpunkt ist es möglich, spontane Kontraktionen zu detektieren.

In ergänzenden Experimenten wurde untersucht, welche Signalkaskaden durch die Gabe der Wnt-Moleküle in Kardiomyozyten ausgelöst werden. Hierbei zeigte sich, dass die Aktivierung der kardiomyozytären Gene durch die Wnt-Moleküle nicht von der Aktivierung von β-Catenin und *lymphoid-enhancing factor 1* (Lef-1) abhängig ist, sondern auf einer Aktivierung der Proteinkinase C beruht.

Auch durch die gleichzeitige Co-Aktivierung verschiedener anderer Signalkaskaden, die bekanntermaßen während der Embryonalentwicklung zur Induktion von Muskelzellen führen, war es in den eigenen Untersuchungen nicht möglich, eine vollständige Differenzierung der mBM-MASCs zu funktionellen Herzmuskelzellen auszulösen. Unter der Annahme, dass in den Stammzellen eventuell durch epigentische Regulationsmechanismen eine Aktivierung von essentiellen Genen verhindert sein könnte (d.h. die Transkription durch Hypermethylierung bzw. Deacetylierung von Genen inhibiert ist), wurde außerdem mittels verschiedener pharmakologischer Interventionen Einfluss auf das Chromatin-Remodeling genommen. Auch hierdurch war es jedoch nur möglich, die Expression einzelner Muskel-spezifischer Marker zu aktivieren, ohne eine echte funktionelle Differenzierung der mBM-MASCs auszulösen.

Zusammenfassend konnte daher festgestellt werden, dass die Behandlung der adulten Knochenmarksstammzellen mit etablierten Myogenese-Induktoren sowie Versuche eines pharmakologischen Chromatin-Remodelings zwar zu einer Aktivierung einzelner Herzmuskel-spezifischer Gene führt, die Induktion eines vollständigen Differenzierungsprozesses hin zu Zellen mit den funktionellen Charakteristika von Kardiomyozyten jedoch nicht möglich ist.

<u>Anlage zu 2.1.:</u>

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Activation of Myogenic Differentiation Pathways in Adult Bone Marrow-Derived Stem Cells†

Fikru Belema Bedada,[1] Antje Technau,[2] Henning Ebelt,[2,3] Manja Schulze,[1,2] and Thomas Braun[1]*

Max Planck Institute for Heart and Lung Research, Parkstr. 1, 61231 Bad Nauheim, Germany[1]; Institute of Physiological Chemistry, University of Halle, Halle, Germany[2]; and Department of Medicine III, University of Halle, Halle, Germany[3]

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During embryogenesis, various cell types can be programmed by potent inducers to follow distinct differentiation paths. In adult life, this ability seems to be restricted to specific multipotent cells. We have identified two cell populations from adult murine bone marrow which express various "stemness" genes. Treatment with Wnt molecules induced transcription of different skeletal muscle marker genes and evoked expression of cardiomyocyte markers. Further characterization of Wnt-induced intracellular signaling cascades revealed that the skeletal muscle program depended on canonical Wnt signaling, while the induction of cardiomyocyte markers seems to require a protein kinase C-dependent pathway. CDO, another component of the machinery directing skeletal muscle induction and expansion, selectively activated skeletal muscle- but not cardiomyocyte-specific genes. Although we were able to turn on various cell-type-specific markers by different induction regimens, we never obtained fully differentiated, functional cells. We conclude that the differentiation of adult stem cells is incomplete and lacks certain cues necessary to acquire a truly functional status.

Tissue-specific stem cells contribute to the regeneration and maintenance of numerous if not all tissues of mammals, including blood, liver, intestine, skeletal muscle, and the central nervous system. It is generally believed that tissue-specific stem cells are determined to follow specific cellular fates and contribute only to the tissue from which they originate. This paradigm has been challenged recently. Several studies postulated the presence of adult stem cells capable of differentiating into a broad spectrum of specialized cells. For instance, it has been claimed that adult neuronal stem cells isolated from the brain tissue of the mouse differentiate into blood, skeletal muscle, and endothelial cells (4, 28, 36), that cells from human adipose tissue differentiate into bone, muscle, and cartilage (39), and that cells from the dermis of mammalian skin differentiate into skeletal muscle, neuron, glial, and fat cells (34). Furthermore, several studies proposed that bone marrow-derived cells have the capacity to differentiate not only into blood but also into various other cell types, such as muscle (11, 13), brain (6), and liver (33), among others. It has also been claimed that a single rare cell population, so-called multipotent adult progenitor cells, is able to differentiate into derivatives of all three germ layers (17). Since such cells can be isolated and handled with relative ease, they have been considered attractive vehicles for somatic gene and cellular therapy.

Although the significance and validity of some of these studies have been questioned, it seems clear that certain stromal cells of the bone marrow (and probably also of other organs) can respond to various stimuli in vitro and in vivo (26) by expression of cell-type-specific marker molecules. Distinct cell types within the rather diverse stromal cell population can be defined by their expressions of specific surface marker molecules, their levels of adherence to various substrates, the ability for self-renewal, and their responses to various stimuli (17).

Unfortunately, several of the parameters, which are of critical importance for the isolation and characterization of distinct stromal cell populations, might vary from one lab to another. Since the stroma consists of various different mesenchymal cell types, it is usually necessary to separate distinct cell populations based on fluorescence-activated cell sorting (FACS) and/or the adherence properties of these cells to cell culture dishes, subsequent culture conditions, and other treatments (17). Obviously, such procedures might lead to the isolation and growth of slightly different cell types with different properties in various assays. In addition, cocultures of different cell types and transplantation of cells into host animals are prone to all types of labeling and detection artifacts. At present, it is not clear whether bone marrow-derived cells or other circulating cells play any significant role that can be attributed to the incorporation of these cells into diseased tissues. Alternatively, it seems possible that some of the beneficial effects observed after the infusion of stem cells rely on the induction of proliferation of resident cells by grafted cells.

A paradigmatic example for the ongoing debate about the specific functions of different cell types in tissue regeneration is the skeletal muscle. Despite a wide agreement that muscle satellite cells represent the main source of muscle stem cells, other cell populations which either reside within skeletal muscles or are derived from the bone marrow have been proposed to contribute to muscle regeneration. These cell types, which have been collectively named adult stem cells, include the so-called side population (13, 16) and $CD45^+$ cells resident in skeletal muscle (25). While satellite cells readily give rise to

* Corresponding author. Mailing address: Max Planck Institute for Heart and Lung Research, Parkstr. 1, 61231 Bad Nauheim, Germany. Phone: 49-0-6032-705-401. Fax: 49-0-6032-705-419. E-mail: thomas.braun@kerckhoff.mpg.de.

† Supplemental material for this article may be found at http://mcb.asm.org/.

differentiated myocytes in culture, adult stem cells behave differently. It has been claimed that side population cells differentiate spontaneously at a low degree into myotubes and hematopoietic cells (2), while $C45^+$ cells depend on Wnt signaling to initiate the myogenic differentiation cascade (25). On the other hand, Zhao and Hoffman (37) reported that none of the Wnt members discussed by Polesskaya et al. (25) are active in vivo, raising doubts whether Wnt signaling significantly contributes to the regeneration of adult muscle tissue in vivo by recruiting nonmuscle cells residing within the muscle tissue.

Here, we have focused on the plasticity of murine bone marrow-derived cells and on signals that are known to be involved in the control of myogenesis, namely, Wnts and CDO, which direct skeletal muscle cell lineage induction and expansion during embryogenesis. We examined the effects of Wnts and CDO on two different murine bone marrow-derived multipotent adult stem cell populations (mBM-MASCs) which we isolated from whole bone marrow and named mBM-MASCs1 ($CD34^-$/Sca-1^{high}) and mBM-MASCs2 ($CD34^+$/Sca-$1^{moderate}$). In addition, we analyzed the inherent multilineage potentials of these cells after epigenetic reprogramming and treatment with different growth factor combinations. Although we were able to induce the expression of molecular markers characteristic of all three germ layers in a subset of treated cells, we did not obtain fully functional cells. Our results suggest that the differentiation of mBM-MASCs depends on stochastic events and is arrested prior to terminal differentiation, probably due to the absence of critical determination events.

MATERIALS AND METHODS

Isolation and cultivation of mBM-MASCs. mBM-MASCs were isolated from the bone marrow of 2-month-old female ICR mice by expansion of individual clones derived from rare, slowly dividing cells. Clones were recovered and subjected to additional rounds of plating and growth until homogenous cell populations were obtained. Details of the procedure are described in the supplemental material. Two cell populations which showed differential expression of a selected cell surface marker, designated mBM-MASCs1 and mBM-MASCs2, were characterized further.

Flow cytometry. mBM-MASCs were characterized at various time points by standard flow cytometry using antibodies against Sca-1, c-Kit, CD34, CD45, Ter119, CD13, SSEA-1 (stage-specific embryonic antigen), CD133/prominin, Flk-1, and H-$2D^d$. Data collected from >10,000 cells were expressed as the percentage of positive cells from the total gated cell population. Raw data were analyzed using the CellQuest Pro software (BD Inc.).

RNA isolation and RT-PCR. Total RNA was isolated from adult mouse skeletal muscle, heart, liver, and brain tissues and differentiated and undifferentiated mBM-MASCs with Trizol (Invitrogen). Reverse transcription-PCR (RT-PCR) analyses were performed using 1 μg of DNase-treated RNA isolated from various tissues and differentiated and undifferentiated mBM-MASCs as described previously (21). Detailed protocols and primer sequences are available from the authors on request. In all cases, a housekeeping gene, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, was used as an internal control. Identities of PCR products were corroborated by DNA sequence analysis and hybridization with radioactively labeled probes.

Construction of recombinant retroviruses. Mouse expressed sequence tag clones containing the complete coding regions of CDO (cell adhesion molecule down-regulated by oncogene) IMAGp998C168558Q3 and Wnt11 (Wingless-related mouse mammary tumor virus integration site 11) IMAGp998G23800 were obtained from Deutsches Ressourcenzentrum für Genomforschung, GmbH. Respective cDNA fragments were released with appropriate restriction enzymes and inserted into pMSCVneo (Clontech). All constructs were verified by sequence analysis. Recombinant viral particles were generated by transfection of either pMSCVneo-Wnt11 or pMSCVneo-CDO together with the *gag-pol* expression constructs pM57 and M108 into the retroviral packaging cell lines C2BAC and Phoenix (ATCC) as described previously (23). The myogenin promoter-enhanced green fluorescent protein (eGFP) lentiviral reporter virus was a kind gift of D. Baltimore (Caltech, Pasadena, Calif.) (20).

Generation of Wnt- and CDO-expressing cells. Retroviral constructs encoding Wnt7A, Wnt7B, and Wnt4 were generously provided by Jan Kitajewski (Columbia University New York, N.Y.) and used to transfect Psi2 packaging cells using standard procedures (23). Stably transfected clones were isolated after G418 treatment and used to generate high-titer virus preparations. For coculture experiments, we used either packaging cells expressing different Wnts and CDO or NIH 3T3 cells which had been infected with different recombinant retroviruses. Infected cells were treated with mitomycin C (GIBCO BRL) to inhibit cell proliferation and to prevent the overgrowth of inducing cells.

Induction of cell differentiation. For coculture experiments, mBM-MASCs1 and -2 were seeded in six-well tissue culture plates (Nunc) at 1×10^5 cells/well. After 24 h, the same number of amitotic feeder cells expressing Wnt signaling molecules or CDO was added. Cultures were maintained in low-glucose Dulbecco's modified Eagle's medium supplemented with 3% (vol/vol) fetal calf serum for 7 or 8 days, with a medium change at day 4. To initiate epigenetic reprogramming, mBM-MASCs1 and -2 were seeded in six-well tissue culture plates (Nunc) at 1×10^5 cells/well. The next day, different concentrations of 5-azacytidine (AZA) (5, 10, and 15 μmol/liter), trichostatin A (TSA; 0.1, 0.3, and 0.9 μmol/liter), or a combination of both 5-AZA and TSA (5 and 0.1, 10 and 0.3, and 15 and 0.9 μmol/liter) (obtained from Sigma) were added. After 24 h, the medium was changed and cells were maintained in low-glucose Dulbecco's modified Eagle's medium supplemented with 3% fetal calf serum (vol/vol) for 10 days with a single medium change at day 4. In some experiments, 5-azacytidine was added as indicated above, but cells were maintained in culture for either 15 or 21 days. In order to explore the effects of various growth factors, cells seeded in six-well tissue culture plates (Nunc) at 1×10^5 cells/well were treated with different concentrations of fibroblast growth factor 2, bone morphogenetic protein 2, hepatocyte growth factor/scatter factor, or combinations of various growth factors as indicated below. Inhibition of protein kinase C (PKC) activity was achieved by treatment of mBM-MASCs with different concentrations of staurosporine and bisindolylmaleimide I as indicated. After 12 h, cells expressing the Wnt11 molecule were added, and the cultures were incubated for an additional 7 to 8 days. Medium including the inhibitors was changed every 3 days.

Immunofluorescence staining. Sarcomeric myosin heavy chain (MHC) was detected with the MF-20 monoclonal antibody isolated from the supernatant of hybridoma cells as described previously (5). Monoclonal antibodies against cardiac troponin T (anti-cTnT; 1:200) and troponin I (anti-cTnI; 1:200) were purchased from DPC Biermann. The use of the myogenin antibody has been described before (12). Staining was accomplished with secondary antibodies coupled with Alexa 488 or Alexa 594 (Chemicon). Nuclear staining was performed using Hoechst 33258 (Dako) at 5 μg/ml for 10 min.

RESULTS

Isolation of two multipotent cell populations (mBM-MASCs) from adult murine bone marrow. Initial attempts to activate myogenic pathways in nonpurified populations of mesenchymal cells derived from the bone marrow proved to be highly variable and biased by occasional contaminations with myoblasts and yielded no conclusive results in our hands (see Fig. S1A in the supplemental material), although it has been claimed by other groups that full myogenic differentiation might be accomplished using unselected bone marrow-derived mesenchymal cells (29). To achieve a reproducible outcome in a defined experimental system, we decided to use individual cell populations which express distinct sets of marker genes. We therefore isolated two separate multipotent mesenchymal cell populations from the bone marrow as described in Materials and Methods. Morphologically, cells from both populations displayed large nuclei and scanty cytoplasms and exhibited roughly the same doubling times. Initially, cultures went through phases of dormancy of nearly a month before homogenous mBM-MASCs were recovered by a series of sequential passages (see Fig. S1B in the supplemental material). FACS analysis (forward and sideward scatter) indicated that the cell population consisted mostly of large and moderately granular

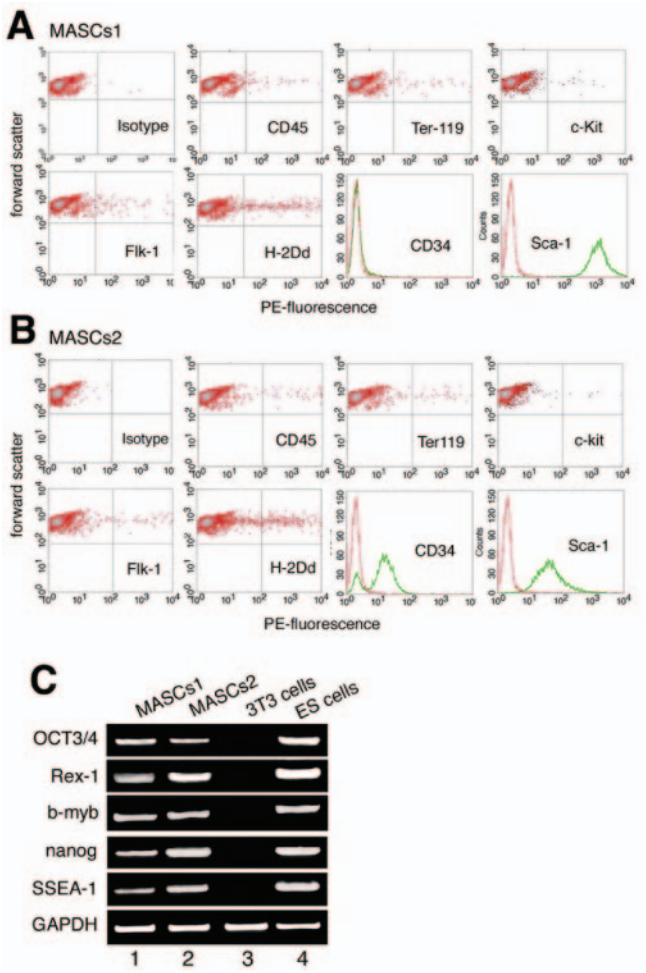

FIG. 1. Analysis of stem cell marker gene expression in mBM-MASCs. (A) FACS analysis of uninduced mBM-MASCs1 and -2. Expression of Sca-1, c-Kit, CD34, CD45, Ter119, Flk-1, and H-2D^d surface markers was analyzed by staining with phycoerythrin (PE)-conjugated monoclonal antibodies. mBM-MASCs1 and -2 differed in their expressions of Sca-1 and CD34. No expression of CD34 was found in mBM-MASCs1. Negative peaks in the CD34 and Sca-1 graphs represent phycoerythrin-conjugated isotype controls. (B) RT-PCR analysis of the expression of alleged stemness genes in mBM-MASCs1 and -2, embryonic stem (ES) cells, and NIH 3T3 cells. mBM-MASCs1 and -2 showed robust expressions of OCT3/4, Rex-1, b-myb, nanog, and SSEA-1 (stage-specific embryonic antigen) (lanes 1 and 2). Embryonic stem cells (lane 4) were used a positive control, and NIH 3T3 cells (lane 3) were used as a negative control.

cells, with a minor portion of small cells. FACS-assisted immunocytometry for expression of Sca-1, c-Kit, CD34, CD45, Ter119, Flk-1, and H-2D^d (Fig. 1) as well as CD13, SSEA-1, and CD133/prominin (data not shown) revealed a clear difference in the expression levels of CD34 and different expression levels of Sca-1 in the two cell populations, while other parameters were virtually identical. In summary, mBM-MASCs1 were CD34$^-$ Sca-1^{high} CD45^{dim} Ter119^{dim} Flk-1^{low} H-2D$^{d\ low}$, and mBM-MASCs2 were CD34$^+$ Sca-1^{moderate} CD45^{dim} Ter119^{dim} Flk-1^{low} H-2D$^{d\ low}$. A dot plot representation of the expressions of c-Kit, CD45, Ter119, Flk-1, and H-2D^d indicated some heterogeneity in the population, with few cells expressing the antigens at high concentrations and a large bulk of cells which showed only minor or no expression. (Fig. 1). CD45, Ter119, c-Kit, and Flk-1 were found in less than 0.5% and H-2D^d in no more than 2% of all cells. Interestingly, this ratio remained constant even after extended subculturing, suggesting a stochastic process that leads to the occasional activation of distinct sets of permissive genes.

Various Wnt signaling molecules share similar potentials to activate the myogenic program in mBM-MASCs. Wnt molecules are well-known inducers of myogenesis during embryonic development. We wanted to explore whether myogenic differentiation might be induced in mBM-MASCs by various Wnt molecules and whether differences in the inductive potentials of different Wnt molecules in this assay system might exist.

We therefore cocultured the two different mBM-MASC populations together with amitotic feeder cell lines that secreted Wnt1, Wnt3, Wnt7a, Wnt7b, Wnt4, or Wnt11. After 7 days, we noted profound morphological changes in mBM-MASCs1 (CD34$^-$ Sca-1^{high}). Cells switched from a fibroblast-like phenotype to an appearance that resembled that of small, mononucleated primary myotubes. mBM-MASCs2 (CD34$^+$ Sca-1^{moderate}) reacted similarly, although it took these cells 1 to 2 days longer to acquire the same differentiation-related morphological changes. Treatment of mBM-MASCs with Wnt molecules also resulted in a clear reduction of the proliferation rate compared to that for nontreated cells (data not shown). We next stained Wnt-treated mBM-MASCs by immunofluorescence with an antibody (MF-20) that detects sarcomeric MHC and with a monoclonal antibody directed against myogenin (data not shown). As shown in Fig. 2, approximately 10% of all mBM-MASCs stained positive for MHC, no matter which cell population and Wnt signaling molecule were used (Fig. 2A through X). We did not observe the formation of fused multinucleated myotubes that is characteristic of the differentiation of bona fide myoblasts, suggesting that we were unable to achieve a complete myogenic programming of mBM-MASCs. Changes in culture conditions, including additions of insulin-like growth factor 1, dexamethasone, insulin, and epidermal growth factor, either alone or in combination, had no effect on this outcome (data not shown). No MHC staining was observed in untreated mBM-MASC controls (Fig. 2Y through Ö). To rule out the (unlikely) possibility that fusion of Wnt-expressing cells to mBM-MASCs is required for the initiation of the myogenic program, we repeated the induction experiments by placing Wnt-secreting cells on one side of a membrane with defined pore sizes and the responding mBM-MASCs on the other side. Initiation of the myogenic program, as indicated by MHC expression, was evident even when 0.4-μm-pore-size membranes, which prevent the transmigration of cells through the membrane, were used (see Fig. S1C in the supplemental material).

To further analyze the initiation of the skeletal muscle program in mBM-MASCs, we examined the expression of the four skeletal muscle-specific myogenic determination factors, i.e., Myf5, MyoD, myogenin, and myogenic regulatory factor 4 (MRF4), by RT-PCR 8 days after the initiation of Wnt treatment. As shown in Fig. 3, we always found expression of at least one myogenic factor after induction with different Wnt molecules, although some differences between the effects of individual Wnt molecules were obvious. Induction of mBM-MASCs1 with Wnt11 resulted in expression of all four myo-

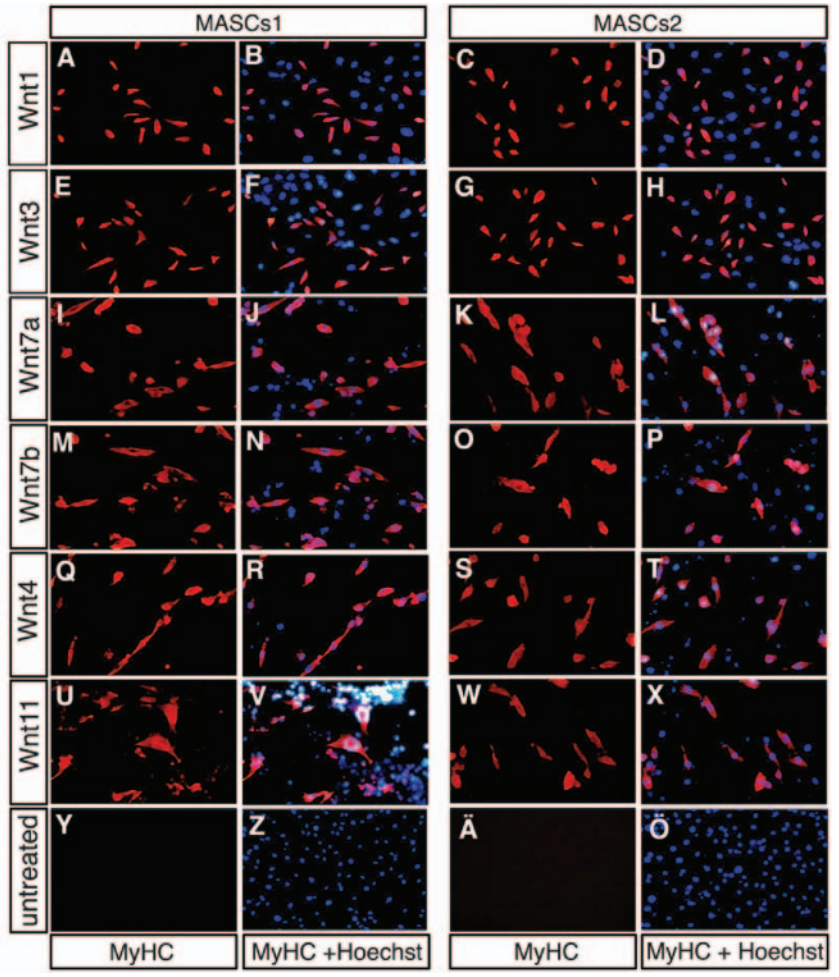

FIG. 2. Different Wnt signaling molecules induce expression of MHC in mBM-MASCs. mBM-MASCs1 and -2 were cocultured with amitotic cells that expressed either Wnt1 (A to D), Wnt3 (E to H), Wnt7a (I to L), Wnt7b (M to P), Wnt4 (Q to T), or Wnt11 (U to X) or with the parental cell line (Y to Ö). After 7 to 8 days, cultures were stained with the monoclonal MF-20 antibody against sarcomeric myosin heavy chain and a secondary antibody coupled to Alexa 594 anti-mouse. MHC expression was indiscriminately activated by all Wnt molecules tested. Nuclei were counterstained with Hoechst 33258 to locate all cells on the plate (B, F, J, N, R, V, and Z [second column] and D, H, L, P T, X, and Ö [fourth column]). MyHC, MHC.

genic factors, while Wnt7a stimulated only the expression of myogenin after continuous treatment. Under our conditions, the expression of Myf5 was activated solely by Wnt11, while the expression of MRF4 depended on Wnt11 and Wnt7b. Even though no systematic study of the temporal expression patterns of MRFs during treatment of mBM-MASCs with different Wnt molecules was performed, changes in the expression profiles of MRFs were observed. Expression of MyoD, for example, was present in most cultures of mBM-MASCs early after the initiation of Wnt treatment but faded at later stages, although this finding varied considerably between individual experiments (data not shown).

We next assessed the number of myogenin-positive cells after treatment with different Wnt molecules by using myogenin antibody staining or FACS analysis of mBM-MASCs infected with a myogenin promoter-eGFP retroviral reporter construct (Fig. 4A). Expression of myogenin protein was found in up to 10% of all cells, which roughly matched the number of MHC-positive cells (Fig. 4C and D). The myogenin promoter-eGFP reporter, which allows analysis of much larger cell numbers, was activated to a similar degree (Fig. 4A). Despite the fact that all Wnts were expressed at comparable levels in inducing feeder cells, as assessed by semiquantitative RT-PCR (Fig. 4B), we noted consistent differences in the efficiencies of Wnts in activating myogenin expression. However, at present it is hard to decide whether this is due to intrinsic biochemical differences between Wnt molecules or different bioavailabilities of Wnt proteins.

Differential activation of cardiomyocyte markers by different Wnt molecules. To investigate the potential of various Wnt molecules to activate the cardiac program in mBM-MASCs, we analyzed the expression of different cardiomyogenic marker genes by RT-PCR after induction with different Wnt molecules. As shown in Fig. 3, all Wnt molecules tested (Wnt4, Wnt7a, Wnt7b, Wnt11) induced expression of the Nkx-2.5, GATA-4, Hand 2 (dHand), TEF1, and tropomyosin cardiac marker genes, while only treatment with Wnt11 resulted in the expression of β-MHC and brain natriuretic protein (BNP)

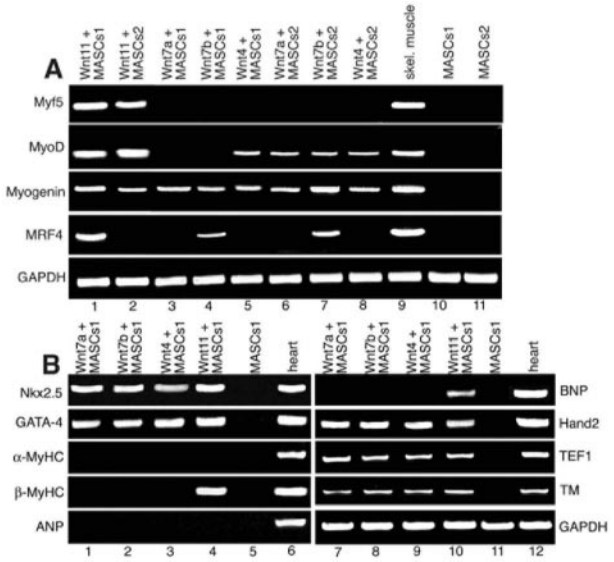

FIG. 3. Induction of skeletal and cardiac muscle marker genes in mBM-MASCs by different Wnt molecules. (A) RT-PCR analysis of the expression of skeletal muscle myogenic factors. RNA was isolated from mBM-MASCs1 and -2, which were cocultured for 7 days with different amitotic Wnt-expressing cells (lanes 1 to 8), with the parental cell line lacking Wnt expression (lanes 10 and 11), and with skeletal (skel.) muscle cells (lane 9). Different expression patterns of myogenic factors were induced. Treatment with Wnt molecules resulted in the activation of at least one myogenic factor. (B) RT-PCR analysis of the expression of cardiac muscle marker genes using the same experimental setup. Wnt11 was the only Wnt molecule that activated expression of β-MHC and BNP (lanes 4 and 10), while other Wnt molecules stimulated Nkx-2.5, Hand2, and GATA-4 expression. All Wnt molecules failed to induce α-MHC and ANP. MyHC, MHC.

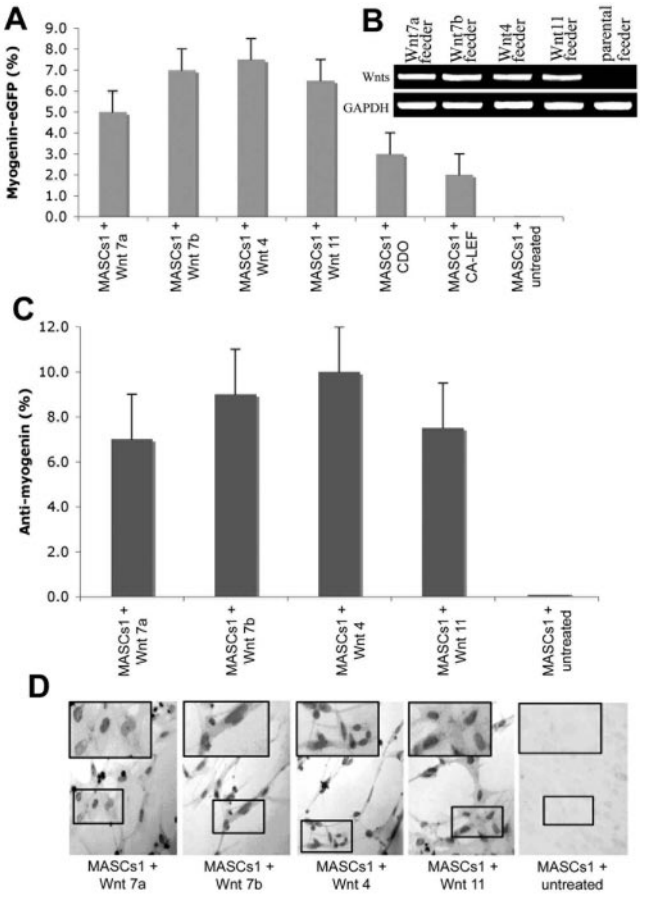

FIG. 4. Comparison of the numbers of myogenin-expressing mBM-MASCs after induction with different Wnts, CDO, and CA-LEF. (A) FACS-assisted cell counts of GFP-positive mBM-MASCs that had been stably infected with a myogenin promoter-eGFP retroviral reporter virus before treatment with various effectors. (B) Semiquantitative RT-PCR of the expression of different Wnt molecules in inducing feeder cells. (C) Number of myogenin-positive cells after staining with an anti-myogenin antibody. Numbers in panels A and C represent the percentages of positive cells within the whole cell population. Error bars indicate the standard deviations. (D) mBM-MASCs were induced with different Wnt-expressing cells and stained with an anti-myogenin antibody to reveal the presence of the nuclear antigen. Representative examples are shown.

(Fig. 3B). However, we were unable to identify reproducible expression of other typical cardiomyocyte genes, such as the α-MHC and atrial natriuretic protein (ANP) genes (Fig. 3B). Similarly, the induction of mBM-MASCs by Wnt11 resulted in the detection of cTnT by immunofluorescence (Fig. 5D), but no organized contractile apparatus or cross-striations were discernible (Fig. 5D). Double immunofluorescence staining of Wnt11-induced mBM-MASCs using antibodies directed against GATA-4 and Nkx-2.5 revealed a high degree of heterogeneity among induced cells. Some cells expressed either GATA-4 or Nkx-2.5, some expressed both antigens, and some expressed neither of them (see Fig. S1D in the supplemental material). No cells that underwent spontaneous contractions were identified. We concluded that treatment of mBM-MASCs with Wnt molecules led only to the activation of certain cardiac marker genes and not to the full differentiation that would be necessary to acquire a truly functional status.

CDO activates skeletal muscle but not cardiac muscle markers in mBM-MASCs. CDO is a ROBO-related cell surface protein that has been proposed to mediate effects of cell-cell interactions between muscle precursor cells and to participate in a positive feedback loop with MyoD to enhance skeletal myogenesis (19). To investigate whether directed expression of CDO might enhance the intrinsic propensity of mBM-MASCs, which express CDO at very low levels (Fig. 5E), to initiate expression of myogenic genes during stem cell differentiation, we cocultured mBM-MASCs1 and mBM-MASCs2 together with mitotically inactive feeder cells that carried high levels of the CDO molecule. As shown in Fig. 5, both mBM-MASCs1 and mBM-MASCs2 expressed MyoD and myogenin after 7 to 8 days of cocultivation, whereas MRF4 was exclusively expressed in mBM-MASCs1 and Myf5 in mBM-MASCs2 (Fig. 5A). In addition, we detected the expression of Wnt4 (Fig. 5F) but not of other Wnt molecules (data not shown) in CDO-stimulated MASCs, further supporting the hypothesis that CDO might enhance positive feedback loops to stimulate differentiation. The induction of mBM-MASCs by CDO also resulted in the expression of some sarcomeric proteins, as demonstrated by immunofluorescence detection of MHC (Fig. 5C). Despite a robust expression of MHC, we again did not detect multinucleated myotubes or organized sarcomeric structures, a result comparable to the observations made

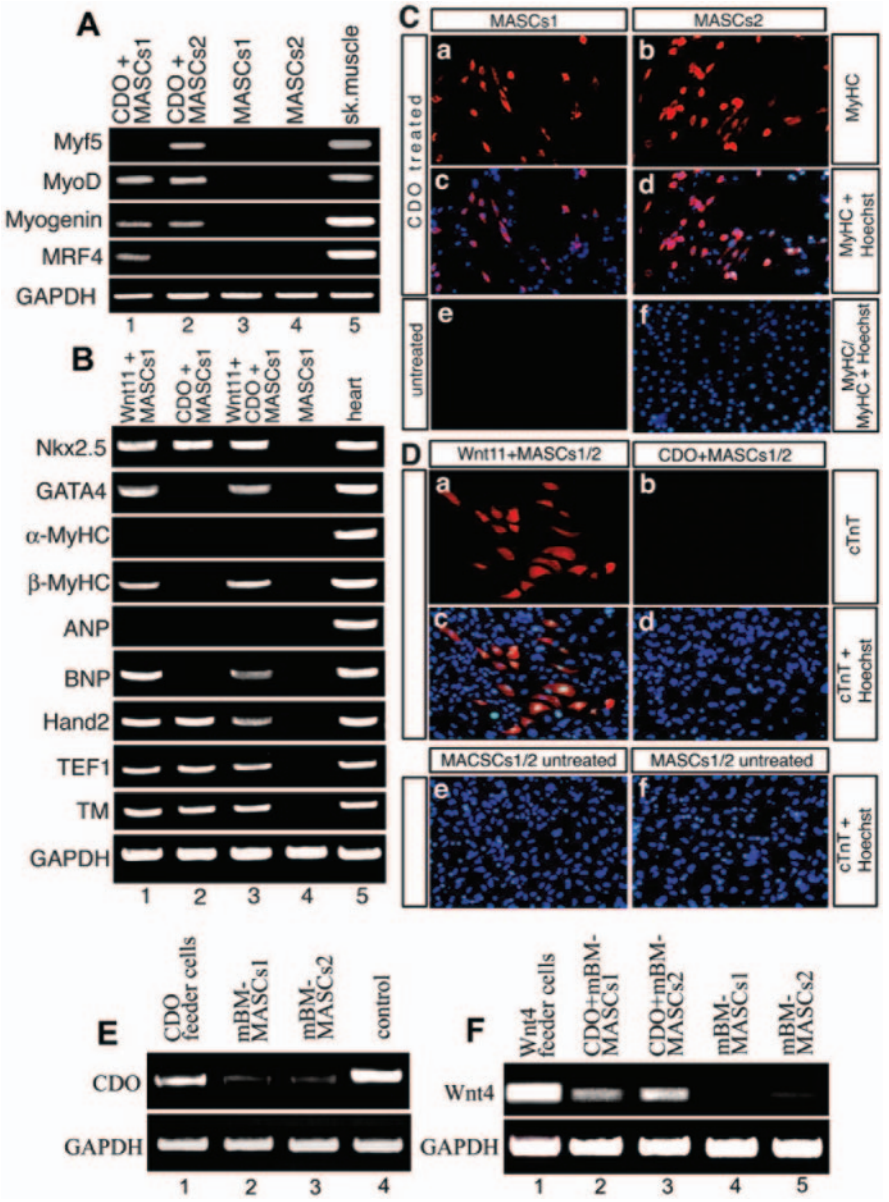

FIG. 5. CDO induces the expression of skeletal muscle but not of cardiac muscle marker genes in mBM-MASCs. (A) RT-PCR analysis of the expression of skeletal muscle myogenic factors. RNA was isolated from mBM-MASCs1 and -2 that were cocultured for 7 days with amitotic CDO-expressing cells (lanes 1 and 2), with the parental cell line lacking CDO expression (lanes 3 and 4), and with skeletal muscle cells (lane 5). CDO treatment led to the robust expression of myogenic regulatory factors. (B) RT-PCR analysis of the expression of cardiac muscle marker genes using the same experimental setup. CDO (lane 2) failed to induce cardiac markers such as GATA4 and β-MHC, while Wnt11 alone (lane 1) or in combination with CDO (lane 4) had this ability. (C) Immunofluorescent staining of MASCs for MHC expression after cocultivation with CDO-expressing cells and with the parental cell line lacking CDO expression. Nuclei were counterstained with Hoechst 33258 (c, d, and f) to locate all cells on the plate. (D) Immunofluorescent staining of MASCs for cTnT expression after cocultivation with Wnt11- and CDO-expressing cells and with the parental cell line lacking CDO or Wnt11 expression. Nuclei were counterstained with Hoechst 3444 (c to f) to locate all cells on the plate. (E) RT-PCR analysis of CDO expression in feeder cells and MASCs. (F) RT-PCR analysis of Wnt4 expression in MASCs after induction with CDO. Note the induction of Wnt4 expression after CDO treatment. MyHC, MHC.

with Wnt-primed mBM-MASCs. Similar results were obtained using human BM-MASCs that were cocultured either with Wnt11- or CDO-expressing cells (see Fig. S1F in the supplemental material). Coculturing with CDO-expressing cells also resulted in a reduction of cell numbers in comparison to untreated plates seeded at the same initial density, most likely indicating cell cycle withdrawal as a consequence of (partial) cellular differentiation. To rule out the option that the initiation of the myogenic program by CDO depended on the fusion of CDO-expressing cells to mBM-MASCs, we performed the same filter experiment described above for Wnt-expressing cells. Similarly, we observed an induction of the myogenic program, as indicated by MHC expression, even when 0.4-μm-pore-size membranes, which prevent the transmigration of cells through the membrane, were used (see Fig. S1E in the supplemental material).

Although no role for CDO in the activation of cardiac mus-

cle cell differentiation has been described so far, the ability of CDO to activate genes characteristic for striated muscle prompted us to analyze whether CDO-mediated cellular signaling processes might also cause the activation of cardiac muscle marker genes in mBM-MASCs. As shown in Fig. 5, CDO treatment led to the activation of the Nkx-2.5 gene but not to the expression of the GATA-4, α-MHC, β-MHC, ANP, or BNP genes. In this respect, the gene expression pattern evoked by CDO resembled that of Wnt4 and Wnt7, with the notable exception of a missing GATA-4 gene expression. Similarly, CDO-induced mBM-MASCs also failed to show cTnT protein expression (Fig. 5D), while Wnt11 (Fig. 5D) but not Wnt4 and Wnt7 (data not shown) clearly resulted in an induction of cTnT in a subset of stimulated cells. Combined application of CDO and Wnt11 did not prevent the activation of cardiac muscle-specific Wnt11 target genes, indicating that CDO does not affect cardiomyogenic pathways in a negative manner (Fig. 5B).

Activation of cardiomyocyte marker gene expression by Wnt molecules in mBM-MASCs depends on PKC but not on LEF signaling. Wnt molecules are known to play a pivotal role in the induction of myogenesis during somitic development. Ectopic expression of Wnt-1 completely represses ventral (sclerotomal) markers and enhances and expands expression of dorsal (myogenic) markers in somites. Similarly, the delivery of an activated form of β-catenin to somitic mesoderm mimics the effects of Wnt-1, suggesting that Wnt signaling is mediated via the so-called canonical Wnt/β-catenin pathway (7). We wanted to explore whether the activation of skeletal and cardiac muscle genes in mBM-MASCs also depends on the canonical Wnt/β-catenin pathway. We therefore expressed a constitutively active form of LEF (CA-LEF) (1), which is an essential part of the β-catenin nuclear complex, by retrovirus-mediated gene transfer in mBM-MASCs and assayed the expression of tissue-specific marker genes. Directed expression of CA-LEF resulted in the activation of several skeletal muscle-specific genes, including the Pax7, Myf5, MyoD, and myogenin genes in mBM-MASCs (Fig. 6A). Likewise, we observed the activation of a myogenin promoter-GFP lentiviral reporter construct in a subset of mBM-MASCs (Fig. 6C). In contrast, we found an absence of cardiomyocyte markers in mBM-MASCs after CA-LEF expression, with the exception of Nkx-2.5 (Fig. 6B), which was also expressed after treatment with Wnt4, Wnt7a, and Wnt7b (Fig. 3B). These results clearly indicate that Wnt-mediated activation of cardiac marker genes in mBM-MASCs did not depend on the canonical Wnt/β-catenin pathway but was relayed by an alternative route. Although the precise mechanisms of noncanonical Wnt signaling have not been unveiled in detail, it seems clear that PKC plays an important role both in the Wnt/Jun N-terminal protein kinase and Wnt/Ca^{2+} pathways (24). We therefore treated mBM-MASCs that were cocultured with Wnt11-expressing cells with different concentrations of staurosporine and bisindolylmaleimide I, two known inhibitors of PKC. As shown in Fig. 7, the expression of Nkx2.5, myocardin, BNP, and β-MHC was completely abrogated after inhibition of PKC, while the expression of TEF-1, tropomyosin, and GAPDH remained unchanged. Surprisingly, the expression of GATA-4 was not affected by staurosporine and bisindolylmaleimide I. We even noted a slight increase in GATA-4 gene transcripts after PKC inhibition (Fig. 7). Taken together, our results suggest the activation of different sets of cardiac muscle-specific genes by multiple Wnt signaling branches.

