Conditional Expression of SV40 T-antigen in Mouse Cardiomyocytes Facilitates an Inducible Switch from Proliferation to Differentiation

Received for publication, December 23, 2002, and in revised form, January 27, 2003
Published, JBC Papers in Press, February 17, 2003, DOI 10.1074/jbc.M213102200

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Studies of cardiac muscle gene expression and signaling have been hampered by the lack of immortalized cardiomyocyte cell lines capable of proliferation and irreversible withdrawal from the cell cycle. With the goal of creating such cell lines, we generated transgenic mice using cardiac-specific cis-regulatory elements from the mouse Nkx2.5 gene to drive the expression of a simian virus 40 large T-antigen (TAg) gene flanked by sites for recombination by Cre recombinase. These transgenic mice developed tumors within the ventricular myocardium. Cells isolated from these tumors expressed cardiac markers and proliferated rapidly during serial passage in culture, without apparent senescence. However, they were unable to exit the cell cycle and failed to exhibit morphological features of terminal differentiation. Introduction of Cre recombinase to these cardiac cell lines by adenoviral delivery resulted in the elimination of TAg expression, accompanied by rapid cessation of cell division, and increase in cell size without an apparent induction of cellular differentiation. Incubation of cells lacking TAg in serum-deficient media with various pharmacological agents (norepinephrine, phenylephrine, or bone morphogenetic protein-2/4) or constitutively active calcium/calmodulin-dependent protein kinase I and/or calcineurin led to the formation of sarcomeres and up-regulation of cardiac genes involved in excitation-contraction coupling. The combination of TAg expression under the control of an early cardiac promoter and Cre-mediated recombination allowed us to derive an immortal cell line from the ventricular myocardium that could be controllably withdrawn from the cell cycle. The conditional expression of TAg in this manner permits propagation and regulated growth termination of cell types that are otherwise unable to be maintained in cell culture and may have applications for cardiac repair technologies.

Studies of cardiac development have been hampered by the lack of immortalized cell lines capable of proliferation and differentiation. The major obstacle to deriving such cell lines is the phenomenon of permanent withdrawal of mammalian cardiac muscle cells from the cell cycle shortly after birth (1, 2). Although a small fraction of adult mammalian cardiomyocytes can re-enter the cell cycle and replicate DNA upon physiological or pathological stimulation in vivo, there is no significant contribution to cardiac repair by hyperplasia of cardiac cells following damage (i.e. myocardial infarction) (3). Neonatal or embryonic cardiac muscle cells will progress through limited rounds of cell division in cell culture, but they ultimately withdraw permanently from the cell cycle, and cultured adult cardiomyocytes will not divide and eventually die.

There have been several attempts to establish cardiac muscle cell lines. Cardiomyocytes have been derived from embryonic stem cells (4), P19 cells (5), and hematopoietic stem cells (6). During the course of differentiation these cells differentiated into different cell types and therefore did not represent a homogeneous cell population. To overcome this problem the cardiomyocytes derived from embryonic stem cells were subjected to enrichment by using a selectable marker driven by a cardiac-specific promoter (7, 8). Nonetheless, all of these approaches failed to provide a sustainable homogeneous cardiac cell line.

Other cell lines, QCE-6 and H9c2, derived from precardiac mesoderm of quail (9) or embryonic rat myocardium (10), respectively, provided useful models for studying early cardiac fate specification and cardiac ion channel function. However, upon induction of differentiation, the QCE-6 cell line transformed into a mixed population of cells and failed to differentiate into mature cardiomyocytes. The H9c2 cell line was shown to express a number of muscle-specific channels but displayed few muscle structural proteins.

Many investigators have used ectopic expression of oncogenes to transform embryonic or adult cardiac muscle cells into immortalized cell lines. Rat embryonic ventricular cardiomyocytes infected with recombinant retrovirus expressing v-myc and v-H-ras resulted in cells exhibiting some myocyte characteristics; however, these cells do not form sarcomeres or contract (11).

