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Conditional differentiation of heart- and smooth muscle-derived cells transformed by a temperature-sensitive mutant of SV40 T antigen

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SUMMARY

To create muscle cell lines that conditionally differentiate in vitro we introduced a temperature-sensitive SV40 T antigen by retroviral infection into rat aortic smooth muscle cells (SMCs) and neonatal heart-derived cells. After G418 selection cell lines isolated were characterized at permissive (33°C) and non-permissive (39°C) temperatures. [^{3}H]Thymidine uptake showed that progression through the cell cycle is greatly reduced at 39°C. Cytoskeletal proteins, such as actins and vimentin did not change significantly after temperature shift, while the number of desmin-positive SMCs significantly increased when cells were switched to 39°C. Heart-derived muscle cells showed sarcomeric myosin heavy chain reactivity only when grown at 39°C. After thrombin stimulation intracellular calcium in both cell types increased severalfold in 39°C-cells but not in 33°C-cells. Whole cell patch-clamp recordings of SMCs and heart-derived cells revealed a strong increase in nicardipine-sensitive Ca^{2+} current when cells were switched to 39°C. Nicardipine-insensitive Ca^{2+} current also increased in both cell types at the non-permissive temperature. Na^+ current in SMCs was large at 33°C and small or not detectable at 39°C and absent in heart-derived cells. Using a cDNA probe specific for the α_1 subunit of the dihydropyridine-sensitive Ca^{2+} channel we demonstrate a temperature-sensitive expression of the dihydropyridine receptor mRNA in smooth muscle-derived cells but not in heart-derived H10 cells. Our results suggest that upon downregulation of SV40 T antigen these cells become quiescent and exhibit a more differentiated phenotype. These cell lines may provide a useful tool to investigate ion channel- and receptor signal transduction, as well as cell cycle control in smooth and possibly cardiac muscle cell differentiation.

Key words: Muscle, Differentiation, Oncogene

INTRODUCTION

Transformation of mammalian cells by certain tumor viruses, such as simian virus 40 (SV40), myelocytomatosis virus 29 (MC29), papillomavirus or Rous sarcoma virus (RSV) is a common approach to establish stable cell lines for in vitro studies (Balk, 1980; Bedard et al., 1987; Perez-Reyes et al., 1992; Engelmann et al., 1993). The phenotype of these cells, however, often changes substantially upon integration of transforming DNA sequences into the cellular genome. These effects are thought to be due to an interference by oncoproteins of cellular factors modulating cellular growth and differentiation (reviewed by Hunter, 1991).

Several in vitro differentiation systems were already tested for effects of oncogenes on myogenesis. In skeletal muscle, RSV prevents myotube formation (Fiszman and Fuchs, 1975; Holtzer et al., 1975). Likewise, v-myc, N-ras and H-ras, but not their cellular counterparts, interfere with myogenesis (Falcone et al., 1985; Endo and Nadal-Ginard, 1986; Olson et al., 1987). Rabbit and human smooth muscle cells transformed by transfection with the early region of SV40 revealed a reduction in the level of smooth muscle α-actin expression, indicating that SV40 T and/or SV40 t antigens may modulate smooth muscle differentiation (Nachtigal et al., 1987, 1990; Reilly, 1990; Legrand et al., 1991).

Conditionally expressed oncogenes, i.e. oncogenes that are expressed only in specific culture conditions, are particular helpful to study the effects of oncoproteins in the context of cell proliferation and differentiation. First, cells can be immortalized and subsequently propagated in culture conditions permissive for the oncoprotein. Second, the option to turn off the oncogene expression enables the control of oncoprotein effects in the same cell and may in turn re-induce a more highly differentiated phenotype. Studies with erythroid cells and myeloblasts using temperature-sensitive (ts) mutants of several viral oncogenes showed that differentiation of cells could be induced by changing the culture conditions to temperatures non-permissive for a functional oncogene (Samarut and Gazzolo, 1982; Beug et al., 1984). Similarly, precursor cells of the mammalian central nervous system immortalized by a ts

mutant of SV40 T antigen differentiate at the non-permissive temperature to neurons and glial cells, respectively (Frederiksen et al., 1988).