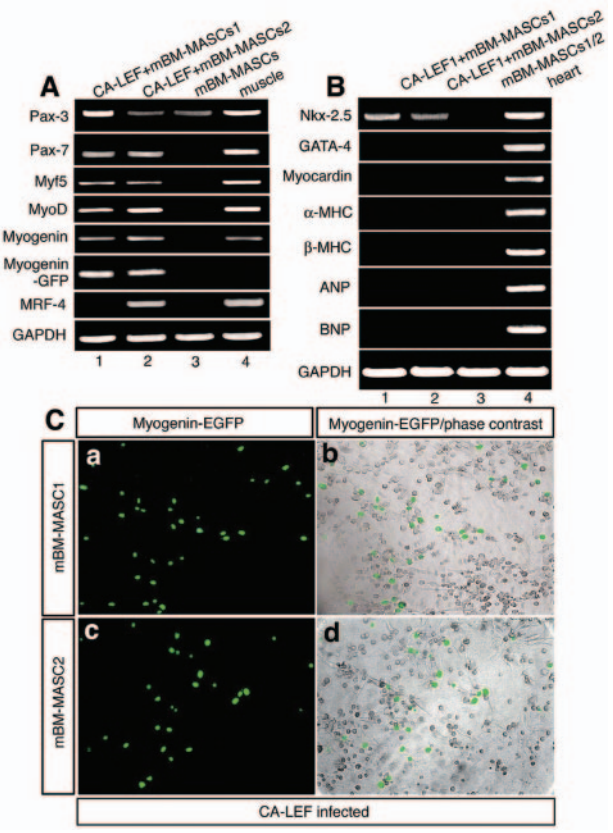

FIG. 6. CA-LEF1 induces the expression of skeletal muscle but not of cardiac muscle marker genes in mBM-MASCs. RT-PCR analysis of the expression of skeletal muscle myogenic factor (A) and cardiac marker genes (B) after infection of mBM-MASCs1 and -2 with a retrovirus encoding CA-LEF. CA-LEF1 led to the activation of several skeletal muscle markers but not of cardiac marker genes, with the exception of the Nkx-2.5 gene. (C) mBM-MASCs1 and -2, each containing a myogenin promoter-eGFP reporter construct, were infected with a retrovirus encoding CA-LEF. Activation of the myogenin-eGFP reporter construct is shown under fluorescent light (a and c) and merged with corresponding phase-contrast images (b and d), indicating the activation of skeletal muscle genes.

Induction of chromatin remodelling leads to spontaneous expression of skeletal and cardiac muscle marker genes. From our previous experiments, it became clear that mBM-MASCs own a considerable plasticity that allows them to activate parts of the skeletal and cardiac muscle cell programs in response to specific cues. Although specific signals are apparently required to efficiently turn on components of defined differentiation networks, a certain propensity for spontaneous determination events seemed inherent to mBM-MASCs. We wanted to investigate whether this plasticity might be enhanced by stimulation of chromatin remodelling and alleviation of gene silencing. We therefore incubated mBM-MASCs with AZA (an inhibitor of DNA methylation), TSA (an inhibitor of histone deacetylases), or a combination of both drugs and analyzed the expression of skeletal and cardiac muscle-specific genes. Treatment of mBM-MASCs with either drug (Fig. 8) resulted in

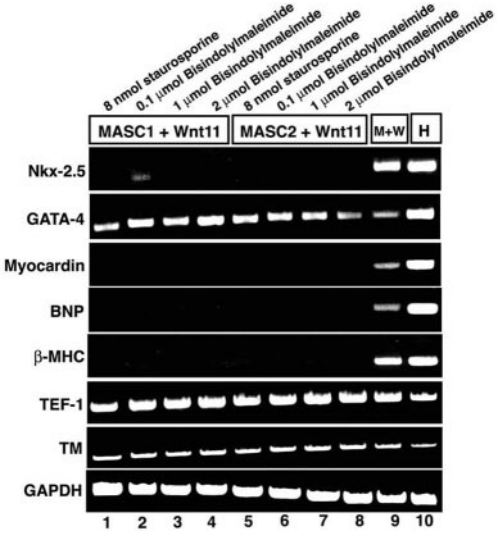

FIG. 7. Inhibition of PKC abrogates activation of most cardiac markers by Wnt11 in mBM-MASCs. RT-PCR analysis of the expression of cardiac markers after coculturing of Wnt11-expressing cells with mBM-MASCs and treatment with various concentrations of bisindolylmaleimide I and staurosporine (lanes 1 to 8). RNAs isolated from cocultures without the addition of inhibitors and from heart tissue were used as controls. Expression of cardiac marker genes, with the exception of the GATA-4 gene, was efficiently repressed by the inhibitors.

activation of the myogenic factors MyoD, myogenin, and MRF4 (Fig. 8B) and of sarcomeric MHC (Fig. 8A). In contrast to the induction of the myogenic program by Wnts, we did not notice expression of Myf5 even after combined treatment with AZA and TSA. After treatment with AZA and TSA, we detected the expression of several cardiac marker genes, including the cTnI (Fig. 8C), GATA-4, Hand-2, and β-MHC (Fig. 8D) genes, in mBM-MASCs. Interestingly, we also found the expression of α-MHC, which was not present after induction of MASCs with Wnt11, and the activation of a transgenic eGFP reporter gene driven by the α-MHC promoter that had been stably introduced into mBM-MASCs (Fig. 8C). However, we failed to score the expression of ANP, indicating a partial recapitulation of the cardiac program despite the activation of the α-MHC gene.

DISCUSSION

Adult mesenchymal stem cells do not consist of a phenotypically uniform cell population. Reports about bone marrow-derived mesenchymal cells that are able to differentiate into numerous different cell types, including cardiac and skeletal muscle cells, neurons, and kidney cells, as well as the description of multipotent adult progenitor cells that are able to form derivatives of all three germ layers, have raised hopes for the use of adult stem cells for therapeutic purposes. It had been suggested that multipotent adult stem cells might be classified by the expression of distinct sets of marker molecules. This concept is derived mainly from highly successful work on hematopoietic stem cells which has led to the identification of pluripotent hematopoietic stem cells, which through a series of developmental events generate cells of the erythroid, myeloid, and lymphoid lineages (reviewed in reference 38). However, it is so far an untested assumption that all adult multipotent progenitor cells consist of a uniform cell type similar to that of pluripotent hematopoietic stem cells that reacts reproducibly upon treatment with certain stimuli. In this study, we have shown that mBM-MASCs might differ in the expressions of two popular stem cell markers, i.e., CD34 and Sca-1, without having major differences in plasticities and differentiation potentials. Minor differences that were found between cell populations seemed due to stochastic deviations rather than to programmed differences between the investigated cells. It also should be kept in mind that the differentiation potential of isolated (stem) cells in vitro might in part be imposed by the cultivation per se and might not necessarily reflect their natural behavior in a stem cell niche.

Although comprehensive surveys of gene activities as obtained by microarray hybridization techniques certainly yield more information than work with single marker genes does (15), they are difficult to interpret, due to the large number of genes affected, the effects of the physiological/metabolic state, and numerous other variables. Most likely, such influences will prevent an unbiased, accurate view of the differentiation potential of a cell. We conclude that an operational definition of mBM-MASCs in response to various inducers defines more precisely the biological potential of a stem cell than does the expression of distinct marker molecules.

Differential induction of cell-type-specific differentiation events by distinct signaling molecules. The pivotal role of Wnt molecules for somitic myogenesis is well established (9, 22). Although individual Wnt molecules have been implicated in the direct specific expression of either Myf-5 or MyoD, the lack of muscle phenotypes in Wnt knockouts argues against a strict linear relationship (14). In support of this notion, we found that Wnt molecules activate myogenesis in MASCs in a rather promiscuous way. This observation is also backed by our finding of the activation of the canonical Wnt pathway by CA-LEF-stimulated differentiation of adult stem cells, although it has been reported that the activation of the canonical Wnt pathway does not promote differentiation and even inhibits differentiation of embryonic stem cells (30).

We also detected a strong activation of skeletal muscle-specific genes by CDO. CDO has been shown to enhance the differentiation of C2C12 myogenic cells, whereas the expression of dominant negative forms of CDO inhibited differentiation (19). Mice that lack CDO display delayed skeletal muscle development and defective muscle stem cell differentiation, indicating that CDO is part of a positive feedback network that maintains the myogenic transcriptional program (8). Our finding that CDO activates the expression of myogenic regulatory factors and subsequently also of structural muscle proteins nicely fits into the picture. We reason that CDO augments an endogenous propensity of mBM-MASCs to activate myogenic genes, thereby stabilizing the positive feedback loop that ultimately drives myogenesis.

The failure of CDO to induce cardiac muscle-specific genes was in contrast to the ability of Wnt molecules, which stimulated both skeletal and cardiac muscle genes. While the role of Wnt molecules for skeletal muscle development is well established, their function in the development of the cardiac lineage is less clear. It has been proposed that Wnt11, which seems to

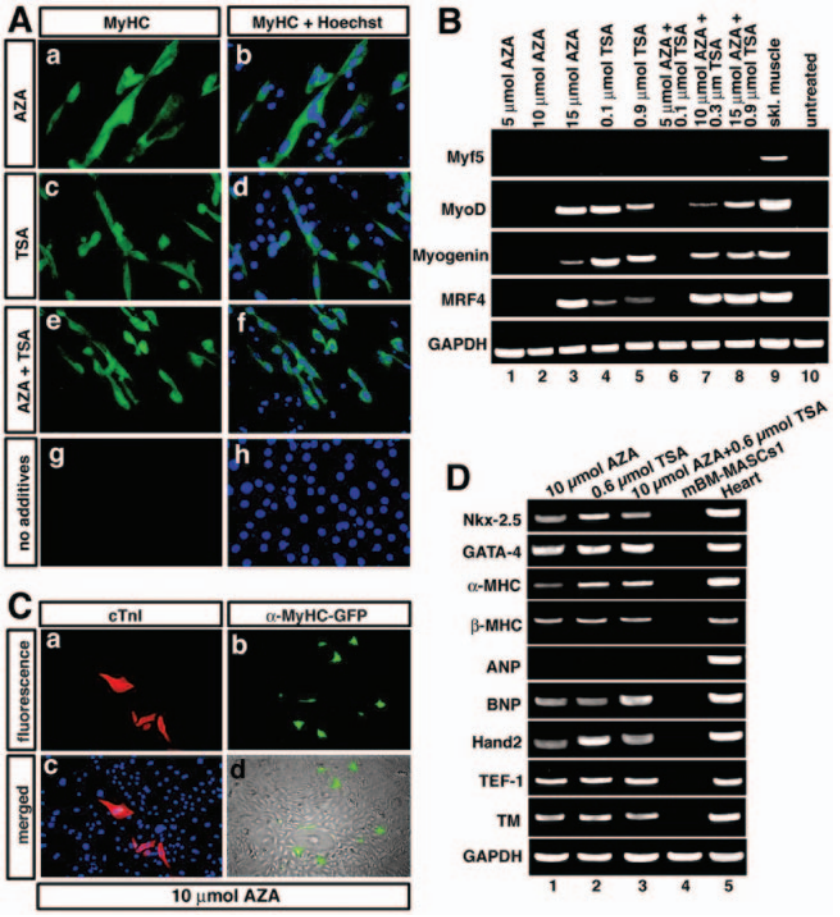

FIG. 8. Epigenetic reprogramming leads to enhanced plasticity and spontaneous differentiation of MASCs. (A) mBM-MASCs2 were stained with the monoclonal MF-20 antibody against sarcomeric myosin heavy chain and a secondary antibody coupled to Alexa 488 after treatment with AZA and/or TSA (a to f) or without treatment (g and h). Nuclei were stained with Hoechst 33258 to locate all cells within a culture (b, d, f, and h). The addition of either drug dramatically increased the number of MHC-positive cells. (B) RT-PCR analysis of the expression of skeletal muscle myogenic factors in mBM-MASCs2 after the addition of AZA, TSA, and AZA/TSA (lanes 1 to 8). RNAs isolated from untreated mBM-MASCs2 (lane 9) and from skeletal muscle tissue (lane 10) were used as negative and positive controls, respectively. Note the lack of Myf5 expression after AZA and AZA/TSA treatment. (C) After treatment with AZA, mBM-MASCs2 were stained with a monoclonal antibody against cTnI and a secondary antibody coupled to Alexa 594 (a and c) in the absence (a) and presence (c) of Hoechst 33258. In panels b and d, treatment with AZA was done with mBM-MASCs2 that contained an α-MHC-eGFP reporter construct. Activation of the α-MHC-eGFP reporter construct is shown under fluorescent light (b) and merged with a corresponding phase-contrast image (d). (D) RT-PCR analysis of the expression of cardiac muscle marker genes in mBM-MASCs1 after the addition of AZA, TSA, and AZA/TSA (lanes 1 to 3). RNAs isolated from untreated mBM-MASCs1 (lane 4) and from heart tissue (lane 5) were used as negative and positive controls, respectively.

act via a noncanonical Ca^{2+}- and PKC-dependent pathway, stimulates cardiomyocyte development (10, 24), while other Wnts appear to inhibit cardiogenesis (35). In our experimental setup, we indeed observed a preferential activation of cardiac marker genes by Wnt11 and only a reduced ability of other Wnt molecules to activate cardiomyogenesis, but we observed no suppression.

Wnt molecules activate cardiac and skeletal muscle-specific gene expression via separate intracellular signaling networks. It is certainly an oversimplification to view skeletal myogenesis as a result of canonical Wnt signaling and cardiogenesis as a result of noncanonical Wnt signaling, in particular since Wnt signals derived from the dorsal ectoderm (Wnt-7a) have also been shown to employ the noncanonical PKC signaling cascade in P19 cells (27). Experiments in chicken embryo demonstrated that Wnt-1 and Wnt-3a induced myogenesis via Frizzled1 and β-catenin/Lef1/T-cell factor signaling (31), while the ability of Wnt11 to induce ectopic cardiogenesis was attributed to the noncanonical Ca^{2+}/PKC-dependent pathway (24). Along the same lines, we demonstrated that the expression of CA-LEF in mBM-MASCs resulted in the activation of skeletal muscle-specific proteins but not in the expression of cardiac muscle-specific genes, with the notable exception of the Nkx-2.5 gene. Inhibition of PKC using the PKC inhibitor bisindolylmaleimide I and the kinase inhibitor staurosporine abrogated Wnt-induced expression of cardiac genes, indicating an important role of noncanonical Wnt signaling in mBM-MASCs for the promotion of the cardiac program.

Plasticity and spontaneous differentiation of MASCs might be enhanced by induction of chromatin remodelling. Normally, stem cells are maintained in a quiescent state and need specific stimuli for renewal and differentiation. When stem cells differ-

entiate, they have to suppress genes that are incompatible with the upcoming cell type. This might be achieved either by an active control mechanism, which depends on dynamic interactions of regulatory proteins present in the cell, or by a passive mechanism that silences unneeded genes so that they are normally not accessible in a given cell lineage. A passive mechanism might rely on promoter/gene hypermethylation and histone deacetylation (3). Release of the passive control mechanism will result in an increase of gene transcription and in the inappropriate expression of individual genes. In the current study, we observed a spontaneous activation of skeletal and cardiac muscle genes in mBM-MASCs after demethylation and acetylation, indicating that hypermethylation and chromatin compaction by histone deacetylation are important mechanisms by which myogenic gene expression is silenced in stem cells. It seems likely that the forced inhibition of these silencing mechanisms increases cellular plasticity by allowing the spontaneous activation of critical control genes. Such a wave of undirected gene activity might eventually result in the formation of self-maintaining regulatory circuits that enable further differentiation events. The MyoD gene has been shown to be particularly susceptible to such regulation (18). It was therefore no surprise to detect an up-regulation of MyoD but not of Myf5 in AZA- and TSA-treated stem cells. Both acetylation and demethylation were able to initiate myogenic gene activation separately. The combined acetylation and demethylation had no synergistic effects and did not increase the expression of muscle-specific genes further, suggesting that the silencing of the stem cell genome might be overcome by either mechanism, which then dominantly affects the other supplementary mechanism.

Activation of cell-type-specific gene expression pattern versus differentiation to fully functional cells. Although several groups have reported the ability of mesenchymal stem cells isolated from the bone marrow and other organs to differentiate into numerous specialized cell types or into derivatives of all three germ layers, no rigorous confirmation of the functional abilities of such cells has been made. Repopulation of the stem cell niche, which is a standard procedure in the hematopoietic field, has not been attempted or has failed. Nevertheless, some progress was made in improving the signature of differentiated cells, e.g., by partially restoring dystrophin expression in mdx mice, an animal model of Duchenne's muscular dystrophy, after bone marrow transplantation studies in the mouse (13). However, the latter findings might also be due to the fusion of bone marrow-derived cells to newly forming myotubes (32).

In our hands, mBM-MASC-derived cells that express marker genes characteristic of cardiac and skeletal muscle after induction with different signaling molecules lacked several features of functional muscle cells. They did not form regular cross-striations or show spontaneous contractions, which are typical for cardiomyocytes. If adult stem cells activate only a subset of cell-type-specific genes, then what is the real nature of adult stem cells during normal development? It seems plausible that they simply represent loose ends or leftovers of various progenitor cells that have preserved the remarkable plasticity of their ancestors but stopped serving an important physiological role. It is also possible, however, that the physiological role of adult stem cells has yet to be unveiled. In any case, adult stem cells are remarkably plastic and easily undergo partial differentiation upon induction, despite the failure to acquire fully functional characteristics. Obvious explanations for an incomplete differentiation might be the absence of activating molecules within the cells or the presence of inhibitory factors. We assume that it might be possible to identify missing components and push the faulty differentiation program to completion, essentially reestablishing the active control mechanism which drives any functionally differentiated cell at any given time.

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2.2 Vergleich der Auswirkungen unterschiedlicher Zelltransplantationen auf die Herzfunktion bei Mäusen mit chronischem Myokardinfarkt

In Übereinstimmung mit den Befunden anderer Arbeitsgruppen [Balsam et al. 2004; Murry et al. 2004; Nygren et al. 2004], stützen die eigenen, unter 2.1. geschilderten Ergebnisse aus Zellkulturexperimenten somit die Hypothese, dass sich aus adulten Knochenmarksstammzellen nicht in relevantem Umfang funktionell intakte Kardiomyozyten differenzieren lassen. Dennoch belegen Daten aus klinischen Studien günstige Effekte der Transplantation von Knochenmarksstammzellen bei der Behandlung des Myokardinfarktes, was sich nicht allein durch die allgemein akzeptierte Induktion der Vaskulogenese erklären lässt [Assmus et al. 2002; Wollert et al. 2004]. Eine mögliche Erklärung für diese scheinbare Diskrepanz ist in der Hypothese zu sehen, dass nicht die Generierung neuer Muskelzellen, sondern vielmehr die Stammzell-vermittelte parakrine Beeinflussung des Myokards positive Auswirkungen begründet. Um diesen Aspekt weiter zu verfolgen, sollten in eigenen Untersuchungen die Auswirkungen verschiedener Zelltransplantationen auf die gestörte Herzfunktion bei Mäusen nach experimentellem Myokardinfarkt verglichen werden [Ebelt et al. 2007]. Hierzu wurde bei adulten männlichen ICR-Mäusen die linke Herzkranzarterie (LAD) operativ verschlossen, was zu einer Infarzierung der freien Wand des linken Ventrikels führt. 7 Tage nach dem LAD-Verschluss wurden den Tieren unterschiedliche Zellpräparationen in die Randzone des Infarktes injiziert: die Tiere erhielten entweder nur PBS (Kontrollgruppe) oder aber Myoblasten der Skelettmuskulatur bzw. murine embryonale Kardiomyozyten, die zuvor mittels eines genetischen Selektionsverfahrens aus embryonalen Stammzellen (ES) *in vitro* differenziert und selektioniert worden waren [Zandstra et al. 2003]. Beide Zellarten wurden vor der Transplantation jeweils mit einem stabilen Fluoreszenz-Farbstoff (DiI) markiert, um eine spätere Identifizierung der transplantierten Zellen zu ermöglichen. Die Auswirkungen der unterschiedlichen Behandlungen wurden mittels serieller echokardiographischer Untersuchungen analysiert, und 28 Tage nach den Transplantationen wurden die Herzen der Tiere mittels standardisierter *in-situ*-Fixierung entnommen und der histologischen Auswertung zugeführt bzw. für molekularbiologische Analysen verwendet.

Die beiden verwendeten Zellpräparationen (Myoblasten bzw. ES-Kardiomyozyten) waren bewusst aus dem Spektrum der theoretisch denkbaren Zellarten ausgewählt worden, da sie sich im Hinblick auf ihre Fähigkeit, in den Verbund der Kardiomyozyten des Rest-Myokards als kontraktile Zellen zu integrieren und damit als ‚neue Muskelzellen' zur Verfügung zu stehen, grundsätzlich unterscheiden. Eine Reihe von Studien hatte im Vorfeld der eigenen Experimente belegt, dass Myoblasten der Skelettmuskulatur in den Herzen von

Versuchstieren stabile Transplantate bilden [Koh et al. 1993; Taylor et al. 1998], ohne dass hierbei jedoch eine funktionelle Integration der Zellen in das Myokard stattfindet [Rubart et al. 2004]. Im Unterschied hierzu handelt es sich dagegen bei den verwendeten ES-Kardiomyozyten bezüglich der funkionellen Integrationsfähigkeit um ‚ideale' Transplantate.

Die eigenen Untersuchungen zeigten, dass sowohl die Transplantation der Myoblasten als auch der ES-Kardiomyozyten zu einer Verbesserung der linksventrikulären Funktion im Herzinfarktmodell der Maus führt. Während bei Tieren, die nach der Infarkt-Induktion keine Zelltransplantation erhalten, sich eine progrediente Dilatation des linken Ventrikels (LV) mit zunehmender Verschlechterung der Ejektionsfraktion (EF) findet, wirken beide Transplantationsprotokolle diesem Funktionsverlust entgegen – auch wenn es in keinem Falle möglich ist, den infarkt-bedingten Ausfall der Herzfunktion wieder vollständig herzustellen. Mittels der echokardiographischen Untersuchungen kann belegt werden, dass die positiven Auswirkungen der Myoblasten-Transplantationen bereits nach 7 Tagen anhand des Rückgangs der LV-Dilatation nachweisbar sind. Dagegen finden sich signifikante Effekte der ES-Kardiomyozyten-Transplantation erst nach 14 Tagen, wobei auffällt, dass hier die systolische LV-Funktion (Ejektionsfraktion) stärker als die Dilatation des linken Ventrikels beeinflusst wird. Im weiteren Zeitverlauf der Untersuchungen kommt es jedoch zu einer zunehmenden Konvergenz der beobachteten Effekte, so dass 28 Tage nach den Zelltransplantationen keine signifikanten Unterschiede zwischen den beiden Transplantationsprotokollen bestehen – gegenüber den unbehandelten Kontrolltieren bleibt der Behandlungseffekt beider Zellarten jedoch weiter signifikant nachweisbar.

Bei der histologischen Analyse der Herzen zeigt sich, dass die Myoblasten-Transplantationen zu einer signifikanten Abnahme der Infarkt-Expansion führen – ein Effekt, der sich in der Gruppe der Tiere, die mit ES-Kardiomyozyten behandelt wurden, nur als Trend findet. In beiden Behandlungsgruppen kann eine Verminderung der reaktiven Hypertrophie des Rest-Myokards anhand einer verringerten Querschnittsfläche der Kardiomyozyten im *Septum interventriculare* nachgewiesen werden. Dagegen wird das Ausmaß der interstitiellen Fibrose nur durch die Transplantation der ES-Kardiomyozyten vermindert.

Aufgrund weiterer Analysen lässt sich überraschenderweise feststellen, dass in den eigenen Experimenten die beobachtete Verbesserung der Herzfunktion nicht von dem langfristigen Überleben der Transplantate abhängig ist. 48 Stunden nach den Transplantationen lassen sich in beiden Behandlungsgruppen wie erwartet die transplantierten Zellen anhand der Fluoreszenz des Zell-Trackers DiI nachweisen. Zu diesem Zeitpunkt finden sich unter den transplantierten Myoblasten nur wenige apoptotische Zellen, während der Anteil

apoptotischer ES-Kardiomyozyten deutlich höher liegt (Nachweis mittels *terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling* (TUNEL) -Assay). Dennoch sind 28 Tage nach den Zellinjektionen nahezu keine Myoblasten in den Herzen der Versuchstiere mehr nachweisbar, wohingegen sich bei den Tieren, die ES-Kardiomyozyten injiziert bekommen hatten, anhand der DiI-Fluoreszenz stets eindeutig Transplantat-Inseln auffinden lassen. Allerdings sind diese ‚neuen Kardiomyozyten' durch Narbengewebe vom Rest-Myokard separiert, so dass die Möglichkeit einer funktionellen Kopplung nicht anzunehmen ist.

Insgesamt sprechen die Befunde somit erneut für die Hypothese, dass die günstigen Effekte der Zelltransplantationen überwiegend durch parakrine Mechanismen vermittelt werden. Um diese Vermutung weiter zu analysieren, wurde zum einen untersucht, welche Zytokine und Wachstumsfaktoren von den für die Transplantationen eingesetzten Zellarten sezerniert werden (Antikörper-Array). Des Weiteren erfolgte zu unterschiedlichen Zeitpunkten nach den Zellinjektionen die Quantifikation verschiedener Zytokine in den Empfängerherzen mittels Western blot. Im Ergebnis kann belegt werden, dass sowohl die Myoblasten der Skelettmuskulatur als auch die ES-Kardiomyozyten zum Zeitpunkt der Transplantation ein breites Spektrum von Signalstoffen sezernieren. Auch noch 28 Tage nach den Zellinjektionen findet sich in den Empfängerherzen ein gegenüber infarzierten Kontrollherzen deutlich differentes Expressionsprofil kardioaktiver Zytokine. So ist beispielsweise die Konzentration von Interleukin-6 und Oncostatin nach beiden Zelltransplantationen signifikant erhöht, wohingegen sich nur nach Gabe der ES-Kardiomyozyten auch eine vermehrte Konzentration von Tumornekrosefaktor-β nachweisen lässt. In ergänzenden Zellkulturexperimenten kann gezeigt werden, dass die gefundenen Zytokine auch tatsächlich eine funktionelle Relevanz besitzen, da sie zu einer Beeinflussung des Metablismus der Kardiomyozyten führen und die Viabilität von Herzmuskelzellen beeinflussen.

Anlage zu 2.2.:

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Cellular Cardiomyoplasty: Improvement of Left Ventricular Function Correlates with the Release of Cardioactive Cytokines

HENNING EBELT,[a,b] MIRCO JUNGBLUT,[b] YING ZHANG,[a] THOMAS KUBIN,[c] SAWA KOSTIN,[c] ANTJE TECHNAU,[b] SVETLANA OUSTANINA,[b,c] SYLVIA NIEBRÜGGE,[d] JÜRGEN LEHMANN,[d] KARL WERDAN,[a] THOMAS BRAUN[b,c]

[a]Department of Medicine III and [b]Institute of Physiological Chemistry, Martin Luther University, Halle, Germany; [c]Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany; [d]Institute for Cell Culture Technology, University of Bielefeld, Bielefeld, Germany

Key Words. Infarction • Stem cells • Myocytes • Cytokines

ABSTRACT

A growing number of studies are reporting beneficial effects of the transplantation of alleged cardiac stem cells into diseased hearts after myocardial infarction. However, the mechanisms by which transplanted cells might help to promote repair of cardiac tissue are not understood and might involve processes different from the differentiation of transplanted cells into cardiomyocytes. We have compared the effects exerted by skeletal myoblasts (which are not able to form new cardiomyocytes) and ESC-derived cardiomyocytes after implantation into infarcted mouse hearts by echocardiographic follow-up and histological analysis and related these effects to the release of cardioactive cytokines. We found that both cell types led to a long-lasting improvement of left ventricle function and to an improvement of tissue architecture. Since no relevant amounts of myoblast-derived cells were present in infarcted hearts 28 days after transplantation, we investigated the release of cytokines from implanted cells both before and after transplantation into infarcted hearts. ESC-derived cardiomyocytes and myoblasts secreted substantial amounts of interleukin (IL)-1α, IL-6, tumor necrosis factor-β, and oncostatin M, which strongly supported survival and protein synthesis of cultured cardiomyocytes. We postulate that the beneficial effects of the transplantation of myoblasts and cardiomyocytes on heart function and morphology only partially (if at all) depend on the integration of transplanted cells into the myocardium but do depend on the release of a complex blend of cardioactive cytokines. STEM CELLS *2007;25:236–244*

INTRODUCTION

Myocardial infarction and the resulting loss of contractile heart muscle is a frequent cause of heart failure and death. Mammalian cardiomyocytes are terminally differentiated cells, which lose their proliferative potential shortly after birth, so that the adult heart is unable to replace dead or damaged cardiomyocytes after myocardial injury [1]. At present, it is not clear whether putative native cardiac precursor cells, which have been identified recently, contribute to a significant degree to the replacement of myocardial cells in particular since they are present only at very low numbers in the adult heart [2]. Therefore, the transplantation of different types of progenitor or stem cells into diseased hearts remains a promising concept to treat heart insufficiency and myocardial infarction (MI).

In recent years, not only experimental studies in animals but also several clinical trials have shown beneficial effects of cell transplantations to improve cardiac repair after MI [3, 4]. However, the mechanisms that cause these effects are still enigmatic. In particular, the acclaimed formation of new cardiomyocytes from noncardiac stem cells [5] has been questioned and is a matter of fierce debate [6]. Alternatively, it might be envisaged that implanted progenitor cells stimulate vasculogenesis or angiogenesis, inhibit apoptosis, activate endogenous repair mechanisms, or otherwise support cells of the diseased host tissue. Some of these effects might be mediated by the release of cytokines and growth factors either from implanted cells or by the reacting host tissue, thereby establishing autocrine and/or paracrine loops. Cytokines have been demonstrated to affect myocardial functions in a complex manner; these functions are strongly context dependent. The relative contribution of activated signaling pathways and the physiological milieu might evoke either stimulatory or depressant contractile responses [7]. Tumor necrosis factor-α (TNF-α), for example, confers resistance to hypoxic injury of adult mammalian cardiac myocytes [8], cardiotrophin-1 shows a protective effect against nonischemic cell death of cardiomyocytes [9], and insulin-like growth factor (IGF)-I has well-known anabolic effects on skeletal muscle cells and acts as a survival factor for the myocardium and other tissues [10]. The effect of other cytokines, such as oncostatin M (OSM), interleukin (IL)-1, and IL-6, on adult cardiomyocytes, however, has been studied only poorly so far.

In our experiments, we compared effects of the transplantation of two different cell types (myoblasts and ESC-derived cardiomyocytes) into infarcted mouse hearts with respect to the improvement of cardiac function and morphology. These two cell types differ fundamentally in their potential to form new cardiomyocytes. Skeletal myoblasts have been used as cardiac transplants for a long time [11] but are unable to form new cardiomyocytes or to integrate functionally into the host myocardium. ESC-derived cardiomyocytes [12], on the other hand, might be considered "ideal transplants," since they have the potential to integrate into the host myocardium. Surprisingly, we found that both cell types improve cardiac function and morphology to approximately the same degree

Correspondence: Thomas Braun, M.D., Ph.D., Max Planck Institute for Heart and Lung Research, 61231 Bad Nauheim, Germany. Telephone: 49-6032-705-401; Fax: 49-6032-705-419; e-mail: thomas.braun@kerckhoff.mpg.de Received June 20, 2006; accepted for publication September 8, 2006; first published online in STEM CELLS *EXPRESS* September 14, 2006. 1066-5099/2007/$20.00/0 doi: 10.1634/stemcells.2006-0374

Table 1. Morphometric and echocardiographic measurements of hearts 28 days after cell transplantations (more details are given in supplemental online Table A)

Parameter	Sham	PBS	Myoblasts	ES-derived CMs
Body weight (g)	38.5 ± 0.9	35.5 ± 1.6	34.4 ± 0.7	36.8 ± 0.8
Heart weight (mg)	270 ± 12	369 ± 22	313 ± 15[a]	311 ± 17[a]
Heart weight/BW (mg/g)	7.1 ± 0.4	10.6 ± 0.9	9.0 ± 0.4	8.4 ± 0.4[a]
Liver weight (mg)	2066 ± 87	1768 ± 113	2061 ± 118	2032 ± 97
Lung weight (mg)	461 ± 37	576 ± 25	494 ± 36	536 ± 51
Heart rate (min^{-1})	529 ± 17	534 ± 11	497 ± 13	531 ± 18
LVIDD (mm)	4.3 ± 0.1	6.2 ± 0.1	5.8 ± 0.1[a]	5.9 ± 0.1[a]
LVIDS (mm)	2.6 ± 0.1	5.6 ± 0.1	4.7 ± 0.2[a]	4.9 ± 0.2[a]
FS	0.40 ± 0.02	0.10 ± 0.01	0.19 ± 0.02[a]	0.18 ± 0.02[a]
FAC (%)	61 ± 1	22 ± 2	35 ± 3[a]	35 ± 3[a]

[a] $p < .05$ in comparison to PBS treatment.
Abbreviations: BW, body weight; CM, cardiomyocyte; ES, embryonic stem; FAC, fractional area change; FS, fractional shortening; LVIDD, left ventricular internal diameter in diastole; LVIDS, left ventricular internal diameter in systole; PBS, phosphate-buffered saline.

after experimental MI in mice, although myoblasts were not present in hearts 28 days after transplantation. Since both cell types secrete an overlapping set of cytokines that inhibit apoptosis and stimulate cardiomyocyte protein synthesis and cell growth in vitro, we suggest that the release of cardioactive substances might explain the beneficial effects of noncardiac stem cells on diseased hearts.

METHODS

Myocardial Infarction, Cell Transplantations, and Determination of Infarct Size

The investigation conforms to the NIH Guide for the Care and Use of Laboratory Animals (NIH publication number 85-23, revised 1996). Adult female CD-1 mice underwent left anterior descending coronary artery (LAD) ligation as described [13]. Briefly, mice were anesthetized with isoflurane and ventilated mechanically, left sided thoracotomy was performed in the fourth intercostal region, and the LAD was ligated proximal to its main bifurcation. In sham-operated animals, thoracotomy was performed without LAD ligation. Seven days after MI, before transplantation of different cell preparations, all animals were subjected to echocardiography to determine the size of the infarcted myocardium. Animals that turned out to have small MIs were excluded from the study (5 of 42 mice). All remaining mice received injections of 3×10^5 ESC-derived cardiomyocytes or myoblasts in phosphate-buffered saline (PBS) or PBS alone directly into infarcted hearts using a Hamilton microsyringe. Injections were targeted to the border zone of the infarcted region, which was readily visible due to its pale color. At the end of the experiments, a retrograde perfusion of the heart with 4% paraformaldehyde in diastole was performed to reliably assess histomorphological endpoints. Four sections dividing the space between apex and mitral valve into five equal parts were selected for determination of infarct size as described [14]. The infarct size was calculated based on the following equation: Infarct size (%) = (circumference of infarcted left ventricle [LV])/(total circumference of LV) × 100.

Histological Analysis of Cardiac Remodeling and Immunohistochemical Stainings

Wall thickness was determined on the same four slides per heart that were used for infarct size measurements (trichrome staining). Myocyte cross-sectional area (MCSA) and interstitial collagen fraction were measured by fluorescence microscopy using either fluorescein-conjugated peanut agglutinin or rhodamine-labeled lectin I from *Griffonia simplicifolia*, respectively, as described [15]. To monitor myofibrillogenesis, cells on chamber slides were fixed with 4% formaldehyde and stained for F-actin with rhodamine-phalloidin and a monoclonal antibody against sarcomeric α-actinin (clone EA-53; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) as described previously [16].

Echocardiography

Echocardiography in spontaneously breathing mice was performed under anesthesia with 1.5% isoflurane as described using a 10-MHz Toshiba ultrasound probe (Toshiba, Neuss, Germany, http://www.toshiba-medical.de) [17]. Two-dimensional images and M-mode tracings were recorded from the parasternal short axis view at midpapillary level to determine left ventricular wall thickness (e.g., thickness of left ventricular posterior wall at diastole) and for quantification of left ventricular dimensions (left ventricular internal diameter in diastole [LVIDD] and left ventricular internal diameter in systole [LVIDS]). Left ventricular pump function was analyzed by independent calculation of both fractional shortening (FS) (one-dimensional estimation of LV function) and fractional area change (two-dimensional estimation of LV function).

Cell Culture

Skeletal myoblasts from CD-1 mice were prepared as described previously [18]. ESC-derived cardiomyocytes were obtained from embryonic stem cells following published procedures [12]. Briefly, genetically engineered embryonic stem cells carrying a fusion gene consisting of the α-major histocompatibility complex-promoter driving the aminoglycoside phosphotransferase (neomycin resistance) were aggregated into EBs, inoculated into stirred suspension cultures, and differentiated for 9 days before selection of cardiomyocytes by the addition of G418. Throughout the culture period, EBs and viable cell numbers were measured. In addition, flow cytometric analysis was performed to monitor sarcomeric myosin (a marker for cardiomyocytes) expression. Based on myosin heavy chain (MyHC) staining, the purity of cardiomyocytes was >99% before transplantation. Directly before transplantation, both myoblasts and ESC-derived cardiomyocytes were labeled with the fluorochrome DiI according to instructions of the manufacturer. Viability of the cells was determined in parallel by propidium iodide (fluorescence-activated cell sorting; supplemental online Fig. A) and trypan blue exclusion [19]. Ventricular cardiac myocytes of 2–3-month-old male Sprague-Dawley rats were isolated, cultured, and labeled as described [16].

Detection of Cytokines

Cytokines secreted by the cells used for transplantations were determined in cell culture supernatants using the mouse cytokine array 3.1 (RayBiotech, Norcross, GA, http://www.raybiotech.com) according to the manufacturer's instructions. Expression levels were normalized to total protein concentrations in supernatants and to the intensity of reference spots included on each array. All supernatants were compared with culture medium to exclude contaminations of serum additives. After transplantation of cells into infarcted hearts, expression of cytokines were detected by Western blot analysis. Tissue samples were homogenized by sonication and then heated for 1 minute at 99°C. Twenty μg of protein samples were resolved on 4%–12% SDS polyacrylamide gradient gel (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) and blotted onto nitrocellulose. Bound antibodies were visualized using Quentix and the Femto detection kit (Perbio Science, Bonn, Germany, http://www.

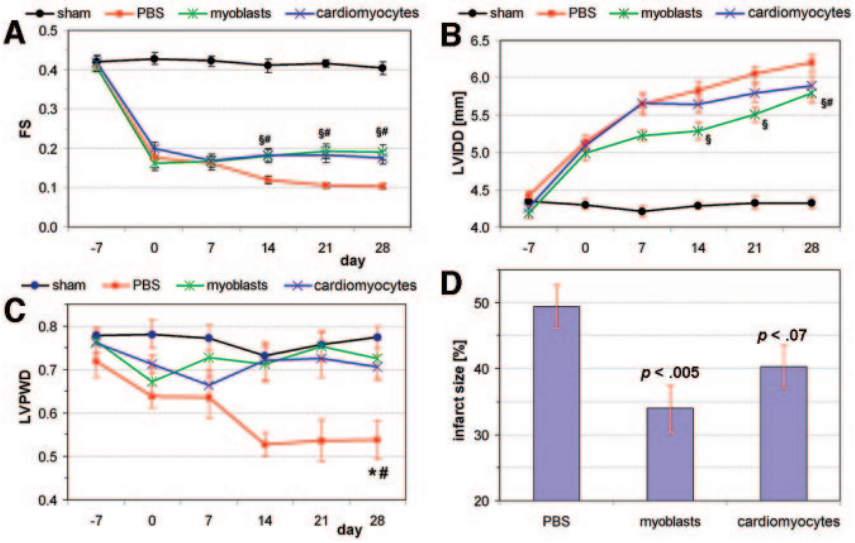

Figure 1. Infarct expansion is restricted by myoblasts or ESC-derived cardiomyocytes. Echocardiographic measurements of FS **(A)** and LVIDD **(B)**. Implantation of myoblasts or cardiomyocytes both improved cardiac function compared with controls. §, $p < .05$ PBS versus myoblasts; #, $p < .05$ PBS versus cardiomyocytes. See text and Table 1 for further details. **(C):** Echocardiographic measurement of the end diastolic thickness of the LVPWD. Seven days after myocardial infarction, only a moderate reduction of LVPWD in all groups was visible, since the posterior wall is not directly affected by left anterior descending coronary artery ligation. In PBS-treated animals, thinning of the LVPWD progressed with time. A reduced thinning was seen in animals that received ESC-derived cardiomyocytes or myoblast transplantation. *, $p < .05$ myoblasts versus PBS; #, $p < .05$ cardiomyocytes versus PBS. **(D):** Histological quantification of the infarct size 28 days after transplantations revealed a restriction of infarct expansion after transplantation of myoblasts or ESC-derived cardiomyocytes in comparison with PBS-treated animals. p values relate to a comparison of the treatment groups versus PBS. Abbreviations: FS, fractional shortening; LVIDD, left ventricular end diastolic diameter; LVPWD, left ventricular posterior wall at diastole; PBS, phosphate-buffered saline.

perbio.com) according to instructions of the manufacturer. Antibodies against cytokines were all purchased from R&D Systems (Minneapolis, http://www.rndsystems.com).