Promising results have come from studies utilizing simian virus 40 (SV40) T-antigen (TAg) as a transforming factor in murine and human primary cells (12). Cell lines derived by

The abbreviations used are: TAg, simian virus 40 large T-antigen; BrdUrd, 5-bromo-2′-deoxyuridine; Ad, adenovirus; Ad-Cre, recombinant adenovirus expressing Cre recombinase; Ad-lacZ, recombinant adenovirus expressing β-galactosidase; BMP, bone morphogenetic protein; FBS, fetal bovine serum; PE, phenylephrine; NE, norepinephrine; TGF-β1, transforming growth factor β1; IGF-I, insulin-like growth factor I; RT, reverse transcriptase; CaMKI, calcium/calmodulin-dependent protein kinase I; HDAC, histone deacetylases.

1 The work was supported by grants from the National Institutes of Health, Bridges Project for the Alliance for Cellular Signaling (NIGMS, National Institutes of Health), the Donald W. Reynolds Foundation, and the Texas Advanced Technology Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
expression of TAg in cardiac, skeletal, and smooth muscle showed rapid proliferation and, in the case of conditional expression, retained some degree of differentiation (13–20). In particular, the AT-1 cell line derived from atrial tumors of transgenic mice expressing SV40 TAg driven by the atrial natriuretic factor promoter was the first cardiac cell line that was able to maintain contractility in vitro after being passaged several times (21, 22). The main drawback of this line is that it must be propagated as ectopic grafts in syngeneic mice and cannot be passaged in vitro or frozen. In contrast, the HL-1 cell line derived from the original AT-1 line can be passaged in conventional cell culture and frozen, although it must be maintained in a proprietary media of unknown composition supplemented with growth factors and hormones such as insulin, retinoic acid, and norepinephrine (23). Neither AT-1 nor HL-1 cells become quiescent by serum deprivation. These drawbacks significantly limit their use in signal transduction and biochemical studies.

The Nkx2.5 gene encodes a homeodomain transcription factor that is among the earliest known markers of cardiogenesis in the vertebrate embryo (24). Recently, a cardiac cell line that expresses TAg under the control of the proximal cardiac enhancer of the Nkx2.5 gene was derived (13). This cardiac cell line exhibited a cardiac embryonic like phenotype. However, it was devoid of striations or contractile properties and unable to exit the cell cycle.

In the present study, we developed immortalized cardiac cell lines from hearts of transgenic mice carrying cardiac-specific regulatory sequences from the mouse Nkx2.5 gene to drive the expression of the TAg coding region flanked by loxP sites. We implemented the Cre-lox system as an efficient method to inactivate genes permanently (25, 26), allowing for Cre recombinase-mediated deletion of the TAg gene. We show that these cell lines proliferate rapidly until they are infected with an adenovirus encoding Cre recombinase, at which time they cease expressing TAg and exit the cell cycle. Cessation of cell division in these cells is accompanied by an alteration of cell morphology, assembly of an organized stress fiber network, and changes in gene expression characteristic of differentiated myocytes. This strategy for generating reversibly transformed cell lines may be widely applicable for the generation of cell lines from a variety of tissues that are otherwise unable to be maintained in proliferative or differentiated states in culture.

EXPERIMENTAL PROCEDURES

Generation of Transgenic Mice—DNA constructs, Nk-TAg (Fig. 1A) and NkL-TAg (Fig. 4A), were used to generate transgenic mice expressing SV40 large TAg under the control of the mouse Nkx2.5 gene heart-specific enhancer (~9435 through ~735 bp) and its proximal promoter (~265 through ~262 bp) (27). NkL-TAg DNA was constructed by inserting 34-bp loxP sequences on either side of the TAg of Nk-TAg DNA. The orientation of the loxP recognition sequences was confirmed by sequencing. Nk-TAg and NkL-TAg DNA fragments were excised from the pBluescript plasmid using XhoI and XbaI and were gel-purified prior to microinjection of the DNA into pronuclei of fertilized B6C3F1 mice. Genotyping of F0 mice was performed by Southern blot and prior to microinjection of the DNA into pronuclei of fertilized B6C3F1 (C57BL/6J × C3H/HeJ) mice. Genotyping of F0 mice was performed by Southern blot and prior to microinjection of the DNA into pronuclei of fertilized B6C3F1 (C57BL/6J × C3H/HeJ) mice.