In skeletal muscle-derived C2C12 cells immortalized by inducible SV40 T antigens, high levels of expression of the oncogene prevented terminal differentiation (Endo and Nadal-Ginard, 1989), or, delayed myotube formation (Iujdivin et al., 1990), suggesting a concentration-dependent mechanism suppressing differentiation. To our knowledge, however, smooth muscle and heart-derived cell lines that express an oncogene conditionally have not been reported.

We here describe the isolation and characterization of smooth muscle and heart-derived cells conditionally immortalized with a ts mutant of SV40 T antigen by retroviral transduction. The phenotype of these cells depends on the level of expression of SV40 T antigen and can easily be regulated by changing the culture temperature. Differentiated cells can re-enter the cell cycle by a temperature shift to the permissive temperature. We demonstrate significant effects of the conditional expression of SV40 T antigen on ion channels, intracellular calcium concentrations after thrombin stimulation and on synthesis of structural proteins in both cell types. Cells were passaged for more than one year without crisis or phenotypic changes throughout subculture, indicating the stable states of their phenotypes. These cell lines may provide useful in vitro models to study interactions between cell cycle regulation and differentiated functions of cardiac and smooth muscle cells

MATERIALS AND METHODS

Cell culture

Primary cultures of neonatal rat hearts were prepared as recently described (Sadoshima et al., 1992). To enrich for cardiac myocytes cells were preplated twice for 30 minutes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FCS). The remaining cell suspension was plated on collagenized tissue culture dishes at a density of 1×10^4 cells/cm^2 in DMEM/F12 in a ratio of 1:1, v/v (Gibco BRL, Gaithersburg, MD) supplemented with 10% FCS. Further reduction of fibroblast contamination was achieved by treating cells with calcium ionophore A23187 (Calbiochem, La Jolla, CA) at 20 μg/ml for 30 minutes, 24 hours after plating (Kaneko and Goshima, 1982). Aortic smooth muscle cells from neonatal rats were isolated by mincing whole segments of thoracic aorta followed by sequential enzymatic digestion with 0.05% trypsin, 0.53 mM EDTA in Hanks' balanced salt solution (Sigma, St Louis, MO) for 30 minutes at 37°C. Smooth muscle cells were plated at a density of 1×10^3 cells/cm^2 in DMEM with 10% FCS. Primary cells were infected in the presence of 8 μg/ml polybrene (Aldrich, Milwaukee, WI) for 2 hours with the recombinant retrovirus pZipSVtsA58, containing the early region of the SV40 temperature sensitive mutant tsA58 and the gene conferring resistance to the neomycin analog G418 (Frederiksen et al., 1988; a gift from Dr R. McKay). The shuttle vector-containing medium was replaced by DMEM, penicillin (100 i.u./ml) and streptomycin (100 μg/ml) with 10% FCS and cells were subsequently cultured at 33°C. Twenty four hours after infection the selection for infected cells was started by adding 400 μg/ml G418 (Gibco BRL) to the medium. After 3 weeks of culture in selection medium we observed very few remaining cells amongst uninfected cells in control dishes while scattered colonies of resistant cells were growing in the dishes with infected cells. Single colonies were picked with metal cloning rings and subcultured in 96-well dishes and expanded into 6-well dishes. As reference, AT-1 cells, derived from right atrial tumors of SV40 T antigen transgenic mice (from Dr W. Claycomb and Dr L. Field) were cultured as recently described (Katz et al., 1992). In some experiments cells were grown in the presence of platelet derived growth factor (PDGF-BB, 10 ng/ml), transforming growth factor beta-1 (TGF-β, 10 ng/ml), fibroblast growth factor (FGF, 10 ng/ml) (all from Collaborative Research), retinoic acid (10^{-7} M), dimethyl sulfoxide (DMSO, 1%) and 5-azacytidine (3 μM) (all from Sigma).