RESULTS

Transplantation of Skeletal Myoblasts or Embryonic Stem Cell-Derived Cardiomyocytes Improves Cardiac Function After MI in Mice

To compare putative beneficial effects of two different cell types (myoblasts and cardiomyocytes) on diseased hearts, we generated a cohort of CD-1 mice ($n = 47$) with a ligation of the LAD. This procedure induced a large MI that led to a severe impairment of LV function (Table 1). Although sham-operated animals showed a stable LV geometry and function, animals with LAD ligation displayed a significant dilation of the LV (LVIDD and LVIDS), as well as a reduced systolic function as measured by FS (echocardiographic one-dimensional estimation of left ventricular pump function) and fractional area change (echocardiographic two-dimensional estimation of left ventricular pump function) 7 days after LAD ligation. Animals that received a mock treatment (injection of PBS) were characterized by a progressive enlargement of the LV and a reduction of systolic function. In most animals, we observed maximal dilatation and minimal systolic pump function 35 days after MI.

Transplantation of both skeletal myoblasts and ESC-derived cardiomyocytes significantly improved cardiac function (Fig. 1A; Table 1). Although both treatments failed to completely restore cardiac function, progression of chronic heart failure was slowed down leading to a significantly reduced LV dilation and improved LV function. Beneficial effects of skeletal myoblasts were already evident 7 days after transplantation, as indicated by a reduction of LV dilation (Fig. 1B, LVIDD). The effects of ESC-derived cardiomyocytes became apparent approximately 1 week later, 14 days after transplantation. In contrast to myoblasts, cardiomyocytes primarily improved the contractility of the heart (as measured by LVIDS and FS) rather than LV dilation (supplemental online Table A). Despite these initial differences, the echocardiographic parameters in both groups converged over time and were similar with respect to LV function 28 days after transplantation.

Effects of Cell Transplantations on Infarct Expansion

It seemed possible that the improvement of LV function observed after myoblast and cardiomyocyte transplantation was caused by a reduction of infarct progression. In agreement with this hypothesis we found that hearts that had received myoblasts or cardiomyocytes showed a significantly reduced infarct expansion in comparison with PBS-treated animals (Fig. 1D). PBS-treated hearts were characterized by a progressive thinning of the left ventricular posterior wall at midpapillary level in echocardiography, although this region of the heart was not directly affected by LAD ligation (Fig. 1C). Histological examinations performed at the end of the experiment 28 days after transplantation revealed that a significant larger part of the LV of PBS-treated animals was occupied by scar tissue than hearts that had received myoblasts or cardiomyocytes.

Transplantation of Myoblasts and ESC-Derived Cardiomyocytes Led to Different Effects on Long-Term Cardiac Remodeling but Both Mitigated Ventricular Wall Thinning After MI

Myocyte hypertrophy and interstitial collagen deposition are hallmarks of chronic remodeling processes after MI. We therefore analyzed whether and to what extent transplantation of myoblasts and ESC-derived cardiomyocytes affected these parameters. As shown in Figure 2A, transplantation of either myoblasts or cardiomyocytes reduced the hypertrophic response of the remote myocardium in the interventricular septum (indi-

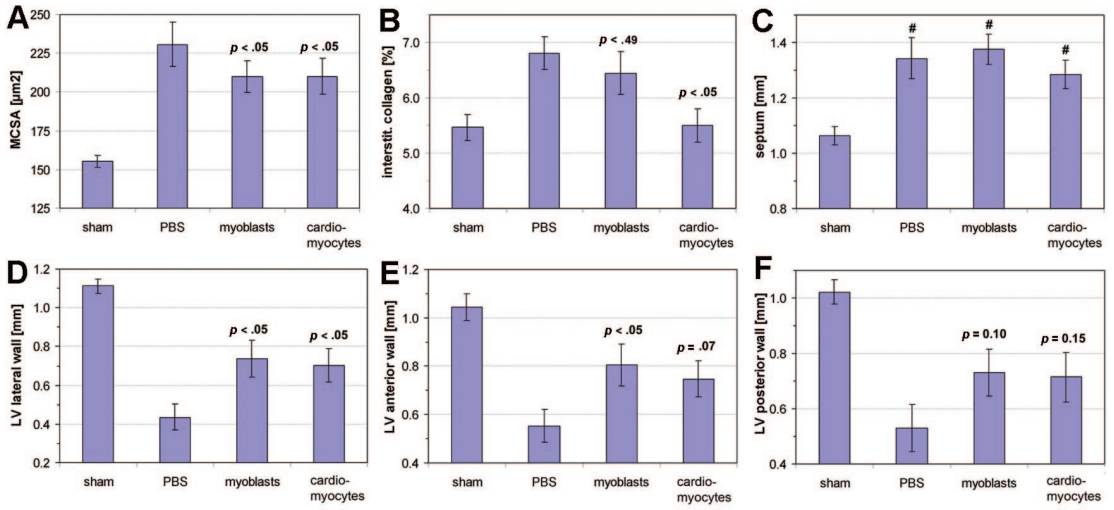

Figure 2. Transplantation of myoblasts or ESC-derived cardiomyocytes limits adverse cardiac remodeling. Effects of cell transplantations on remodeling of the remote myocardium in the interventricular septum were determined by the extent of MCSA **(A)** and interstit. collagen deposition **(B)**. *p* values relate to a comparison of the treatment groups versus PBS. Thickness of the interventricular septum **(C)** and of free left ventricular walls **(D–F)** was analyzed histologically 28 days after cell transplantations. Transplantation of myoblasts or ESC-derived cardiomyocytes constrained the reduction of the thickness of left ventricular free walls after infarction. #, $p < .05$ in comparison with noninfarcted hearts (sham). *p* values correspond to the comparative treatment with PBS. Abbreviations: interstit., interstitial; LV, left ventricle; MCSA, myocyte cross-sectional area; PBS, phosphate-buffered saline.

cated by a reduced MCSA). In addition, transplantation of cardiomyocytes decreased the reactive collagen deposition in the interventricular septum (Fig. 2B). Surprisingly, myoblast engraftment did not reduce interstitial fibrosis after MI, although the infarct size in myoblast-treated animals was diminished significantly in comparison with the PBS control group.

It is well known that due to LaPlace's law, thinning of the left ventricular walls after MI enhances adverse cardiac remodeling and LV dilatation. We therefore determined the thickness of the left ventricular free walls and the interventricular septum at the end of the experiment to analyze a potential contribution of ventricular wall thinning to adverse cardiac remodeling and LV dilatation. As expected, all animals that underwent LAD ligation and MI showed a hypertrophy of the interventricular septum (IVS). Interestingly, animals that received a PBS injection displayed a significantly increased thinning of the free left ventricular walls, whereas animals that were treated with either myoblasts or cardiomyocytes showed a only a moderate reduction of the diameter of the free left ventricular walls (Fig. 2C–2F). It seems likely that the reduced thinning of the free left ventricular walls accounts, at least in part, for the reduced LV dilatation after myoblast or cardiomyocyte treatment.

Improvement of Cardiac Function After Cardiomyoplasty Did Not Depend on the Fate of Transplanted Cells

In principle, it might be possible that the improvement of cardiac function and the suppression of adverse cardiac remodeling in animals that have received cellular transplants is caused by an integration of contractile cells into the remaining myocardium (coupled or uncoupled), which results in improved contraction force. Alternatively, it might be envisioned that transplanted cells nourish or support the diseased myocardium in a paracrine manner that is not dependent on the contractile status of transplanted cells. To distinguish between these two possibilities, we followed the fate of transplanted cell after MI using DiI labeling. We chose DiI labeling since the expression of enhanced green fluorescent protein impairs contractile functions of muscle cells [20] and

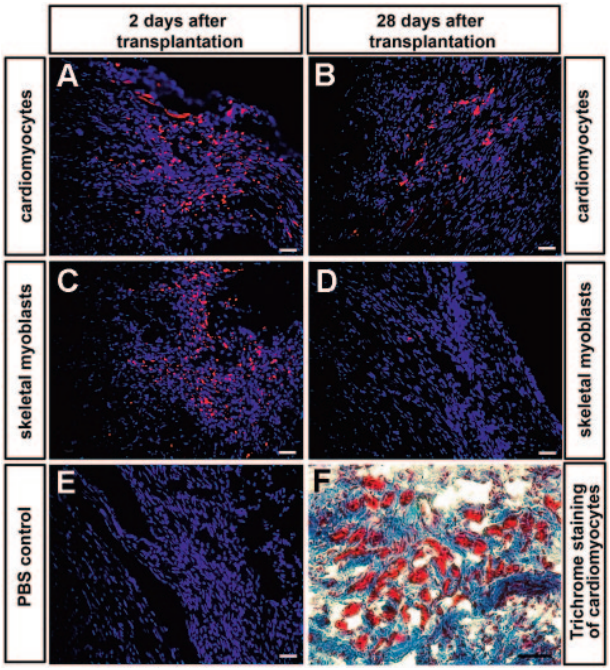

Figure 3. ESC-derived cardiomyocytes, but not myoblasts, are present in host hearts 28 days after transplantation. The fate of DiI-labeled transplanted ESC-derived cardiomyocytes **(A, C, F)** and myoblasts **(B, D)** in recipient hearts was analyzed 2 days **(A, C, E)** and 28 days **(B, D, F)** after transplantation. **(A–E):** Fluorescence microscopic pictures: red, DiI (cell tracker); blue, nuclear staining (Hoechst 33258). After transplantation of ESC-derived cardiomyocytes, DiI-labeled cells were easily located 2 days **(A)** and 28 days **(B)** after engraftment. Skeletal myoblasts were present only after 2 days **(C)**. Virtually no DiI-positive myoblasts were found 28 days after transplantation **(D)**. No DiI-labeled cells were found in PBS-treated animals **(E)**. Islets of ESC-derived cardiomyocytes 28 days after transplantation embedded in fibrous tissue (trichrome staining) **(F)**. Scale bars = 75 µm. Abbreviation: PBS, phosphate-buffered saline.

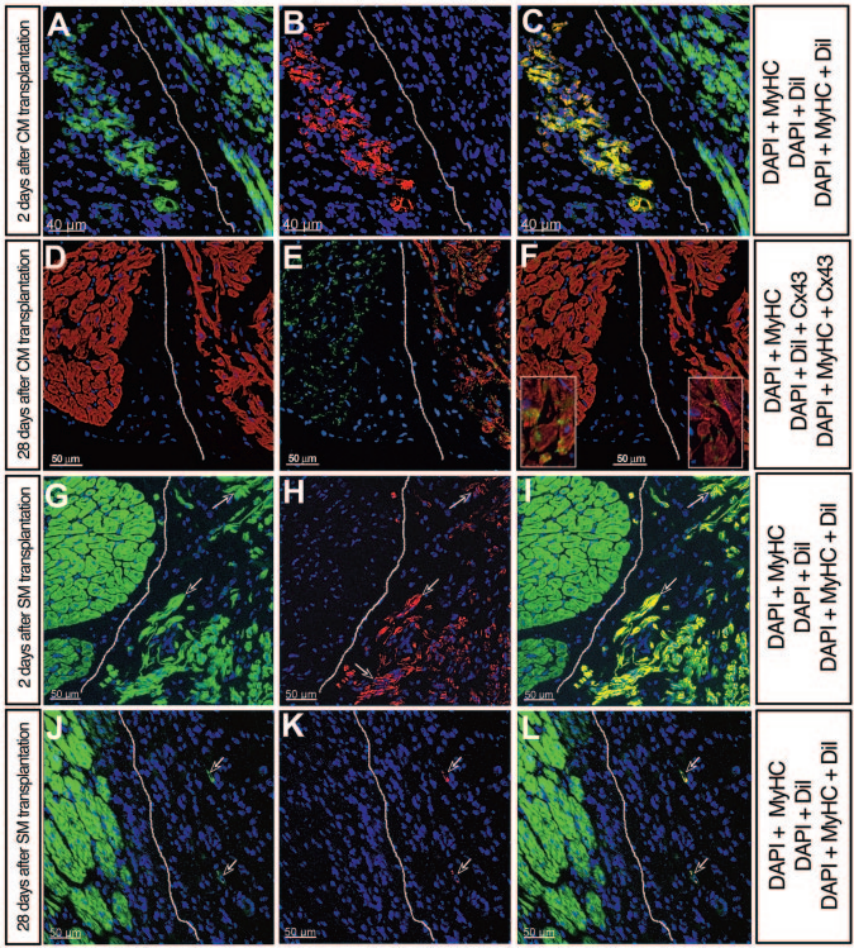

Figure 4. Transplanted ESC-derived CMs and primary skeletal muscle cells are separated by scar tissue from the remaining myocardium. Shown is fluorescence labeling for sarcomeric myosin (MF20 antibody [green in **(A, C, G, H, J, L)**; red in **(D, F)**], connexin 43 [green staining in **(E, F)**], DiI [red staining in **(B, E, H, K)**], and DAPI [blue nuclear staining in **(A–L)**]). White lines in **(A–L)** indicate the border zone of the infarcted myocardium. All transplanted ESC-derived CMs stained positive for MyHC but were separated from the remaining myocardium by scar tissue **(A–C)**. ESC-derived CMs expressed connexin 43 suggesting coupling to each other but not to the remaining myocardium **(D–E)**. Insets in **(F)** reveal expression of connexin 43 in healthy myocardium (left) and in transplanted ESC-derived CMs (right). Two days after transplantation, large numbers of primary skeletal muscle cells, which expressed MyHC, were present in the infarcted hearts **(G–I)**. After 28 days, only a very few skeletal muscle-derived cells remained in the infarcted area (arrows in **[J–L]**). Note the stable labeling of transplanted cells by DiI without transfer to neighboring cells, which are also negative for MyHC. Abbreviations: CM, cardiomyocyte; DAPI, 4,6-diamidino-2-phenylindole; MyHC, myosin heavy chain; SM, skeletal myoblast.

might even lead to cardiomyopathy [21]. Twenty-eight days after transplantation, we detected several regions in the border zone of the MI, which contained islets of new muscle cells derived from cardiomyocytes, as indicated by DiI fluorescence (Fig. 3). "New" cardiomyocytes were isolated from the host myocardium by fibrous tissue, making it unlikely that the majority of transplanted cardiomyocytes became part of the functional myocardial syncytium (Figs. 3F, 4A–4C). High-resolution laser scan microscopy revealed that all transplanted cells expressed MyHC (Fig. 4A–4C). Moreover, we found that transplanted cardiomyocytes expressed connexin 43, the major gap junction protein of cardiomyocytes. Although the expression pattern of connexin 43 was not as regular as in the endogenous myocardium, we concluded that the transplanted cardiomyocytes were able to couple functionally to each other (Fig. 4A–4F). We excluded, however, a coupling of transplanted cells with the remaining host myocardium, since all transplanted cardiomyocytes were well separated from endogenous cardiomyocytes by fibrous tissue. None of the animals that received differentiated embryonic stem (ES)-derived cardiomyocytes showed any signs of tumor formation.

Surprisingly, we only very rarely detected new muscle cells in hearts that had received skeletal myoblasts 28 days after transplantation (Figs. 3, 4J–4L). Although some hearts contained some DiI-labeled skeletal muscle cells, the number of surviving transplanted myoblast-derived cells was low in the vast majority of animals (Figs. 3, 4J–4L). To answer the question whether host animals initially received a sufficient number of viable myoblasts, which later disappeared from the host tissue during cardiac remodeling, we analyzed hearts at earlier time points after transplantation. Forty-eight hours after transplantation, the presence of myoblasts and ESC-derived cardiomyocytes were easily discernable by the DiI label (Figs. 3, 4G–4L). High-resolution confocal microscopy revealed that most skeletal muscle-derived cells expressed MyHC (Fig. 4G–4I) and differentiated in situ to myocytes. The number of contaminating fibroblasts in recipient hearts was very low (Fig. 4I), although we did not reach the purity of ESC-derived cardiomyocytes, which were derived by genetic selection.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining revealed only a small number of apoptotic myoblasts (Fig. 5E–5H) but a considerably higher number of apoptotic cells derived from ESC-derived cardiomyocytes (Fig. 5A–5D). Our findings suggest that most myoblasts initially engrafted into the host myocardium without signs of massive apoptosis but later disappeared from the tissue, probably by nonapoptotic cell death.

Myoblasts and ESC-Derived Cardiomyocytes Secrete a Complex Mixture of Growth Factors and Cytokines

Since the transplantation of myoblasts led to a significant improvement of cardiac function after MI despite the absence of stable long-term grafts, we reasoned that myoblasts (and probably also cardiomyocytes) might activate endogenous signal cascades within the host tissue most likely by the release of cardioactive molecules. We therefore analyzed various cytokines and growth factors secreted by myoblasts and cardiomyocytes at the time of transplan-

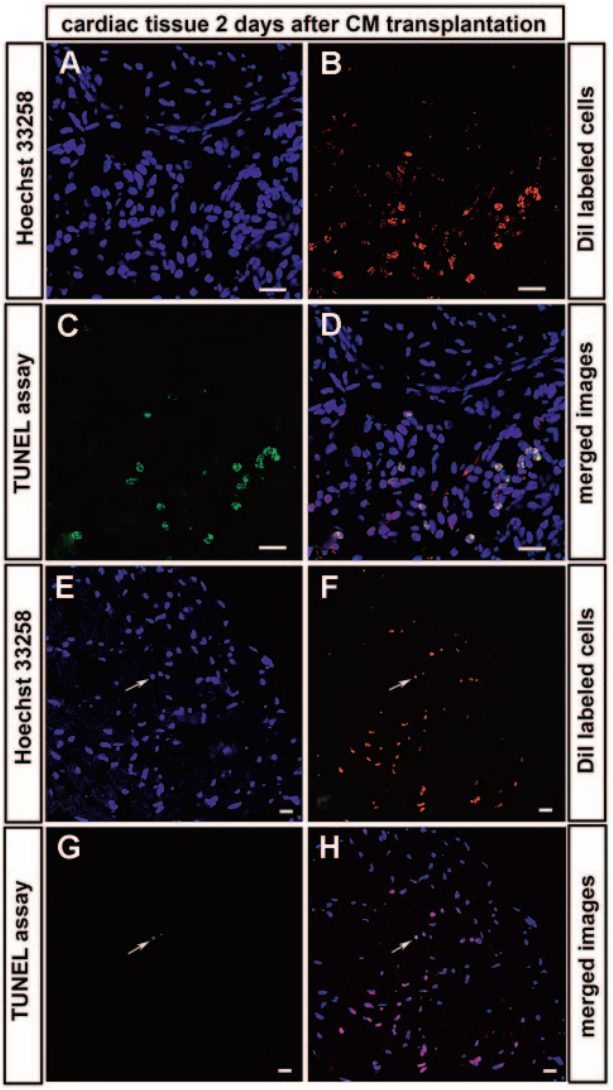

Figure 5. Skeletal myoblasts and ESC-derived CMs show a different degree of apoptosis 2 days after transplantation into infarcted hearts. Apoptotic cells were detected using the TUNEL assay 48 hours after transplantation of ESC-derived CMs **(A–D)** and primary skeletal muscle myoblasts **(E–H)**. Blue **(A, E)**, nuclear staining (Hoechst 33258); red **(B, F)**, DiI (cell tracker); green **(C, G)**, TUNEL-positive cells. **(D, H):** merged images. Only a very few transplanted skeletal muscle cells (red staining in **[F]**) were TUNEL-positive (arrow in **[G]**), whereas many more transplanted CMs underwent apoptosis (green staining in **[C]**). Scale bars = 25 μm. Abbreviations: CM, cardiomyocyte; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

tation using a semiquantitative Western blot assay. As shown in Table 2, both skeletal myoblasts and ESC-derived cardiomyocytes released a complex, partially overlapping blend of growth factors and leukocyte-attracting cytokines. Cardiomyocytes secreted high amounts of proinflammatory cytokines, such as GRO, IL-6, MCPs, and TNF-α, whereas myoblasts released mitogen-inducible gene, chemokine CXC motif ligand 9, stem cell factor, and IGF-I, which are known to promote cell motility and stimulate cardiomyocyte growth. Both types of cells produced vascular endothelial growth factor and platelet-derived growth factor-B, which stimulate angiogenesis; proinflammatory cytokines, such as IL-1α, IL-4, TNF-β, and macrophage colony-stimulating factor; and stimulants of cell motility and interstitial matrix remodeling (OSM and epidermal growth factor) (Table 2). It seems clear that such a complex

Table 2. Secretion of growth factors and cytokines by skeletal myoblasts and ESC-derived cardiomyocytes (semiquantitative assay)

Cytokine	ES-derived CMs	Myoblasts
GM-CSF	+	0
GRO	++	0
IL-1α	++	++
IL-1β	+	0
IL-2	+	0
IL-3	+	0
IL-4	+	++
IL-5	+	0
IL-6	++	0
IL-7	+	0
IL-8	+	0
Il-10	+	0
IL-12	+	0
IFN-γ	+	0
MCP-1	++	0
MCP-2	++	0
MCP-3	+	0
MCSF	+	+
MIG	0	++
MIP-1δ	+	0
RANTES	0	+
SCF	0	+
SDF-1	+	0
TARC	+	0
TGF-β 1	+	0
TNF-α	++	0
TNF-β	++	0
EGF	+	+
IGF-1	0	++
Ang	+	+
OSM	++	+
TPO	+	0
VEGF	+	++
PDGF-B	+	+
Leptin	+	++

Cell culture supernatants were analyzed using the mouse cytokine array 3.1. (RayTest) and normalized to tissue culture medium containing all serum additives. 0, not detected; +, weak signal; ++, strong signal. Abbreviations: CM, cardiomyocyte; EGF, epidermal growth factor; ES, embryonic stem; GM-CSF, granulocyte macrophage-colony-stimulating factor; GRO, growth-regulated protein; IFN, interferon; IGF, insulin-like growth factor; IL, interleukin; M16, mitogen-induced gene; MCP, monocyte chemotactic protein; MCSF, macrophage colony-stimulating factor; OSM, oncostatin M; PDGF, platelet-derived growth factor; RANTES, regulated on activation normal T cell expressed and secreted; TGF, transforming growth factor; SCF, stem cell factor; SDF, stromal-derived factor; TARC, thymus- and activation-regulated chemokine; TNF, tumor necrosis factor; TPO, thrombopoietin; VEGF, vascular endothelial growth factor.

composition of cytokines and growth factors will have profound effects on cardiomyocyte survival, remodeling of the myocardium, and cardiac function. To explore whether transplantation of myoblasts and ESC-derived cardiomyocytes resulted in a significant increase of cytokine levels within infarcted hearts in vivo, we performed a Western blot analysis. Extracts were prepared from the infarct area of hearts 2 or 21 days after injection of myoblasts, ESC-derived cardiomyocytes, and PBS. Hearts that received a PBS injection were devoid of IL-6, OSM, or TNF-β expression both 2 and 21 days after injection (Fig. 6). In contrast, transplantation of cardiomyocytes resulted in a robust presence of OSM, IL-6, and TNF-β 2 days after the injection. We observed a fading of IL-6 and TNF-β signals 21 days after injection, whereas the concentration of OSM remained stable for up to 3 weeks (Fig. 6). Infarcted hearts that received myoblasts did contain significant amounts of IL-6 (which declined after 21 days) and OSM but lacked expression of TNF-β. In addition, we found expression of IGF-I in infarcted hearts that received cardiomyocytes and myoblasts but also in

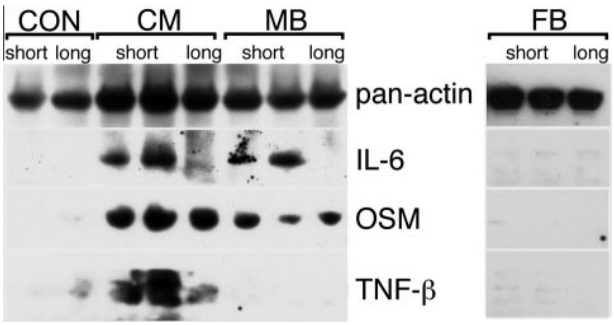

Figure 6. Skeletal myoblasts and ESC-derived CMs but not primary fibroblasts release a mixture of different cytokines after transplantation into infarcted hearts. Western blot analysis of extracts prepared from the infarct area of hearts 2 (short) or 21 days (long) after injection of phosphate-buffered saline (PBS) (CON), ESC-derived CMs, MBs, and primary FBs. Left: CON hearts did not express IL-6, OSM, or TNF-β. CM transplantation resulted in a robust presence of OSM, IL-6, and TNF-β 2 days after injection. The expression of IL-6 and TNF-β declined after 21 days. MB transplantation led to an expression of IL-6 and OSM but not of TNF-β. Staining for pan-actin was used to assure equal loading. Right: Transplantation of FBs did not result in an expression of OSM, IL-6, or TNF-β hearts 2 days (short) or 21 days (long) after injection and was undistinguishable from PBS treatment. The position of OSM (and all other cytokines analyzed) within the gel matched the expected molecular weight for each individual molecule. Abbreviations: CM, cardiomyocyte; CON, control; FB, fibroblast; IL, interleukin; MB, myoblast; OSM, oncostatin M; TNF, tumor necrosis factor.

mock-transplanted infarcted and healthy control hearts (data not shown). To address the question of whether expression of cytokines in the manipulated myocardium was an unspecific phenomenon resulting from implantation of any cell type, we also used primary fibroblasts for transplantation. Western blot analysis indicated that transplantation of fibroblasts did not result in a significant expression of OSM, IL-6, and TNF-β in hearts 2 days (short) or 21 days (long) after injection (Fig. 6). Because of the low expression level of cytokines, however, which prevented an in situ detection of cytokine expression in transplanted cells, we cannot exclude the possibility that transplantation of cardiomyocytes and skeletal muscle cells but not of fibroblasts triggered cytokine expression in endogenous cardiomyocytes.

Cytokines Improve the Viability of Isolated Cardiomyocytes In Vitro

To further delineate the impact of individual growth factors and cytokines released by ESC-derived cardiomyocytes and myoblasts on cardiomyocytes, we incubated isolated adult rat cardiomyocytes for 5 and 10 days with selected growth factors that were either released by ESC-derived cardiomyocytes (IL-6), myoblasts (IGF-I), or both cell types (OSM, TNF-β, and IL-1α). All growth factors clearly increased the number of surviving cardiomyocytes in culture by approximately 30% after 10 days in culture (Fig. 7; data not shown) compared with nontreated controls. In addition, we observed a considerable induction of the rate of protein synthesis within cardiomyocytes after 5 days, which probably reflected adaptive patterns of protein expression inflicted by the different cytokines used. The strongest effect was elicited by IGF-I (2.1-fold) and OSM (1.8-fold), followed by slightly lower rates (1.5-fold) in TNF-β-, IL-1α-, and Il-6-treated cultures (Fig. 7). Remodeling of myocytes and synthesis of new sarcomers was monitored by staining of cultures for F-actin and sarcomeric α-actinin (Fig. 7). In comparison with control cultures, IGF-I showed the highest density of myofibrils, ranging from the center of the cell down to the end of newly built-up extensions. In contrast, OSM-treated cardiomyocytes were characterized by a substantial cell lengthening and a limited appearance of new cross-striations, which probably reflects reduced myofibrillogenesis. IL-1α and IL-6 induced a phenotype that was similar to but less pronounced than that of OSM-treated myocytes.

DISCUSSION

Transplantation of stem cells seems a promising concept for the treatment of myocardial infarction and heart failure. However, despite encouraging results from first clinical trials that demonstrated beneficial effects of different transplantation procedures [3, 4], the mechanisms that underlie the improvement of cardiac function are still enigmatic. In particular, it has been questioned whether and to what extent transplanted cells might acquire characteristics of functional cardiac muscle cells and improve the contractile force of damaged hearts.

Our experimental set-up was based on the usage of two completely different cell types, which intentionally are either unable to form cardiomyocytes (skeletal myoblasts) or ES-derived cardiomyocytes that are already differentiated along the "correct" cellular lineage and hence do not depend on reprogramming or (trans)differentiation. Our results show that despite the different origin of the cells a similar improvement of cardiac function was achieved, although some significant differences were noted.

Skeletal myoblasts have been proposed to improve heart function after myocardial infarction in several animal models and in humans [11, 22, 23]. However, the exact mechanisms that eventually lead to functional improvements are still under debate. Although initial reports favored the idea of the formation of cardiomyocyte-like cells that might be implemented in the concerted process of systolic contraction [24], recent studies clearly demonstrated that skeletal myoblasts remain functionally isolated from the host myocardium. Only a very few transplanted myoblasts fuse with residing cardiomyocytes and are therefore able to contribute to "synchronized" contractions [25].

As anticipated, we were able to corroborate the positive effects of myoblast transplantations after MI. Both echocardiography and histological analysis revealed an improved heart function after myoblast engraftment. Surprisingly, this effect occurred even in the absence of a reasonable number of stable long-term grafts. Furthermore, we found that transplanted cells (both cardiomyocytes and skeletal muscle cells) were always well separated from the remaining intact myocardium by scar tissue preventing functional coupling of transplanted cells. Engraftment of myoblasts was accompanied by several characteristic changes of organ function and morphology that differed from effects exerted by ESC-derived cardiomyocytes. (a) Improvement of LV function (better: reduction of LV decline) occurred rapidly. Myoblasts seemed to exert their beneficial effects immediately after engraftment, whereas protective effects of the transplantation of cardiomyocytes became apparent after 14 days. Morphologically this effect was reflected by an (early) inhibition of infarct expansion, which might, at least partially, be explained by the release of survival-promoting growth factors and cytokines. Since the number of skeletal muscle cells decreased considerably over time, it seems reasonable to assume that the reduced presence of myoblasts went along with incremental lower concentrations of secreted molecules, further strengthening the argument that the cytokines released from skeletal muscle cells acted at early stages of MI-induced cardiac remodeling. (b) In contrast to ESC-derived cardiomyocytes, only a very few skeletal muscle-derived cells were still present in the host myocardium 4 weeks after transplantation, although a large number of MyHC-positive skeletal muscle cells were detected shortly after transplantation into recipient hearts. The lasting improvement of LV function, despite the disappearance of myoblasts from the host tissue,

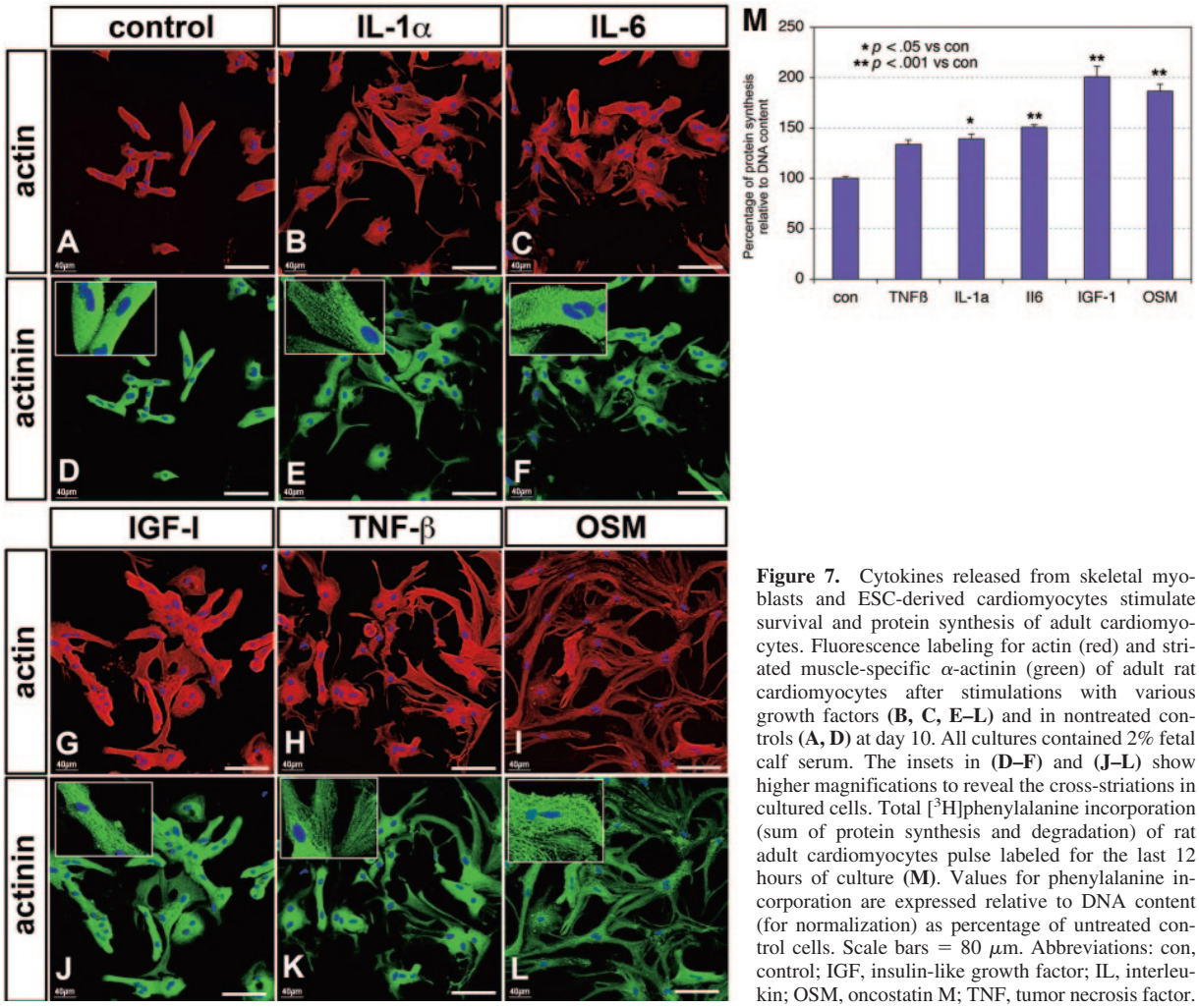

Figure 7. Cytokines released from skeletal myoblasts and ESC-derived cardiomyocytes stimulate survival and protein synthesis of adult cardiomyocytes. Fluorescence labeling for actin (red) and striated muscle-specific α-actinin (green) of adult rat cardiomyocytes after stimulations with various growth factors **(B, C, E–L)** and in nontreated controls **(A, D)** at day 10. All cultures contained 2% fetal calf serum. The insets in **(D–F)** and **(J–L)** show higher magnifications to reveal the cross-striations in cultured cells. Total [^{3}H]phenylalanine incorporation (sum of protein synthesis and degradation) of rat adult cardiomyocytes pulse labeled for the last 12 hours of culture **(M)**. Values for phenylalanine incorporation are expressed relative to DNA content (for normalization) as percentage of untreated control cells. Scale bars = 80 μm. Abbreviations: con, control; IGF, insulin-like growth factor; IL, interleukin; OSM, oncostatin M; TNF, tumor necrosis factor.

clearly indicated that a therapeutic intervention by cardiomyoplasty with myoblasts during the first days or weeks after MI sufficed to prevent adverse cardiac remodeling and to establish long-lasting effects. Despite the virtually complete disappearance of myoblasts from host myocardium, the number of apoptotic cells was higher in animals that received cardiomyocytes, which suggests that myoblasts disappeared by various routes, probably including nonapoptotic modes of cell death. (c) Transplantation of myoblasts led to an enhancement of interstitial fibrosis of the remote myocardium. Obviously, the containment of infarct expansion (and eventually infarct size) by grafted myoblasts, which resulted in a "mechanical advantage," did not result in a reduction of interstitial fibrosis of the IVS. It seems that profibrotic effects exerted by transplanted myoblasts overcame the potential mechanical benefits, which should have alleviated the fibrotic response. The detection of several proinflammatory and profibrotic cytokines secreted by myoblasts nicely supported this notion. It should be pointed out that the profibrotic effects of myoblasts might not necessarily be harmful or unfavorable, since the formation of stable scar tissue in LV walls might inhibit progressive wall thinning and adverse LV remodeling.

Several groups have reported an improvement of cardiac function after transplantation of ESC-derived cardiomyocytes (a recent review is given in [26]). Since ESC-derived cardiomyocytes show most of the characteristics of native primary cardiomyocytes, it was generally assumed that transplanted ESC-derived cardiomyocytes directly contribute to the contractile force of recipient hearts, although the extent of tissue colonization by grafted cells did not always match the improvement of cardiac function. We have also observed a clear improvement of LV function after transplantation of ESC-derived cardiomyocytes into infarcted hearts although new cardiomyocytes were always clearly isolated from the host myocardium by fibrous tissue. Echocardiographic monitoring of transplanted animals indicated that beneficial inputs of predifferentiated cardiomyocytes were delayed in comparison with myoblast treatments but reached a similar level after 28 days. In agreement with this observation, ESC-derived cardiomyocytes seemed less effective in restricting infarct expansion but were more successful in preventing adverse chronic remodeling of the heart, indicated by the deposition of collagen and fibrosis. Our data suggest that at least some effects of ESC-derived cardiomyocytes were achieved by the release of cardioactive growth factors and cytokines.

In vitro IGF-I, OSM, IL-1α, IL6, and TNF-β all improved cardiomyocyte survival. Similar observations had been made for CT-1, IL-6, and TNF-α, which confer cytoprotective effects to adult cardiac myocytes [8, 9], whereas others, such as insulin and IGF-I, stimulate the metabolism of cardiomyocytes [27, 28]. Interestingly, the same proinflammatory cytokines, such as granulocyte colony-stimulating factor [29], TNF-α, and IL-6, which were released by ESC-derived cardiomyocytes and/or myoblasts, have profound effects on LV contractility, although this effect strongly depends on the concentration of cytokines and exposition time of the heart (reviewed in [7]). In addition to an improvement of cardiomyocyte survival, several of the cytokines and growth factors

released by ESC-derived cardiomyocytes and myoblasts do have effects on macrophages and mesenchymal stem cells [30] and modulate the inflammation reaction, interstitial matrix composition, scar formation, and other processes related to cardiac remodeling, which might explain the reduced level of adverse remodeling after cardiac body engraftment.

Taken together, our experiments demonstrate that transplantation of either skeletal myoblasts or ESC-derived cardiomyocytes, most probably by using overlapping paracrine signaling pathways, improves heart function. A careful delineation of the effects of individual cytokines (or certain cytokine combination) on post-MI remodeling might lead to the formulation of growth factor cocktails that do not rely any longer on potentially "hazardous" cells. Such cocktails when applied locally to the diseased myocardium possibly with the help of recently developed nanofibers [31] or other carriers might eventually be implemented into future MI treatment strategies to achieve maximal protection of cardiomyocytes from apoptosis and optimization of scar composition and cardiac remodeling.

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DISCLOSURES

The authors indicate no potential conflicts of interest

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2.3 Etablierung eines *in vivo*-Modells zur gezielten Expression von Fremdgenen in den Herzen neonataler Mäuse

Die bisherigen Untersuchungen hatten gezeigt, dass sich aus adulten Knochenmarksstammzellen nicht in relevantem Umfang funktionell intakte Kardiomyozyten differenzieren lassen und dass im eigenen Herzinfarktmodell die positiven Effekte von Zelltransplantationen in erster Linie auf parakrinen Effekten basieren. In der Folge sollte deshalb stattdessen versucht werden, durch die gezielte Beeinflussung der Genexpression den Wiedereintritt von Herzmuskelzellen in den Zellzyklus auszulösen, um so die verfügbare Anzahl von Kardiomyozyten wieder zu vermehren.

Die Etablierung transgener Tiermodelle stellt ohne Zweifel in der Mehrheit der Fälle die Methode der Wahl dar, um Gene experimentell zu inaktivieren bzw. gezielt zu exprimieren [Hunter et al. 1993]. Die phänotypischen Veränderungen, die aus der veränderten Genexpression resultieren, gestatten in der transgenen Situation detaillierte Rückschlüsse auf die Funktion des jeweiligen Genproduktes und erlauben die Untersuchung von andernfalls nur schwer zugänglichen Interaktionen. Andererseits ist die Generierung transgener Modelle zeit- und kostenintensiv, und gelegentlich ist die intendierte Aktivierung eines Gens zu einem beliebigen Zeitpunkt mittels transgener Verfahren nur schwierig zu realisieren. Die Verwendung viraler Vektoren kann in solchen Situationen eine alternative Möglichkeit darstellen, um die zeitlich exakt planbare Expression eines Gens mit hoher Expressionsstärke sicherzustellen (aktuelle Übersicht in [Lyon et al. 2008]).

Verschiedenen Strategien sind in der Vergangenheit zum Einsatz gekommen, um Einfluss auf die genetische Information des postnatalen Herzmuskels zu nehmen. Rekombinante Adenoviren sind die am häufigsten verwendeten Vektoren, um Fremdgene in Kardiomyozyten sowohl in Zellkulturexperimenten [Kirshenbaum et al. 1993] als auch *in vivo* [Kass-Eisler et al. 1994] zu exprimieren. Adenovirale Vektoren sind in der Lage, Kardiomyozyten auch noch nach Sistieren der Teilungsaktivität sehr effektiv zu infizieren und bieten so einen deutlichen Vorteil gegenüber retroviralen Expressionssystemen [Rebolledo et al. 1998; Zhao et al. 2002; Fleury et al. 2003].

Es ist bekannt, dass bei adulten Mäusen die direkte intramyokardiale Injektion von adenoviralen Vektoren *in vivo* nur zu einer räumlich begrenzten Expression des Zielgens im Herzen führt [Kass-Eisler et al. 1994; Kass-Eisler und Leinwand 1997]. Für verschiedene Fragestellungen ist es jedoch erstrebenswert, möglichst eine über das gesamte Herz verteilte Expression des interessierenden Gens mit ausreichender Expressionsstärke zu erreichen. Insbesondere für die später geplanten eigenen Untersuchungen zur Beeinflussung der

Zellzyklusblockade von Kardiomyozyten war aufgrund verschiedener Überlegungen darüber hinaus die Anwendung der Vektoren bei neonatalen Tieren wünschenswert. Zum einen war davon auszugehen, dass bei neonatalen Mäusen nur sehr geringe immunologische Abwehrvorgänge als Antwort auf die adenovirale Infektion induziert werden, was zu einer verlängerten Expressionsdauer der Ziel-Gene führen sollte. Zum anderen vollzieht sich bei Mäusen – wie bei Säugetieren allgemein – der Zellzyklusaustritt der Kardiomyozyten in der Perinatalperiode, so dass sich neonatale Kardiomyozyten im Gegensatz zu adulten Zellen noch empfänglicher gegenüber proliferationsfördernden Interventionen verhalten sollten.

Im Folgenden konnte erfolgreich ein Modell der adenoviralen Expression von Fremdgenen in den Herzen neonataler Mäuse etabliert werden [Ebelt und Braun 2003]. Hierfür wurden rekombinante Adenoviren eingesetzt, die für unterschiedliche Markergene kodieren und somit eine eindeutige und leichte Identifizierung erfolgreich transduzierter Zellen gestatten. So wurde ein Adenovirus verwendet, der die Expression des Enzyms β-Galaktosidase in den Zellkernen von infizierten Zellen auslöst (Ad-nLacZ). Ausserdem fand ein mittels des AdEasy-Systems [He et al. 1998] selbst konstruierter Reportervirus Verwendung, der in transduzierten Zellen zur Expression von *enhanced green fluorescent protein* (EGFP) führt (Ad-EGFP).