Cloning cylinders were used to harvest cell clones. Cell growth curves were generated by plating 1.5 × 10^4 or 2 × 10^5 cells (as indicated) in growth medium onto 100-mm plates and counting total cell number every 24 h for 5 days. Differentiation medium contains Dulbecco's modified Eagle's medium/F-12 supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml 0.2% collagen type II (Worthington) and 0.6 mg/ml pancreatin (Sigma). Cardiac cells derived from Nk-TAg and NkL-TAg mice were maintained in Dulbecco's modified Eagle's/F-12 media supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml 0.2% collagen type II (Worthington) and 0.6 mg/ml pancreatin (Sigma). Cardiac cells derived from Nk-TAg and NkL-TAg mice were maintained in Dulbecco's modified Eagle's/F-12 media supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml 0.2% collagen type II (Worthington) and 0.6 mg/ml pancreatin (Sigma).

Histochemical Analysis—Heart tissue was fixed in 10% phosphate-buffered formalin and stained with hematoxylin-eosin. Nk-TAg and NkL-TAg cells were cultured on coverslips and fixed for 10 min with either −20 °C methanol or 4% paraformaldehyde for immunohistochemistry. Blocking was performed by incubating fixed cells with 1.5% bovine serum albumin and 0.1% normal goat serum in phosphate-buffered saline for 30 min. Primary antibodies were incubated for 30–60 min in 1.5% bovine serum albumin in phosphate-buffered saline as follows: monoclonal anti-myosin (smooth) (1:100, Sigma), polyclonal anti-myosin (skeletal) (1:100, Sigma), monoclonal anti-α-smooth muscle actin (1:100, Sigma), monoclonal anti-skeletal myosin (slow) (1:100, Sigma), polyclonal anti-connexin-43 (1:100, Sigma), monoclonal anti-α-sarcomeric actin clone 5C5 (1:100, Sigma), monoclonal anti-desmin (1:100, Sigma), monoclonal anti-calcinon (1:100, Sigma), monoclonal anti-α-actinin (sarcomeric) clone EA-53 (1:200, Sigma), and monoclonal anti-SV40 T-antigen (1:100, Santa Cruz Biotechnology). Secondary antibodies conjugated to either fluorescein isothiocyanate or Texas Red (Jackson-Western) were diluted in phosphate-buffered saline and incubated for 30 min at room temperature. In some cases, nuclei were co-stained with 4,6-diamidino-2-phenylindole (10 μg/ml) for 1 min. Coverslips were mounted with Vectashield (Vector Laboratories), and fluorescein or confocal images were captured using Leica DMRXE or Zeiss 3.95 microscopes, respectively.

Measurements of Contractility and Calcium Transients in Response to Electrical Stimulation—Excitation-contraction coupling function was assessed as described previously (29, 30) by measuring cell contractility and calcium transients. Briefly, cells were grown in 35-mm tissue culture dishes for 2–3 days until 70–80% confluent and loaded with 2 μM fura 2-AM (Molecular Probes) in minimum Eagle's medium containing 1 mM MgCl2 and exposed to 37 °C. A platinum ring was placed in the tissue culture dish. Myocyte contractions and calcium transients were elicited by field stimulation at 1.5 Hz (Ion Optix) with current pulses of 4 ms duration and voltages of 40 V. The polarity of the stimulating electrodes was alternated at every pulse to prevent accumulation of electrochemical byproducts. Myocyte contractions were imaged and scanned at a rate of 240 Hz (Ion Optix). Calcium transients were observed by exciting the fura 2-AM-loaded cells with alternating wavelengths of 340 and 380 nm and recording the emission intensity at 510 nm. Contraction and calcium transient data for each myocyte were recorded from a minimum of 12 consecutive stimuli.