Immunocytochemical characterization of cell lines

Isolated cell lines were grown on coverslips or alternatively on LabTek 8 chamber tissue culture slides (Nunc, Napperville, IL) at 33°C (permissive temperature) and in parallel at 39°C (non-permissive temperature). For immunocytochemical characterization cells were permeabilized in methanol at –20°C for 5 minutes followed by a brief dip in –20°C acetone. After air drying primary antibodies were incubated for 30 minutes in a humid chamber. After three washes in phosphate buffered saline (PBS, pH 7.4) for 5 minutes each secondary antibodies were applied for 30 minutes. Secondary antibodies were Texas red- or fluorescein isothiocyanate-coupled, affinity purified donkey anti-mouse or anti-rabbit IgG (Jackson Immuno Research, West Grove, PE). Coverslips were washed three times in PBS, rinsed with deionized water, dehydrated in ethanol for 3 minutes, air dried and mounted in Mowiol (Calbiochem). The following antibodies were used: Pab 416 against SV40 T antigen (hybridoma cells were obtained from the American Type Culture Collection); MF20, a murine monoclonal antibody against sarcomeric myosin heavy chain (Bader et al., 1982; a gift from Dr D. Bader); monoclonal antibodies against the intermediate filament proteins vimentin (3B4 from Progen, Heidelberg, FRG; V9 from Boehringer Mannheim, Indianapolis, IN), cytokeratins 8 and 18 (K_S 8.17.2 and K_S 18.174, from Progen), and desmin (DE-B-5, Boehringer Mannheim); monoclonal antibodies against desmoplakins (DP1 and 2-2.15, from Boehringer Mannheim) smooth muscle α-actin (asm-1; from Progen), smooth muscle myosin heavy chain (SMMS-1; Lazard et al., 1993) and skeletel muscle α-actin (from Sigma, St Louis, MO). To identify endothelial cells we used rabbit antibodies against factor VIII related antigen (from Behringwerke, Marburg, Germany). For references of antibodies, see also Jahn et al. (1987).

Bromodeoxyuridine incorporation and thymidine uptake

Bromodeoxyuridine (BrdU) was added to the medium at 10 μM for 5 hours. Slides were rinsed with PBS and fixed in methanol for 10 minutes at 4°C. After air drying cells were rehydrated in PBS, incubated for 1 hour in 2 M HCl at 37°C and neutralized in 0.1 M $Na_2B_4O_7{\cdot}10H_2O$, pH 8.5. Incorporated BrdU was detected by indirect immunofluorescence microscopy (see above) using a monoclonal antibody against BrdU (Progen) as primary antibody. For thymidine uptake cells were grown in the presence of [^{3}H]thymidine (1 μCi/ml, 80 Ci/mmol, from NEN, Boston, MA) for 24 hours and processed as described previously (Sadoshima et al. 1992).

Measurement of intracellular calcium

Intracellular calcium concentrations were obtained by fura-2 loading of cells that were kept for 5 days either at the permissive (33°C) or the non-permissive temperature (39°C) essentially as previously described (DeFeo et al., 1987; Papageorgiou and Morgan, 1991) with minor modifications: cells were exposed to 5 μM of the acetomethoxymethylester form of fura-2 in Hanks' solution containing 1 mM calcium and 1.2 mM Mg^{2+} for 30 minutes. To calculate absolute concentrations of [Ca^{2+}] the dissociation constant for Ca^{2+} was assumed to be 210 nM. Background autofluorescence was negligible. Values for [Ca^{2+}] were determined from multiple cytoplasmic and nuclear areas. For [Ca^{2+}] free measurement medium contained 2 mM EGTA.