Mittels quantitativer histologischer Verfahren kann gezeigt werden, dass es möglich ist, durch die herznahe intrathorakale Injektion von rekombinanten Adenoviren eine zuverlässige Transduktion des Herzmuskels bei neonatalen Mäusen zu realisieren. Die Gabe von ansteigenden Virusmengen führt dabei zu einer exponentiellen Zunahme der transduzierten Zellen, nach Injektion von 10^8 *plaque forming units* (pfu; maximal eingesetzte Dosis) findet sich eine Expression des Zielgenes in 71±8% der kardialen Zellen.

Die adenoviralen Reportergene lassen sich über mehr als 50 Tage in unveränderter Expressionsstärke nachweisen. Anzeichen für eine Störung der Herzfunktion oder relevante Inflammations- oder Immunprozesse ergeben sich nicht, und auch 150 Tage nach der einmaligen Virusapplikation zeigen noch 12±2% der Zellen eine Expression der Markergene. Ausserdem kann gezeigt werden, dass die Effizienz der myokardialen Transduktion sehr einfach durch die Co-Injektion geringer Mengen eines EGFP-Adenovirus (10^6 pfu) anhand der resultierenden epikardialen Fluoreszenz vorhergesagt werden kann.

Anlage zu 2.3.:

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Optimized, highly efficient transfer of foreign genes into newborn mouse hearts in vivo

Henning Ebelt[a,b] and Thomas Braun[a,*]

[a] *Institute of Physiological Chemistry, University of Halle-Wittenberg, Hollystr. 1, 06097 Halle, Germany*
[b] *Department of Medicine III, University of Halle-Wittenberg, Hollystr. 1, 06097 Halle, Germany*

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Abstract

Expression of foreign genes in vivo is a standard method to disclose functions of specific genes and to alter physiological conditions in distinct cell types and tissues. Virus-mediated gene transfer has proved to be a valuable tool for directed gene expression in vivo complementary to transgenic approaches. However, several problems associated with routes of application, endurance of gene expression, and efficiency of infections still have to be solved. We have optimized a gene transfer protocol into hearts of newborn mice to achieve widespread long-lasting expression using adenoviral vectors. Intrathoracic injection of high-titer adenoviral preparations (10^8 pfu) led to expression of foreign genes in $>71 \pm 8\%$ of all heart cells for >50 days after infection without any morphological signs of cardiac malfunction, inflammation, or immune response. This approach might be adapted to long-term cellular studies in vivo since 5 months after infection up to 20% of all cardiac cells still expressed virally encoded genes. Successful and efficient expression of other gene of interest can be easily controlled by co-injection of low titers of a reporter vector encoding EGFP (10^6 pfu).

Transgenic techniques in experimental murine animals are the methods of choice to abrogate functions of distinct genes and to express genes ectopically in tissues of interest such as the heart [1]. The analysis of phenotypic changes arising from attenuated or enhanced gene expression will reveal functions and interactions of different gene products in various settings [2–5]. On the other hand, the use of transgenic techniques is expensive, laborious, and time-consuming. In addition, it is sometimes difficult to achieve voluntary expression in a given tissue at the right time and to avoid potential adversary effects due to untimely or premature expression. The use of viral vectors provides an alternative means to avoid such problems and to accomplish high-level, timed expression.

So far a number of different strategies have been devised to attain gene delivery into postnatal cardiac muscle. The most widely applied tool to introduce and overexpress genes in cardiomyocytes in vitro and in vivo are adenoviruses [6], which readily infect cells after cessation of cell division. Recently, lentivirus-based vectors have also been successfully used to infect cardiomyocytes in vitro [7,8] although the efficiency of infection did not reach adenoviral infection rates in vivo [9].

In contrast to spatially restricted infections in vivo, which can be achieved by administration of adenoviral vectors into adult hearts of mice by direct intramyocardial injection [10] it is often favorable to achieve widespread expression of the gene of interest in most cardiac cells. To reach this goal we devised a technique using high-titer adenoviral vectors that allowed us to express genes of interest in >78% of all heart cells for >50 days after infection of postnatal mouse embryos without any signs of cardiac malfunction, inflammation, or immune response. We anticipate that this technique is highly useful to avoid early lethality inflicted by forced transgenic expression of dominant negative or active molecules during cardiac development.

* Corresponding author. Fax: +49-345-557-3811.
E-mail address: thomas.braun@medizin.uni-halle.de (T. Braun).

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Materials and methods

Construction of recombinant adenoviral vectors. The adenovirus encoding nuclear β-galactosidase (Ad-nlsLacZ) was kindly provided by T. Eschenhagen (University of Hamburg). The adenovirus encoding EGFP was generated using the AdEasy-System by co-transfection of pCMV-GFP and pAdEasy1 into BJ5183 cells as described [11].

Amplification of adenoviral vectors. Adenoviral vectors were amplified on monolayers of HEK293 cells in DMEM 2.5% FCS until complete cytopathic effect was observed. Virus preparations and determination of virus titers were performed as described [11].

Adenovirus-mediated gene transfer into hearts of newborn mice. At the day of birth neonate ICR-mice were anesthetized by cooling on ice for approximately 2 min and put in front of a cold light source to visualize the silhouette of the heart. Using a Hamilton syringe with a 26-gauge needle a total volume of 10 μl was injected into the thoracic cavity beside the heart at a left parasternal position. Finally, animals were re-warmed and put back to their mothers.

Examination and quantification of reporter gene activity and quantification of inflammatory responses. At different time-points after virus injection, animals were sacrificed by cervical dislocation. Organs (heart, lung, liver, kidney, and musculus iliopsoas) were removed, embedded, sectioned, and stained with X-Gal or with hematoxylin/eosin (H&E), respectively. Using imaging software (Scion Image), both the total area of nuclei (AN) from H&E stained sections and area of LacZ-positive nuclei (AZ) were measured on corresponding slides. The percentage of LacZ-positive nuclei was calculated as percentage pos. nucl. = AZ/AN × 100. Cryosections were stained with H&E and examined for the presence of infiltrating cells. The percentage of nuclei per area was quantified by an imaging software (Scion Image) to rule out any increase of mononuclear cells due to possible cardiac inflammation.

Echocardiography. For echocardiography, animals were anesthetized with 1.25% isofluorane [12]. Data acquisition was performed using a 10 MHz transducer (Toshiba) at the midpapillary level in parasternal short axis. M-Mode measurements were used to determine left ventricular diameters (LVID) and fractional shortening (FS), fractional area change (FAC) as a parameter of ejection fraction was calculated from B-mode as FAC % = LVAD − LVAS/LVAD × 100, where LVAD (LVAS) is the endocardiac area of the left ventricle at the end of diastole (and systole, respectively).

Results

Survival rates after neonatal injection of recombinant adenoviruses did not depend on virus titer or virus-mediated effects

As much as 40.6 + 6.1% of all animals that had received an intrathoracic injection at their day of birth survived the procedure without any signs of clinical illness or retarded postnatal development. Lethality was confined to the first 8 h after injection of recombinant adenoviruses, all other animals survived until adulthood. No correlation was spotted between early lethality and different reporter constructs or of the concentration of injected virus solutions.

The degree of myocardial gene expression correlates to the number of virus particles in a semi-logarithmic way

To determine the ratio of injected viral particles and myocardial reporter gene expression, increasing amounts of virus solutions ranging from 1×10^6 to 1×10^8 pfu in a final volume of 10 μl were injected into the thoracic cavity of newborn mice as described in Materials and methods. Fifteen days after the injection hearts were removed and β-gal activity was examined on cryosections (Fig. 1). Quantitative evaluation of β-gal activity revealed a clear exponential correlation between the number of virus particles injected and the number of β-gal-expressing cells (Fig. 1). After injection of 10^8 pfu of an Ad-nlsLacZ virus 71 ± 8% of all cardiac cells expressed the reporter gene. It appears likely that higher concentrations of virus might result in an even higher rate of cardiac infection although we did not attempt to concentrate the virus further and cytopathic effects resulting from excessive virus application might limit such an approach.

Though in our hands infection rates showed no substantial differences between different experiments, it might be an advantage to control the success of the injection procedure directly. In many cases it is not possible to include a reporter gene in the virus expressing

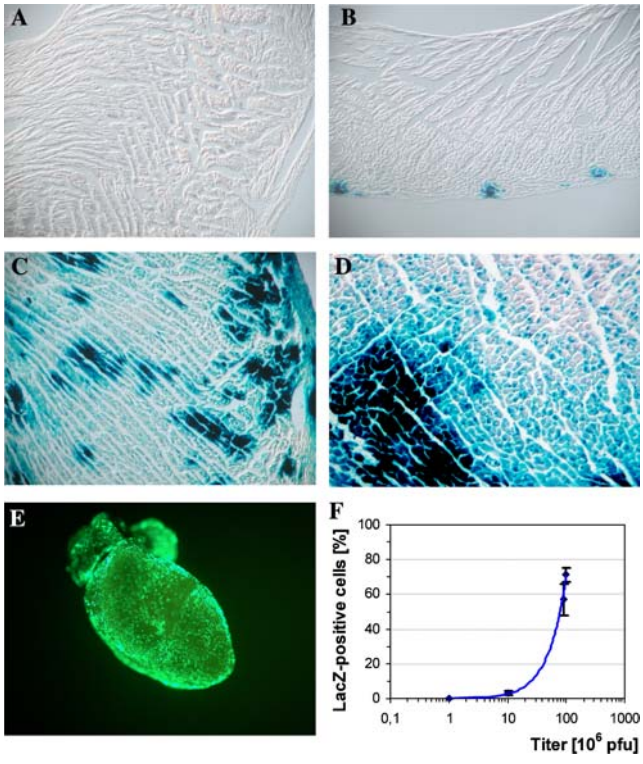

Fig. 1. The degree of myocardial gene expression correlated to the number of virus particles in an exponential way. Increasing numbers of the adenovirus Ad-nlsLacZ were injected into the thoracic cavity of newborn mice. After 15 days, hearts were removed and cryosections were stained for β-gal activity. (A) 10^6 pfu; (B) 10^7 pfu; (C) 9×10^7 pfu; (D) 10^8 pfu; (E) co-injection of 10^6 pfu Ad-EGFP as a tracer to validate successful injection events; and (F) dose–response curve between adenovirus-mediated β-gal activity and number of virus particles injected. β-Gal expression was quantified using imaging software (mean ± SEM).

the gene of interest. In such cases the addition of a separate adenovirus encoding the EGFP gene to the virus preparation might represent a versatile tool to prove the success of the infection procedure. We therefore co-injected low titers of a reporter vector encoding EGFP (10^6 pfu) together with high titers of the vector of interest into the thoracic cavity. As shown in Fig. 1, the fluorescence emitted from the EGF-protein allows an immediate control of the infection efficiency. Hence, it is possible to predict the myocardial expression level of the gene of interest simply by analyzing pericardial EGFP-fluorescence.

Adenovirus-mediated expression in the heart can be observed for more than 5 months

Hearts and other organs (lung, liver, kidney, and M. iliopsoas) were removed after different time intervals from animals that had received an intrathoracic injection of 10^8 pfu Ad-nlsLacZ virus at their day of birth, and β-gal activity was visualized on cryosections. Up to 50 days after infection, no significant loss of reporter gene activity was found in the heart. One hundred and fifty days after infection $12.1 \pm 1.8\%$ of cardiac cells were still positive for LacZ (Fig. 2). In contrast, in the other organs the percentage of cells expressing β-gal clearly declined strongly between day 15 and 50. One hundred and fifty days after virus administration we found only very rarely β-gal positive cells in these organs (Fig. 3). We reason that the differences between heart versus lung, liver, and kidney tissues are due to enhanced proliferation and/or replacement of those cells compared to cardiomyocytes, which are already predominantly postmitotic at this developmental stage.

It should be emphasized that the intrathoracic administration regimen of adenoviral particles leads to a very efficient short-term expression of virus-encoded genes in the liver and the lung. Quantitative analysis of β-gal activity on cryosections from the lung and liver 15 days after infection revealed that virtually all cells in these organs expressed virally encoded genes (Fig. 3). In contrast, the kidney and the M. iliopsoas, which have no direct contact to the thoracic cavity (or are separated from it by the comparatively thin diaphragm), do only

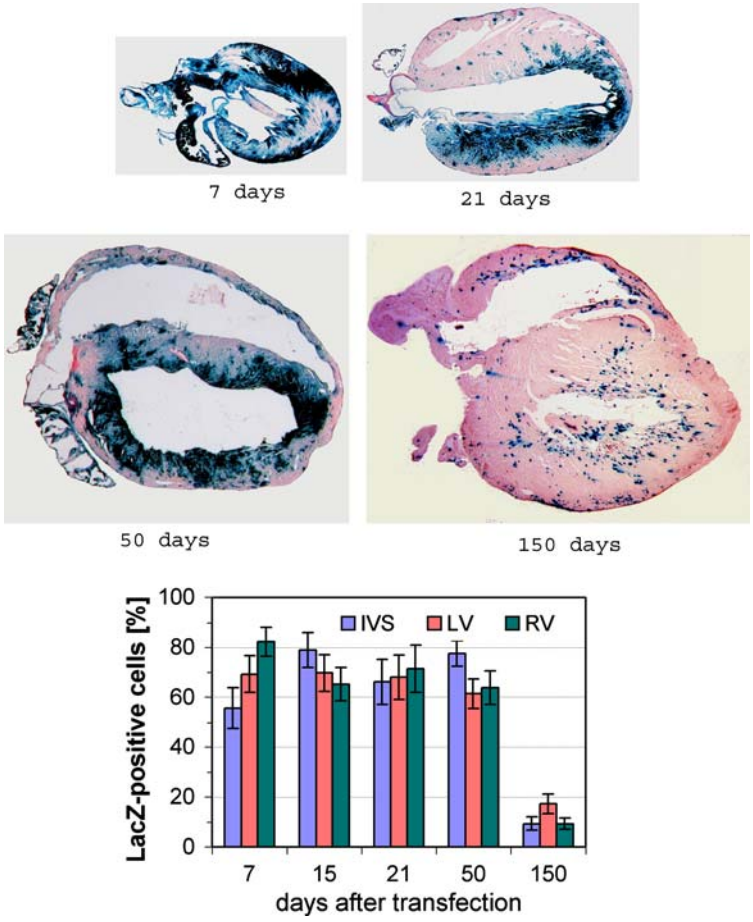

Fig. 2. Adenovirus-mediated expression in the heart can be observed for more than 5 months. As much as 10^8 pfu of an adenovirus Ad-nlsLacZ preparation was injected into the thoracic cavity of newborn mice. Hearts were removed at different time-points as indicated and stained for β-gal activity. Expression in the IVS (interventricular septum), LV (left ventricle), and RV (right ventricle) differed only slightly in the groups that received the same virus dose (mean ± SEM).

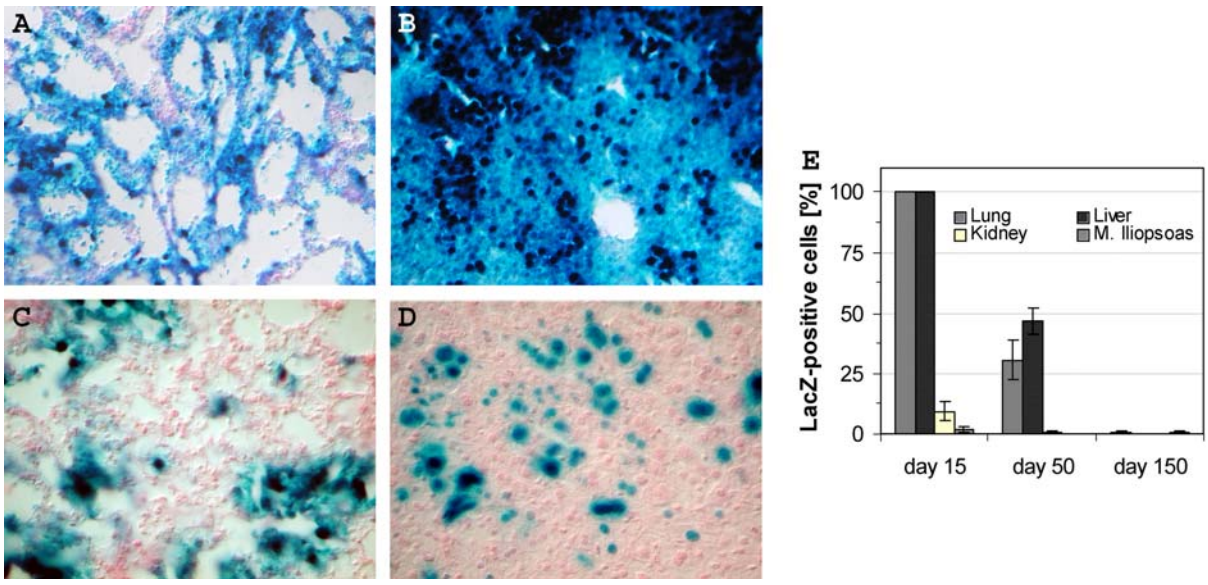

Fig. 3. Highly efficient short-term adenovirus gene expression in organs adjacent to the thoracic cavity. Administration of 10^8 pfu of the adenovirus Ad-nlsLacZ led to a complete infection of the lung (A and E) and the liver (B and E) 15 days after injection, whereas organs, which do not have close contact to the thoracic cavity such as kidney and M. iliopsoas, showed only a minor expression rate. At day 50 after injection expression in the lung (C and E) and liver (D and E) had already decreased significantly while expression in the heart remained unchanged at this time-point. Quantification of lacZ-expressing cells in different organs at various time-points after infection (E) (mean ± SEM).

show expression of virus proteins in a minor fraction of the organ (10% and 2%, respectively).

Injections of high concentrations of adenovirus particles do not affect cardiac morphology and function

To investigate whether adenoviral gene delivery leads to changes in the morphology of the heart such as infiltration with mononuclear cells, deposition of extracellular matrix, cell swelling, and other signs of inflammation, we prepared sections from hearts at various time-points after infection and stained them with H&E. No infiltrating cells or other pathological signs were observed at any time-point (Fig. 4). Using an imaging program the percentage of nuclei per area was quantified on sections derived from animals, which had received the highest viral titer (10^8 pfu). As shown in Fig. 4 no significant differences in nuclear density or other pathological signs were found. Additionally, 150 days after intrathoracic injection of the highest viral titer cardiac function was examined by echocardiography. No signs of altered cardiac function nor significant differences in left ventricular wall thickness, left ventricular diameters, fractional shortening, or ejection fraction were observed between infected animals and untreated littermates (Table 1).

Discussion

In this study, we present an optimized approach to direct expression of foreign genes in the heart of newborn mice by using recombinant adenoviral vectors. In the past several attempts have been made to influence mammalian gene expression in vivo using adenoviral vectors [13]. To deliver viral vectors, different strategies ranging from intravenous application [14], intraplacental injections [15], or direct injection into the ventricular cavity of newborn mice or embryos were applied [16]. However, the percentage of infected cardiomyocytes reported so far did not exceed 20%. In our experiments, we demonstrate that intrathoracic injection of an adenovirus encoding the reporter gene β-galactosidase leads to infection rates of 71 ± 8% of cardiac cells in a dose-dependent manner. While the use of low viral titers favors the infection of pericardium and atria, titers of more than 10^7 pfu allow infection of cardiomyocytes of the free ventricular walls and the interventricular septum.

Other authors claimed that intrathoracic injection of adenoviruses result in a high variability of the rate of infected cardiomyocytes [16]. In our hands injections into the thoracic cavity were highly reproducible and showed only little variations. It is possible that differences in the injection procedure, handling of virus solutions, and virus concentrations might account for these divergent findings. In addition, we devised an experimental approach that allows, by co-injection of low titers of an EGFP adenovirus (10^6 pfu) together with high titers of the vector of interest, to control infection rates in an easy and very efficient way. Based on pericardial EGFP-fluorescence accomplished by the co-injection procedure it is possible to predict the myocardial expression level of the gene of interest. Thus

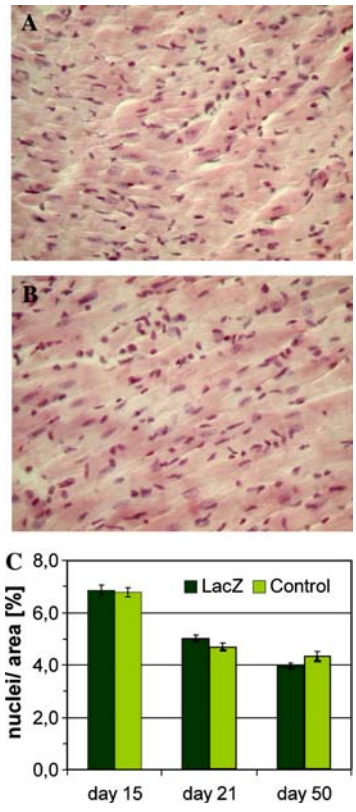

Fig. 4. Injections of high concentrations of adenovirus particle do not affect cardiac morphology. As much as 10^8 pfu of the adenoviral reporter construct Ad-nlsLacZ was injected into the thoracic cavity of newborn mice. No morphological differences were discernable between uninfected controls (A) and animals infected with 10^8 pfu 150 days after injection with Ad-LacZ (B). Hearts were removed at different time-points as indicated and stained with H&E. Columns indicate the percentage of nuclei per area (C) (mean ± SEM).

one can select hearts with highest infection rates directly after opening the animal's thorax (data not shown).

To infect a target cell by adenoviral vectors, a critical number of viral particles have to be present in the direct surrounding of the cell. Cell specific thresholds seem to depend on several parameters as expression of the Coxsackie virus–adenovirus receptor and related integrins [17]. Intrathoracic administration of low viral titers favors infection of atria, pericardium, and cells of the outer myocardial layers, while an increase in the number of injected viral particles in our experiments resulted in infection of additional cardiomyocytes in the inner myocardial layers. It seems reasonable to assume that a relatively high concentration of adenoviral particles is able to build up a gradient that allows efficient penetration of newborn mouse tissue, which is still relatively loose at this time-point of development. Thus, deeper layers of the target tissue might be reached if the initial virus concentration is sufficiently high. This view is also supported by the very high infection rates observed in the liver after intrathoracic injection of high-titer virus preparations. In this case the virus had to cross a natural barrier, the diaphragm, in order to make contact with the liver.

Beside virus spread per diffusionem within organs and adjacent tissue, a number of adenoviruses have apparently entered blood circulation and were distributed across the animal leading to infections in remote organs such as skeletal muscle and kidney. The overall rate of such events, however, was relatively low compared to topically applied virus suspensions. Infected cells in peripheral organs were found in direct neighborhood to blood vessels. In the kidney, for example, locations of infected cells were mostly confined to the glomeruli.

In accordance with data from previous studies [18], we found no significant decrease of reporter gene expression in our experiments within 50 days after virus administration. After 150 days, the percentage of LacZ-expressing cells in the heart was reduced to 12.1 ± 1.8%, indicating that more than 80% of the initially infected cells had lost their reporter gene expression. The mechanisms leading to this inactivation of the LacZ-gene are not known so far. Several explanations might be envisaged to explain this observation: (i) degradation of the viral genome or inactivation of adenoviral gene transcription [19], (ii) removal of infected cells by innate or adaptive immune responses [20,21]. Since we did not find signs of inflammation in the heart destruction of infected cells or virus-mediated cell lysis seems less likely. Adaptive immunity has not fully developed in newborn mice hence an immune response against a

Table 1
Injection of adenovirus particles does not affect cardiac function

Parameter	Uninjected control	Injected with Adeno-LacZ
Left ventricular wall thickness		
Diastolic (mm)	1.09 ± 0.08	1.01 ± 0.04
Systolic (mm)	1.47 ± 0.07	1.52 ± 0.07
Left ventricular diameter		
Diastolic (mm)	3.21 ± 0.12	3.30 ± 0.05
Systolic (mm)	1.84 ± 0.11	1.87 ± 0.14
Fractional shortening	0.42 ± 0.01	0.43 ± 0.04
Fractional area change (%)	63 ± 3	67 ± 3

Animals injected with 10^8 adenovirus particles and uninjected control animals were subjected to echocardiographic analysis at the age of 5 months. No signs of cardiac malfunction were observed in injected animals (mean ± SEM).

single round of adenovirus infection will almost certainly not occur. This presumption is supported by our finding that no signs of cytopathic effects were observed neither histologically nor by echocardiography, and (iii) continuous replacement of cardiomyocytes. Adenoviruses are the vectors of choice to manipulate gene expression in cardiomyocytes in vitro. Our optimized in vivo expression approach allows the rapid shift from an in vitro cell culture application to a more complex in vivo situation using the same vector system. Intrathoracic injection of high-titer adenovirus preparation allows rapid, high efficiency expression of a gene of interest in a high percentage of cardiac cells and thus offers an alternative to more tedious and time-consuming transgenic approaches, which sometimes are not practicable due to early lethality or lack of suitable promoters.

Acknowledgments

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2.4 Einfluss der adenoviralen Expression verschiedener E2F-Transkriptionsfaktoren auf die Zellzyklusarretierung von neonatalen Kardiomyozyten *in vitro*

Kardiomyozyten von Säugetieren verlieren in der Perinatalperiode die Fähigkeit, sich zu teilen [Soonpaa et al. 1996; Soonpaa und Field 1997]. Daher ist das adulte Herz nicht in der Lage, nach schwerer Schädigung (etwa nach einem Koronararterienverschluß) nekrotische Zellen durch neue Kardiomyozyten zu ersetzen. Im Prinzip sollte es jedoch möglich sein, die nach einer Herzschädigung verbliebenen Kardiomyozyten zur Zellteilung anzuregen und so wieder zu vermehren. Diese Hypothese gründet vor allem auf der Beobachtung, dass – insbesondere im höheren Alter und nach extremer hämodynamischer Belastung – durchaus vereinzelte Kern- und Zellteilungen von Kardiomyozyten festgestellt werden konnten [Anversa et al. 1991; Anversa und Kajstura 1998; Anversa et al. 1998].

Seit den 1990er Jahren sind verschiedentlich Versuche unternommen worden, Kardiomyozyten von Säugetieren zur Teilung zu bringen. Als Vorraussetzung hierfür ist es erforderlich, den Restriktionspunkt im Zellzyklus am G1/S-Übergang zu überwinden. Dies wurde versucht, indem Oncogene wie E1A [Kirshenbaum und Schneider 1995], Komponenten der Zellzyklusmaschinerie wie Cyclin D1 [Soonpaa et al. 1997] oder Schlüssel-Regulatoren des S-Phase-Eintritts wie der Transkriptionsfaktor E2F1 [Agah et al. 1997; von Harsdorf et al. 1999] gezielt überexprimiert wurden. Zwar war es in den zitierten Untersuchungen tatsächlich möglich, die DNA-Synthese von Kardiomyozyten zu induzieren, der forcierte Zellzykluseintritt war jedoch meist von einer gleichzeitigen Apoptose-Induktion begleitet [Agah et al. 1997; von Harsdorf et al. 1999].

Der Zellzyklus unterliegt bei Säugetieren der Regulation durch ein komplexes Netzwerk von Faktoren, die das kontrollierte Durchlaufen der verschiedenen Zyklusphasen bzw. die Arretierung an einer bestimmten Position sicherstellen. Die Stimulation mit Wachstumsfaktoren führt in zur Proliferation fähigen Zellen zur Bildung von aktiven Komplexen aus D-Typ-Cyclinen und den *cyclin dependent kinases 2*/ *-4* (cdk-2/ -4). Als Folge werden sogenannte *pocket proteins* wie das Retinoblastoma-Protein (pRb) phosphoryliert. In der hypo-phosphorylierten Form bindet pRb an E2F-Transkriptionsfaktoren, die dadurch inaktiviert werden. Durch pRb-Phosphorylierung werden die E2Fs frei gesetzt und aktivieren die Transkription von wichtigen Genen des Nucleotid-Metabolismus und der DNA-Synthese, was zur Überwindung des Restriktionspunktes und der Einleitung der S-Phase in den Zellen führt [DeGregori et al. 1995].

Der Name „E2F“ leitet sich ursprünglich von der Beobachtung ab, dass das Adenovirus-Protein E1A einen zellulären Co-Faktor benötigt, um den Promotor des adenoviralen E2-Gens

zu aktivieren – den „E2-Faktor“ (E2F). Gegenwärtig sind 8 verschiedene Mitglieder bekannt, die zur Familie der E2F-Transkriptionsfaktoren (im engeren Sinne) gehören: E2F1 bis E2F8.

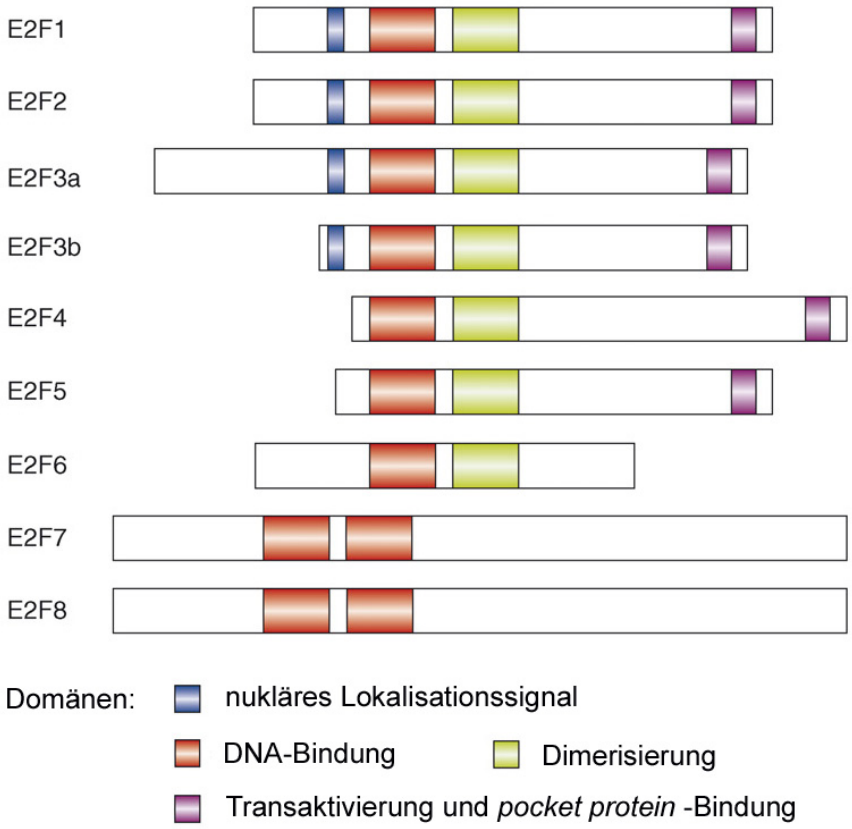

Abb. 1: Schematische Darstellung der E2F-Transkriptionsfaktoren (nach [Iaquinta und Lees 2007]

Während die Faktoren E2F1 bis E2F5 alle jeweils über Domänen zur DNA-Bindung, Dimerisierung und Transaktivierung verfügen, haben E2F6 bis E2F8 keine transaktivierenden Eigenschaften. Aufgrund weiterer Sequenzhomologien, der Interaktion mit unterschiedlichen *pocket proteins* (pRb, p107, p130) und der Fähigkeit, in ruhenden Zellen die Proliferation auszulösen, werden E2F1 bis -5 des weiteren üblicherweise in „aktivierende“ (E2F1, E2F2, E2F3) bzw. „reprimierende“ E2Fs (E2F4 und -5) klassifiziert (Übersicht in [Iaquinta und Lees 2007]).

Frühere Experimente hatten bereits zeigen können, dass die Expression [Kirshenbaum et al. 1996] oder gezielte Aktivierung [Kirshenbaum und Schneider 1995] von E2F1 in Kardiomyozyten zur Induktion der S-Phase führt, aber gleichzeitig auch die Apoptoserate der Zellen deutlich erhöht [Agah et al. 1997]. Da jedoch weitere Mitglieder der E2F-Familie in anderen Zelltypen ebenfalls pro-proliferative Effekte auszuüben vermögen, ohne gleichzeitig

zu vermehrtem Zelltod zu führen [DeGregori et al. 1997; Wang et al. 2000], sollten in den eigenen Untersuchungen die Effekte einer gezielten Expression von E2F1 bis -5 in primären neonatalen Kardiomyozyten im Hinblick auf Zellzyklusstímulation und Apoptose-Induktion untersucht und verglichen werden. Außerdem sollten die zellulären Abläufe analysiert werden, die in Kardiomyozyten entweder zur E2F-induzierten Proliferation oder aber zur Apoptose führen.

Rekombinante Adenoviren, die für die humanen Isoformen der Transkriptionsfaktoren E2F1, E2F2, E2F3, E2F4 oder E2F5 kodieren, wurden freundlicherweise von J.R. Nevins (Howard Hughes Medical Institute, Durham, USA) zur Verfügung gestellt. In isolierten Kardiomyozyten von neonatalen Ratten oder Mäusen wurden die einzelnen E2F-Transkriptionsfaktoren gezielt exprimiert, und die Effekte bezüglich Proliferation wurden mittels Durchflusszytometrie (FACS) und der immunhistologischen Quantifizierung des Bromodesoxyuridin- (BrdU-) Einbaus bestimmt; die apoptotischen Kardiomyozyten wurden ebenfalls mittels FACS bzw. durch TUNEL-Assay quantifiziert [Ebelt et al. 2008].

Die Untersuchungen zeigen, dass sowohl E2F1, E2F2, E2F3 als auch E2F4 die DNA-Synthese in neonatalen Kardiomyozyten induzieren, während dies nach Expression von E2F5 oder einem LacZ-Kontrollvirus nicht zu beobachten ist. Gleichzeitig führen jedoch E2F1, E2F3 und E2F5 – nicht jedoch E2F2 oder E2F4 – auch zu vermehrten Kardiomyozyten-Apoptosen. Die Induktion von Kardiomyozyten-Mitosen ist nur nach Expression von E2F1 oder E2F2 nachweisbar.

Mittels quantitativer *real time*-PCR kann gezeigt werden, dass der E2F-induzierte Wiedereintritt in den Zellzyklus von der transkriptionellen Aktivierung der Cycline A und E abhängig ist, während die D-Typ-Cycline in dieser Situation nur von untergeordneter Bedeutung sind. Nur nach Expression von E2F1, E2F3 oder E2F5 findet sich eine Induktion zahlreicher pro-apoptotischer Gene wie bax, p21WAF und caspase 6, wohingegen die Expression dieser Gene nach Aktivierung von E2F2 oder E2F4 reduziert ist. Die Aktivierung von p19ARF – ein in der Literatur mehrfach diskutierter Schritt der E2F-induzierten Apoptose-Kaskade [DeGregori et al. 1997; Zhu et al. 1999; Tolbert et al. 2002; Lindstrom und Wiman 2003] – findet sich in den Herzmuskelzellen ausschließlich nach Expression von E2F1, nicht jedoch nach den anderen E2F-Transkriptionsfaktoren.

Die Mechanismen, die dafür verantwortlich sind, dass die einzelnen E2Fs zu einer unterschiedlichen Gen-Aktivierung und den damit verbundenen Auswirkungen auf Zellzyklusaktivität und Apoptose führen, können durch die Experimente nicht vollständig geklärt werden. Aus früheren Untersuchungen war bereits bekannt, dass bestimmte E2Fs

spezifische Interaktionen mit weiteren zellulären Co-Faktoren einzugehen vermögen und so selektiv die Transkription beeinflussen [Giangrande et al. 2003]. In den eigenen Untersuchungen liegen die exprimierten E2Fs erwartungsgemäß überwiegend in der freien Form in den Herzmuskelzellen vor, was mittels EMSA (*electrophoretic mobility shift assay*) und ELISA (*enzyme linked immunosorbent assay*) nachgewiesen werden kann. Dennoch ist ein Teil der E2F-Transkriptionsfaktoren auch an Pocket-Proteine gebunden, und Unterschiede in der Pocket-Protein-Bindung können als ein Erklärungsansatz für die differenten Wirkungen der individuellen E2Fs angesehen werden. So zeigt sich, dass E2F1 und E2F3 ausschließlich mit dem Retinoblastoma-Protein interagieren, während die anti-apoptotischen E2Fs (E2F2 und E2F4) vornehmlich an p107 gebunden sind.

Zusammenfassend belegen die eigenen Experimente, dass durch die gezielte Expression von E2F2 und E2F4 die Proliferation von Kardiomyozyten angeregt werden kann, ohne dass gleichzeitig eine unerwünschte Steigerung der Apoptoserate zu beobachten ist.

Anlage zu 2.4.:

Ebelt H, Hufnagel N, Neuhaus P, Neuhaus H, Gajawada P, Simm A, Muller-Werdan U, Werdan K, Braun T: Divergent siblings: E2F2 and E2F4 but not E2F1 and E2F3 induce DNA synthesis in cardiomyocytes without activation of apoptosis. Circ Res 96 (2005) 509-517

Divergent Siblings

E2F2 and E2F4 but not E2F1 and E2F3 Induce DNA Synthesis in Cardiomyocytes Without Activation of Apoptosis

Henning Ebelt, Nadine Hufnagel, Petra Neuhaus, Herbert Neuhaus, Praveen Gajawada, Andreas Simm, Ursula Müller-Werdan, Karl Werdan, Thomas Braun

Abstract—Proliferation of mammalian cardiomyocytes ceases around birth when a transition from hyperplastic to hypertrophic myocardial growth occurs. Previous studies demonstrated that directed expression of the transcription factor E2F1 induces S-phase entry in cardiomyocytes along with stimulation of programmed cell death. Here, we show that directed expression of E2F2 and E2F4 by adenovirus mediated gene transfer in neonatal cardiomyocytes induced S-phase entry but did not result in an onset of apoptosis whereas directed expression of E2F1 and E2F3 strongly evoked programmed cell death concomitant with cell cycle progression. Although both E2F2 and E2F4 induced S-phase entry only directed expression of E2F2 resulted in mitotic cell division of cardiomyocytes. Expression of E2F5 or a control LacZ-Adenovirus had no effects on cell cycle progression. Quantitative real time PCR revealed that E2F1, E2F2, E2F3, and E2F4 alleviate G0 arrest by induction of cyclinA and E cyclins. Furthermore, directed expression of E2F1, E2F3, and E2F5 led to a transcriptional activation of several proapoptotic genes, which were mitigated by E2F2 and E2F4. Our finding that expression of E2F2 induces cell division of cardiomyocytes along with a suppression of proapoptotic genes might open a new access to improve the regenerative capacity of cardiomyocytes. (***Circ Res*****. 2005;96:509-517.)**

Key Words: E2F ■ cardiomyocyte ■ cell cycle ■ proliferation

Differentiation of cardiomyocytes is inversely correlated with proliferation. During embryogenesis precursor cells of cardiomyocytes within the precardial mesoderm show a very high rate of cell proliferation of approximately 70%, which drops to 45% on onset of cardiomyocyte differentiation.[1] Around birth, a transition from hyperplastic to hypertrophic myocardial growth occurs, and cytokinesis and cell proliferation, which are hallmarks of fetal development, are superseded by hypertrophy and binucleation of cardiomyocytes.[2] The proliferation block of cardiomyocytes prevents efficient replacement of functional myocardial on tissue damage, although some reports demonstrated division of cardiomyocytes in the failing heart, indicating that cardiomyocytes retain at least some proliferative capacity[3]

The mammalian cell cycle is tightly regulated by a complex network of factors that either promote cell cycle progression or arrest cells at a certain cycle position. Stimulation by growth factors in proliferating cells leads to formation of complexes of D-type cyclins and cdk2/4 and phosphorylation of pocket proteins such as the retinoblastoma protein (pRb). pRb binds and inactivates E2F transcription factors in its hypophosphorylated form, whereas phosphorylated pRb releases E2Fs, which then activate genes required for nucleotide metabolism and DNA synthesis.[4] In addition to the established role in regulation of cell proliferation, some E2Fs, in particular E2F1 and E2F3, have been shown to induce apoptosis[5] and cause, at least in part, the $Rb^{-/-}$ phenotype in mice, which is characterized by excessive apoptosis.[6]

The family of E2F transcription factors comprises seven individual members: E2F1 to E2F7. Although E2F1 to E2F5 all have both a DNA binding domain, a dimerization domain, and a transactivating domain (see review[7]), E2F6 and E2F7 have no transactivating properties. Based on sequence homologies, binding of pocket proteins, and the ability to induce S-phase entry in quiescent cells, E2F1 to 5 can be further distinguished into "activating" E2Fs (E2F1, E2F2, and E2F3) and "repressing" E2Fs although such a classification holds true only to specific experimental setups.[7]

Previous studies have demonstrated that activation of E2F1 in cardiomyocytes stimulates DNA synthesis but in parallel increases apoptosis.[8] Because individual members of the E2F family serve distinct roles in different cell types and proapoptotic effects are a special hallmark of E2F1,[5,9] we decided to exploit discrete properties of other E2F family members in control of cell proliferation to overcome the cell cycle block of cardiomyocytes without induction of apoptosis. Further-

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From the Institute of Physiological Chemistry (H.E., N.H., P.N., H.N., P.G., T.B.) and Departments of Medicine III (H.E., U.M.-W., K.W.) and Cardio-Thoracic Surgery (A.S.), University of Halle-Wittenberg, Germany; and the Max-Planck-Institute for Heart and Lung Research (T.B.), Bad Nauheim, Germany.

Correspondence to Thomas Braun, Max-Planck-Institute for Heart and Lung Research, Bad Nauheim, Germany. E-mail thomas.braun@kerckhoff.mpg.de

Circulation Research **is available at http://www.circresaha.org** **DOI: 10.1161/01.RES.0000159705.17322.57**

more, we began to analyze the regulatory network downstream of E2F, which controls whether a cardiomyocyte is prone to die or to proliferate.