Drug Treatment—Drugs were added into cell culture media as indicated. The following concentrations were used: 100 μM phenylephrine (PE), 10 μM norepinephrine (NE), 10 ng/ml recombinant human transforming growth factor-β (TGF-β1) (R & D Systems), 1 μg dynorphin-β (Peninsula Laboratories), 1 μM trans- or cis-retinoic acid (Sigma), 15 ng/ml bone morphogenetic protein (BMP)-2/4 (Genetics Institute, Cambridge, MA), 1 μM angiotensin II (R & D Systems), 20 μM endothelin-1 (R & D Systems), 100 ng/ml insulin-like growth factor I (IGF-I) (Roche Molecular Biochemicals), 5-aza-cytidine (Sigma), and 10 μg/ml mitomycin C (Sigma).

Viral Infection—Cells were infected with recombinant adenoviruses (Ad) at a multiplicity of infection of 100 for 3–12 h. The medium was replaced with growth medium, and NkL-TAg cells were cultured for the next 5 days. Cells were rinsed in PBS, and RNA was obtained from the following sources: Ad-Cre recombinase (Ad-Cre) was provided by Dr. Frank Graham (McMaster University) (25); GATA4 (Ad-GATA4), Nkx2.5 (Ad-Nkx2.5), MEK6 (Ad-MEK6), and green fluorescent protein (Ad-GFP) were generated using the “Easy-Track” system as described (31); antisense HDAC4 and HDAC5 (Ad-HDAC4 or -5), and CRE specific coding regions (Ad-CRE) were generated using the Easy-Track system as described (31); vectors encoding HDAC4 or HDAC5 (Ad-CRE) were generated using the Easy-Track system as described (31); vectors encoding HDAC4 or HDAC5 (Ad-CRE) were generated using the Easy-Track system as described (31). Ad-hCMV-lacZ were generated by Dr. Robert Gerard (University of Texas Southwestern) and were constructed using an adenovirus vector as described above and cultured in growth medium for 2 more days. Ad-hCMV-lacZ were generated by Dr. Robert Gerard (University of Texas Southwestern) and were constructed using an adenovirus vector as described above and cultured in growth medium for 2 more days.

DNA Synthesis Assay—NkL-TAg cells were infected with Ad-Cre virus as described above and cultured in growth medium for 2 more days followed by incubation with BrdUrd for 2 h. DNA synthesis was
RESULTS

Generation of Nhx2.5-TAg Transgenic Mice with Cardiac Tumors—The Nhx2.5 cis-regulatory sequences consisting of the early cardiac-specific enhancer region (−9435 to −7353 bp) fused to the endogenous promoter (−265 to +262 bp) were linked to the coding region for SV-40 large T-antigen and GATA4 by Western blot analysis in isolated Nk-TAg cell lines. All of the established cell lines expressed Nhx2.5, GATA4, and TAg genes as detected by RT-PCR (data not shown), although the level of expression varied for each cell line.

Many loci of myocardial hyperplasia were noted, none of which involved the endocardium. The architecture of the remaining myocardium was preserved although many cardiomyocytes had excessively large hematoxylin-rich nuclei as a possible sign of polyploidy. It was not possible to determine the cause of death of the animal; however, it is plausible that either outflow obstruction or ventricular arrhythmia led to sudden death.

Isolation of Immortalized Cardiac Cells—A heart harvested from a 3.5-week-old (F₂) transgenic Nhx2.5-TAg mouse displaying mild cyanosis exhibited protruding masses in the left ventricle. These tumors were excised from the myocardium and grown in medium containing 20% FBS, we observed a doubling time of less than 24 h. Decreasing the serum content to 15% slightly reduced the doubling time indicating that Nk-TAg cells respond to factors in serum (data not shown).
and cardiac-enriched gene expression. The majority of Nk-TAg cell lines expressed transcripts encoding proteins characteristic of cardiomyocytes, such as the Nkx2.5, GATA4, and MEF2C transcription factors (Fig. 2A; and see Table I for a list of other genes). However, many structural proteins that are essential for contraction of cardiac myocytes, including titin and sodium, calcium-exchanger (Ncx-1), were not detected even by RT-PCR (Table I).