RNA preparation and RNA blotting

Total cellular RNA was isolated by the acidic guanidinium thio-

cyanate method (Chomczynsky and Sacchi, 1987). Poly(A)$^+$ RNA was isolated using oligo(dT)-cellulose columns; 10 μg of total RNA or 5 μg of poly(A)$^+$ RNA per lane were electrophoreticaly separated on denaturing 1% agarose gels containing 0.5 ng/ml ethidium bromide and blotted to GeneScreen membrane (New England Nuclear) by capillary transfer, and UV crosslinked. Prehybridization and hybridization were done according to the manufacturer's protocol (Method I). Membranes were hybridized with a *Pst*I/*Hin*dIII fragment of the murine thrombin receptor cDNA (Vu et al., 1991; a gift from Dr S. Coughlin) or a cardiac DHP receptor cDNA (Takahashi et al., 1992), washed at 65°C in 0.5× SSC (1× SSC is 0.15 M NaCl, 15 mM trisodium citrate) containing 0.1% SDS for 20 minutes and exposed to Kodak X-Omat X-ray film.

Whole-cell patch-clamp recording

The standard patch-clamp technique was used (Hamill et al., 1981). The whole-cell voltage-clamp recordings were performed as described previously (Akaike et al., 1989). The extracellular solution contained 20 mM $BaCl_2$, 104 mM NaCl, 2 mM KCl, 15 mM tetraethylammonium chloride, 5 mM 4-aminopyridine, 10 mM glucose, 10 mM Hepes-Tris-HCl (pH 7.35). The patch-pipette solution contained 110 mM *N*-methyl-D-glucamine, 5 mM Na_2ATP, 5 mM $MgCl_2$, 20 mM CsCl, 10 mM EGTA, 10 mM Hepes (pH 7.2). Before experiments were started cells were kept at 33°C or 39°C for at least 3 days. Nicardipine was dissolved in 99% ethanol to make a 1 mM stock solution.

Transfection of cardiac-specific promoter-*lacZ* constructs

Two promoter-*lacZ* constructs were designed using the cardiac α-actin promoter and the cardiac troponin C promoter. Both promoters have been reported to be expressed at high levels in cardiac muscle (Parmacek et al., 1992; Sartorelli et al., 1992). Expression vectors were constructed by cloning the fragment of pCH110 (Pharmacia) containing the *lacZ* gene and SV40 poly(A) signal into the corresponding *Hin*dIII and *Bam*HI sites in pGEM-4 (Promega) to create pGEM-LacZ. The promoter for the mouse cardiac troponin C gene (−176 to the cap site) and the mouse cardiac α-actin gene (−120 to the cap site) were each linked 5′ to the bacterial *lacZ* gene encoding β-galactosidase. The specific sequences chosen were the minimum 5′ upstream sequences shown to confer cardiac-specific expression of these two genes (Parmacek et al., 1992; Sartorelli et al., 1992). We cloned a 191 bp (−176 to +45) fragment of the mouse troponin C promoter and a 185 bp (−120 to +64) fragment of the mouse cardiac α-actin promoter by using pairs of 21mer PCR primers to amplify these sequences from genomic DNA. PCR fragments were purified on agarose gels and cloned into the *Hin*dIII site at the 5′ end of the *lacZ* gene in pGEM-LacZ to create TNC-LacZ and CA-LacZ, respectively. H10 cells were transfected with TNC-LacZ and CA-LacZ by the modified calcium phosphate method (Chen and Okayama, 1987) and LacZ activity was assayed by staining for β-galactosidase with X-gal.