Materials and Methods

Adenoviruses, Cell Culture, and FACS

Adenoviruses expressing E2F1, E2F2, E2F3, E2F4, or E2F5 (Ad-E2F1/-E2F5) were kindly provided by J.R. Nevins (Howard Hughes Medical Institute, Durham, NC); nuclear β-galactosidase (Ad-nlsLacZ) was a gift from T. Eschenhagen (University of Hamburg, Germany). Adenoviruses were used as described previously.[10]

Cardiomyocytes from newborn rats and mice were prepared using standard procedures. In all experiments, cells were transferred to serum-free DMEM containing 25 mg/L BSA, 2.5 mg/L transferring, and 25 μg/L insulin 24 hours before virus administration and maintained in this medium until fixation. Animals were bred at the animal facility of the Martin-Luther-University Halle-Wittenberg (MLU) or purchased from Harlan Winkelman GmbH (Borchen, Germany). All animal experimentations were endorsed by the local government and performed according to guidelines of the MLU.

DNA content measurements by FACS analysis were performed essentially as described.[11] The cell cycle profile was calculated using MultiCycle software (Phoenix Flow Systems). Proliferation of cardiomyocytes was determined using the BrdU-Hoechst method,[11] which allows enumeration of cells at different stages of three consecutive cell cycles including mitotic cell division.[12] All FACS analyses were restricted to MF20-positive cells to exclude contaminating fibroblasts.

Immunhistochemistry and TUNEL Analysis

To detect DNA-synthesizing nuclei, cells were incubated with BrdU for 24 hours and stained according to the manufacturer's instructions (BrdU Immunohistochemistry System; Oncogene). Finally, cells were stained for myosin heavy chain (MyHC) using the MF20 antibody as described previously.

DNA double strand breaks were marked by fluorescein-conjugated dUTP using an in situ Cell Death Detection Kit (Roche). To calculate the percentage of TUNEL-positive cells, both the total area of nuclei ($Area^{nuclei}$) from Hoechst fluorescence and the area of TUNEL-positive nuclei ($Area^{TUNEL}$) were measured using imaging software (Scion Image). Percentage of TUNEL-positive nuclei was calculated as $[\%\ \text{TUNEL-pos.}] = Area^{TUNEL}/Area^{nuclei} \times 100$.

Quantitative Real-Time PCR

Total RNA was isolated using Trizol reagent according to the instructions of the manufacturer. cDNAs were synthesized using oligo-dT-primers as

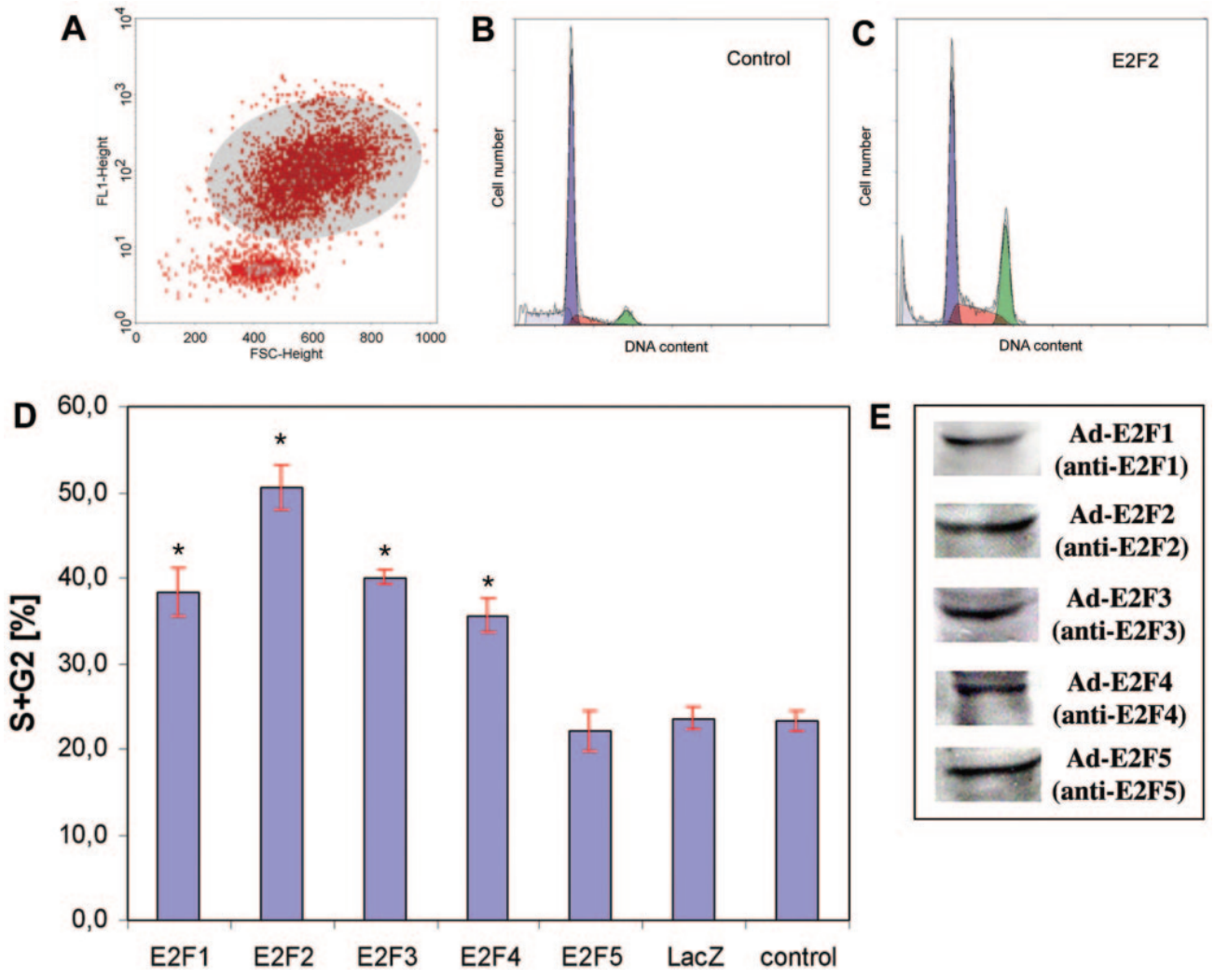

Figure 1. E2F1, E2F2, E2F3, and E2F4 stimulate cell cycle progression in primary cardiomyocytes. Cell cycle analysis of cardiomyocytes by FACS after expression of E2F transcription factors. A, Gating of cardiomyocytes after MyHC-staining (gray area). B and C, Representative cell cycle profiles of an untreated control (A) and after Ad-E2F2 infection (B). D, Percentage of cardiomyocytes in S- or G2-phase after expression of E2F1–5, LacZ, or without treatment was calculated using the MultiCycle software. *$P<0.05$.

described.[13] Real-time PCR was performed as described.[13] Primer sequences are listed in the expanded Materials and Methods section in the online data supplement available at http://circres.ahajournals.org. Expression levels are shown as follows: expression level=(copy number geneA per μL cDNA)/(copy number G3PDH per μL cDNA). Relative expression levels in different experiments were compared based on the expression of a gene of interest ("geneX") with and without overexpression of E2F: relative expression level=($GeneX^{E2F}$/$G3PDH^{E2F}$)/($GeneX^{control}$/$G3PDH^{control}$)).

Protein Isolation, Western Blots, EMSA, and ELISA

Protein isolation, subcellular fractioning, and Western blot analysis were performed according to standard techniques. Antibodies against E2Fs (sc-22820, sc-22820, sc-22822, sc-866, and sc-999) and pocket proteins (sc-50-G, sc-7986, sc-250, and sc-317-G) were purchased from Santa Cruz Biotech. Interactions between E2Fs and pocket proteins were analyzed using the "Mercury TransFactor Profiling Kit - Oncogenesis 1" according to the instructions supplied by the manufacturer (Clontech). Band-shift assays were done as described[14] using the E2F binding site TCCGTTTTCGCGCTTAAATTTGAGAAAGGGCGCGAAACTGGA.[5]

Results

E2F1, E2F2, E2F3, and E2F4 but not E2F5 Stimulate S-Phase Entry of Cardiomyocytes

To analyze distinct effects of individual members of the E2F-transcription factor family on cell cycle progression of cardiomyocytes, E2F1, E2F2, E2F3, E2F4, and E2F5 were expressed in serum-starved neonatal rat cardiomyocytes using adenoviral vectors (Figure 1E). After 48 hours, DNA content of cardiomyocytes was analyzed by FACS (Figure 1A through 1C). As shown in Figure 1D, E2F1, E2F2, E2F3, and E2F4 but not E2F5 or LacZ control virus significantly increased the number of cardiomyocytes in S- and G2-phase although all E2Fs were expressed at similar degrees in infected cardiomyocytes as indicated by Western blot analysis (Figure 1E). To confirm these results, we prepared primary cardiomyocytes from newborn mice, infected them with E2F1, E2F2, E2F4, or LacZ adenoviruses and counted the number of DNA-synthesizing cardiomyocytes that had incorporated BrdU. In agreement with our findings obtained by FACS analysis using neonatal rat cardiomyocytes E2F1, E2F2, and E2F4 but not LacZ clearly increased the number of DNA-synthesizing cardiomyocytes (Figure 2A and 2B). Additionally, histological examination revealed that the number of binucleated cardiomyocytes was elevated after directed expression of E2F1 and E2F2 (Figure 2C).

E2F1, E2F3, and E2F5, but not E2F2 or E2F4 Exert Proapoptotic Effects

Parallel to the analysis of cell cycle progression, we quantified the extent of apoptosis of neonatal rat cardiomyocytes using FACS analysis. With some but not all E2F-adenoviruses, we noted a clear increase of the number of cells with a DNA content lower that G1-phase cells, which is indicative for cells undergoing programmed cell death. Interestingly, expression of E2F2 and E2F4 did not result in induction of apoptosis of neonatal rat cardiomyocytes concomitant with stimulation of S-phase entry, whereas E2F1, E2F3, and E2F5 all lead to a significant increase of apoptotic cells. E2F1, which has a unique ability to induce apoptosis when accumulating in cells devoid of proliferative signals, evoked the strongest apoptotic response relative to other E2F

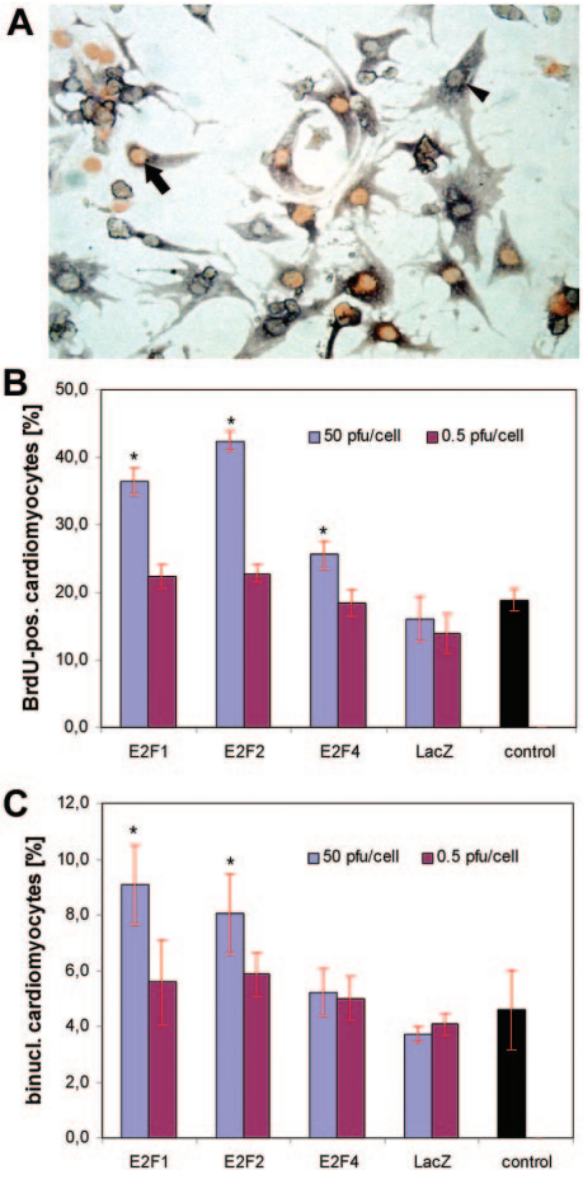

Figure 2. E2F1 and E2F2 strongly induce BrdU incorporation and binucleation of primary murine cardiomyocytes. A, Double staining with anti-BrdU (yellow-brown nuclei) and anti-myosin heavy chain (gray cytoplasm). Arrow, BrdU-positive cardiomyocytes; arrowhead, BrdU-negative cardiomyocyte (staining after expression of E2F2). B, Percentage of BrdU-positive cardiomyocytes after expression of E2F1, E2F2, and E2F4 at different MOI (50 and 0.5 pfu/cell, respectively). C, Percentage of binucleated cardiomyocytes. *$P<0.05$.

family members (Figure 3A and 3B). Surprisingly, we also noted a modest increase of apoptosis with the LacZ control virus when compared with uninfected cardiomyocytes or cells infected with E2F2 and E2F4. Toxic effects of reporter genes including LacZ, which lead to apoptosis, have been reported before and, in the case of EGFP, have occasionally even resulted in dilatative cardiomyopathy in transgenic animals.[15]

Similar results were obtained when we quantified cells undergoing apoptosis using the TUNEL technique. After E2F1 expression the number of primary cardiomyocytes from neonatal mice labeled by TUNEL staining was elevated up to

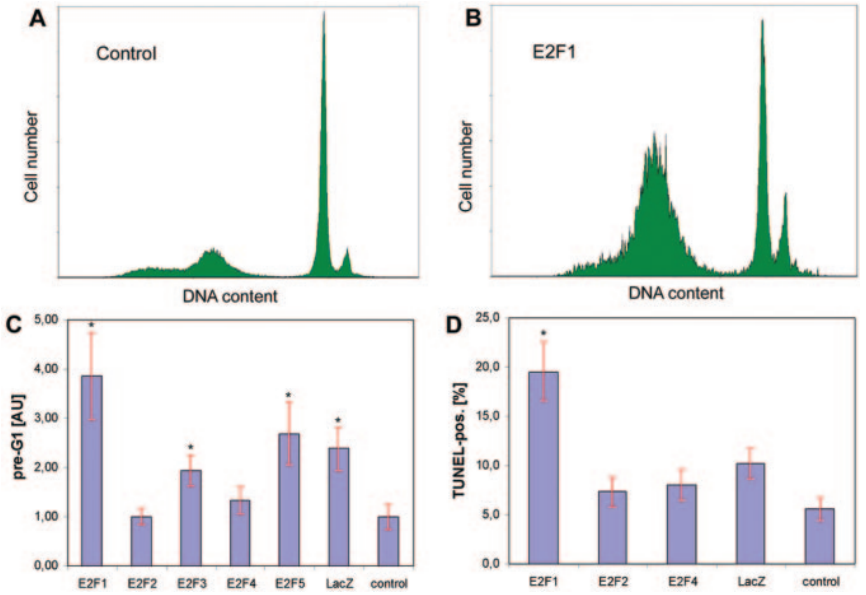

Figure 3. E2F1, E2F3, and E2F5 but not E2F2 and E2F4 induce apoptosis of cardiomyocytes. Rate of apoptotic cardiomyocytes was determined after expression of E2F transcription factors by FACS (A and B) and by TUNEL (C and D). A and B, Cell cycle profiles of untreated control (A) and E2F1 expressing rat cardiomyocytes cells (B). C, Quantification of pre-G1 peak particles after expression of E2F1–5 and LacZ and in untreated controls. D, Percentage of TUNEL-positive murine cardiomyocytes after E2F treatment. *$P<0.05$.

23%. In marked contrast, treatment of cardiomyocytes with E2F2 or E2F4 did not result in an elevated rate of TUNEL-positive cells and remained close to 5% of all cardiomyocytes in culture. We again detected a slight increase of apoptotic cells after LacZ expression using this alternative technique (Figure 3C).

Expression of E2F1 and E2F2, but not of E2F4, Induces Mitosis of Cardiomyocytes

The experiments described earlier clearly showed that both E2F2 and E2F4 induce DNA synthesis in cardiomyocytes without provoking apoptosis. S-Phase entry, however, does not prove that cells complete the cell cycle and divide. It remained possible that cardiomyocytes arrested either in G1-[16] or in G2-phase and thereby mimicked a hypertrophic phenotype as described earlier for E2F1.[17] We therefore used the flowcytometric BrdU-Hoechst assay, which allows retrospective assessment of G1, S, and G2/M cell cycle phases of synchronous and asynchronous cell populations[11,12] (Figure 4). In the absence of BrdU, all cells within G1-phase (either growth arrested or after mitosis) showed the same intensity of fluorescence for PI and Hoechst 33258 (Figure 4B). BrdU, which is incorporated into the DNA, quenches selectively the Hoechst fluorescence but does not interfere with PI. Hence, cardiomyocytes that have passed the cell cycle completely (S-phase and mitosis), will have the same PI fluorescence but a reduced Hoechst fluorescence compared with G1 arrested cells, which did not proliferate (Figure 4A). As seen in Figure 4C through 4E, the number of dividing cardiomyocytes increased significantly after expression of E2F1 and E2F2 but remained unchanged after expression of E2F4. Thus, only E2F1 and E2F2, but not E2F4, stimulated mitotic cell division of cardiomyocytes although all three E2Fs induced S-phase entry.

S-Phase Entry of Cardiomyocytes After E2F-Expression Does Not Seem to Depend on D-Type Cyclins but on A- and E-Type Cyclins Whereas Induction of Mitosis Coincides With Expression of Cyclins B1 and B2

We next decided to identify cell cycle–related genes, which might be indispensable for E2F-induced S-phase entry. Because not all E2Fs induced S-phase entry of cardiomyocytes, we were able to distinguish potential E2F target genes that correlated with cell cycle progression of cardiomyocytes from those, which were upregulated by certain E2Fs but apparently dispensable for cell cycle progression.

mRNA expression levels of a panel of cell cycle-related genes were determined by quantitative real-time RT-PCR after infection with adenoviruses coding for individual E2F family members. As shown in Table 1, D-type cyclins, which are considered to be major players to overcome the G1 restriction point in various cell types, were not consistently upregulated in cardiomyocytes infected with S-phase promoting E2Fs. Clearly, D-type cyclins were not stimulated in cardiomyocytes infected with E2F2, which strongly induced S-phase entry in our experimental system. Along the same line, E2F5, which does not promote DNA-synthesis in cardiomyocytes, led to a robust induction of cyclinD2.

In contrast to D-type cyclins, the induction of A-type and particularly of E-type cyclins clearly correlated with DNA-synthesis in cardiomyocytes. As shown in Table 1, only those E2Fs, which significantly stimulated DNA synthesis, led to a parallel increase of these S-phase related cyclins. Whereas E2F1, E2F2, and E2F3 elevated cyclinA mRNA level, induction of cyclin E was primarily directed by E2F2 and E2F4, which also lacked apoptosis-inducing activities. Progression through M-phase in dividing cells is known to depend on activation of B-type cyclins.[18] The finding that both cyclin B1 and B2 are induced only after expression of E2F1 and -2 is in line with our data that only E2F1 and E2F2 but not E2F4 lead to a full completion of the cell cycle and a division of cardiomyocytes.

Because cell cycle progression does not only require the presence of cell cycle inducers but also the absence of cell cycle inhibitors such as cyclin-dependent kinase inhibitors (CKIs), which might be activated in cardiomyocytes in response to untimed proliferation signals, we measured changes in transcriptional activation of p15INK, p16INK, and p19INK after directed E2F expression in primary cardiomyo-

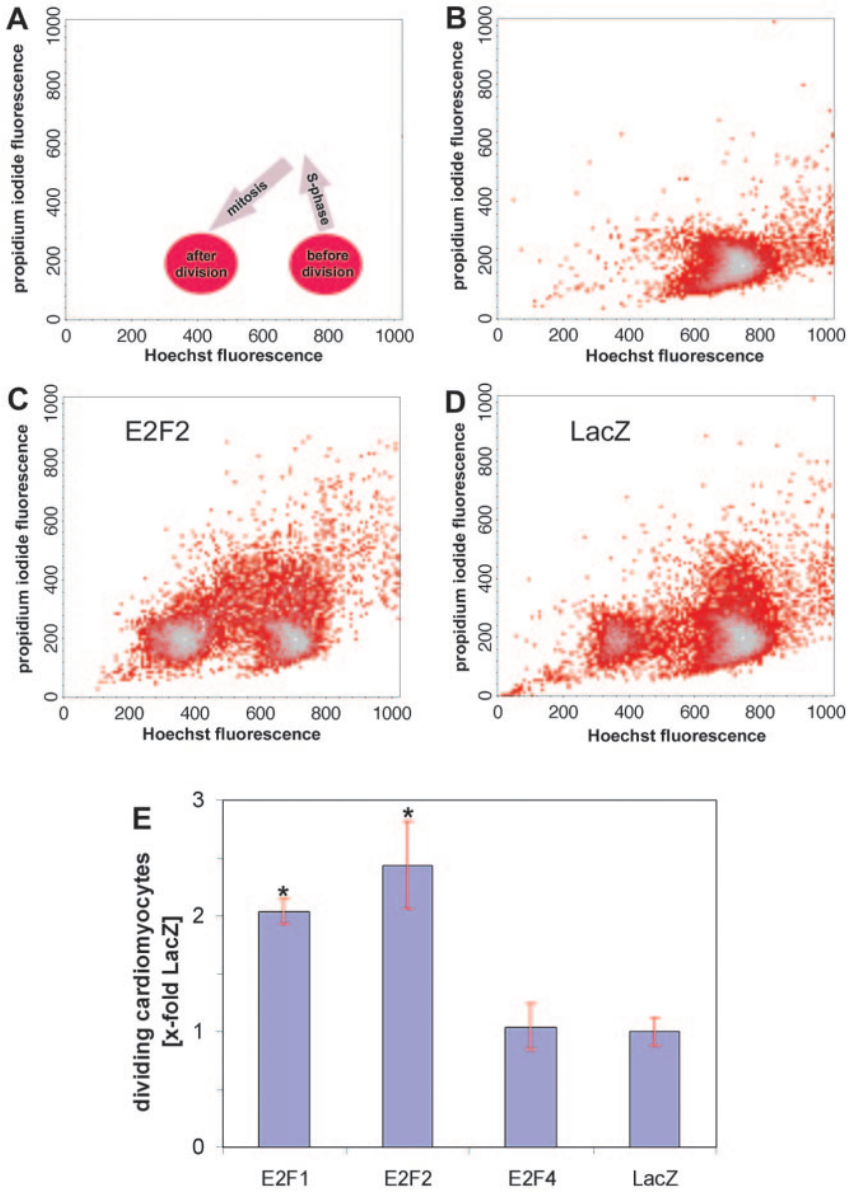

Figure 4. Expression of E2F1 and E2F2 but not E2F4 induces mitosis of cardiomyocytes. Cell cycle progression of cardiomyocytes was analyzed using the flowcytometric BrdU-Hoechst assay (see text for details). Analysis was restricted to MF20-positive cells to exclude contaminating fibroblasts (not shown; compare Figure 1A). A, Schematic bivariate diagram of cycling cardiomyocytes. B, In cultures lacking BrdU, cells, which had completed mitosis, were undistinguishable from growth-arrested cells in G1-phase (B; negative control). C and D, Exposure to BrdU and subsequent incorporation into DNA quenches Hoechst fluorescence and allows identification of cardiomyocytes, which have completed mitosis. C, Number of dividing cells increased after expression of E2F2 but not after infection with a LacZ adenovirus (D). E, Bars indicate the fold induction of mitosis after expression of E2F1, E2F2, and E2F4 normalized to lacZ-infected cells. *$P<0.05$.

cytes (Table 1). Despite an upregulation of p15INK after E2F2, of p16INK after E2F1 and E2F5, and of p19INK after E2F1 expression, no striking correlation between E2F expression, INK-type CKI activation, and S-phase entry or induction of apoptosis was noted.

Differential Induction of Apoptosis Promoting Genes by E2F Transcription Factors in Cardiomyocytes

The striking differences between individual E2Fs in induction of apoptosis suggested that the activation of apoptosis promoting genes by distinct E2Fs differ significantly. We therefore investigated whether p21CIP/WAF and Apaf-1, which are known to be direct targets of E2F1 in some cell types are also activated in cardiomyocytes and whether other E2F family members evoked similar responses. As shown in Table 2, E2F1, but also its proapoptotic relatives E2F3 and E2F5, strongly stimulated p21CIP/WAF expression. The induction of p21CIP/WAF expression was even 3-times higher using E2F5 compared with E2F1 (Table 2). In addition, we noted a robust induction of bax and caspase6 after expression of E2F1 and E2F5. Interestingly, expression of the proapoptotic genes p21CIP/WAF and caspase6 were decreased after treatment with E2F2 and E2F4 reflecting selective induction of cell cycle progression without induction of apoptosis by these E2F family members.

Previous studies demonstrated that E2F1 has a special ability to induce apoptosis and that stimulation of expression of $p19^{ARF}$ is an early step in this process in several cell types. Therefore, we analyzed activation of $p19^{ARF}$ in cardiomyocytes after overexpression of individual E2Fs to disclose whether induction of $p19^{ARF}$ is a common feature of E2F-induced cell death or occurs only in E2F1 expressing cells. Interestingly, $p19^{ARF}$ mRNA was significantly upregulated only after E2F1 expression (Table 2) highlighting the distinctive apoptotic potential of E2F1, which needs to be sup-

TABLE 1. mRNA Expression of Cell Cycle–Related Genes After Expression of E2F Transcription Factors in Cardiomyocytes

Gene	E2F1	E2F2	E2F3	E2F4	E2F5
Cyclin A	**4.50**	**1.82**	1.41	*0.48*	*0.08*
Cyclin B1	**1.68**	**1.77**	0.39	0.67	0.06
Cyclin B2	**1.78**	**1.55**	0.47	0.52	0.15
Cyclin D1	1.16	0.70	**5.59**	**2.30**	1.22
Cyclin D2	**>100**	0.64	**>100**	**>100**	**>100**
Cyclin D3	0.82	0.81	0.88	0.96	1.06
Cyclin E	**30.4**	**82.6**	**19.9**	**98.4**	**6.1**
cdk2	0.60	**2.57**	**4.92**	**1.55**	0.64
cdk4	1.13	0.92	**1.75**	1.39	0.89
$p15^{INK}$	0.60	**3.30**	0.64	*0.38*	*0.44*
$p16^{INK}$	**3.59**	1.06	1.26	1.24	1.55
$p19^{INK}$	**1.70**	0.97	*0.25*	*0.42*	*0.07*

Mean values, n≥3. Bold indicates more than 150% of control; italic, less than 50% of control. Relative expression level=$(GeneX^{E2F}/G3PDH^{E2F})/(GeneX^{control}/G3PDH^{control})$.

pressed during normal proliferation. We also observed a strong stimulation of mdm2 by E2F1. Mdm2 is generally believed to inhibit apoptosis and hence might counteract the capacity of E2F1 to trigger apoptosis. Clearly, however, this mechanism did not suffice to balance the apoptotic potential of E2F1 in cardiomyocytes. Because our cultures did contain a minor population of noncardiomyogenic cells, it has to be kept in mind that such cells might also contribute to a small degree to the transcriptional changes measured by RT-PCR.

Differential Association of Adenoviral Delivered E2F2s With Pocket Proteins in Cardiomyocytes

E2F-dependent transcription is negatively regulated by three different pocket proteins (Rb, p107, and p130), which also serve as adaptors to recruit various histone modifying enzymes to E2F bindings sites. E2Fs bound to Rb/p107/p130 are not only incapable to stimulate cell cycle progression but also actively repress transcription of several genes containing E2F binding site.[19] We therefore wanted to analyze to what extent adenoviral-delivered E2Fs interacted with different pocket proteins in cardiomyocytes using band-shift and ELISA based complex formation tests. As shown in Figure 5A, Rb complexed primarily with E2F1, E2F2, and E2F3,

TABLE 2 mRNA Expression of Apoptosis-Related Genes After Expression of E2F-Transcription Factors in Cardiomyocytes In Vitro

Gene	E2F1	E2F2	E2F3	E2F4	E2F5
p53	1.43	0.89	**2.12**	1.36	**1.51**
p21CIP/WAF	**11.83**	*0.25*	**19.76**	0.96	**32.26**
Apaf-1	1.37	1.05	1.20	1.07	1.43
caspase6	**1.52**	0.85	1.14	0.95	**1.62**
bax	**1.85**	1.37	1.37	1.26	**1.75**
mdm2	**19.68**	*0.32*	**2.05**	0.54	1.40
p19ARF	**5.99**	1.08	0.93	0.69	1.40

Mean values, n≥3. Bold indicates more than 150% of control; italic, less than half of control. Relative expression level=$(GeneX^{E2F}/G3PDH^{E2F})/(GeneX^{control}/G3PDH^{control})$.

whereas p107 only showed a significant interaction with E2F2 and E2F4. Interestingly, the majority of E2F proteins were not bound to pocket proteins as indicated by the much higher concentration of extract needed to detect Rb and p107 compared with E2F1 and E2F2 in the ELISA assay (Figure 5B). Based on the different concentrations needed to yield similar signal strength, we calculated that the concentration of unbound E2F1 and E2F2 exceeded the bound fraction by ≈10-fold probably owing to the massive delivery of E2F by the adenoviral vectors. Similarly, we readily detected individual E2F-complexes in conventional band-shift experiments using a radioactively labeled E2F binding site with extracts from E2F-transfected cardiomyocytes (Figure 6) that were apparently devoid of bound pocket proteins. Correspondingly, we were unable to super-shift the E2F-complexes using antibodies against Rb, p107, and p130, respectively, most probably due to the limited sensitivity of this system (data not shown).

Discussion

Replacement of dead or dying cardiomyocytes would certainly herald a new era of chronic heart failure treatment. Although autologous cell transplantations using, for example, muscle progenitor cells or adult stem cells[20] show some promise, it is currently unclear whether the benefit, mode of action, and potential side effects of stem cell transplantations justify a broad application. An alternative approach is the stimulation of proliferation of remaining cardiomyocytes. Such a strategy would be particularly useful in situations of localized myocardial damage as, eg, after myocardial infarction.

The E2F-transcription factors are key regulators of S-phase entry in a wide variety of cell types. In the last years, several attempts were made to increase E2F-activity or to express different oncogenes to bypass the cardiomyocyte's restriction point and stimulate proliferation. It has been shown that both overexpression of E2F1[8] and E1A-induced elimination of pRb, which in turn leads to increased E2F-activity,[21] can reactivate DNA synthesis in cardiomyocytes. Unfortunately, however, E2F1-induced cell cycle reentry was always accompanied by a severe increase of apoptosis of cardiomyocytes. Surprisingly, no attempts have been made to use other members of the E2F transcription factor family, which also stimulate DNA synthesis in quiescent cells but show no (or less) effects on apoptosis, for induction of cell cycle entry of cardiomyocytes. Beside E2F1, especially E2F2 and E2F3 have been demonstrated to induce S-phase strongly[5] and are therefore often classified as "activating" E2Fs. Unlike E2F1, which seems to have a special role to induce apoptosis, E2F2 and E2F3 have not been linked to increased apoptosis in the majority of experimental systems, although there are reports describing a role of E2F3 in induction of programmed cell death.[5] Additionally, in a transgenic mouse model in which E2F4 was expressed by the keratin5 promoter in the epidermis Wang et al[22] found an increase of proliferation similar to E2F1 but no significant increase of apoptosis. Interestingly, these in vivo results differ from findings in cell culture, which originally reported only very poor transactivating and S-phase promoting activities for E2F4.[23]

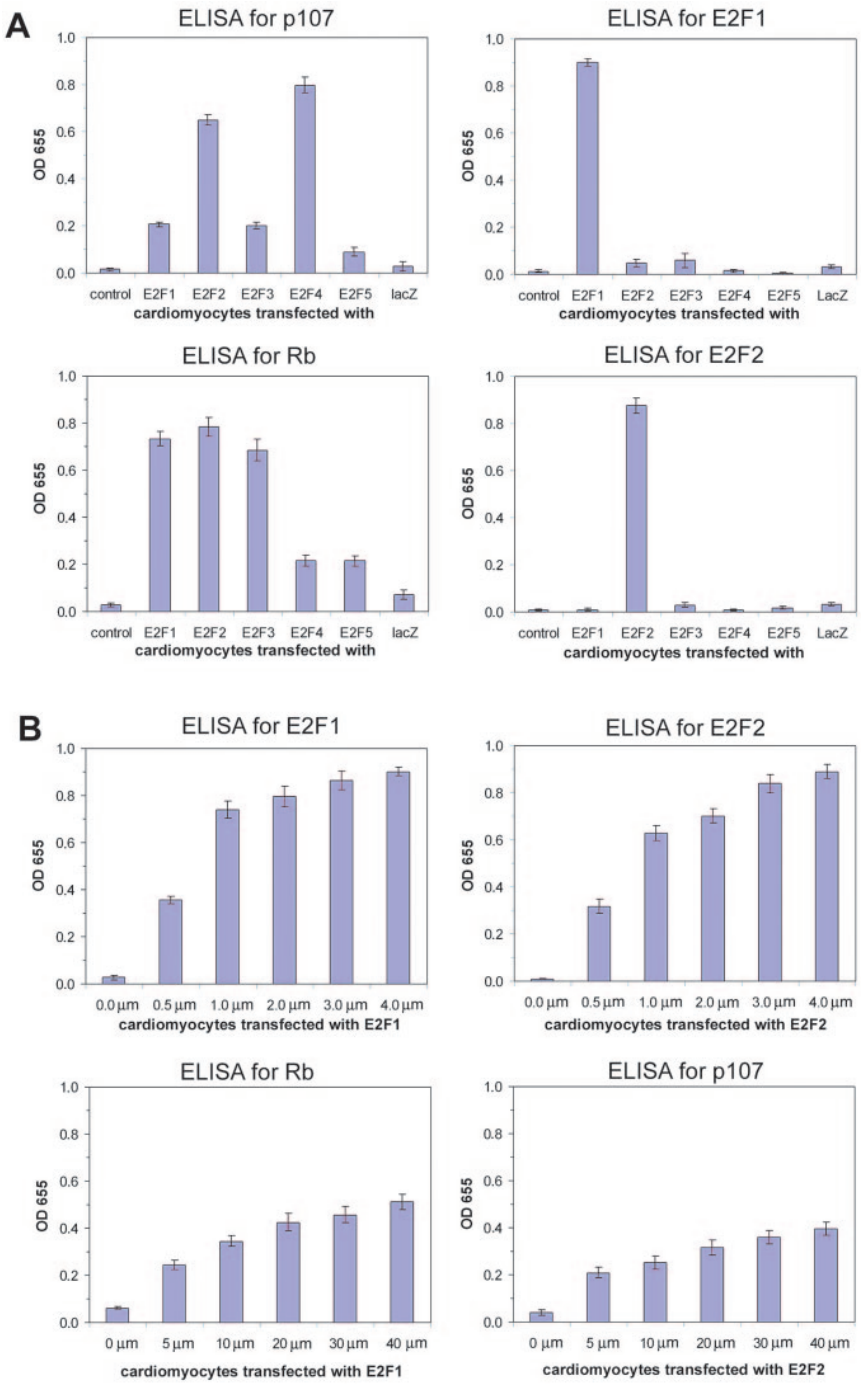

Figure 5. Differential interaction of E2F transcription factors with pocket proteins. Nuclear extracts isolated from different uninfected and infected cardiomyocytes were loaded on microtiter plates coated with an E2F binding site and reacted with antibodies against p107, E2F1, Rb, and E2F2. Bound antibodies were visualized with a secondary antibody coupled to HRP using TMB as a substrate. Absorbance was measured at 655 nm after 45 minutes (A) and 30 minutes (B). A, p107 interacted with E2F2 and E2F4, whereas Rb formed complexes with E2F1, E2F2, and E2F3. B, Different amounts of extracts were used to determine the relative abundance of E2Fs and pocket proteins capable to form complexes at the E2F binding site. Significant higher extract concentrations were necessary to detect bound pocket proteins, indicating that the concentration of pocket proteins did not suffice to bind all E2F proteins.

In this study, we have identified E2F2 and E2F4 as factors that can induce S-phase entry of cardiomyocytes without provoking apoptosis. In particular, E2F2 is a promising candidate to stimulate proliferation of cardiomyocytes because E2F2 was most potent to simulate cell cycle progression including mitosis of cardiomyocytes but did not cause any signs of apoptosis. These findings overcome restrictions of a potential therapeutic use of E2Fs that are associated with a concomitant induction of apoptosis as seen for E2F1 and E2F3. Initially, our finding that directed expression of E2F5-induced apotosis in cardiomyocytes came as a surprise because several reports demonstrated that E2F5 expression correlates with the differentiated phenotype of growth arrested cells during development.[24,25] However, none of the studies cited analyzed effects of E2F5-overexpression, which obviously is an artificial situation that might lead to effects different from the physiological role of E2F5 during development. Another observation, which also does not fit the role of E2F5 as a solitary repressor, was recently made by Ruutu and colleagues, who reported a strong correlation between elevated E2F5 levels and selective growth advantage in a HPV-33–positive cell line.[26]

The differential induction of apoptosis by distinct E2Fs in cardiomyocytes argues against a model that proposes that

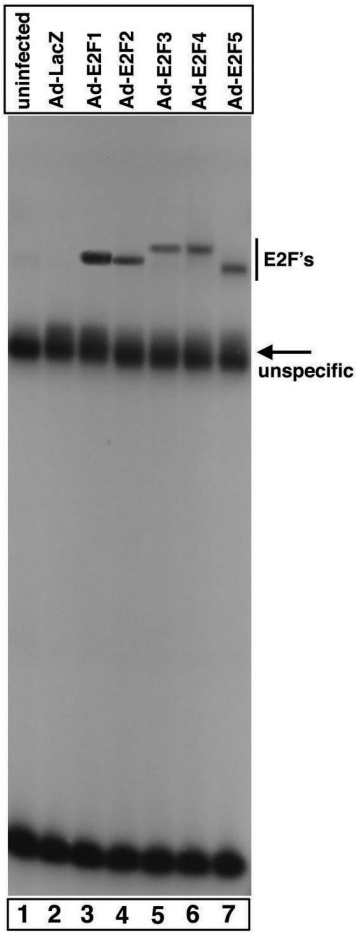

Figure 6. Binding of different E2Fs to a E2F binding site. Nuclear extracts isolated from different uninfected and infected cardiomyocytes were incubated with a radioactively labeled E2F site and analyzed by band shift analysis. Presence of unspecific complexes is indicated by an arrow. No complex formation was detected in uninfected or lacZ-infected cells, whereas E2F-infected cells gave rise to slower migrating complexes.

E2F1, E2F2, and E2F3 indiscriminately provoke S-phase entry and apoptosis depending on expression levels.[7] In contrast, directed expression of E2F2 in cardiomyocytes resulted in a reduced expression of various apoptosis-related genes including p21CIP/WAF and caspase 6 restraining the activity of proapoptotic pathways. According to our knowledge, this is the first example of suppression of proapoptotic genes by a member of the E2F-family.

In line with our findings, several studies demonstrated that E2F transcription factors are involved in cardiac hypertrophy.[17] Hyperplasia and hypertrophy are relatively common reactions of myocardial cells to adverse conditions depending on the developmental stage of the cells.[27] The overall effect of E2Fs might strongly depend on the current status of the cell, the concentration level of E2Fs, and their interaction partners and of pro- and antiapoptotic effectors, which thereby determine whether a cardiomyocyte undergoes hypertrophy, cell division, or programmed cell death.

The divergent effects of individual E2Fs on cell cycle and apoptosis in cardiomyocytes were reflected by a differential stimulation of several cell cycle control genes because we observed induction of different cyclins and CKIs depending on the respective E2F. Interestingly, we found that mRNA levels of D-type cyclins in our experiments did not parallel S-phase entry. Although surprising, the finding that expression of E2F2 leads to S-phase entry of cardiomyocytes without induction of D-type cyclins confirms previous reports demonstrating that E2F1 can overcome growth arrest in the absence of D-type cyclin/cdk-activity[28] and that alternative pathways are able to direct proliferation of cells in absence of cyclin-D activity.[29]

On the other hand, transgenic overexpression of D-type cyclins was reported to induce DNA synthesis in cardiomyocytes,[30] which seems to be in conflict with our data. However, it is not unlikely that a long-term exposure to increased cyclinD levels in transgenic animals results in stronger effects, compared with a short-term exposure, and to activation of further growth promoting pathways.

In contrast to D-type cyclins, A- or E-type cyclins were always found elevated after infection with S-phase inducing E2Fs. We did not find evidence that induction of cyclin A participates in programmed cell death of cardiomyocytes despite a reported role of cyclin A in hypoxia-induced apoptosis of cardiomyocytes.[31]

A favored model is that E2F triggers apoptosis via transcriptional activation of $p19^{ARF}$[32] preferably by E2F1, although E2F1 can provoke apoptosis in the absence of $p19^{ARF}$, indicating the presence of additional pathways for E2F-induced apoptosis.[33,34] In addition, it has been described that E2F2 and E2F3 also activate the ARF gene in fibroblasts without obligatory induction of programmed cell death.[35,36] We only found a relevant induction of $p19^{ARF}$ in cardiomyocytes after expression of E2F1 but not of E2F2 and E2F3. This finding suggests (1) that E2F1 specifically activates the ARF gene as already proposed by DeGregori et al[5] and (2) that alternative pathways mediate E2F3 and E2F5-induced apoptosis. Beside other effects that are caused by E2Fs such as induction of p73 and inhibition of TNFR-associated survival response,[7] the induction of p21CIP/WAF, which has been shown to induce apoptosis in various cells,[37] is a likely explanation.

The specificity by which individual E2Fs activate putative downstream target genes remains an unsolved issue. In the course of our experiments, we found that proapoptotic E2Fs induced mdm2, p21CIP/WAF, caspase 6, and Apaf-1, whereas E2F2 and E2F4 had the opposite effect and repressed transcription of these genes. Likewise, it has been shown that E2F1 can bind directly and activate p21CIP/WAF and apaf-1 promoters[38] while we also observed an induction by E2F3 and E2F5 but not by E2F2 or E2F4, respectively. Further experiments will reveal the cause for specific effects of individual E2Fs on target promoters.

In summary, we found that the transcription factor E2F2 is an excellent candidate to stimulate proliferation of cardiomyocytes. Induction of cell cycle progression by E2F2 and E2F4 is not accompanied by increased death rates of cardiomyocytes. In contrast, both E2F2 and E2F4 seemed to confer increased resistance to apoptosis, further supporting a potential application in repair of damaged myocardium and treatment of heart failure.