Immunohistochemistry analysis of Nk-TAg cells showed a low level of expression of α-actinin, a prototypical Z-line protein in cardiomyocytes (data not shown). Growing the cells on plates coated with laminin or type II collagen at normal (10%) or low serum content (2 or 5%) did not increase expression of α-actinin (Fig. 3A). Mitomycin C treated with mitomycin C were stained with 4,6-diamidino-2-phenylindole (DAPI) and with anti-α-actinin antibody (B and C). Images were taken at ×40 magnification (A and B) and ×100 magnification (C).

A diagram of the NkL-TAg transgenic construct containing the Nkx2.5 cis-regulatory sequences consisting of the early cardiac-specific enhancer region (∼9435 to ∼7353 bp), fused to the endogenous promoter (∼265 to ∼262 bp), and linked to the SV-40 large T-antigen coding region flanked by loxP sites (red diamonds). B, Immunocytochemistry using TAg antibody of NkL-TAg cells (control) and NkL-TAg cells infected with Ad-Cre. An equivalent number of cells was placed on plates. All the cells stained for TAg in the absence of Cre recombinase (left panel), whereas less than 1% of cells stained for TAg after addition of Cre recombinase (right panel); C, BrdUrd incorporation was measured in NkL-TAg cells (control) and NkL-TAg cells infected with Ad-Cre. Equivalent numbers of cells were counted at ×20 magnification. D, NkL-TAg (control) or NkL-TAg cells infected with Ad-LacZ or Ad-Cre were plated at 2 × 10^5 cells per 100-mm plate. Cells were counted for 5 subsequent days. NkL-TAg cells stop proliferating in response to excision of the TAg gene (data not shown). However, addition of mitomycin C to the growth medium abated proliferation of the Nk-TAg cells and enhanced expression of α-actinin in the cytoplasm as unassembled Z-lines (Fig. 3). This finding suggested that inhibition of DNA synthesis in Nk-TAg cells promoted a cardiogenic phenotype. Addition of hypertrophic agents including endothelin-1, PE, and angiotensin II did not further induce the assembly of sarcomeres in Nk-TAg cells (data not shown).

Generation of Conditional TAg-transformed Cardiomyocyte Cell Line—Based on the finding that mitomycin C enhanced α-actinin expression in Nk-TAg cells, we postulated that the lack of growth control is counterproductive to establishing a cardiomyocyte cell line. Therefore, in an effort to induce cellular differentiation, we sought to generate cardiac cell lines capable of terminating cell division. We chose the Cre-lox system as an efficient method to permanently remove and thereby inactivate TAg.

Two loxP sites were added to flank the TAg-encoding region in the Nk-TAg DNA construct (Fig. 4A), and this construct, NkL-TAg, was used to generate transgenic mice. Dissection of the hearts of NkL-TAg transgenic mice at 3.5 weeks of age revealed one mouse with gross cardiomegaly due to multiple ventricular myocardial tumors similar to those found in Nk-TAg mice (data not shown, see Fig. 1). Histological analysis of the transgenic heart revealed a phenotype similar to that observed in the hearts of Nk-TAg transgenic animals (data not shown).
Cells were dissociated from the tumor regions of the NkL-TAg transgenic hearts and plated onto fibronectin/gelatin-coated dishes at a low density. Following 10 days in growth medium containing 10% FBS, the NkL-TAg cells showed different characteristics than the Nk-TAg cells. In contrast to the Nk-TAg cells, NkL-TAg cells did not form well defined colonies and did not grow on top of each other. Remarkably, some of the plated cells maintained contractile activity during the initial passages, although this characteristic gradually disappeared after a few cell passages. Immunohistochemistry confirmed TAg expression in the NkL-TAg cells and also showed that some cells expressed α-actinin in a non-striated pattern (data not shown).