RESULTS

Immunocytochemical characterization of cell lines derived from heart tissue

Primary cultures of neonatal rat heart tissue were enriched for myocytes by serial plating followed by the Ca^{2+} ionophore treatment to remove non-myocytes (Kaneko and Goshima, 1982). By immunofluorescence microscopy the majority of cells were positive for sarcomeric myosin heavy chain, smooth muscle and sarcomeric α-actins, desmin, and desmoplakins (not shown). Only a few cells were positive for von Willebrand factor or cytokeratins, indicating a very low level of contamination with endothelial cells and pericardial cells, respectively (less than 1% of each cell type). Cells were infected with a recombinant retrovirus containing the tsA58 mutant of SV40 T antigen and the neomycin resistance gene. After selection in G418 containing medium 72 single resistant colonies were picked, expanded and analyzed for SV40 T antigen expression by immunostaining using monoclonal antibody Pab 416. When grown at 33°C, which is a permissive temperature for the tsA58 mutant of SV40 T antigen, the majority of cells showed a bright nuclear staining in all cell lines, although the intensity varied from cell to cell (see Fig. 1a). The number of SV40 T antigen-positive cells within a given cell line was in the range of 80-100%. To test the temperature dependence of SV40 T antigen concentrations, cells were grown in parallel cultures at 39°C, which is known to be a non-permissive temperature for this SV40 mutant (Frederiksen et al., 1988). After five days at 39°C the majority of cells were either negative for SV40 T antigen or showed only a faint staining (Fig. 1b) with an average of 10-20% of cells still showing very faint SV40 T antigen reactivity.

Further characterization showed that all cell lines expressed smooth muscle α-actin with a wide range of smooth muscle α-actin positive cells (5-95%; see Fig. 1c). All cell lines isolated synthesized vimentin, as shown in Fig. 1d. Only one cell line showed staining with monoclonal antibody MF20 against sarcomeric myosin heavy chain when grown at the permissive temperature. However, the pattern of staining was not fibrillar, as it is in cultured neonatal cardiocytes, but dotted or rodlike (see below). Only less than 1% of cells of this particular cell line (H61) showed this pattern. All other cell lines grown at the permissive temperature were absolutely negative for MF20 staining. However, when cells were grown for five days at the non-permissive temperature, a varying number of cells in 17 out of 72 cell lines revealed sarcomeric myosin-positive cells. In some lines only scattered cells were sarcomeric myosin-positive, in others up to 40% of the cells revealed this dotted or patchy myosin staining pattern, which was found exclusively in the cytoplasm. In confluent cultures myosin-positive cells were often arranged in circles with myosin-negative cells in the center (Fig. 2a). We examined the relation of SV40 T antigen and myosin heavy chain expression by double immunolabeling (Fig. 2b). Most of the myosin positive cells were negative or only very weakly positive for SV40 T antigen. Extensive cytoplasmic staining for myosin heavy chain was never observed in SV40 T antigen-positive cells.

We also grew cells in medium supplemented with growth factors or substances known to induce differentiation in other in vitro systems. Growth factors tested included platelet derived growth factor (PDGF-BB, 10 ng/ml), transforming growth factor beta-1 (TGF-β, 10 ng/ml), basic fibroblast growth factor (bFGF, 10 ng/ml), and retinoic acid (10^{-7} M), dimethyl sulfoxid (1%, v/v) and 5-azacytidine (3 μM). Additionally, PDGF and TGF-β were applied simultaneously at 10 ng/ml each. After growth for 5 days at the non-permissive temperature all cell lines isolated were tested by immunocytochemistry for sarcomeric myosin heavy chain and desmin expression. We never observed desmin staining in these growth factor treated cells. We also did not find significant changes regarding sarcomeric myosin staining pattern and morphology as compared to untreated cells. Contracting cells were not observed either.

The sarcomeric myosin-positive material never corresponded to structures that would be visible in phase contrast optics. When we stained primary cultures from heart tissue we