Acknowledgments

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2.5 Auswirkungen der gezielten Expression von E2F2 und E2F4 auf die Zellzyklusaktivität terminal differenzierter Kardiomyozyten *in vivo*

Im Rahmen eigener Untersuchungen konnte gezeigt werden, dass in Zellkulturexperimenten die Expression der Transkriptionsfaktoren E2F2 bzw. E2F4 die Proliferation von Kardiomyozyten induziert, ohne gleichzeitig zu vermehrten Apoptosen zu führen (siehe 2.4.; [Ebelt et al. 2005]). Eine Limitation der genannten Experimente war jedoch in dem Umstand zu sehen, dass sämtliche Analysen bislang in neonatalen Herzmuskelzellen durchgeführt worden waren. In diesen Zellen ist die Zellzyklusarretierung noch nicht vollständig abgeschlossen, so dass – anders als in terminal differenzierten adulten Kardiomyozyten – noch in geringem Umfang die Fähigkeit zur Proliferation besteht. Außerdem wurden alle Ergebnisse ausschließlich anhand von Zellkulturexperimenten gewonnen, wohingegen bislang keine Daten aus *in vivo*-Untersuchungen vorlagen. Vor diesem Hintergrund sollten deshalb in einer weiteren Serie von Experimenten die Auswirkungen der gezielten adenoviralen Expression von E2F2 und E2F4 in den Herzen von Mäusen jenseits der Perinatalperiode *in vivo* analysiert werden.

Unter Verwendung der zuvor etablierten Technik (siehe 2.3.; [Ebelt und Braun 2003]) wurden E2F2 und E2F4 in den Herzen von Mäusen durch intrathorakale Injektion der beschriebenen adenoviralen Vektoren exprimiert. Als Kontroll-Gruppe wurden sowohl unbehandelte Mäuse als auch Tiere, die eine Injektion eines Kontroll-Vektors (Ad-EGFP) erhalten hatten, verwendet. Zu unterschiedlichen Zeitpunkten nach den Virusinjektionen wurden die Herzen der Tiere entnommen und histologisch analysiert bzw. molekularbiologischen Analysen zugeführt. In weiteren Experimenten erfolgte darüber hinaus die Virus-Injektion bei adulten Mäusen unter mikroskopischer Kontrolle direkt in das Myokard entsprechend eines bereits zuvor mehrfach beschriebenen Vorgehens [Kass-Eisler et al. 1993; Kass-Eisler et al. 1994; Agah et al. 1997].

Für die Analyse der Zellzyklusaktivität (DNA-Synthese, Mitose) von Herzmuskelzellen anhand histologischer Schnitte ist es von entscheidender Bedeutung, eine eindeutige Zuordnung vornehmen zu können, ob ein bestimmter Zellkern tatsächlich zu einem Kardiomyozyten oder aber zu einer kardialen Nicht-Muskelzelle gehört. Da dies mittels konventioneller mikroskopischer Verfahren in Wildtyp-Mäusen nicht mit ausreichender Sicherheit möglich ist (Übersicht in [Soonpaa und Field 1998]), wurde in den eigenen Untersuchungen ein transgener Reporter-Mausstamm verwendet, bei dem die Zellkerne aller Kardiomyozyten selektiv durch das Enzym β-Galaktosidase (LacZ) markiert sind (αMHC-nLacZ, [Soonpaa und Field 1994]).

Es konnte gezeigt werden, dass die Injektion der adenoviralen Vektoren zu der intendierten Expression von E2F2 bzw. E2F4 in den Herzen der Versuchstiere führt, während dies erwartungsgemäß in den Herzen von Kontrolltieren nicht zu beobachten ist. Histologische Analysen belegen, dass die exprimierten E2Fs nukleär lokalisiert sind, wie dies für Transkriptionsfaktoren zu fordern ist.

14 Tage nach den Virus-Injektionen sind die relativen Herzgewichte der E2F2-exprimierenden Versuchstiere signifikant größer als die unbehandelter Kontrolltiere, nach Expression von E2F4 oder EGFP ist dieser Effekt nicht zu beobachten. Anhand histologischer Untersuchungen kann gezeigt werden, dass sich nur nach E2F2-Expression eine signifikante Zunahme von Kardiomyozyten mit Zeichen der Zellzyklusaktivität findet. Sowohl der Anteil von Kardiomyozyten, die in den 24 Stunden vor Versuchsende die S-Phase durchlaufen haben (nachweisbar am stattgehabten BrdU-Einbau), als auch der Anteil der Herzmuskelzellen, die sich zum Zeitpunkt der Organentnahme in der M-Phase bzw. der Phase der Zytokinese befinden, ist nach E2F2-Expression signifikant erhöht. Der Zellzykluseintritt ist dabei in Übereinstimmung mit den zuvor erhobenen Zellkulturdaten nicht von einer Zunahme von Kardiomyozyten-Apoptosen begleitet. Die genannten Effekte lassen sich auch dann beobachten, wenn die Virusinjektionen bei adulten Tieren erfolgen, so dass E2F2 offenbar nicht nur zu einer Verzögerung des Zellzyklusaustritts, sondern tatsächlich zu einer Induktion von Proliferationsvorgängen von Kardiomyozyten führt. Die absolute Anzahl von Herzmuskelzellen, bei denen durch die Expression von E2F2 Zeichen der Zellzyklusaktivität induziert werden, ist jedoch zahlenmäßig nur sehr gering. So findet sich bei adulten Tieren insgesamt nur bei 33 von 54904 untersuchten Kardiomyozyten (=0.06%) der Nachweis einer stattgehabten S-Phase-Induktion – obwohl durch die verwendete Methodik die Expression von E2F2 wie erwähnt in nahezu 80% der Zellen sichergestellt ist.

Die Experimente zeigen des weiteren, dass sowohl die Expression von E2F2 als auch von E2F4 zu einer signifikanten Zunahme der Kardiomyozyten-Querschnittsfläche (*myocyte cross sectional area*, MCSA) bei 14 Tage alten Tieren führt, was als Ausdruck einer Hypertrophie-Induktion anzusehen ist.

Mittels quantitativer mRNA- und Protein-Bestimmung kann belegt werden, dass die gezielte Aktivierung von E2F2 nicht eine veränderte Expression von cdk-Inhibitoren (*cyclin dependent kinase inhibitors*, CKI) nach sich zieht. Dies stellt insofern einen interessanten Befund dar, als der sich unter physiologischen Bedingungen einstellende Zellzyklusarrest in Kardiomyozyten maßgeblich durch die Aktivierung des cdk-Inhibitors p21 getragen wird [Engel et al. 2003]. Dagegen findet sich in Übereinstimmung mit den zuvor beschriebenen

Zellkulturexperimenten eine signifikante Induktion der Cycline A und E, was die Bedeutung dieser Faktoren bei der Zellzyklusregulation von Herzmuskelzellen erneut unterstreicht.
Zusammenfassend bestätigen die *in vivo*-Experimente das Potential von E2F2, in Kardiomyozyten den Zellzyklusarrest aufzuheben, ohne unerwünschte Effekte im Hinblick auf Kardiomyozyten-Apoptosen zu induzieren. Für eine hypothetische therapeutische Anwendung im Hinblick auf die effektive Vermehrung von Herzmuskelzellen erscheint dieser Effekt jedoch bislang quantitativ nicht ausreichend.

Anlage zu 2.5.:

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E2F2 expression induces proliferation of terminally differentiated cardiomyocytes *in vivo*

Henning Ebelt[1]*, Ying Zhang[1], Alexander Kampke[1], Jia Xu[1], Axel Schlitt[1], Michael Buerke[1], Ursula Müller-Werdan[1], Karl Werdan[1], and Thomas Braun[2]

[1]*Department of Medicine III, University of Halle-Wittenberg, Ernst-Grube-Strasse 40, 06097 Halle, Germany; and* [2]*Max-Planck-Institute for Heart and Lung Research, Parkstrasse 1, 61231 Bad Nauheim, Germany*

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Aims In previous experiments we have demonstrated that expression of the transcription factors E2F2 and E2F4 is sufficient to induce proliferation of isolated primary cardiomyocytes from newborn rats and mice. We now wanted to analyse whether E2F2 or E2F4 are also able to promote cell cycle progression of adult cardiomyocytes *in vivo*, which unlike cardiomyocytes from newborn rodents lack the ability to undergo cell proliferation.

Methods and results E2F2 or E2F4 was expressed in hearts of mice at different developmental stages using adenoviral vectors. Effects regarding proliferation, hypertrophy, and apoptosis were analysed on histological sections, and quantitative assessment of cell cycle regulatory genes was performed by real-time PCR (polymerase chain reaction) and western blot. We found that both E2F2 and E2F4 can stimulate hypertrophic cell growth of cardiomyocytes. However, only directed expression of E2F2 but not of E2F4 was sufficient to induce proliferation of cardiomyocytes. Expression of E2F2 *in vivo* did not increase the percentage of apoptotic cardiomyocytes but down-regulated the expression of the pro-apoptotic genes caspase-6 and apaf-1. Further analysis of the cell cycle regulatory machinery revealed that expression of E2F2 caused a strong induction of cyclin A and E while the expression of cyclin-dependent kinase inhibitors (CKIs) such as p21 was not affected.

Conclusion We conclude that a limited induction of cardiomyocyte cell proliferation can be achieved by E2F2-mediated stimulation of cyclin A and E expression without a reduction of CKIs.

1. Introduction

A broad spectrum of human heart diseases is based on the inability to replace dead or damaged cardiomyocytes during adulthood. In the last few years several attempts have been made to induce heart regeneration. The majority of experiments were based on stem cell transplantation strategies, which has resulted in clinical studies yielding partially encouraging outcomes.[1–3] Other experimental approaches demonstrated that it is feasible to induce proliferation of cardiomyocytes by targeted interference with the mechanisms that normally maintain the blockade of the cell cycle. Pioneering experiments have shown that the activation of E2F transcription factors—either by oncoprotein-induced inhibition of pocket proteins[4] or by directed expression of E2F1[5,6]—is sufficient to induce (limited) proliferation of heart muscle cells. However, cell cycle re-entry was most often accompanied by induction of apoptosis thereby limiting a potential therapeutic value.[6,7] In a previous study we revealed that it is feasible to induce proliferation of cardiomyocytes isolated from newborn rats and mice by targeted expression of E2F2 or E2F4 without stimulation of apoptosis.[8] A clear limitation of our preceding study was the use of neonatal cardiomyocytes, which still show a certain level of proliferation since cardiomyocytes lose their ability to divide only within the first days after birth.[9,10] Additionally, all results were obtained from cell culture experiments, which left open the validity of this approach for the intact heart. To overcome these obstacles we now analysed the effects of overexpression of E2F2 and E2F4 in the adult mouse heart *in vivo*. Here, we demonstrate that both E2F2 and E2F4 induce hypertrophic cell growth but only E2F2 not E2F4 is able to induce proliferation of terminally differentiated cardiomyocytes *in vivo*. E2F2 does not induce but inhibits apoptosis probably by reduction of the expression of the pro-apoptotic genes caspase-6 and apaf-1.

* Corresponding author. Tel: +49 345 557 2113; fax: +49 345 557 2684.
E-mail address: henning.ebelt@medizin.uni-halle.de

2. Methods

2.1 Adenoviral expression constructs

Adenoviruses encoding E2F2, E2F4, and EGPF have been described before.[8] They were handled according to standard procedures with HEK293 as the packaging cell line. Viral titers were determined by plaque assay.[8,11]

2.2 Adenoviral transfection of mouse hearts *in vivo*

For all *in vivo* experiments transgenic mice carrying a nuclear localized LacZ-reporter gene under the control of the αMHC-promotor[12] were used to enable definitive identification of cardiomyocytes on histological sections (kindly provided by Dr. L.J. Field). Transfection of hearts of newborn mice has been described before in detail.[11] Newborn mice were anaesthetized by cooling on ice for $\sim$2 min and put in front of a cold light source to visualize the silhouette of the heart. Using a Hamilton syringe with a 26-gauge needle a total volume of 10 μL was injected into the thoracic cavity beside the heart at a left parasternal position. Finally, animals were re-warmed and put back to their mothers. After 13 days, the mice received a single i.p.-injection of BrdU [100 mg/kg body weight (BW)] and were then sacrificed the next day. The hearts were removed and either embedded for cryosectioning or shock-frozen in liquid nitrogen for RNA (ribonucleic acid) and protein isolation, respectively. Adult mice ($>$3 months) were anaesthetized with 2.5% isofluorane and mechanically ventilated, left-sided thoracotomy was performed in the fourth intercostal space. Under microscopic control the heart was gently uncovered and 10 μL of the virus suspension was injected two times in the left ventricular free wall and once in the interventricular septum.[6,13,14] On day 3 and 4 after surgery, the mice received an i.p.-injection of BrdU (100 mg/kg BW) before they were finally sacrificed on day 5.

The investigation conforms to 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).

2.3 Histological analysis

Ten micro meter cryosections were obtained from hearts of neonatal and adult mice and stained either by X-gal or anti-LacZ to identify nuclei of cardiomyocytes. Immunoassays to detect BrdU incorporation (anti-BrdU; Vector Laboratories), phosphorylated histone-3 (anti-PhoshoH3; Upstate), AuroraB-kinase (anti-AuroraB, Abcam), and activated caspase-3 (anti-activ. casp3, Promega) were performed according to the manufacturer's specifications. For terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labelling (TUNEL) cryosections were incubated with TUNEL labelling mixture (TdT and biotin-11-dUTP; Fermantas) at 37°C for 60 min. The resulting signal was visualized using VECTASTAIN Elite ABC Kit (Vector Laboratories).

For all quantifications using immunocytochemistry (ICC), a minimum of four slides containing cryosections from distinct regions of the heart were analysed. All hearts were embedded in long axis orientation to facilitate identification of right ventricular and left ventricular free walls and the interventricular septum after sectioning.

Expressions of E2F2 and E2F4 were confirmed by western blot and ICC (antibodies: sc-632 and sc-866, Santa Cruz Biotechnology). Hypertrophy of cardiomyocytes was determined by measuring myocyte cross-sectional area as described.[15]

2.4 Ribonucleic acid isolation and quantitative real-time polymerase chain reaction

Total RNA was isolated using Trizol according to standard techniques. Complementary deoxyribonucleic acid (cDNAs) were synthesized using oligo-dT-primers as described.[8] Real-time polymerase chain reaction (PCR) was performed in 25 μL total volume containing 1 μL cDNA, 0.1U *Taq*-polymerase, 5 nmol forward primer, 5 nmol reverse primer, 10 μmol dNTPs, 0.25 μL $\times$100 fluorescein, and 0.5 μL $\times$10 SybrGreen I (Sigma) as described.[8] Primer sequences and specific amplification conditions can be obtained from the authors upon request.

2.5 Protein isolation and western blot

For protein isolation, the hearts were lysed in RIPA-buffer [50 mM Tris–HCl, pH 7.4, 1% Triton X-100, 0.2% sodium deoxycholate, 0.2% sodium dodecyl sulphate (SDS), 1 mM ethylenediaminetetraacetic acid containing 1 mM phenylmethylsulphonyl fluoride], 5 μg/mL Aprotinin and 5 μg/mL Leupeptin (all from Sigma). Thirty microgram of protein lysates were separated on 10% SDS–PAGE (polyacrylamide gel electrophoresis) gels and transferred onto nitrocellulose membranes (Invitrogen Life Technologies, Groningen, The Netherlands). Immunoreactive proteins were visualized with corresponding horseradish peroxidase-conjugated secondary antibodies on Hyperfilm (GE Healthcare) using the SuperSignal West Pico or West Femto detection solutions (Perbio Science). Blots were scanned and analysed using ImageJ software (NIH). The following antibodies were used: anti-cyclin A (sc-751), anti-cyclin D2 (sc-593), and anti-cyclin E (sc-481; all from Santa Cruz Biotechnology); anti-cyclin D1, anti-cyclin D3, anti-cyclin-dependent kinase (CDK4), anti-$p15^{INK4}$, and anti-$p27^{Kip1}$(all from Cell Signaling Technology); anti-$p21^{Waf}$ (BD).

3. Results

3.1 Targeted expression of E2F2 counteracts cell cycle exit of cardiomyocytes and re-induces proliferation of adult cardiomyocytes

In a first series of experiments we determined whether expression of E2F2 or E2F4 counteracts the cell cycle exit of cardiomyocytes in the perinatal period. Adenoviral vectors encoding E2F2, E2F4, or a control vector (EGFP) were injected into the thoracic cavity of newborn mice, which results in a lasting and efficient gene expression in the heart.[11] The directed expression of E2F2, E2F4, or EGFP was confirmed by ICC and western blot, respectively (*Figure 1*). Fourteen days after application of the viruses we found a significant increase in the heart weights from animals that received Ad-E2F2 (*Table 1*). Unexpectedly, this phenomenon was not observed after E2F4 expression although E2F4 is sufficient to induce S-phase entry in cultured cardiomyocytes from newborn rodents *in vitro*.[8] In order to further explore the mechanisms responsible for the increase in heart weight induced by E2F2, we determined the number of proliferating and apoptotic cardiomyocytes in histological sections (*Figure 2*). Fourteen days after targeted expression of E2F2 we detected a significant increase in the number of cardiomyocytes within S-phase as demonstrated by incorporation of BrdU (*Table 2*). We also found a significant increase in the number of phosphorylated histon-H3 positive cardiomyocytes (*Table 2*) and cardiomyocytes expressing AuroraB-kinase (*Table 2*), which suggested that these cardiomyocytes completed the entire cell cycle including mitosis and cytokinesis. Expression of E2F4 resulted only in a slight increase in BrdU- and phoshorylated histon-H3 positive cardiomyocytes in comparison with untreated control animals and EGFP-treated mice, which was statistically not significant. We also investigated whether the E2F transcription factors have an impact on hypertrophic cell growth of cardiomyocytes. Interestingly, both E2F2- and E2F4-induced hypertrophy of cardiomyocytes resulting in enlarged cross-sectional areas of myocytes (*Figure 3*).

Since forced induction of proliferation often coincides with increased rates of apoptotic cell death[5–7] we determined the effects of E2F2 and E2F4 on apoptosis. Apopotic cells were visualized by staining of activated caspase-3, which identifies cells that are irrevocably committed to apoptosis.[16] Histological analysis revealed that E2F2 significantly reduced the number of apoptotic cells in comparison with EGFP-expressing mice while directed expression of E2F4 led to a marked increase in apoptosis (*Table 3*). To further distinguish between apoptotic cardiomyocytes and non-muscle cells, we used the TUNEL assay in combination with cardiomyocyte specific X-gal staining. This approach revealed that pro-apoptotic effects of E2F4 were restricted to non-cardiomyocytes while cardiomyocytes were not adversely affected by E2F2 or E2F4 (*Table 3*). To define the molecular pathway that might control E2F4-mediated apoptosis we analysed the expression of apoptosis-related genes. Interestingly, we detected a clear increase in the mRNA expression of caspase-6 and apaf-1 in hearts infected with Ad-E2F4 and Ad-EGFP, which was not seen in E2F2-treated animals (*Table 4*). Furthermore, we found a repression of the pro-aopototic gene bax in hearts infected with adenoviruses encoding E2F-transcription factors and the EGFP-expressing control vector compared with uninfected controls animals (*Table 4*). Apparently, E2F2 specifically repressed transcription of the pro-apoptotic genes caspase-6 and apaf-1 while repression of the pro-apoptotic gene bax was due to an unspecific effect of the adenoviral backbone.

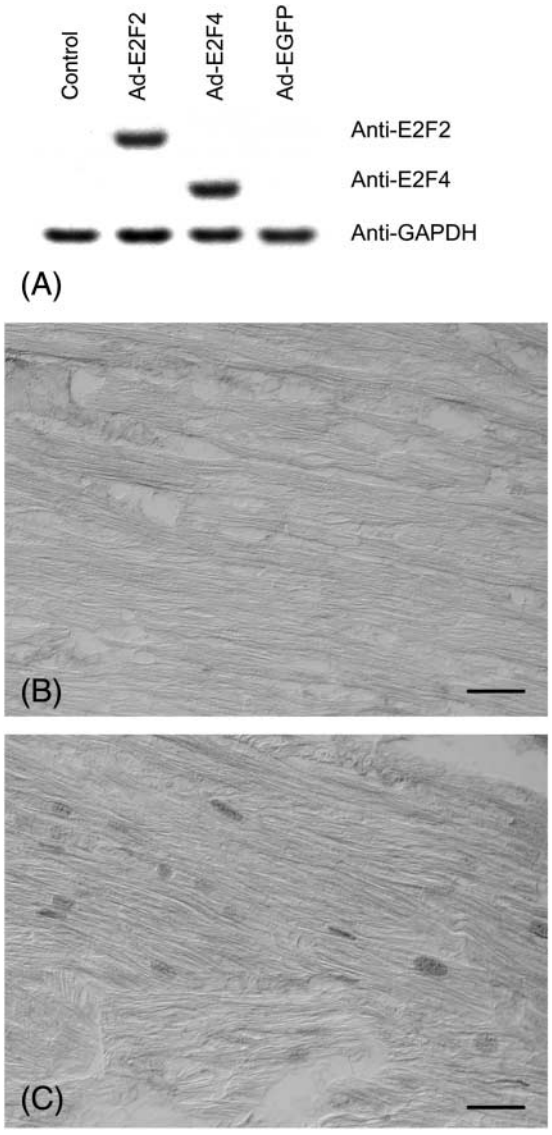

Figure 1 Directed expression of E2F transcription factors in hearts of mice 14 days after injection of the indicated adenoviruses: (*A*) western blot; (*B* and *C*) examples of cryosections stained with anti-E2F2 (peroxidase/diaminobenzidine). (*B*) Untreated control; (*C*) intrathoracic injection of Ad-E2F2 at day of birth. Scale bar: 10 μm.

So far, our results clearly demonstrated that E2F2 led to an increase in cardiomyocyte proliferation in 14-day-old mice without a concomitant increase in apoptosis. Although no signs of cardiomyocyte proliferation (neither S-phase activity nor mitosis) can be detected in hearts of untreated control mice at P14 (*Table 2*) it was not possible to distinguish whether the expression of E2F2 led to a delay of cell cycle exit or to a real re-induction of proliferation. Therefore, we turned to the adult situation and tested the effects of E2F2-expression on proliferation of cardiomyocytes in 3-month-old mice. As shown in *Table 5*, directed expression of E2F2 was sufficient to induce DNA-synthesis in terminally differentiated adult cardiomyocytes although the increase in the number of mitotic cells induced by E2F2 did not reach statistical significance. Similar to the situation in young mice at P14 we did not find signs of increased apoptosis induced by E2F2-activity.

3.2 E2F2 induces expression of cyclins D3, A, and E but does not affect expression of CDK inhibitors

In principle, it was possible that induction of cell cycle activity in cardiomyocytes after E2F2-expression was due to

Table 1 Body and organ weights of mice 14 days after intrathoracic injection of the indicated adenoviruses

Parameter	Control ($N = 7$)	E2F2 ($N = 6$)	E2F4 ($N = 9$)	EGFP ($N = 6$)
Body weight (g)	6.9 ± 0.2	6.7 ± 0.6	7.5 ± 0.5	6.4 ± 0.3
Heart (mg)	44.8 ± 1.3	$54.8 \pm 3.6^{*,\#}$	49.2 ± 3.2	48.1 ± 2.5
Lungs (mg)	90.7 ± 2.1	85.7 ± 10	93.4 ± 4.8	97.7 ± 8.5
Liver (mg)	262.1 ± 10.5	265.6 ± 24.9	293.4 ± 28.7	231.5 ± 22.1
Rel. heart (mg/g)	6.4 ± 0.2	$8.4 \pm 0.5^{*}$	6.6 ± 0.2	7.5 ± 0.2
Rel. lung (mg/g)	12.5 ± 0.4	12.8 ± 0.6	12.7 ± 0.7	15.2 ± 1.0
Rel. liver (mg/g)	37.9 ± 0.7	39.8 ± 0.9	38.2 ± 1.9	35.7 ± 1.8

$^{*}P < 0.05$ vs. control.
$^{\#}P < 0.05$ vs. EGFP.

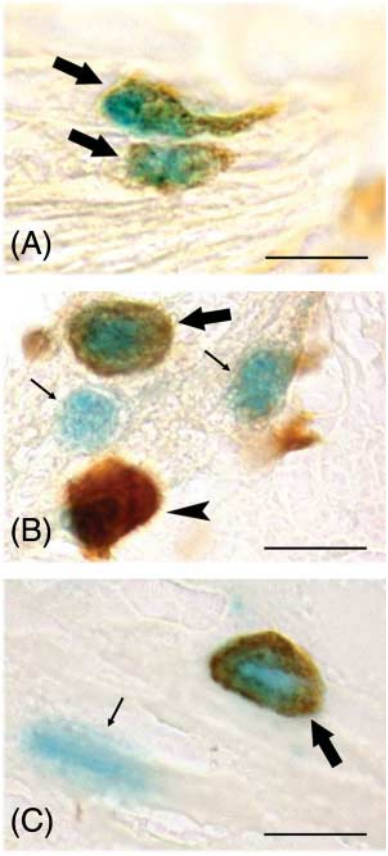

Figure 2 E2F2 stimulates cell cycle progression of cardiomyocytes *in vivo*. Histological determination of parameters of proliferation and apoptosis in cardiomyocytes (examples). All sections were first stained with X-gal to visualize the nuclei of the cardiomyocytes (transgenic reporter mouse strain αMHC-nlsLacZ) followed by specific antibodies (peroxidase). (*A*) Anti-BrdU staining (14 days after injection of Ad-E2F2), bold arrows: two neighbouring BrdU-positive cardiomyocytes. (*B*) Staining for AuroraB as a marker of cytokinesis (14 days after injection of Ad-E2F2), bold arrow: cardiomyocyte positive for AuroraB, thin arrows: cardiomyocytes negative for AuroraB, arrowhead: non-cardiomyocyte expressing AuroraB. (*C*) Terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labelling (TUNEL) assay (14 days after injection of Ad-EGFP), bold arrow: apoptotic cardiomyocyte (TUNEL-positive), thin arrow: non-apoptotic cardiomyocyte (TUNEL-negative). Scale bars: 10 μm.

stimulation of pro-proliferative cyclins or to suppression of CDK inhibitors. Especially p21WAF has been reported to act as one of the major mediators of cell cycle arrest under physiological conditions in cardiomyocytes.[17] p21WAF is also a known downstream target of p53[18], which is of particular importance since E2F transcription factors induce expression of p53 in various settings.[19]

To determine the relative ratios of pro- and anti-proliferative effectors in cardiomyocytes we measured the mRNA expression levels of several cyclins in E2F-infected and control animals. We found that several cyclins were induced after directed expression of E2F2 (*Table 6*). Interestingly, we also found a slight increase in cyclins A, E, and D3 after injection of the control vector – although the changes induced by Ad-EGFP were clearly lower compared with Ad-E2F2 and did not reach statistical significance.

Western blot analysis revealed that the protein concentrations of cyclin A, D3, and E were significantly elevated in hearts of E2F2-expressing mice (*Figures 4* and 5) while no clear differences in the expression of any cyclin-dependent kinase inhibitors (CKIs) (p15, p21, p27) were found. Taken together our results clearly argue for a stimulation of cyclin gene expression by E2F2 and against an active repression of inhibitory cell cycle regulators (*Figure 5*).

4. Discussion

Our experimental data clearly demonstrate that E2F2 is able to stimulate *de novo* proliferation of adult cardiomyocytes, which might have an impact on the therapeutic strategies to stimulate cardiac regeneration. Conceptually, our approach differs from previous studies using neonatal cells[8] or transgenic mice, which constantly express pro-proliferative effectors and hence might generate populations of continuously cycling cardiomyocytes, which have never ceased cell division. Yet, a *de novo* induction of cell proliferation in the adult heart appears to be instrumental for any therapeutic intervention.

Previously, we reported that E2F1, E2F2, E2F3, and E2F4 were all able to stimulate S-phase entry in primary cultures of neonatal cardiomyocytes although induction of DNA synthesis was accompanied by increased apoptosis in case of E2F1 and E2F3 but not E2F2 and E2F4.[8] These findings raised the question whether E2F2 or E2F4 might be used *in vivo* to unlock the cell cycle in adult cardiomyocytes. Surprisingly, we found that only E2F2 was sufficient to induce proliferation in postmitotic cardiomyocytes while E2F4 was not. None of the parameter that were increased after expression of E2F2 such as elevated DNA-synthesis, enhanced expression of mitotic markers on histological sections and increase in heart weight was seen in E2F4-treated animals. In parallel, expression of E2F2 did not induce apoptosis of cardiomyocytes but on the contrary led to the mRNA down-regulation of caspase-6 and apaf-1, which resembled the situation in cultured cardiomyocytes.

In our analyses, we have used nuclear antigens to determine both the number of apoptotic (TUNEL) and proliferating (BrdU, phosphoH3, AuroraB) cardiomyocytes (*Table 3*). Since we have used transgenic reporter mice in which the nuclei of all cardiomyocytes were labelled by the LacZ gene (αMHC-nlsLacZ) we were able to unequivocally differentiate between cardiomyocytes and non-muscle cells, which is indispensable to enable a reliable analysis of the cell cycle activity of cardiomyocytes. It is interesting to note that we detected a similar percentage of cardiomyocytes which have passed through S-phase in control samples (control: 0.002% at day 14; $<$0.002% in adult mice) as the group of L.J. Field who has established this transgenic reporter mouse strain ($<$0.003% in adult mice[20]). In contrast, other groups that relied only on the less strict histological identification of cardiomyocytes occasionally reported mitotic indices several magnitudes higher ($\approx$2% at day 14;[21] see also[22]).

In our experiments, the percentage of double-stained cardiomyocytes (e.g. all nuclei double-positive for LacZ and BrdU in relation to all LacZ-positive nuclei) describes the amount of cardiomyocytes which underwent S-phase (BrdU), mitosis (phoshoH3), cytokinesis (AuroraB), or apoptosis (TUNEL). The unequivocal identification of cardiomyocytes, which undergo cell cycle progression and apoptosis, represents a clear advantage compared with studies that used less robust antibody labelling techniques and cytoplasmatic antigens such as activated caspase-3 to detect mitotic

Table 2 Proliferation

Group	Left ventricle	Septum	Right ventricle	Total
Number of BrdU-positive cardiomyocytes 14 days after intrathoracic injection of the indicated adenoviruses (*$P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. EGFP; $^{\S}P < 0.05$ vs. E2F4)				
Control (5 mice)	1/31387	0/17451	0/14751	1/63589 (=0.002%)
E2F2 (5 mice)	6/28173	8/22904	5/16445	19/67522 (=0.028%)$^{*,\#,\S}$
E2F4 (7 mice)	2/23506	1/23727	0/13732	3/60965 (=0.005%)
EGFP (6 mice)	0/22682	0/21179	0/13056	0/56917 (=0%)
Number of phosphoH3-positive cardiomyocytes 14 days after intrathoracic injection of the indicated adenoviruses (*$P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. EGFP)				
Control (5 mice)	0/41825	0/28983	0/16841	0/87649 (=0%)
E2F2 (5 mice)	1/45911	4/34088	0/18291	5/98290 (=0.005%)$^{*,\#}$
E2F4 (7 mice)	2/64068	2/45338	0/25496	4/134902 (=0.003%)
EGFP (6 mice)	0/43487	0/36518	0/19552	0/99557 (=0%)
Number of AuroraB-positive cardiomyocytes 14 days after intrathoracic injection of the indicated adenoviruses (*$P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. EGFP)				
Control (5 mice)	0/25810	0/23734	0/10792	0/60336 (=0%)
E2F2 (5 mice)	4/30195	1/27681	0/9659	5/67535 (=0.007%)$^{*,\#}$
E2F4 (7 mice)	1/33522	0/28377	0/6792	1/68691 (=0.001%)
EGFP (6 mice)	0/33748	0/21922	0/8364	0/64034 (=0%)
Number of BrdU-positive non-cardiomyocytes 14 days after intrathoracic injection of the indicated adenoviruses (*$P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. EGFP; $^{\S}P < 0.05$ vs. E2F4)				
Control (5 mice)	1388/5184	719/3082	407/1848	2514/10114 (=24.9%)
E2F2 (5 mice)	1742/5809	991/3066	483/2216	3216/11091 (=29%)$^{*,\#,\S}$
E2F4 (7 mice)	1098/3958	849/3278	347/2114	2294/9350 (=24.5%)$^{\#}$
EGFP (6 mice)	1371/5300	696/3368	396/2438	2463/11106 (=22.2%)*

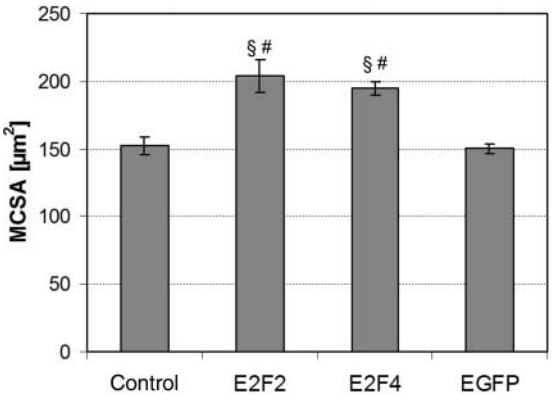

Figure 3 E2F2 and E2F4 stimulate cardiomyocyte growth *in vivo*. Myocyte cross-sectional areas (MCSA) of cardiomyocytes from hearts of 14-day-old mice treated with the indicated adenoviral expression constructs: $^{\S}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. EGFP.

and apoptotic cardiomyocytes, respectively. On histological sections the majority of nuclei of cardiomyocytes are not in the same plane as the cytoplasm as pointed out before.[20] This leads to the effect that significantly higher numbers of individual cardiomyocytes will be counted when cyoplasmic rather than nucleic markers are used (factor: 3.4 in Ref.[20]). This phenomenon makes it difficult to calculate reliably the 'net effect' of (nuclear) proliferation and (cytoplasmic) apoptosis. We have eliminated this restriction by using exclusively nuclear parameters to quantify all aspects of cell cycle activity and apoptosis. Hence it became possible to calculate the 'net effect' between proliferation and cell death indicating that expression of E2F2 favours proliferation of cardiomyocytes to a certain extent.

Another example for complex interaction of cellular processes is the increase in heart weight after expression of E2F2 but not E2F4. It seems likely that the increase in heart weights was caused by increased proliferation of both cardiomyocytes and non-muscle cells in combinaton with hypertrophy of individual cardiomyocytes since the relatively low increase in the number of cardiomyocytes was not sufficient to explain the total increase in heart mass. In the case of E2F4 this effect was apparently neutralized by enhanced apoptosis although it is possible that the observed differences in apoptosis between E2F2 and E2F4 may indicate variations in the immune response against the viral infections. In this context it seems noteworthy that we also found signs of increased apoptosis after injection of the control Ad-EGFP virus, which might be due to certain toxicity of the adenoviral vector and to the cytopathogenic effect of EGFP, which is able to cause cardiomyopathy after continous high-level expression in transgenic models.[23]

Cell cycle progression depends on the activation and inactivation of cyclin-dependent kinases, which in turn are affected by the concerted induction and degradation of regulatory cyclins. Proliferation of isolated cardiomyocytes does not appear to depend strictly on the induction of D-cyclins since directed expression of E2Fs was sufficient to overcome the restriction point even in the absence of a noteworthy stimulation of cyclin D-expression[8] – a finding which had also been described in other cells and settings before.[24] In the *in vivo* experiments presented here, E2F2 induced a strong increase in cyclin D3 expression both on the mRNA and protein level in postmitotic hearts. Nevertheless it is difficult to say whether

Table 3 Apoptosis

Group	Left ventricle	Septum	Right ventricle	Total
Number of apoptotic cells (CM and non-CM) as indicated by expression of activated caspase-3 at day 14 after intrathoracic injection of the indicated adenoviruses (normalized per 100 cardiomyocyte nuclei) (*$P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. EGFP; $^{\S}P < 0.05$ vs. E2F4)				
Control (5 mice)	11/42891	9/31821	2/18071	22/92783 (=0.024 per 100 CM)
E2F2 (5 mice)	4/43783	7/32842	4/23789	15/100414 (=0.015 per 100 CM)$^{\#,\S}$
E2F4 (7 mice)	37/57189	34/29800	12/26074	83/113063 (=0.073 per 100 CM)$^{*,\#}$
EGFP (6 mice)	17/45988	13/29662	12/29338	42/104988 (=0.04 per 100 CM)*
Number of apoptotic cardiomyocytes as indicated by TUNEL assay at day 14 after intrathoracic injection of the indicated adenoviruses (*$P < 0.05$ vs. control)				
Control (5 mice)	0/37584	0/29207	0/12234	0/79025 (=0%)
E2F2 (5 mice)	1/36828	1/35699	0/9302	2/81829 (=0.002%)
E2F4 (7 mice)	2/34150	1/31416	0/10304	3/75870 (=0.004%)
EGFP (6 mice)	3/40926	1/34786	1/7254	5/82966 (=0.006%)*
Number of apoptotic non-cardiomyocytes as indicated by TUNEL assay at day 14 after intrathoracic injection of the indicated adenoviruses (*$P < 0.05$ vs. control; $^{\S}P < 0.05$ vs. E2F4)				
Control (5 mice)	2/4292	1/4116	0/2557	3/10965 (=0.03%)
E2F2 (5 mice)	2/5772	4/4738	0/2028	6/12538 (=0.05%)§
E2F4 (7 mice)	10/5302	6/4536	1/2157	17/11995 (=0.14%)*
EGFP (6 mice)	7/5374	2/4153	2/2459	11/11986 (=0.09%)*

Table 4 Messenger ribonucleic acid expression of apoptosis-related genes at day 14 after intrathoracic injection of the indicated adenoviruses. Expression level is shown as copies per 1000 copies glyceraldehyde phosphate dehydrogenase (and as relative expression level in comparison with untreated control). Bold: >150% of control

Gene	Control	E2F2	E2F4	EGFP
Caspase-6	3.0 ± 0.3 (100%)	3.7 ± 0.6 (123%)	**5.3 ± 1.4 (177%)**	**5.9 ± 2.4 (197%)**
Apaf-1	1.9 ± 0.6 (100%)	1.5 ± 0.4 (79%)	**3.1 ± 1.0 (163%)**§	**3.7 ± 2.1 (195%)**
Bax	43 ± 13 (100%)	22 ± 3 (51%)	24 ± 2 (56%)	26 ± 5 (60%)

$^{\S}P < 0.05$ E2F2 vs. E2F4.

Table 5 Parameters of proliferation and apoptosis in adult mouse hearts after expression of E2F2

Group	BrdU-positive CM	PhoshoH3-positive CM	Activated caspase-3-positive cells
Control (4 mice)	0/58501 (=0%)	1/46742 (=0.002%)	2/48805 (=0.004 per 100 CM)
E2F2 (3 mice)	33/54904 (=0.06%)$^{*,\#}$	2/48671 (=0.004%)	2/42375 (=0.005 per 100 CM)
EGFP (4 mice)	0/43527 (=0%)	0/42075 (=0%)	2/42734 (=0.005 per 100 CM)

*$P < 0.05$ vs. control.
$^{\#}P < 0.05$ vs. EGFP.

Table 6 Messenger ribonucleic acid expression at day 14 after intrathoracic injection of the indicated adenoviruses. Expression level is shown as copy number per 1000 copies glyceraldehyde phosphate dehydrogenase (and as relative expression level in comparison with untreated control). Bold: >150% of control

Gene	Control	E2F2	EGFP
Cyclin A	0.8 ± 0.2 (100%)	**1.2 ± 0.3 (150%)**	1.0 ± 0.5 (125%)
Cyclin E	0.01 ± 0.01 (100%)	**0.19 ± 0.04 (1900%)**$^{\#}$	**0.14 ± 0.07 (1400%)**
Cyclin B1	0.21 ± 0.13 (100%)	**0.76 ± 0.19 (362%)**$^{\#,\S}$	*0.05 ± 0.02 (24%)*
Cyclin B2	1.4 ± 0.4 (100%)	**4.1 ± 0.7 (293%)**$^{\#,\S}$	1.4 ± 0.4 (100%)
Cyclin D1	0.25 ± 0.11 (100%)	0.37 ± 0.15 (148%)	0.23 ± 0.13 (92%)
Cyclin D2	1.7 ± 0.7 (100%)	2.5 ± 1.2 (147%)	1.1 ± 0.4 (65%)
Cyclin D3	3.7 ± 3.7 (100%)	**6.7 ± 5.8 (181%)**	5.2 ± 5.2 (141%)

$^{\#}P < 0.05$ vs. control.
$^{\S}P < 0.05$ vs. EGFP.

this stimulation of D-cyclins was indispensable for the induction of proliferation of cardiomyocytes. Interestingly, we again observed a striking correlation between the ability of individual E2Fs to induce proliferation of cardiomyocytes and the expression levels of cyclin A and E as described in our cell culture experiments before. Our results suggest that the increased expression of cyclins A, D, and E and not a repression of CKIs are responsible for the E2F-induced proliferation of cardiomyocytes since we did not find evidence for a reduced expression of p21WAF, which is considered to be one of the major components responsible for cell cycle arrest in cardiomyocytes.[17] This was a somewhat surprising finding because p21WAF is a known downstream target of E2F transcription factors[25] and of p53,[18] which can be induced and activated by E2Fs.[19] However, it is not possible to confine these results solely to cardiomyocytes since our protein lysates also contain material from cardiac non-muscle cells.

Taken together our results indicate that E2F2 is an appropriate candidate to stimulate proliferation of cardiomyocytes *in vivo* in adult hearts. E2F2 does not only induce proliferation of cardiomyocytes but also reduces expression of pro-apoptotic genes such as caspase-6 and apaf-1. These features clearly favour E2F2 for potential therapeutic strategies in comparison with other S-phase inducing genes described so far.

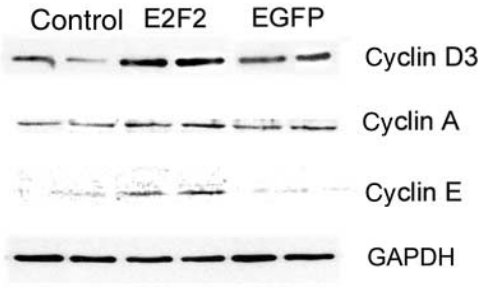

Figure 4 E2F2 induces expression of cyclin D3, A, and E *in vivo*. Western blot analysis (examples) of control hearts and hearts infected with Ad-E2F2 and Ad-EGFP. Proteins were extracted from hearts of 14-day-old mice after adenoviral expression of the indicated genes.