**NkL-TAg Cells Exit the Cell Cycle following Cre-recombinase Expression**—NkL-TAg cells grew without apparent senescence even after 50 serial cell passages. Immunocytochemistry using TAg antibody showed that all NkL-TAg cells expressed TAg protein regardless of the passage number. Infection of NkL-TAg cells with recombinant adenovirus expressing Cre recombinase (Ad-Cre) effectively removed the TAg gene from the cellular genome as confirmed by PCR (data not shown) and immunocytochemistry using TAg antibody (Fig. 4B). Deletion of the TAg gene was followed by a dramatic decrease in BrdUrd incorporation (Fig. 4C) and eventual cessation of cell growth of the NkL-TAg cells (Fig. 4D). In contrast, infection of NkL-TAg cells with a recombinant adenovirus expressing β-galactosidase (Ad-LacZ) initially caused a decrease in the cell growth rate, but ultimately resulted in no change in cell proliferation rate when compared with non-infected cells (Fig. 4D).

**Characterization of NkL-TAg Cells before and after Removal of Tag**—Although NkL-TAg cells showed a gene expression pattern similar to Nk-TAg cells (Table I), additional cardiac-specific genes were expressed in NkL-TAg cells including myoglobin, α-myosin heavy chain, FHL2/DRAL, MCIP1, MEF2D, and atrial natriuretic factor. Immunocytochemistry revealed expression of desmin and cadherin (Fig. 5, A and B) as well as connexin-43 (data not shown) that was localized to the cell junctions.

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**Fig. 5. Immunocytochemistry of NkL-TAg cells.** A. NkL-TAg cells were immunostained with antibodies recognizing desmin or cadherin. B. NkL-TAg cells non-infected (control) or infected with Ad-Cre were immunostained with antibody recognizing α-smooth muscle actin. Magnification at ×40.

**Fig. 6. Effect of different treatments on the morphology of NkL-TAg cells before and after deletion of TAg.** Uninfected NkL-TAg cells (control) or NkL-TAg cells infected with Ad-Cre were cultured in differentiation media containing PE, NE, BMP2/4, Ad-CaMKI, or Ad-calcineurin. Immunocytochemistry was performed using anti-α-actinin (sarcomeric) antibody. Magnification at ×40.
Deletion of the TAg and subsequent withdrawal from the cell cycle resulted in a dramatic decrease in cell surface area (up to 10-fold and dependent on cell density). Immunocytochemistry showed that NkL-TAg cells depleted of TAg exhibited pronounced stress fibers that were immunoreactive for smooth muscle actin (Fig. 5, C and D). After removal of TAg, many of the NkL-TAg cells become binucleated. However, depletion of TAg had no apparent effect on the level of expression of selected cardiac-specific genes as tested by RT-PCR and/or immunocytochemistry (data not shown).

**Induction of Differentiation of NkL-TAg Cells by Various Stimuli**—We surveyed the effect of different drugs, hormones, and overexpression of signaling proteins in attempts to induce further differentiation of NkL-TAg cells. Differentiation was assessed using immunocytochemistry with an antibody against sarcomeric α-actin. Prior to depletion of TAg, addition of caffeine, potassium chloride, 5-azacytidine, low oxygen, PE, NE, IGF-I, cis- or trans-retinoic acids, dynorphin, TGF-β1, ionomycin, basic fibroblast growth factor, or recombinant adenovirus containing various transcripts (CMV-LacZ, CMV-Cal-calcineurin A, CMV-GATA4, CMV-Nkx2.5, CMV-IGF1, CMV-HDAC5, CMV-CaMKI, CMV-MEK6, and CMV-calcineurin A) had no effect on inducing sarcomere formation as assessed by α-actin immunocytochemistry. However, expression of α-actin was increased in NkL-TAg cells upon removal of TAg and switching the medium to contain low serum (5% horse serum), insulin, transferrin, and selenium (Fig. 6). Moreover, addition of PE, BMP2/4, or NE, to the media led to further induction of sarcomere formation in some cells (Fig. 6). A similar induction was seen when NkL-TAg cells depleted of TAg were grown in differentiation media and infected with recombinant adenovirus expressing constitutively active CaMKI or constitutively active calcineurin A (Fig. 6), known effectors of cardiomyocyte hypertrophy (34, 35).