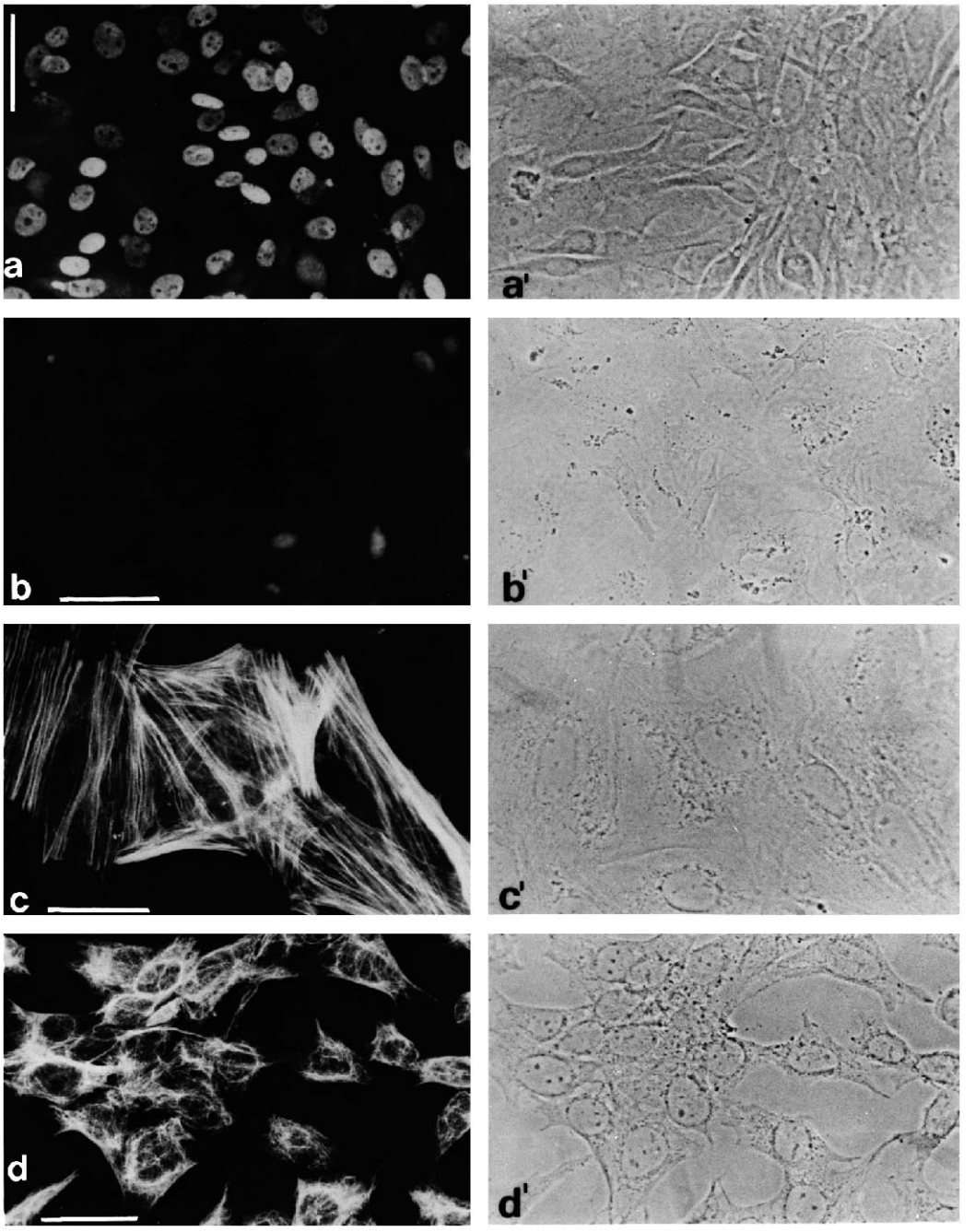

Fig. 1. Immunofluorescence microscopy performed on heart-derived H10 cells. The right panel shows phase contrast micrographs corresponding to the field shown in epifluorescence on the left panel. (a and b) Staining of monoclonal antibody Pab 416 against SV40 T antigen on cells growing at the permissive temperature (a) as compared to cells that were kept for 5 days at the non-permissive temperature (b). Note that almost all nuclei of cells grown at the permissive temperature are positive for SV40 T antigen, although the intensity of the signal somewhat varies from cell to cell. In contrast, at the non-permissive temperature only a few cells are SV40 T antigen-positive, while the majority of cells do not show any reaction. Cytoplasmic staining is not detectable at either temperature. (c and d) Immunostainings using monoclonal antibodies against smooth muscle α-actin (c) and vimentin (d), respectively. Cells shown in (c,c′) were grown at the non-permissive temperature, in (d,d′) at the permissive temperature. Bars: (a,b), 50 μm; (c,d), 25 μm.