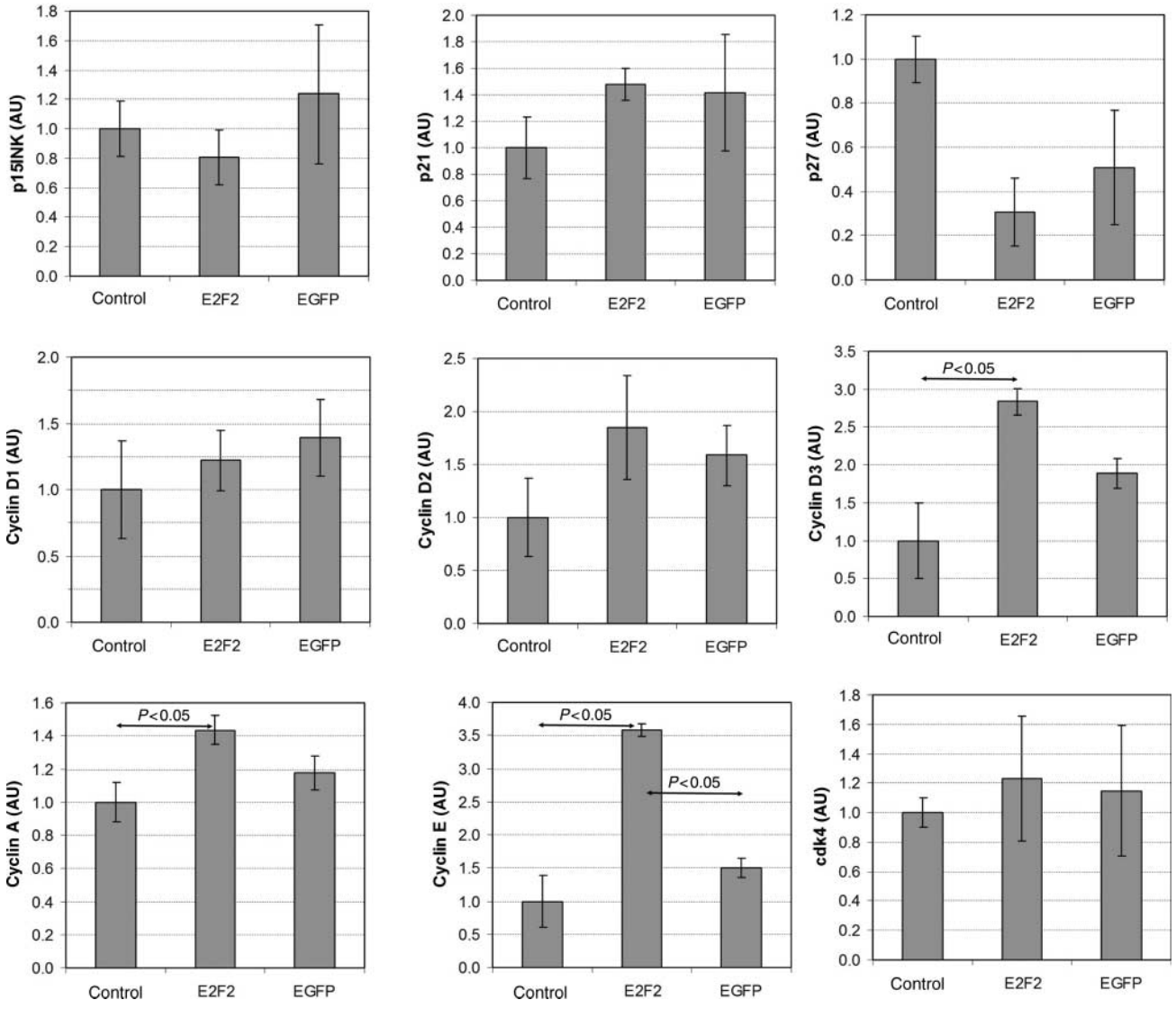

Figure 5 Quantitative assessment of cell cycle regulatory proteins. Western blot analysis of control hearts and hearts infected with Ad-E2F2 and Ad-EGFP. Proteins were extracted from hearts of 14-days-old mice after adenoviral expression of the indicated genes. ($N = 4$ per group).

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Conflict of interest: the authors wish to declare that there are no real or apparent conflicts of interests associated with this work.

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2.6 Beeinflussung der kardiomyozytären Zellzyklusblockade durch Expression einer dominant-negativen p73-Isoform (p73DD)

Die bisherigen Untersuchungen konnten belegen, dass sich durch die gezielte Expression des Transkriptionsfaktors E2F2 in Kardiomyozyten die Zellzyklusblockade zwar prinzipiell überwinden lässt, Zeichen der Proliferation sind aber dennoch in einem zahlenmäßig nur sehr geringen Anteil der Herzmuskelzellen nachweisbar. Aus diesem Grund sollte nach Möglichkeiten gesucht werden, die Häufigkeit des Zellzykluseintritts weiter zu steigern, um so einer hypothetischen therapeutischen Anwendung näher zu kommen. Untersuchungen in der Literatur hatten zeigen können, dass in anderen Zelltypen die kombinierte Beeinflussung verschiedener Signalkaskaden zu synergistischen Effekten in Bezug auf die Proliferationsaktivität führt. So war es beispielsweise möglich, durch die simultane Aktivierung von ras, T-Antigen und der katalytischen Untereinheit der Telomerase (hTERT) die Alterungskrise menschlicher Fibrozyten zu überkommen und den Zellen eine permanente Teilungsfähigkeit zu verleihen [Hahn et al. 1999].

Neben der Aktivierung von E2F-Transkriptionsfaktoren waren in jüngerer Zeit auch weitere Signalkaskaden als mögliche Angriffspunkte für die Zellzyklusstimulation von Kardiomyozyten erkannt worden. In transgenen Mäusen, bei denen p53 ausgeschaltet worden war, wurde eine verstärkte Proliferation von Kardiomyozyten nach Myokardinfarkt beschrieben [Nakajima et al. 2004]. Nicht untersucht wurde bisher jedoch, ob vergleichbare Effekte auch nach Ausschaltung des strukturell und funktionell verwandten p73 zu beobachten sind. p73 stellt ein erst in jüngerer Zeit identifiziertes Mitglied der p53-Familie dar, das in struktureller und funktioneller Hinsicht p53 sehr ähnlich ist (mehr als 60% Übereinstimmung auf Aminosäure-Ebene). Wie p53, so kann auch p73 eine Vielzahl von p53-responsiven Genen wie beispielsweise p21 und bax induzieren und dadurch Zellzyklusarrest oder Apoptose auslösen [Blint et al. 2002; Melino et al. 2002]. Im Gegensatz zu p53 stellt p73 jedoch keinen typischen Tumor-Suppressor im engeren Sinne dar, da sich kaum Mutationen von p73 in humanen Tumoren finden und der Verlust von p73 bei Mäusen nicht zur erhöhten Tumorinzidenz führt [Ikawa et al. 1999; Yang et al. 2000]. Ein weiterer Unterschied zwischen p53 und p73 besteht darin, dass p73 natürlicherweise in unterschiedlichen Isoformen vorkommt: zum einen existieren verschiedene transaktivierende Varianten (sog. TA-Isoformen), zum anderen werden auch mehrere N-terminal verkürzte (ΔN-) Isoformen exprimiert [Melino et al. 2002]. Funktionell zeigen die ΔN-Formen dabei dominant-negative Eigenschaften sowohl gegenüber TAp73 als auch gegenüber p53 [Yang et al. 2000; Nakagawa et al. 2002; Nakagawa et al. 2003].

Um in eigenen Experimenten gezielt mit p53-/p73-abhängigen Signalkaskaden zu interferieren, sollten dominant-negative Formen von p73 (p73DD) in Kardiomyozyten überexprimiert werden. Hierzu wurden auf Grundlage von zuvor bereits publizierten Plasmiden [Irwin et al. 2000] rekombinante Adenoviren generiert.

Der Transkriptionsfaktor p73 besteht funktionell aus drei Domänen (schematische Darstellung siehe Abb. 2), die biologische Wirkung wird von Homotetrameren entfaltet. Bei der p73DD-Form wurden – in Analogie zu den oben beschriebenen, natürlicherweise vorkommenden ΔN-Isoformen – die für die Wirkung als Transkriptionsfaktor erforderlichen Domänen für DNA-Bindung und Transaktivierung entfernt, die so verkürzte Version behält jedoch die Fähigkeit zur Oligomerisierung [Irwin et al. 2000]. Nach Expression der trunkierten Form (p73DD) bilden sich Tetramere aus dem endogen vorkommenden p73 und p73DD, diese Komplexe können jedoch nicht die Transkription von Zielgenen aktivieren. Somit wird endogen vorkommendes p73 in funktionsuntüchtige Komplexe gebunden und inaktiviert. Aufgrund der strukturellen Ähnlichkeit von p73 und p53 konnte davon ausgegangen werden, dass p73DD ebenfalls die Aktivität von p53 inhibiert, wie dies für andere trunkierte Versionen von p73 bereits bekannt war [Pozniak et al. 2000; Nakagawa et al. 2002].

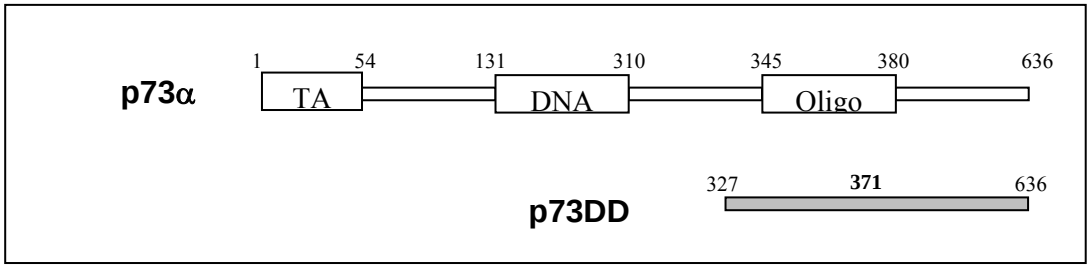

Abb. 2: Schematische Darstellung von p73α und p73DD. Zahlen geben die Position der Aminosäuren an (nach [Irwin et al. 2000]). Domänen: TA = Transaktivierung; DNA = DNA-Bindung; Oligo = Oligomerisierung. Bei p73DD(mut) wurde durch die Punktmutation (L371P) die Oligomerisierungsfähigkeit und damit die dominant-negative Eigenschaft inaktiviert.

Neben dem beschriebenen Konstrukt mit dominant-negativen Eigenschaften p73DD(wt) wurde auch ein Kontroll-Konstrukt generiert (Ad-p73DD(mut)), bei dem durch eine Punktmutation die dominant-negativen Eigenschaften der Wildtyp-Form eliminiert wurden [Irwin et al. 2000]. Sowohl p73DD(wt) als auch p73DD(mut) wurde am N-Terminus eine Markierungssequenz (T7-Tag) angefügt, um später die eindeutige Detektion der rekombinanten Proteine und die Unterscheidung von endogenem p73 zu ermöglichen.

Die eigenen Adenoviren wurden als bi-cistronische Konstrukte konzipiert: durch Verwendung einer *internal ribosome entry site* (IRES) wurde jeweils ein EGFP eingefügt, um eine einfache Identifizierung transfizierter Zellen zu ermöglichen (Abb. 3).

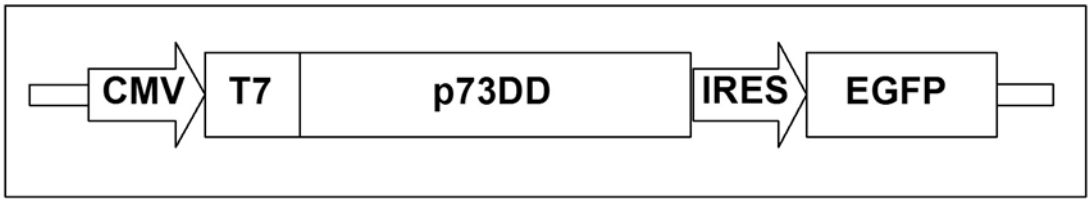

Abb. 3: Schematische Darstellung von Ad-p73DD. CMV: CMV-Promotor. T7: T7-Tag (Markierungssequenz). IRES: *internal ribosome entry site*. EGFP: *enhanced green fluorescent protein*

Die neu generierten Adenoviren wurden zunächst in Zellkulturexperimenten eingesetzt, um die korrekte Funktion der Konstrukte zu überprüfen [Ebelt et al. 2008]. Mittels Western blot kann belegt werden, dass die Adenoviren wie intendiert zur Expression einer verkürzten p73-Isoform mit einem Molekulargewicht von 38kd führen. Der überwiegende Anteil der rekombinanten Proteine findet sich dabei in der nukleären Fraktion, wie dies für Transkriptionsfaktoren zu erwarten ist. Mittels eines Luciferase-Reporterassays kann des weiteren gezeigt werden, dass Ad-p73DD(wt), nicht jedoch p73DD(mut) zur Inhibierung sowohl der p53- als auch der p73-abhängigen Transkription führt. In Primärkulturen von neonatalen Rattenkardiomyozyten wird nur durch p73DD(wt) die Protein-Expression von endogenem p53 und p73 im Sinne einer *feed back*-Regulation induziert, die mutante p73DD-Isoform hat diesbezüglich keinen Effekt.

Anhand von cDNA-Microarray-Hybridisierungen zeigt sich, dass die gezielte Expression von p73DD(wt) in den Herzen neonataler Mäuse die Aktivität einer Vielzahl von Genen verändert. In der Zusammenschau fällt insbesondere die Induktion von proliferations-fördernden Cyclinen und *cyclin dependent kinases* (cdk) sowie mehrerer MAP-Kinasen (*mitogen activated protein kinases*) auf, der Expressionslevel der E2F-Transkriptionsfaktoren sowie verschiedener Caspasen unterliegt dagegen keiner eindeutigen Regulation durch p73DD. Neben den genannten, bekanntermaßen in die Zellzyklusregulation involvierten Genen findet sich außerdem eine deutliche Änderung der mRNA-Expression histon-modifizierender Enzyme: während mehrere Histon-Acetyl-Transferasen unter Einwirkung von p73DD vermehrt exprimiert werden, findet sich gleichzeitig eine – in funktioneller Hinsicht synergistische – Herabregulation von Histon-Deacetylasen, was als Hinweis auf eine

Beeinflussung des Chromatin-Remodelings durch p73DD in Richtung einer vermehrten transkriptionellen Genaktivierung angesehen werden kann.

Histologische Untersuchungen belegen, dass die adenovirale Expression von p73DD(wt) zur vermehrten Zellzyklusaktivität von Kardiomyozyten führt, und drei Tage nach Beginn der Expression von p73DD(wt) findet sich eine signifikante Zunahme der relativen Herzgewichte der Versuchstiere. Dies kann insofern auf die Vermehrung von Kardiomyozyten zurückgeführt werden kann, da zum einen der Anteil proliferierender Kardiomyozyten deutlich erhöht ist, während gleichzeitig eine signifikante Verringerung der mittleren Zellgröße der einzelnen Herzmuskelzellen nachweisbar ist. Zu einem späteren Beobachtungszeitpunkt (14 Tage nach Injektion der Viren) zeigt sich jedoch, dass sich ein Wechsel von der vornehmlichen Proliferation hin zur vermehrten Hypertrophie der Kardiomyozyten vollzieht. Zwar ist auch zu diesem Zeitpunkt der Anteil von Kardiomyozyten mit Zeichen der Zellzyklusaktivität signifikant erhöht, gleichzeitig lässt sich jetzt jedoch auch eine Zunahme der Zellgröße ((*myocyte cross sectional area*, MCSA) beobachten. Molekularbiologische Untersuchungen können belegen, dass die zu diesem Zeitpunkt stattfindende Größenzunahme der Zellen typische Charakteristika der ‚pathologischen' Hypertrophie aufweist: zum einen findet sich begleitend auch eine Vermehrung des interstitiellen Kollagen-Gehaltes (*interstitial collagen fraction*, ICF), außerdem ist die gesteigerte Aktivierung embryonaler Gene wie beispielsweise β-MHC nachweisbar. Bei der Interpretation dieses Befundes muss jedoch berücksichtigt werden, dass der Begriff ‚pathologische' Hypertrophie für gewöhnlich verwendet wird, um die Maladaptation von adulten Kardiomyozyten an Zustände von erhöhtem Stress zu beschreiben. Im Rahmen pathophysiologischer Belastungen der Myozyten ändert sich deren Genexpression dahingehend, dass bei einer Reihe von Proteinen (z.B. Myosin) die adulten durch embryonale Isoformen ersetzt werden. Im Rahmen der eigenen Experimente führt die gezielte Expression von p73DD – wie anhand der Microarray-Analysen nachweisbar – zur Aufrechterhaltung der embryonalen Genexpression und damit auch der Teilungsaktivität der Kardiomyozyten über die Perinatalperiode hinaus. Es liegt daher die Vermutung nahe, dass die verlängerte Persistenz des embryonalen Expressionsmusters den unter normalen Bedingungen nach der Geburt stattfindenden Reifungsprozess der Kardiomyozyten verzögert, so dass Analogien zur ‚pathologischen' Hypertrophie entstehen. Diese Sichtweise wird durch den Befund unterstützt, dass neben der Aktivierung der genannten embryonalen Hypertrophie-Gene durch p73DD(wt) auch eine Expressionssteigerung von Proteinkinase B und β-Catenin als Marker des ‚physiologischen' Wachstums induziert wird.

In einer zuvor publizierten Arbeit war gezeigt worden, dass die Ausschaltung von p53 in einem transgenen Mausmodell zwar zur vermehrten Proliferation von Kardiomyozyten nach Myokardinfarkt, nicht jedoch unter basalen Bedingungen führt [Nakajima et al. 2004]. Die eigenen Untersuchungen können jedoch wie beschrieben belegen, dass die Expression der dominant-negativen p73-Isoform den Zellzykluseintritt von Kardiomyozyten auch ohne vorausgegangene Herzschädigung induziert. Für diesen Befund sind verschiedene Erklärungsansätze denkbar. Zum einen wurden alle Untersuchungen in der zitierten Arbeit ausschließlich an adulten Kardiomyozyten vorgenommen, während die hier beschriebenen Untersuchungen vornehmlich an neonatalen Zellen durchgeführt wurden. Es muss jedoch betont werden, dass sich auch in den eigenen Untersuchungen am Tag 14 nach der Geburt der Zellzyklusaustritt der Herzmuskelzellen bereits vollzogen hat, was in dem sehr geringen BrdU-Labeling-Index der Kontrolltiere von 0,001% sichtbar wird. Andererseits könnte p73DD tatsächlich einen ‚stärkeren' Antagonisten im Hinblick auf die Zellzyklusblockade von Kardiomyozyten darstellen als das in der zitierten Arbeit verwendete CB7-Allel von p53. Es ist mittlerweile gezeigt worden, dass p73 und p53 zwar beide über das *p53 response element* (p53RE) die Transkription von Zielgenen aktivieren können, darüber hinaus vermag p73 aber auch an DNA-Bereiche mit einer abweichenden Sequenz zu binden und so die Aktivität von Genen zu beeinflussen, die keiner Regulation durch p53 unterliegen [Sasaki et al. 2005].

Wie bereits erwähnt, stellt die Aktivierung des cdk-Inhibitors p21 einen wesentlichen Mechanismus der Zellzyklusblockade in Kardiomyozyten dar [Engel et al. 2003]. Mittels Western blot kann gezeigt werden, dass – entsprechend der Arbeitshypothese – p73DD(wt) in den Herzmuskelzellen zu einer Herabregulation des bekanntermaßen p53-/ p73-responsiven p21 führt, was als ein wichtiger Mechanismus der Zellzyklusstimulation angesehen werden kann. Des Weiteren sind in p73DD(wt)-exprimierenden Kardiomyozyten die Cycline A, E, B1 und B2 vermehrt nachweisbar.

Für die Generierung der eigenen p73DD-Adenoviren sind cDNA-Konstrukte verwendet worden, die ursprünglich zur Untersuchung und Inhibierung der E2F1-induzierten Apoptose eingesetzt worden waren [Irwin et al. 2000]. In Übereinstimmung hierzu finden sich nach Expression von p73DD auch in den eigenen Untersuchungen keine Hinweise für eine vermehrte Apoptose von Herzmuskelzellen.

Perspektivisch erscheint die kombinierte Inhibierung von p53-/p73-abhängigen Signalkaskaden gemeinsam mit einer Erhöhung der E2F-Aktivität als ein viel versprechender Ansatz, um Regenerationsvorgänge von Kardiomyozyten zu induzieren. Durch diese

Kombination sind zum einen synergistische Effekte im Hinblick auf den S-Phase-Eintritt und die Zellzyklusprogression der Herzmuskelzellen zu erwarten. Zum anderen ist davon auszugehen, dass gleichzeitig auch der E2F-initiierten Apoptose, die zumindest teilweise über p53- und p73-abhängige Kaskaden vermittelt wird, weiter entgegengesteuert werden kann [Irwin et al. 2000; Zaika et al. 2001].

Anlage zu 2.6.:

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Original article

Directed expression of dominant-negative p73 enables proliferation of cardiomyocytes in mice

Henning Ebelt [a,*], Ying Zhang [a], Katharina Köhler [a], Jia Xu [a], Praveen Gajawada [a,c], Thomas Boettger [c], Thomas Hollemann [b], Ursula Müller-Werdan [a], Karl Werdan [a], Thomas Braun [c]

[a] Department of Medicine III, University of Halle-Wittenberg, Ernst-Grube-Str. 40, 06097 Halle, Germany
[b] Institute of Physiological Chemistry, University of Halle-Wittenberg, Hollystr. 1, 06097 Halle, Germany
[c] Max-Planck-Institute for Heart and Lung Research, 61231 Bad Nauheim, Parkstr. 1, Germany

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ABSTRACT

Previous studies have shown that p53 plays an important role in maintaining cell cycle arrest of cardiomyocytes, which might account for the inability of human hearts to regenerate adequately after injury. Therefore, inhibition of p53 represents an attractive strategy to restore cell cycle progression in cardiomyocytes although such an approach is hampered by the potential danger of concomitant tumor induction. During normal development, N-terminal truncated isoforms of the p53-related protein p73 are naturally occurring antagonists of p53 and p73, which are not related to tumor induction. We have generated recombinant adenoviruses encoding dominant-interfering p73 (Ad-p73DD) to inhibit p53/p73 in murine hearts at different developmental stages. We found that the expression of p73DD(wt) in newborn mice led to the increase of the relative heart weights after 14 days which is paralleled by a significant increase of proliferating cardiomyocytes as seen by ICC (BrdU-incorporation, phosphorylation of histone3, expression of AuroraB) without induction of apoptosis. Stimulation of cell cycle progression in cardiomyocytes went along with a significant down-regulation of the p53-dependent cdk-inhibitor p21WAF both on mRNA and protein level. Furthermore, mRNA levels and protein expression of D-type cyclins and cyclins A, B2, and E were selectively increased after expression of p73DD. We further show that the cell cycle entry of cardiomyocytes is not restricted to neonatal hearts but is also found in adult mouse hearts 5 days after intramyocardial injection of Ad-p73DD. Taken together we reason that directed expression of dominant-negative p73 might be utilized to stimulate proliferation of cardiomyocytes to improve cardiac regeneration.

1. Introduction

Regeneration of myocardial tissue after damage has been observed in newts and fish [1,2] but not in human patients where extensive or progressive loss of cardiomyocytes frequently leads to heart failure and death. In the last years a number of studies have shown that the tumor-suppressor p53 is instrumental to direct cell cycle exit of cardiomyocytes, which occurs in mammals in the perinatal period. Initial studies using viral oncogenes such as E1A and SV40 large T-antigen demonstrated that it is possible to re-induce proliferation of postmitotic cardiomyocytes [3,4] — a phenomenon which finally has been shown to be dependent on the inactivation of p53 and related cell cycle regulators like p193 and p107 [5,6]. More recent studies showed that transgenic expression of dominant-negative isoforms of p53 or p193 enables cell cycle entry of cardiomyocytes which in the case of p193-inhibition are sufficient to counteract adverse cardiac remodelling in a mouse model of myocardial infarction [7] However, beside the permanent expression of a dominant-interfering p53 in the transgenic situation mentioned above [7] so far no attempt has been made to analyze whether an acute interference with the action of p53 may induce proliferation of cardiomyocytes. A general concern to apply inhibition of p53 for therapeutic purposes is based on the well-known tumor-suppressor function of p53 and the finding that reduced activity of p53 is associated with nearly all kinds of malignancies [8]. An artificial down-regulation of p53 clearly bears the potential of unintended induction of tumor formation. In fact, transgenic over-expression of the SV40 large T-antigen in the heart under control of the ANF promotor [4] resulted in enhanced tumor formation.

In the last years, it has been found that p53 together with p63 and p73 constitutes a family of genes which share a similar structure and can function as transcription factors [review: [8]]. Despite obvious similarities there are also significant differences between the three genes. For instance, p73 (unlike p53) is not considered a typical tumor suppressor as it is usually not found mutated in malignancies and mice lacking p73 do not show signs of increased tumor formation [9,10].

* Corresponding author.
E-mail address: henning.ebelt@medizin.uni-halle.de (H. Ebelt).

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Under physiological conditions and especially during embryonic development various p73 isoforms had been found. This diversity arises not only from alternative splicing but is also a consequence of the activation of an alternative promoter located in intron 3 leading to N-terminally truncated p73 variants (ΔNp73). The ΔNp73 isoforms (in contrast to the TAp73 isoforms) lack transactivating properties and therefore act in a dominant-negative manner inhibiting functionally active p53 and p73 [10–12]. The ability of the naturally occurring N-terminally truncated p73 variant to interfere with p53/p73 signaling raised the question whether directed expression of such a dominant-negative p73 would also be sufficient to relax the cell cycle blockade in cardiomyocytes.

Here we demonstrate that recombinant adenoviruses encoding a dominant-negative isoform of p73 (p73DD), which suppresses the activity of endogenous p53 and p73, induces proliferation of cardiomyocytes both in newborn and in adult mice without concomitant induction of apoptosis. Forced cell cycle entry of cardiomyocytes was accompanied both by down-regulation of several histone deacetylases and the cell cycle inhibitor p21WAF, which is an established target of p53, and an induction of cyclins A, B2, D3, and E.

2. Methods

2.1. Adenoviral expression constructs

Plasmids encoding truncated mutants of human p73 linked to an N-terminal T7-tag (p73DD) [13] were kindly provided by Dr. M. Irwin (Harvard Medical School, Boston, Massachusetts, USA). It has been shown that p73DD(wt) exerts dominant-negative effects by recruitment of full-length p73/p53 isoforms into non-transactivating complexes [13]. Both the wildtype p73DD (p73DD(wt)) and a mutant p73DD which harbours a point mutation (L371P) leading to the loss of the dominant-negative properties (p73DD(mut)) were first cloned into the EcoRI-site of pIRES2-EGFP (BD Clontech). Then the cassette containing p73DD-IRES-EGFP was excised and further cloned into pShuttle-CMV (XhoI/XbaI) of the AdEasy system for generation of recombinant adenoviruses as described [14]. Adenoviruses were handled as described with HEK293 as the packaging cell line, and viral titers were determined by plaque-assay [15,16].

mRNA expression of p73DD was confirmed by RT-PCR (forw: ATGACTGGTGGACAGCAAATGG; rev: GCTCTCTTTCAGCTTCATCAGG) with the forward primer designed to bind at the sequence of the T7-tag to avoid interference with endogenous p73. For determination of subcellular localization of p73DD, cytoplasmic and nuclear proteins were isolated using BD™ Transfactor Extraction Kit according to the manufacturer's instructions, untreated cells were used as a control.

2.2. p53 reporter assay

HEK293 or H1299 ($p53^{-/-}$) cells were plated in 6 well plates at a density of 5×10^6 cells per well, respectively, and transfected according to standard protocols with 4 μg of a p53-dependent luciferase plasmid containing a p53-response element (Mercury™ Profiling Vectors; Clontech) and 1 μg of a β-galactosidase plasmid to allow normalization for transfection efficiency. After 24 h the cells were then infected for 1 h with 10^8 pfu Ad-p73DD(mut), Ad-p73DD(wt), or Ad-EGFP, respectively. 24 h later, 150 μl cell lysis buffer (BD luciferase reporter assay kit) was added to each well and luciferase assay was performed according to the manufacturer's instructions (BD Luciferase reporter assay kit). In parallel, β-galactosidase assay utilizing CPRG was performed in order to normalize for transfection efficiency as described [17].

2.3. Adenoviral transfection of mouse hearts in vivo

For all in vivo experiments transgenic mice carrying a nuclear localized LacZ-reporter gene under the control of the aMHC-promotor [18] were used to enable the definite identification of cardiomyocytes on histological sections (kindly provided by Dr. L. J. Field).

Transfection of hearts of newborn mice was performed by intrathoracic injection of adenoviral vectors which is known to lead to a lasting transfection of cardiomyocytes as described before in detail [15]. At the day of birth the mice were anaesthetized by cooling on ice for approximately 2 min and put in front of a cold light source to visualize the silhouette of the heart. Using a Hamilton syringe with a 26-gauge needle a total volume of 10 μl was injected into the thoracic cavity beside the heart at a left parasternal position. Finally, animals were re-warmed and put back to their mothers. After 13 days, the mice received a single i.p.-injection of BrdU (100 mg/kg BW) and were then sacrificed the next day. The hearts were removed and either embedded for cryosectioning or shock-frozen in liquid nitrogen for RNA and protein isolation, respectively.

Adult mice were anaesthetized with 2.5% isofluorane and mechanically ventilated, left sided thoracotomy was performed in the 4th ICR. Under microscopic control the heart was gently uncovered and 10 μl of the different virus suspensions were injected 2 times in the left ventricular free wall and once in the interventricular septum. On day three and four after surgery, the mice received an i.p.-injection of BrdU (100 mg/kg BW) before they were finally sacrificed on day 5.

2.4. Histological analysis

The hearts from the neonatal and the adult mice were cut into 10 μm cryosections. All immunohistochemical staining procedures included the initial identification of nuclei of the cardiomyocytes by X-gal staining. Immunoassays to detect BrdU-incorporation (Anti-BrdU; Vector Labs.), phosphorylated histone3 (Anti-PhoshoH3; Upstate), AuroraB kinase (anti-AuroraB, Abcam) and activated caspase3 (anti-activ. casp3, Promega) were performed according to the manufacturer's specifications. Determination of myocyte cross sectional area (MCSA) and interstitial collagen fraction was performed as described [19].

2.5. RNA isolation, microarray gene expression analysis, and quantitative real-time PCR

Total RNA was isolated using Trizol according to standard techniques. Affymetrix Mouse Genome 430 2.0 microarrays were used to determine differences in mRNA expression levels in neonatal mice 7 days after expression of p73DD(wt) or p73DD(mut), respectively. For quantitative real-time PCR, cDNAs were synthesized using oligo-dT-primers as described [20]. Real-time PCR was performed in 25 μl total volume containing 1 μl cDNA, 0,1U Taq-polymerase, 5 nmol forward primer, 5 nmol reverse primer, 10 μmol dNTPs, 0,25 μl 100 times fluorescein, and 0.5 μl 10 times SybrGreen I (Sigma) as described [20] (for primer sequences see Supplemental data and [16,21,22]). Expression levels of cell cycle related genes are shown as: expression level = (copy number geneA per μl cDNA)/(copy number G3PDH per μl cDNA * 1000) [16].

2.6. Protein isolation and Western blot

For protein isolation and Western blot analysis hearts were dissolved in RIPA-buffer (50 mM Tris–HCl, pH 7.4, 1% Triton X-100, 0.2% sodium deoxycholate, 0.2% SDS, 1 mM EDTA) containing 1 mM PMSF, 5 μg/ml Aprotinin and 5 μg/ml Leupeptin (all from Sigma). 30 μg of protein lysates were separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes (Invitrogen Life Technologies, Groningen, The Netherlands). Immunoprecipitation was achieved by overnight incubation of 100 μg protein lysate from primary cultures of neonatal cardiomyocytes [16] with a p73 antibody

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(Epitomics) followed by precipitation with Dynabeads protein A (Invitrogen) according to the manufacturer's protocol. Immunoreactive proteins were visualized with corresponding HRP-conjugated secondary antibodies on Hyperfilm (GE Healthcare) using the SuperSignal West Pico or West Femto detection solutions (Perbio Science). Blots were scanned and analyzed using ImageJ (NIH, USA). The following antibodies were used in this study: anti-T7-tag (Novagen); anti-cyclin A (sc-751), anti-cyclin B2 (sc-22776), anti-cyclin D2 (sc-593), and anti-cyclin E (sc-481; all from Santa Cruz Biotechnology); and anti-cyclin D1, anti-cyclin D3, anti-CDK4, anti-$p15^{INK4}$, anti-$p27^{Kip1}$, anti-PKB, anti-β-catenin, and anti-GAPDH (all from Cell Signaling Technology); anti-$p21^{Waf}$, and anti-p53 (BD).

2.7. Statistics

All data are shown as means ± SEM. Comparisons between multiple groups were performed using ANOVA or *H*-test (Kruskal–Wallis). $p<0.05$ was considered statistically significant.

3. Results

3.1. Generation of recombinant adenoviruses expressing p73DD(wt) and p73DD(mut)

In order to interfere with p53/p73 signaling in cardiomyocytes a truncated form of p73 (p73DD) was cloned into an adenoviral expression vector. The ability of p73 to block efficiently p53/p73 mediated signaling was initially analyzed in HEK293 cells. Both RT-PCR (not shown) and Western blot analysis indicated a robust expression of recombinant p73DD (molecular weight of 38kd). As expected, p73DD was not found in the cytosolic but exclusively in the nuclear fraction where it should interfere with p53- and p73-dependent transcription (Fig. 1A).

A p53-luciferase reporter assay was performed to confirm that the adenoviral p73DD-constructs inhibited p53/p73-dependent transcription. The assay relied on inhibition of a luciferase reporter gene driven by a p53-responsive promoter. As shown in Fig. 1B, expression of p73DD(wt) strongly inhibited the expression of the luciferase reporter indicating a reduced activation of the p53-responsive promoter while expression of an inactive p73 mutant (p73DD(mut)), which carries a point mutation that impairs oligomerization with endogenous p53 and p73, had only a minor effect on reporter gene activity. Similar findings were also made using the $p53^{-/-}$ cell line H1299, in which induction of the luciferase gene solely depends on p73 clearly demonstrating that the p73DD-construct inhibited both p53 and p73 signaling.

In order to determine whether the recombinant adenoviruses do have an impact on the regulation of endogenous p53 and p73 we next analyzed the expression level of both genes in isolated cardiomyocytes from neonatal rats by Western blot analysis Expression of p73DD(wt) led to an up-regulation of p53 indicating the activation of a compensatory feed-back loop induced by functional abrogation of p53 (Fig. 1C, lower panel). Western blot analysis using an antibody, which does not react with the truncated p73DD constructs revealed the absence of significant amounts of full-length p73 in neonatal rat cardiomyocytes although immunoreactive isoforms of a reduced molecular weight were detected (Fig. 1C, upper panel). The presence of these p73 derivatives was suppressed both by p73DD(wt) and p73DD(mut), which most likely is due to unspecific effects of the viral constructs. Interestingly, we detected an induction of full-length p73 after infection with the p73DD(wt) adenovirus while the p73DD(mut) virus did cause only a minor up-regulation of full-length p73. We concluded that expression of p73DD(wt) led also to an up-regulation of active p73 similar to the compensatory up-regulation of p53 described above.

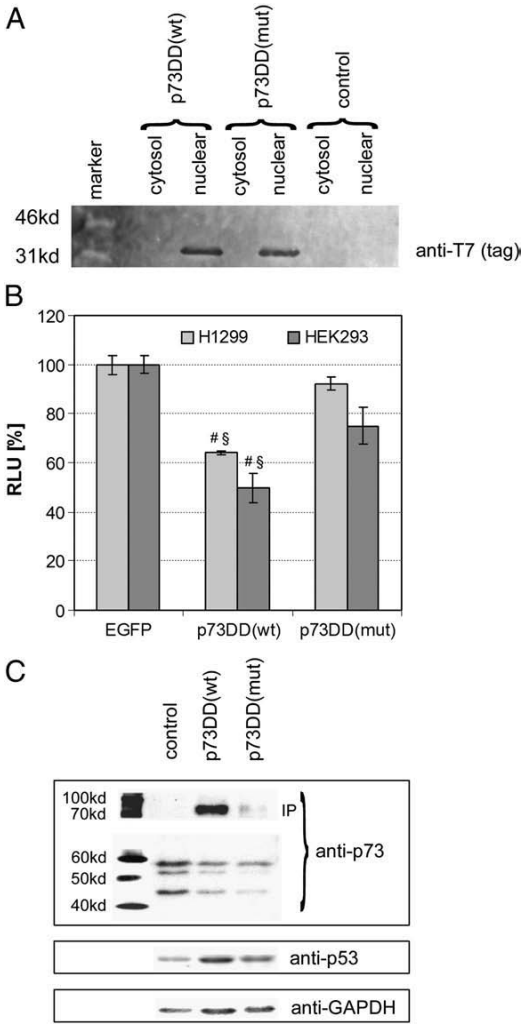

Fig. 1. Adenoviral expression of p73DD(wt) and p73DD(mut). (A) HEK293 cells were infected with Ad-p73DD(wt), Ad-p73DD(mut), or mock infected (culture medium). Cytosolic and nuclear fractions were separated and stained with anti-T7 antibody. Anti-T7 immunoreactivity corresponding to the p73DD isoforms (T7-tag) was found in the nuclear but not the cytosolic fractions of infected cells. (B) Ad-p73DD(wt) significantly reduced p53-/p73-dependent transcription of the luciferase reporter gene leading to reduced light emission both in HEK293 as well as in $p53^{-/-}$ H1299 cells ($N \geq 4$ per group). (C) p73DD(wt) induces the expression of p53 and p73 in CM from neonatal rats (representative examples). IP = immunoprecipitation with anti-p73.

3.2. Expression of p73DD(wt) activates pro-proliferative genes and counteracts transcriptional silencing in neonatal hearts

Next, we used our adenoviral vectors to express p73DD in the hearts of newborn mice. Since activation of p53 is known to be a key event during the cell cycle exit of cardiomyocytes in the perinatal period we decided to analyze first the effects of targeted expression of dominant-negative p73DD(wt) on cardiomyocyte cell cycle activity early after birth. Neonatal mice received a single intra-thoracic virus injection on their day of birth which has been shown to result in a long term transfection of more than 70% of all cardiac cells [15]. After 7 days, mRNA expression in these hearts was analyzed by microarray hybridisation (Fig. 2). Inhibition of p53/p73 by p73DD(wt) led to a sustained up-regulation of key regulators of cell cycle activity such as A- and D-type cyclins and various cyclin dependent kinases (cdk). In parallel, the expression of p73DD(wt) had a severe impact on the expression of histone modifying enzymes. Both class I and II histone

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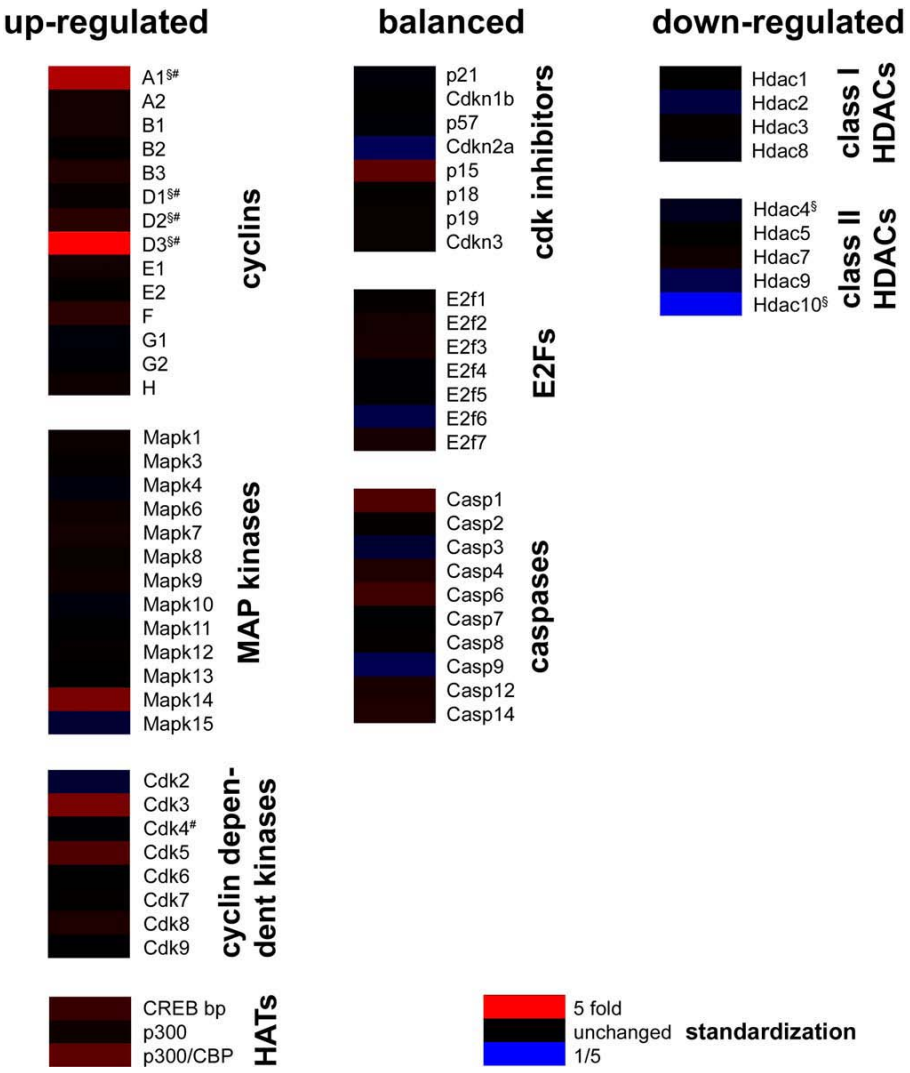

Fig. 2. mRNA expression in hearts of neonatal mice 7 days after expression of p73DD(wt) in comparison to p73DD(mut) (mean values of $n=3$; Affymetrix Mouse Genome 430 2.0 Microarray). § confirmed by real-time PCR. # confirmed by Western blot.

deacetylases were widely suppressed while histone acyltransferases were up-regulated indicating positive chromatin modelling and enhanced transcriptional activity. Unexpectedly, however, we did not detect a striking effect on the transcriptional activity of E2F genes although it has been reported that E2Fs have a profound impact on the proliferative capacity of cardiomyocytes and is linked to p53 activity in various settings [16,23,24].