**NkL-TAg Cells Exhibit Calcium Transients in Response to Electrical Stimulation**—Calcium current and cell contractility were analyzed to determine whether NkL-TAg cells were excitable. NkL-TAg cells expressing TAg showed no response to electrical stimulation. However, when NkL-TAg cells depleted of TAg and cultured for 7 days in differentiation medium containing PE were subjected to electrical stimulation, calcium transients were readily detected in ~30% of the cells examined (Fig. 7A). This implies that the sarcoplasmic reticulum of NkL-TAg cells releases calcium in response to electrical stimulation. Despite this fact, cells failed to contract. In comparison to freshly isolated adult cardiac myocytes (Fig. 7B), NkL-TAg cells showed a diminished response. In addition, calcium transients in NkL-TAg cells were elicited in response to every other stimulus at the 50-Hz stimulation frequency, whereas freshly isolated cardiac myocytes responded to each electrical stimulus. The inability of NkL-TAg cells to respond to every stimulus may be attributable to delayed restoration of excitability because intracellular calcium in these cells may return to the basal level more slowly than in normal adult myocytes.

**Microarray Analysis of Nk-TAg and NkL-TAg Cell Lines**—Gene expression profiles were examined using microarray analysis comparing RNA transcripts of NkL-TAg cells with NIH/3T3 fibroblasts. Scatter plot analysis of the microarray

![Fig. 7. Measurement of calcium transients in NkL-TAg cell lines](image)

**Fig. 7. Measurement of calcium transients in NkL-TAg cell lines.** A, NkL-TAg cells infected with Ad-Cre were subjected to an electrical stimulation at 1.5 Hz (Ion Optix) with current pulses of 4-ns duration and voltages of 40 V. Calcium transients were observed by exciting the fura 2-AM-loaded cells with alternating wavelengths of 340 and 380 nm and recording the emission intensity at 510 nm. Calcium transient data for each myocyte were recorded from a minimum of 12 consecutive stimuli. B, similar measurements were performed on mouse adult cardiomyocytes. Pulses are indicated with arrowheads.

![Fig. 8. Microarray analysis of gene expression profiles of the NkL-TAg cell line](image)

**Fig. 8. Microarray analysis of gene expression profiles of the NkL-TAg cell line.** Scatter plot of microarray analysis using RNA isolated from uninfected NkL-TAg cells (control) and NkL-TAg cells infected with Ad-lacZ (A); uninfected NkL-TAg cells (control) and NkL-TAg cells infected with Ad-Cre (B); C, NkL-TAg cells infected with Ad-LacZ and NkL-TAg cells infected with Ad-Cre. Each dot represents a gene on the microarray slide. **Block dots** denote genes that are expressed in both samples. **Green dots** represent genes exclusively expressed in NkL-TAg cells infected with Ad-lacZ (A), NkL-TAg cells infected with Ad-Cre (B), and NkL-TAg cells infected with Ad-Cre (C). **Blue dots** represent genes exclusively expressed in A, and NkL-TAg cells (control) in B, and NkL-TAg cells infected with Ad-lacZ in C. **Diagonal lines** on the graphs show 2-, 5-, and 10-fold differences.
results revealed three distinct groups of genes. The first and largest group contains genes that are equally represented in both of the cell lines, consisting primarily of housekeeping genes. The second group contains genes that are predominantly or exclusively expressed in NIH/3T3 cells and consists of non-muscle genes. The third group of genes is predominantly or exclusively expressed in NkL-TAg cells and consists mainly of cardiac-specific genes, supporting the premise that the NkL-TAg cell line is a cardiomyocyte cell line (see Supplemental Material, Table S2). Notably, many of the genes revealed by microarray analysis, such as those encoding calponin, smooth muscle actin, skeletal actin, and atrial natriuretic factor, are characteristic of embryonic cardiomyocytes. Furthermore, analysis of the microarray data revealed several uncharacterized ESTs expressed in NkL-TAg cells. These ESTs were shown to be selectively expressed in heart by Northern blot and in situ hybridization (data not shown).