never observed such a staining pattern. However, in cells derived from atrial tumors of SV40 T antigen transgenic mice (AT-1 cells; Steinhelper et al., 1990) we observed a similar staining pattern in scattered cells (not shown). An overview of the immunostaining results of a cell line with a high portion of sarcomeric myosin expressing cells (H10 cells) is shown in Table 1, the left column.

Transfection of cardiac-specific promoter-*lacZ* constructs into H10 cells

To confirm the cardiac origin of H10 cells the cardiac-specific promoter-*lacZ* constructs were made (see Materials and Methods) and transfected into H10 cells. The cardiac troponin C promoter construct, whose activity is highly restricted to cardiomyocytes (Parmacek et al., 1992) was able to drive LacZ expression in H10 cells as indicated by ~25-fold higher number of LacZ expressing cells as compared to cells transfected with promoterless *lacZ*. On the other hand the cardiac α-actin promoter, which is active both in cardiac and embryonic skeletal muscle cells, was not active. Both promoters were unable to express the *lacZ* gene in COS7 cells, but were active when transfected into primary neonatal cardiocytes (not shown).

Characterization of cell lines derived from the thoracic aorta by immunofluorescence microscopy and [^{3}H]thymidine incorporation

In order to derive smooth muscle cell lines that conditionally

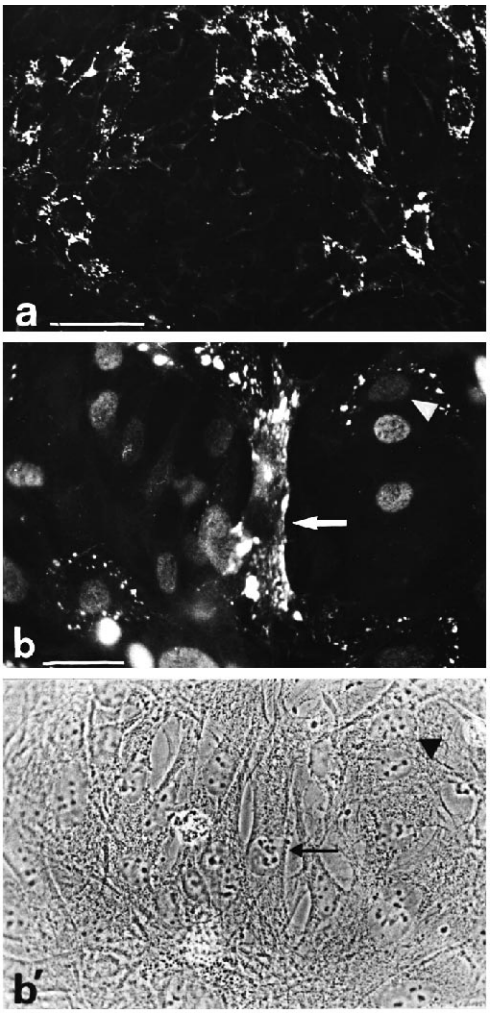

Fig. 2. Immunofluorescence microscopy of H10 cells using monoclonal antibody MF20 against myosin heavy chain alone (a) or in combination with monoclonal antibody Pab 416 against SV40 T antigen (b). Cells were grown for five days (a) and three days (b), respectively, at the non-permissive temperature. (a) A subset of cells shows a bright dotted or rodlike staining pattern in the peripheral cytoplasmic regions. The nuclei are negative for sarcomeric myosin heavy chain. (b) Few cells show both nuclear staining of SV40 T and dotted cytoplasmic myosin staining (arrowhead). One cell with the bright myosin staining (arrow) does not show any nuclear reaction for SV40 T antigen. In this specific cell myosin immunoreactive material is aligned, although real filaments are not detectable. (b′) The same field as a phase contrast micrograph. Bars: (a) 100 μm; (b) 40 μm.