3.3. S-phase entry and mitosis of neonatal cardiomyocytes are stimulated by directed expression of p73DD(wt) without induction of apoptosis

We next examined whether the induction of pro-proliferative factors eventually translates into amplification of cardiomyocytes. Analysis of relative heart weights and immunohistological parameters of proliferation in neonatal mice 3 days after the virus injections revealed that expression of p73DD(wt) but not of the mutant p73DD-isoform led to a significant increase of the relative heart weight (Table 1). This phenomenon was clearly related to the induction of cardiomyocyte proliferation as we found prove of increased cell cycle activity of cardiomyocytes on histological sections (BrdU-uptake as a marker of DNA synthesis, activation of AuroraB kinase indicative for cytokinesis) while the mean size of the individual cardiomyocytes was significantly smaller than in control animals (Table 1).

We also investigated whether p73DD(wt)-induced proliferation of cardiomyocytes persists at later developmental stages. Expression of p73DD(wt) led to a moderate but significant increase of the relative heart weights (6.8 ± 0.2 mg/g) 14 days after the virus injections (Fig. 3) in comparison to untreated control mice (6.1 ± 0.2 mg/g) whereas the relative weights of liver and lungs did not change. No relevant changes

Table 1
Relative heart weights, cardiomyocyte cross sectional area (MCSA), and parameters of cell cycle activity 3 days after expression of the indicated adenoviruses in neonatal mice

Parameter	Control	p73DD(wt)	p73DD(mut)
Relative heart weight [mg/g]	6.9 ± 0.2	7.7 ± 0.3§	7.2 ± 0.2
MCSA [μm^2]	12.8 ± 0.4	10.4 ± 0.6§	11.2 ± 0.3
BrdU-positive CM	42/6275 (=0.67%)	180/9450 (=1.9%)§,#	78/8224 (=0.95%)
Aurora B-positive CM	0/14,218 (=0%)	6/14,607 (=0.04%)§	0/14,868 (=0%)

§$p<0.05$ vs. control. #$p<0.05$ vs. p73DD(mut).

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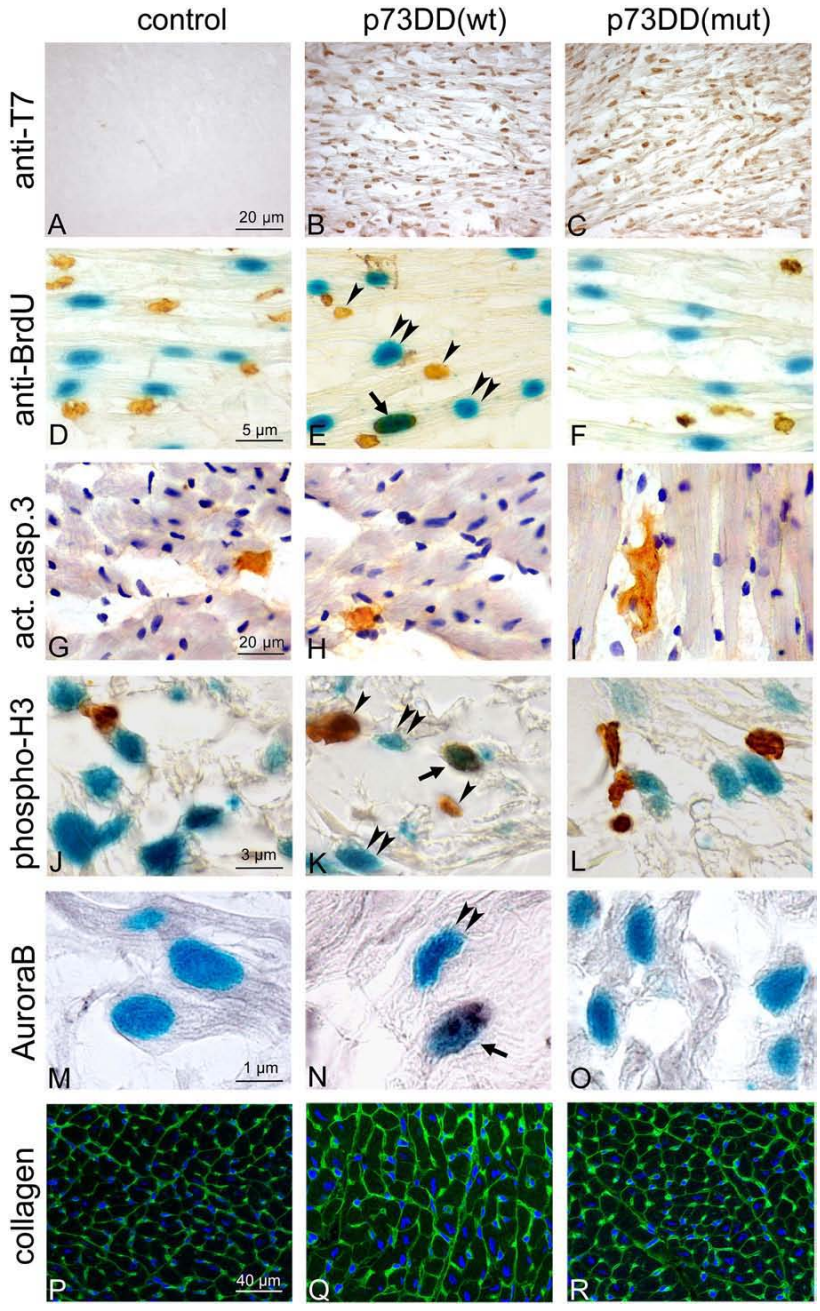

Fig. 3. Examples of cryosections from mouse hearts after expression of the indicated adenoviruses. (A–C) Anti-T7 (tag) to identify transfected cells expressing the p73DD-isoforms. (D–F) BrdU staining. Hearts were first stained with X-gal to visualize the nuclei of the cardiomyocytes (transgenic reporter mouse strain αMHC-nlsLacZ) and then stained with Anti-BrdU/peroxidase. Single arrowhead: BrdU-positive non-cardiomyocyte, double arrowhead: BrdU-negative cardiomyocyte. Arrow: BrdU-positive cardiomyocyte. (G–I) Staining for activated caspase3 (nuclear counter staining: hematoxyline. (J–L) Staining for phosphorylated histone H3 (bluish X-gal stain marks nuclei of CM). Single arrowhead: pH3-positive non-cardiomyocytes, double arrowhead: pH3-negative cardiomyocytes. Arrow: pH3-positive cardiomyocyte. (M–O) Staining for AuroraB (X-gal stain marks CM-nuclei). Double arrowhead: AuroraB-negative cardiomyocyte. Arrow: AuroraB-positive cardiomyocyte. (P–R) Staining with peanut-agglutinin to visualize interstitial collagen. Images A–C and M–O were taken from 3 day old mice, images D–L and P–R were from 14 day old mice.

of relative heart weights were noted after expression of mutant p73DD (6.5±0.2 mg/g). We also found signs of increased cardiomyocyte proliferation on histological sections indicated by a 9fold increase of BrdU-positive CM and a significant number of CM, which stained positive for phosphorylated histone H3, a marker for mitosis (Table 2). Interestingly, we noted a switch from hyperplastic to a combined hyperplastic and hypertrophic cell growth of the heart at this stage since the mean cell size of the cardiomyocytes was significantly increased after infection with p73DD(wt) but not with p73DD(mut).

As stated before, it is well know that activation of oncogenes, which stimulate cell cycle activity of cardiomyocytes often results in induction of apoptosis. We therefore analyzed whether directed expression of p73DD induced programmed cell death. As shown in Table 2 staining for activated caspase3 revealed no significant

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Table 2
Relative heart weights, cardiomyocyte cross sectional area (MCSA), and parameters of cell cycle activity and apoptosis 14 days after expression of the indicated adenoviruses in neonatal mice

Parameter	Control	p73DD(wt)	p73DD(mut)
Relative heart weight [mg/g]	6.1 ± 0.2	6.8 ± 0.2§	6.5 ± 0.2
MCSA [μm²]	73.1 ± 3.2	88.9 ± 0.8§,#	72.1 ± 1.6
BrdU-positive CM	1/120,182 (=0,001%)	17/191,109 (=0,009%)§,#	3/141,922 (=0,002%)
PhosphoH3-postive CM	0/109,030 (=0%)	10/166,718 (=0,006%)§	2/124,326 (=0,002%)
Activated caspase3-positive cells	23/153,790	23/168,726	28/162,236

§$p<0.05$ vs. control. #$p<0.05$ vs. p73DD(mut).

induction of apoptosis in p73DD expressing hearts compared to uninfected controls (Table 2).

Further analysis of the hypertrophic cell growth 14 days after p73DD(wt)-expression revealed that the increase of cardiomyocyte size was accompanied by an increase of the expression of BNP and the β-MHC-isoform (Table 3) as well as a significant increase of interstitial fibrosis (Fig. 4), which are hallmarks of "pathological" hypertrophy. However, we also detected an increase of the expression of PKB (160 ± 31%, n.s.) and β-catenin (173 ± 17%, $p<0.05$) by Western blot analysis after directed expression of p73DD(wt) which are generally considered to represent markers of "physiological" cell growth (p73DD(mut): 114 ± 4% and 149 ± 35%, respectively; n.s.).

3.4. p73DD(wt) induces the expression of growth-stimulating cyclins and inhibits expression of p21WAF

Progression through the cell cycle is dependent on the regulated activation of cyclins and cyclin dependent kinases. Since directed expression of dominant-negative p73DD(wt) leads to S- and M-phase inductions in cardiomyocytes we wanted to know whether this is achieved by a direct transcriptional activation of cyclins or by suppression of inhibitory CKIs. Previous studies have revealed that the p53-dependent cell cycle inhibitor p21WAF is a key mediator of cell cycle arrest in terminally differentiated cardiomyocytes [25], which suggests that expression of p21WAF might be affected by dominant-negative p73DD(wt).

Expression of p73DD(wt) led to a change in the mRNA levels of several cell cycle related genes (Table 4) while the administration of mutant p73DD (where the domain essential for interaction with p53/p73 has been destroyed) was undistinguishable from untreated controls in most cases. We also found a p73DD(wt)-induced stimulation of cyclins related to G1/S-transition (cyclin D2) and S-phase progression (cyclins A/E) as well as of M-phase associated cyclins (cyclin B1/B2). Additionally, the expression of the potent cell cycle inhibitor p21WAF was clearly reduced after expression of p73DD(wt) as expected.

Since changes in mRNA levels do not necessarily lead to changes in corresponding protein levels, we quantified the key components of the cell cycle machinery by Western blot analysis. Indeed, as seen in Fig. 4, the majority of genes which were induced by p73DD(wt) showed increased protein levels accordingly. Only Cyclin D2, which expression was induced by both wildtype and mutant p73DD, showed no difference at the level of protein relative to untreated control hearts.

Table 3
mRNA expression 14 days after expression of the indicated adenoviruses in neonatal mice

Gene	Control	p73DD(wt)	p73DD(mut)
ANP [deltaCT GAPDH]	1.41 ± 0.70	1.65 ± 0.14	1.13 ± 0.62
BNP [deltaCT GAPDH]	0.12 ± 0.04	0.23 ± 0.04	0.18 ± 0.05
bMHC/aMCH [ratio × 10^3]	1.61 ± 0.18	2.71 ± 0.40§	2.29 ± 0.34

Data from quantitative real-time PCR ($N=5$). §$p<0.05$ vs. control.

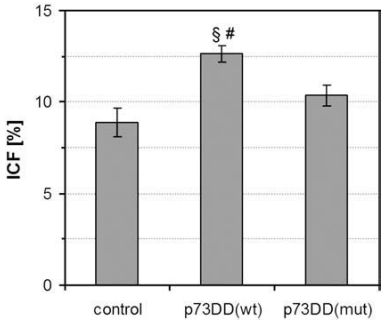

Fig. 4. Determination of interstitial collagen fraction (ICF) in hearts at day 14 after injection of the indicated adenoviruses ($N\geq5$ per group).

On the other hand, the transcriptional down-regulation of p21WAF induced by p73DD(wt), is followed by lower levels of the corresponding protein (Fig. 5).

3.5. p73DD(wt) stimulates S-phase entry of cardiomyocytes in adult mouse hearts

The potential of mammalian cardiomyocytes to divide during embryonic development drops in the perinatal period, which raises the question whether the elevated rate of cardiomyocyte proliferation in neonatal mice (which still could show some proliferation in the first days after birth) was due to an inhibition or delay of cell cycle exit rather than to a real induction of cell cycle re-entry. Hence, we investigated whether expression of the dominant-negative p73DD (wt) does also enable S-phase entry of adult cardiomyocytes, which are well beyond the restriction point. We found that injection of Ad-p73DD(wt) into the myocardium of adult mice led to a significant increase of BrdU-incorporation into cardiomyocytes indicative for successful induction of DNA synthesis (Table 5). We also observed an increase of the number of phosphoH3-positive cardiomyocytes after expression of p73DD(wt) although this elevation did not reach statistical significance ($p=0.09$) in adult hearts. Again, the rate of apoptosis was not different between p73DD-expressing hearts and untreated controls.

4. Discussion

We here show that directed expression of a dominant-negative isoform of p73 enables proliferation of cardiomyocytes and also induces hypertrophic growth resulting in an increased cross sectional area of myocytes. Both mechanisms seem to contribute to the increase of the relative heart weights 14 days after injection of p73DD adenoviruses. Although the principal and fundamental role of p53-mediated signaling pathways in the regulation of cell cycle activity of

Table 4
mRNA expression of cell cycle related genes given as copy number per 1000 copies GAPDH and as percent of untreated control 14 days after intra-thoracic injection of the indicated adenoviruses

Gene	Control	p73DD(wt)	p73DD(mut)
p21WAF	4.1 (100%)	1.9 (46%)	3.8 (94%)
Cyclin A	0.79 (100%)	2.06 (263%)	0.97 (124%)
Cyclin B1	0.22 (100%)	0.39 (176%)	0.05 (25%)
Cyclin B2	1.37 (100%)	4.44 (325%)	1.35 (99%)
Cyclin D1	0.25 (100%)	0.25 (101%)	0.23 (94%)
Cyclin D2	3.2 (100%)	7.52 (235%)	5.64 (176%)
Cyclin D3	8.97 (100%)	9.5 (106%)	5.18 (58%)
Cyclin E	0.25 (100%)	0.4 (157%)	0.16 (61%)

mRNA induction (more than 150% of control) is highlighted by light gray color while mRNA down-regulation (less than 50% of control) is marked by dark gray color. Mean values of $n=4$ per group.

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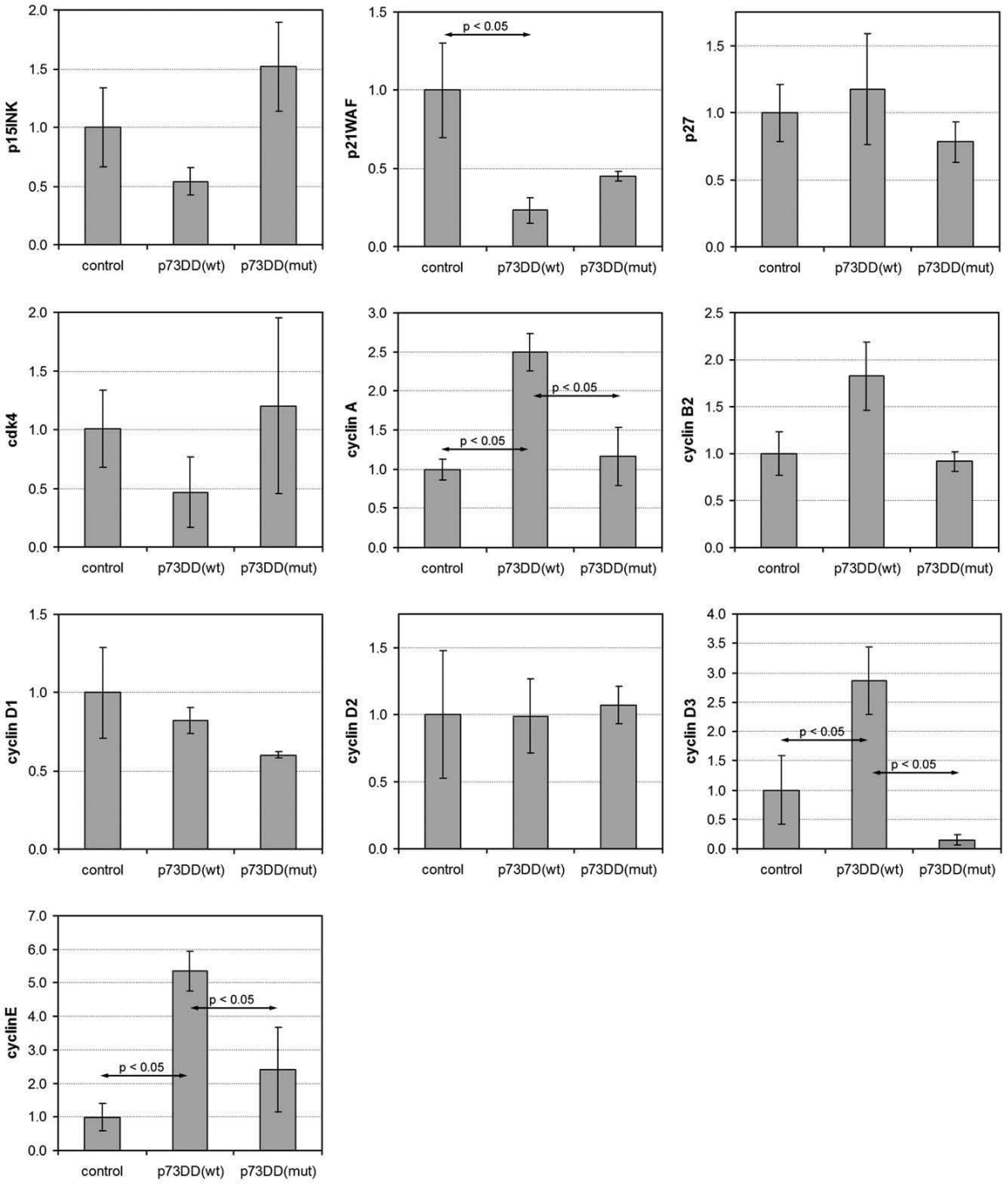

Fig. 5. Protein expression of cell cycle related genes in hearts of newborn mice 14 days after administration of the indicated adenoviruses (quantification of Western blots; $n=4$).

cardiomyocytes has been addressed before [5,7], our study reveals important new aspects that further improve the understanding of the cell cycle arrest in cardiomyocytes what perhaps might help to come closer to the vision of therapeutic heart regeneration.

p53-dependent pathways are certainly the most important anti-tumor defence lines of mammals known today [26,27]. It was therefore no surprise that expression of the SV40 large T-antigen, which suppresses p53-activity in the heart, leads to increased frequency of tumor formation [4]. Hence, it seems difficult to separate the improvement of cardiac regeneration by inactivation of the p53 gene, from the increased incidence of cancer. Manipulation of the p53-related p73 gene might offer a solution to this dilemma. Unlike p53, p73 is not a classical tumor suppressor since mutations of p73 are not typically found in malignancies and deletion of p73 does not lead to an increased tumor incidence in mice [9,10]. Furthermore, several truncated forms of p73 are expressed under physiological conditions in various tissues such as the brain, which interfere in a dominant-negative manner with p73 and p53 [10,12]. Apparently, expression of

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Table 5
Parameters of proliferation and apoptosis in adult mouse hearts after expression of p73DD(wt)

Group	BrdU-positive CM	PhosphoH3-positive CM	Activated caspase3-positive cells
Control (6 mice)	6/97830 (0.006%)	2/88675 (0.003%)	2/148293
p73DD(wt) (5 mice)	25/106,811 (0.023 %)*	8/104,740 (0.008%)	0/265949

*$p<0.05$.

truncated forms of p73 does not completely abrogate all functions of p53, since the appearance of these dominant-negative forms of p73 does not coincide with tumor formation. Inspired by these findings, we decided to assess the potency of p73DD to release cell cycle arrest of cardiomyocytes to avoid the potential pitfalls that are associated with a direct inactivation of the tumor-suppressor gene p53.

In our experiments, we clearly demonstrated that expression of p73DD(wt) has a strong impact on gene expression and cell cycle regulation of cardiomyocytes. Surprisingly, we also found a similar tendency (although not significant) after the expression of the mutant p73DD isoform in the majority of assays although p73DD(mut) should not interact with p53 or p73. One possible explanation of this finding might be due to a residual binding activity of mutant p73DD, which might cause a mild inhibition of p53/p73. However, we cannot exclude the possibility that the p73DD-isoforms exert other activities in addition to the inhibition of p53/p73.

We observed the strongest effect of dominant-negative p73 on induction of S-phase entry and mitosis in hearts of newborn mice without inducing major adverse side effects regarding apoptosis. Since cardiomyocytes lose their proliferative potential in the perinatal period [28] hearts of newborn mice are clearly more susceptible to proliferative stimuli than cardiomyocytes in adult hearts. Nevertheless, it has to be pointed out that cell cycle progression of neonatal cardiomyocytes is strictly controlled leading to the low labeling index of 0.001% in unstimulated hearts at day 14. Expression of a control virus, which encodes a mutant p73DD harbouring a point mutation that blocks the dominant-negative effect of p73DD had only very little effect on cell cycle parameters indicating that the effect of p73DD are mediated by interference with p53/p73 signaling and not by other, more unspecific effects.

According to our concept, it seemed likely that p73DD(wt) interferes primarily with the expression of p53- and p73-dependent genes. Hence, we analyzed the expression of cell cycle related genes, which are supposed to be under the direct control of p53 and found a strong down-regulation of the cdk-inhibitor p21WAF [29]. The decreased expression of p21WAF, which is a major component of the cell cycle lock in cardiomyocytes [25], might in part explain the increased proliferative activity of neonatal cardiomyocytes after p73DD(wt) expression. Regulation of cell cycle activity depends on the coordinated action of numerous genes including several cyclins. In our experiments we found an mRNA induction of cyclins A, E, B1, and B2 expression upon directed expression of p73DD(wt), which finally resulted in increased protein levels as revealed by Western blots. However, our experiments do not allow to distinguish whether these cyclins were directly induced by p73DD(wt) or whether their elevation rather reflected the regular course of cell cycle progression and was caused by indirect means.

In our experiments we found that expression of p73DD(wt) in the early postnatal period leads to hyperplastic cell growth without signs of cardiomyocyte hypertrophy. We detected an increased number of proliferating cardiomyocytes, which showed a reduced cell size in comparison to untreated control hearts. This situation changed at later time points (P14d). It seems reasonable to assume that arrest of cell cycle progression of cardiomyocytes, which becomes more fixed in the first 2 weeks after birth, caused a shift from hyperplastic to more hypertrophic cell growth. This finding is in line with previous reports showing that the targeted expression of cell cycle activators like D-type cyclins in terminally differentiated cardiomyocytes leads to induction of hypertrophy rather than to proliferation [30]. A closer analysis of the mode of hypertrophy induced by p73DD(wt) in our experiments revealed several aspects of 'pathological' cell growth including expression of BNP and bMHC and increased deposition of interstitial collagen. Historically, the term "pathological" hypertrophy was introduced to describe a pattern of maladaptation of adult cardiomyocytes caused by increased stress. From the molecular point of view, this process is characterized by the re-induction of fetal genes (e.g. bMHC), which substitute for the mature isoforms (e.g. aMHC). Using microarray hybridisation and real-time PCR we demonstrated that expression of p73DD(wt) delays the transition from the proliferating phenotype of fetal cells to mature 'terminally differentiated' cardiomyocytes. It is tempting to speculate that extension of the phase of embryonic gene expression caused by p73DD(wt) collides with the endogenous program of cardiomyocyte maturation thereby enhancing increased maladaptive (-like) cell growth.

Previously, it has been reported that expression of a dominant-interfering p53-isoform in a transgenic model does not lead to enhanced cell cycle activity of cardiomyocytes under basal conditions without further stimulation [7]. Our finding that expression of p73DD promotes proliferation of cardiomyocytes without previous injury might be explained in the following ways: (i) p73DD might act as a 'stronger' antagonist of cell cycle arrest as the CB7 allele of p53 used previously so that no additional stimuli were necessary to induce cell cycle re-entry. p73 is also able to bind DNA and activate transcription via response elements slightly different from p53RE and hence might affect genes which do not respond to p53 although both p53 and p73 are able to act via the p53 response element (p53RE) [31]. (ii) Neonatal cardiomyocytes are more susceptible to an induction of cell cycle progression than adult cardiomyocytes, which have been exclusively used in the study by Nakajima et al. (iii) The expression of p73DD in adult hearts in our experiments was realized by direct intramyocardial injection of the adenoviruses, which also generates an environment of myocardial injury that might stimulate regenerative processes although the presence of cardiomyocytes that incorporated BrdU was not strictly confined to the injection site.

The plasmids encoding p733DD which we have employed for the generation of our adenoviruses were originally used in experiments to explore and suppress the pro-apoptotic mechanisms of E2F1 [13]. In agreement with this study we did not detect an increase in the rate of apoptosis after forced expression of p733DD. Since overexpression of E2F does also stimulate cell cycle entry of cardiomyocytes but often coincides with induction of apoptosis [3,16,24,32,33] it seems promising to combine p73DD(wt) and E2Fs to achieve synergistic effects on cardiomyocyte proliferation and heart regeneration in future experiments. However, we have to point out that the degree of proliferation of cardiomyocytes, which we achieved so far by manipulation of the p73/p53 pathway, was relatively low. It is interesting to note that we observed a strong induction of hypertrophic growth of cardiomyocytes after expression of p73DD, which might reflect the higher propensity of cardiomyocytes to undergo hypertrophy rather than cell division in response to proliferative signals. It will be interesting to determine whether the ability to undergo proliferation might be reached by any cardiomyocyte after appropriate cellular reprogramming or resides only in a rare (sub)-population of cardiomyocytes.

In summary, our data demonstrate that transient expression of a dominant-negative p73DD induces cell cycle activity of both neonatal and adult cardiomyocytes and moderate hypertrophy without provoking apoptosis. This effect seems to be mediated by inhibition of the cdk-inhibitor p21WAF and the induction and stabilization of S- and M-phase cyclins. Prospective investigations are mandatory to

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further explore the potential of p73DD as a candidate to be used for the stimulation of cardiac regeneration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be, found in the online version, at doi:10.1016/j.yjmcc.2008.06.006.

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3 Zusammenfassung und Ausblick

Das adulte Herz ist unter normalen Bedingungen nicht in der Lage, die nach einer Schädigung untergegangenen Kardiomyozyten durch neue kontraktile Zellen zu ersetzen. Da sich eine Herzinsuffizienz in vielen Fällen als Folge des Verlustes von kontraktilem Myokard entwickelt, erscheint es als sinnvoller Therapieansatz, die Menge an Herzmuskelzellen wieder durch therapeutische Eingriffe zu vermehren, um so der Entwicklung bzw. dem Voranschreiten des kardialen Funktionsverlustes zu entgegnen.

Prinzipiell sind verschiedene Ansätze als denkbar beschrieben, die zu einer Vermehrung von Kardiomyozyten beitragen können – in den vergangenen Jahren wurde insbesondere die Möglichkeit von Stammzelltransplantationen intensiv untersucht. Eigene Zellkulturexperimente zeigen jedoch, dass in adulten murinen Knochenmarksstammzellen durch gezielte Interventionen zwar einzelne Herzmuskel-spezifische Gene aktiviert werden können, die Induktion eines vollständigen Differenzierungsprozesses hin zu Zellen mit den funktionellen Charakteristika von Kardiomyozyten ist allerdings nicht möglich. Übereinstimmend stützen Untersuchungen im Herzinfarktmodell der Maus die Hypothese, dass die – zweifellos zu beobachtenden – günstigen Effekte von Zelltransplantationen überwiegend durch parakrine Mechanismen vermittelt werden und nicht auf der Generierung neuer kontraktiler Herzmuskelzellen beruhen.

In der Folge wurde deshalb versucht, durch die gezielte Beeinflussung der Genexpression die Proliferation von Herzmuskelzellen zu induzieren, um so die verfügbare Anzahl von Kardiomyozyten zu vermehren. Es zeigt sich, dass die Expression des Transkriptionsfaktors E2F2 bei Kardiomyozyten den Wiedereintritt in den Zellzyklus auszulösen vermag. Interessanterweise ist dies von einer transkriptionellen Inhibierung pro-apoptotischer Gene begleitet und führt tendenziell zu einer Reduktion der Apoptoserate. Des Weiteren wurde die Bedeutung von p53- und p73-anhängigen Signalkaskaden untersucht, da laut Angaben in der Literatur die Ausschaltung von p53 in einem transgenen Mausmodell zur Proliferation von Kardiomyozyten nach Myokardinfarkt führt. Mittels neu generierter rekombinanter Adenoviren wurden die Auswirkungen der gezielten Expression von p73DD – einer trunkierten Version von p73 mit dominant-negativen Eigenschaften gegenüber p73 und p53 – auf Proliferation und Apoptose von neonatalen und adulten Kardiomyozyten analysiert. Hierbei kann nachgewiesen werden, dass p73DD tatsächlich die Zellzyklusblockade von terminal differenzierten Kardiomyozyten aufzuheben vermag – allerdings finden sich Zeichen der Zellzyklusaktivität (wie auch im Falle der E2F2-Expression) nur bei einer sehr geringen Anzahl von Kardiomyozyten.

Insgesamt belegen die Untersuchungen, dass es prinzipiell möglich ist, den Zellzyklusarrest von Kardiomyozyten zu überwinden – sie zeigen somit eine hypothetische Möglichkeit zur Vermehrung von Herzmuskelzellen im Rahmen regenerativer Therapieansätze auf. Dennoch ist es durch die geschilderten Interventionen bislang nicht gelungen, eine für therapeutische Zielstellungen quantitativ ausreichende Vermehrung von Kardiomyozyten zu induzieren. Bei der geplanten Fortführung der Experimente soll deshalb untersucht werden, ob und wie die Häufigkeit des Zellzyklus-Eintritts weiter gesteigert werden kann. Studien in der Literatur haben zeigen können, dass in anderen Zelltypen die gleichzeitige Beeinflussung verschiedener Signalkaskaden zu synergistischen Effekten in Bezug auf die Proliferationsaktivität führt. Aufgrund der eigenen Daten erscheint die kombinierte Expression von E2F2 und p73DD in Kardiomyozyten diesbezüglich als ein viel versprechender Ansatz. Zukünftige Experimente sollen außerdem die Frage klären, ob sich prinzipiell bei allen Herzmuskelzellen der Wiedereintritt in den Zellzyklus forcieren lässt, oder ob die empfänglichen Zellen eine definierte Sub-Popultion von Kardiomyozyten darstellen.

Verschiedene eigene Befunde legen die Hypothese nahe, dass bei der Zellzyklus-Blockade von Kardiomyozyten epigenetische Phänomene eine wesentliche Rolle spielen. Dies soll im Rahmen weiterer Untersuchungen ebenfalls näher untersucht werden, besondere Berücksichtigung wird hierbei die vermutete Inaktivierung des Cyclin E-Promoters finden.

In den bisherigen Experimenten wurde durch genetische Manipulationen unter Verwendung rekombinanter Adenoviren gezielt Einfluss auf die Zellzyklusarretierung von Kardiomyozyten genommen. Aus den vorliegenden Ergebnissen lassen sich jetzt jedoch auch mögliche Angriffspunkte für pharmakologische Interventionen ableiten. Zum einen haben die bisherigen Arbeiten verschiedene Hinweise darauf ergeben, dass die Inaktivierung von Histon-Deacetylasen (HDACs) eine wichtige Rolle bei der Zellzyklus-Reaktivierung von Kardiomyozyten spielt. Durch den Einsatz chemischer HDAC-Inhibitoren ist dieses Phänomen prinzipiell auch einer pharmakologischen Beeinflussung zugänglich. Außerdem stehen mittlerweile potente synthetische p53-Inhibitoren (z.B. Pifithrin-α) zur Verfügung, deren therapeutisches Potential zur Unterstützung von Regenerationsvorgängen des Herzmuskels im Tiermodell untersucht werden soll.

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5 Thesen

1.) In Deutschland und in vielen anderen europäischen Ländern hat die veränderte Altersstruktur der Bevölkerung in Verbindung mit dem verbesserten Überleben von akuten Myokardinfarkten zu einer deutlichen Zunahme von Patienten geführt, die an chronischer Herzinsuffizienz leiden.

2.) Kardiomyozyten verlieren in der Perinatalzeit ihre Fähigkeit zur Proliferation. Daher ist das adulte Herz nicht in der Lage, die nach einer Schädigung untergegangenen Kardiomyozyten durch neue kontraktile Zellen zu ersetzen. Es erscheint als sinnvoller Therapieansatz, die Menge an Herzmuskelzellen durch therapeutische Eingriffe wieder zu vermehren, um so der Entwicklung bzw. dem Voranschreiten des kardialen Funktionsverlustes zu entgegnen.

3.) Im Rahmen der eigenen Untersuchungen werden verschiedene Fragen zu dem Einsatz von Stammzelltransplantationen im Rahmen der ‚zellulären Kardiomyoplastie' untersucht. Es wird analysiert, inwiefern es möglich ist, durch gezielte Interventionen den Wiedereintritt von Herzmuskelzellen in den Zellzyklus auszulösen, um so die Anzahl von Kardiomyozyten zu vermehren.

4.) Die Behandlung adulter Knochenmarksstammzellen der Maus mit etablierten Myogenese-Induktoren sowie Versuche eines pharmakologischen Chromatin-Remodelings führen zwar zu einer Aktivierung einzelner Herzmuskel-spezifischer Gene, die Induktion eines vollständigen Differenzierungsprozesses hin zu Zellen mit den funktionellen Charakteristika von Kardiomyozyten ist jedoch nicht möglich.

5.) Übereinstimmend stützen Untersuchungen im Herzinfarktmodell der Maus die Hypothese, dass günstige Effekte von Zelltransplantationen überwiegend durch parakrine Mechanismen vermittelt werden und nicht durch die Generierung neuer kontraktiler Herzmuskelzellen zustande kommen.

6.) Um postnatal die Genexpression von Kardiomyozyten gezielt *in vivo* beeinflussen zu können, wird erfolgreich ein Modell der adenoviralen Expression von Fremdgenen in den Herzen neonataler Mäuse etabliert. Mittels quantitativer histologischer Verfahren kann gezeigt werden, dass es möglich ist, durch die herznahe intrathorakale Injektion von rekombinanten Adenoviren eine stabile Transduktion von 71±8% der kardialen Zellen zu erzielen.

7.) Zellkulturexperimente belegen, dass durch die gezielte Expression von E2F2 und E2F4 die Proliferation von Kardiomyozyten angeregt werden kann, ohne dass gleichzeitig eine unerwünschte Steigerung der Apoptoserate zu beobachten ist. Die Stimulation des

kardiomyozytären Zellzykluseintritts in dabei abhängig von der Induktion der Cycline A und E, während D-Typ-Cycline nur von untergeordneter Bedeutung sind.

8.) Auch *in vivo* führt die Expression von E2F2 in Kardiomyozyten zur Überwindung des Zellzyklusarrests. 14 Tage nach den Virus-Injektionen sind die relativen Herzgewichte von E2F2-exprimierenden Versuchstieren signifikant größer als die von Kontrolltieren, nach Expression von E2F4 ist dieser Effekt nicht zu beobachten. Anhand histologischer Untersuchungen kann gezeigt werden, dass sich nur nach E2F2-Expression eine signifikante Zunahme von Kardiomyozyten mit Zeichen der Zellzyklusaktivität findet.

9.) Die Expression sowohl von E2F2 als auch von E2F4 führt zu einer signifikanten Zunahme der Kardiomyozytengröße bei 14 Tage alten Mäusen, was als Ausdruck einer Hypertrophie-Induktion anzusehen ist.

10.) Mittels quantitativer mRNA- und Protein-Bestimmung kann belegt werden, dass die gezielte Aktivierung von E2F2 keine veränderte Expression von cdk-Inhibitoren (*cyclin dependent kinase inhibitors*, CKI) nach sich zieht. Dagegen findet sich in Übereinstimmung zu den Zellkulturexperimenten eine signifikante Induktion der Cycline A und E.

11.) Die Experimente bestätigen insgesamt das Potential von E2F2, in Kardiomyozyten den Zellzyklusarrest aufzuheben, ohne gleichzeitig unerwünschte Effekte bezüglich der Apoptose von Herzmuskelzellen zu induzieren. Für eine hypothetische therapeutische Anwendung im Hinblick auf die effektive Vermehrung von Herzmuskelzellen erscheint dieser Effekt jedoch bislang quantitativ nicht ausreichend.

12.) Untersuchungen in der Literatur hatten belegen können, dass die kombinierte Beeinflussung verschiedener Signalkaskaden zu synergistischen Effekten bezüglich der Teilungsaktivität von Zellen führt. Für Kardiomyozyten war gezeigt worden, dass die Ausschaltung von p53 zur verstärkten Proliferation von Kardiomyozyten nach Myokardinfarkt führt. Nicht untersucht wurde bisher jedoch, ob vergleichbare Effekte auch nach Ausschaltung des strukturell und funktionell verwandten p73 zu beobachten sind.

13.) Um die Aktivität von p53 und p73 gezielt inhibieren zu können, werden rekombinante Adenoviren generiert, die eine p73-Isoform mit dominant-negativen Eigenschaften exprimieren (p73DD). In Zellkulturexperimenten inhibiert p73DD sowohl die p53- als auch die p73-abhängige Transkription und induziert die Protein-Expression von endogenem p53 und p73 im Sinne einer *feed back*-Regulation.

14.) p73DD induziert in den Herzen neonataler Mäuse die Expression von proliferationsfördernden Cyclinen und *cyclin dependent kinases* (cdk). Ausserdem werden Histon-Acetyl-Transferasen unter Einwirkung von p73DD vermehrt exprimiert, und es findet sich eine in funktioneller Hinsicht synergistische Herabregulation von Histon-Deacetylasen als Hinweis auf eine Beeinflussung des Chromatin-Remodelings in Richtung einer vermehrten transkriptionellen Genaktivierung.

15.) Weitere Untersuchungen belegen, dass die adenovirale Expression von p73DD zur vermehrten Zellzyklusaktivität von Kardiomyozyten und zur signifikanten Zunahme der relativen Herzgewichte der Versuchstiere führt. 14 Tage nach Injektion der Viren zeigt sich jedoch ein Wechsel von der vornehmlichen Proliferation hin zur vermehrten Hypertrophie der Kardiomyozyten.

16.) Mittels Western blot kann gezeigt werden, dass entsprechend der Arbeitshypothese p73DD in den Herzmuskelzellen zu einer Herabregulation des bekanntermaßen p53- und p73-responsiven Zellzyklus-Inhibitors p21 führt, was als ein wichtiger Mechanismus der Zellzyklusstimulation angesehen werden kann. Des Weiteren sind in p73DD-exprimierenden Kardiomyozyten die Cycline A, E, B1 und B2 vermehrt nachweisbar.

17.) Nach Expression von p73DD finden sich keine Hinweise für eine vermehrte Apoptose von Herzmuskelzellen.

18.) Perspektivisch erscheint die kombinierte Inhibierung von p53-/p73-abhängigen Signalkaskaden gemeinsam mit einer Erhöhung der E2F-Aktivität als ein viel versprechender Ansatz, um Regenerationsvorgänge von Kardiomyozyten zu induzieren. Hierdurch sind zum einen synergistische Effekte im Hinblick auf den S-Phase-Eintritt und die Zellzyklusprogression der Herzmuskelzellen zu erwarten. Zum anderen ist davon auszugehen, dass gleichzeitig auch der E2F-initiierten Apoptose, die zumindest teilweise über p53- und p73-abhängige Kaskaden vermittelt wird, weiter entgegnet werden kann.

Lebenslauf

29.05.1974	in Hohenmölsen geboren
1980 – 1988	Polytechnische Oberschule „PAUL WEGMANN“, Zeitz
1988 – 1992	Spezialschule mathematisch-naturwissenschaftlich-technischer Richtung „ERNST HAUSMANN“ in Halle/S. (spätere Umbenennung in „GEORG-CANTOR-GYMNASIUM“); Erlangen der Hochschulreife mit der Durchschnittsnote 1,0
1992 – 1993	Zivildienst
1993 – 1999	Studium der Humanmedizin an der MARTIN-LUTHER-UNIVERSITÄT Halle-Wittenberg
01.11.1997	Aufnahme in die „Studienstiftung des deutschen Volkes“ (Förderung bis Ende des Studiums)
1996 – 1999	Arbeit an Promotion unter Leitung von Herrn Prof. Dr. E. Peschke im Insitut für Anatomie und Zellbiologie der MARTIN-LUTHER-UNIVERSITÄT Halle-Wittenberg; Thema: „Radikal-induzierte Veränderungen an pankreatischen B-Zellen neonater Wistar-Ratten *in vitro* – Einfluß von Melatonin“; Erlangen des Grades „doctor medicinae“ mit dem Prädikat: „summa cum laude (ausgezeichnet)“
1999	Approbation
2000 – 2001	Arzt im Praktikum an der Klinik und Poliklinik für Innere Medizin III der Martin-Luther-Universität Halle-Wittenberg (Direktor: Prof. Dr. Karl Werdan)
2001 – 2004	Forschungsaufenthalt bei Prof. Dr. Dr. T. Braun (Institut für Physiologische Chemie der Martin-Luther-Universität; Thema: „Herzmuskelregeneration“)
seit 2004	Assistenzarzt an der Klinik und Poliklinik für Innere Medizin III der Martin-Luther-Universität Halle-Wittenberg (Direktor: Prof. Dr. Karl Werdan) in Weiterbildung zum Facharzt für Innere Medizin und Kardiologie

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Selbstständigkeitserklärung

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Erklärung über weitere Habilitationsverfahren

Ich erkläre, dass an keiner anderen Fakultät oder Universität ein Habilitationsverfahren anhängig ist.

15.09.2008 Henning Ebelt

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