Microarray analysis was also performed on RNA transcripts isolated from NkL-TAg cells (grown in differentiation media with PE) expressing or depleted of TAg. Infection of NkL-TAg cells with Ad-LacZ showed an up-regulation of 298 genes; 197 of these genes were also up-regulated in NkL-TAg cells infected with Ad-Cre, and 232 genes were down-regulated, including 74 genes that were down-regulated in Ad-Cre-infected cells (Fig. 8A). Further studies were not done on these genes, viewing them as a cellular response to adenovirus infection. Deletion of the TAg gene by Cre-recombinase led to significant alterations of gene expression in NkL-TAg cells; 313 genes were down-regulated (>2-fold), and 214 genes were up-regulated (>2-fold) (Fig. 8, B and C). Specifically, the majority of genes down-regulated in TAg-deleted NkL-TAg cells were involved in cell cycle regulation, including those encoding cyclin A2, cyclin E2, cyclin B1, cyclin B2, cdc45, cdc46, cdc6, cdc7, Mcc2, cdc25, and cdk2. In contrast, many of the transcripts that were up-regulated upon deletion of TAg were cardiogenic-associated genes. These findings as well as increased expression of cell cycle inhibitor genes (data not shown) indicate that these cells could be used as a reagent to study cell cycle events in cardiomyocytes.

Expression of TAg is compatible with the expression of many early cardiac lineage markers but is incompatible with growth arrest and formation of mature sarcomeres. The use of a “floxed” TAg transgene circumvents these effects and allows for a rapid and reversible switch in the proliferative properties of cardiac muscle cells. To our knowledge, this type of conditional Cre-lox approach has not been utilized previously to expand and control the differentiation of specific populations of progenitor cells in vitro. It may be useful in the future to use similar Cre-lox methods to expand and study many progenitor or mature cell lines depending on the promoter or transforming agent applied.

The “floxed” TAg approach proved to be highly efficient and efficacious for the control of proliferation of the derived NkL-TAg cell lines. However, withdrawal of NkL-TAg cells from the cell cycle was not sufficient to induce differentiation as assessed by sarcomere formation. It required cultivation of TAg-depleted NkL-TAg cells in low serum media with addition of several factors or overexpression of genes to induce formation of sarcomeres. This was accompanied by dramatic changes in gene expression as revealed by the microarray data. Subpopulation of these cells exhibited Ca²⁺ current upon electrical stimulation. Although TAg-depleted NkL-TAg cells do not contract spontaneously or upon electrical stimulation, further studies may focus on this process, perhaps utilizing different culture strategies.

In summary, we have created cardiomyocyte cell lines using the Nkx2.5 promoter/enhancer and TAg. These cell lines provide interesting opportunities for the study of cardiomyocytes particularly because the NkL-TAg cell line can be expanded in culture and induced to a degree of differentiation with expression of Cre recombinase. In general, the Cre-lox method we have utilized may be useful for the development of different cell lines capable of proliferation followed by differentiation. NkL-TAg cells may serve as a useful tool for studies of cardiac gene regulation and cellular signaling, as well as for novel gene discovery.

Acknowledgments—We thank John McAnally for generating transgenic mice; Dr. Ralph Shohet and Teresa Gallardo for assistance with microarray data analysis; Dr. Robert Gerard for providing adeno-
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