differentiate in vitro, primary cultures obtained from the newborn rat thoracic aorta were infected with a recombinant retrovirus containing tsA58 mutant of SV40 T antigen and the gene conferring neomycin resistance. Primary cultures contained less than 1% endothelial cells as judged by immunofluorescence microscopy using a rabbit serum against von Willebrand factor. All cells were vimentin-positive, 95% were smooth muscle α-actin-positive, and 10% revealed desmin immunoreactivity. Very few cells were positive with antibodies against cytokeratins 8 and 18 (not shown; for cytokeratin expression in smooth muscle cells see Jahn and Franke, 1989). After infection and selection in G418 containing

Table 1. Immunocytochemical characterization of H10 and S7 cells grown at the indicated temperatures

	H10 cells		S7 cells	
	33°C	39°C	33°C	39°C
SV40 large T	++	<15% +	++	<5% +
Vimentin	++	++	++	++
Desmin	–	–	10% +	30% +
Cytokeratins	–	–	–	–
Smooth muscle α-actin	50% ++ 20% +	50% ++ 20% +	90% ++	90% ++
Skeletal muscle α-actin	–	–	–	–
Smooth muscle myosin	–	–	++	++
Sarcomeric MHC	–	<40% ++	–	–
von Willebrand factor	–	–	–	–

%, percentage of cells showing strong (++) or significant (+) immunostaining; ++, virtually all cells show strong immunostaining; –, no immunostaining; MHC, myosin heavy chain.

medium 12 single neomycin resistant colonies were picked, expanded and characterized. In none of the isolated clones did we find any von Willebrand factor-synthesizing or cytokeratin-positive cells. Cells of all clones were spindle-shaped when grown at the permissive temperature.

When switched to the non-permissive temperature, the rate of DNA synthesis decreased significantly as manifested by the reduced [^{3}H]thymidine (Fig. 3a) and BrdU uptakes (not shown) and cells started to become flatter and larger (compare Fig. 3b and c). Similar effects in other rodent cells were already described by Jat and Sharp (1989). Immunostaining of SV40 T antigen showed a strong nuclear signal in almost all cells grown at the permissive temperature. When cells were grown for at least five days at the non-permissive temperature only a few nuclei were stained (not shown).

Regarding the composition of their cytoskeleton, we found most cells expressing smooth muscle α-actin (70-80%) at either temperature although often at variable intensities (Fig. 4a,a′). All cell lines revealed at least a few desmin-positive cells. The number of desmin-expressing cells increased by 20-30% after switching cells to the non-permissive temperature. The desmin staining pattern of S7 cells grown at the non-permissive temperature is shown in Fig. 4b and b′. Vimentin was found in all cells regardless of the culture temperature (Fig. 4c and c′). Smooth muscle myosin heavy chain staining was observed in several cell lines at both temperatures in a subset of cells often arranged in clusters (Fig. 4d and d′). For an overview of the immunofluorescence data for S7 cells, see Table 1, the right column.

Intracellular [Ca^{2+}] measurements

In order to examine the effect of the SV40 T oncoprotein on the physiological properties of H10 and S7 cells, intracellular [Ca^{2+}] was measured by loading cells with the calcium indicator dye fura-2. Intracellular [Ca^{2+}] of heart-derived H10 cells and SMC-derived S7 cells revealed calcium concentrations between 100 nM and 200 nM. [Ca^{2+}] in nuclear areas (closed triangles, Fig. 5a,b) was approximately 50 nM lower than that in cytoplasmic areas. Stimulation of cells grown at the permissive temperature with 10 U/ml thrombin did not have any effect on intracellular [Ca^{2+}] (Fig. 5a and c).

When cells grown at the non-permissive temperature were stimulated with thrombin (10 U/ml) intracellular [Ca^{2+}]

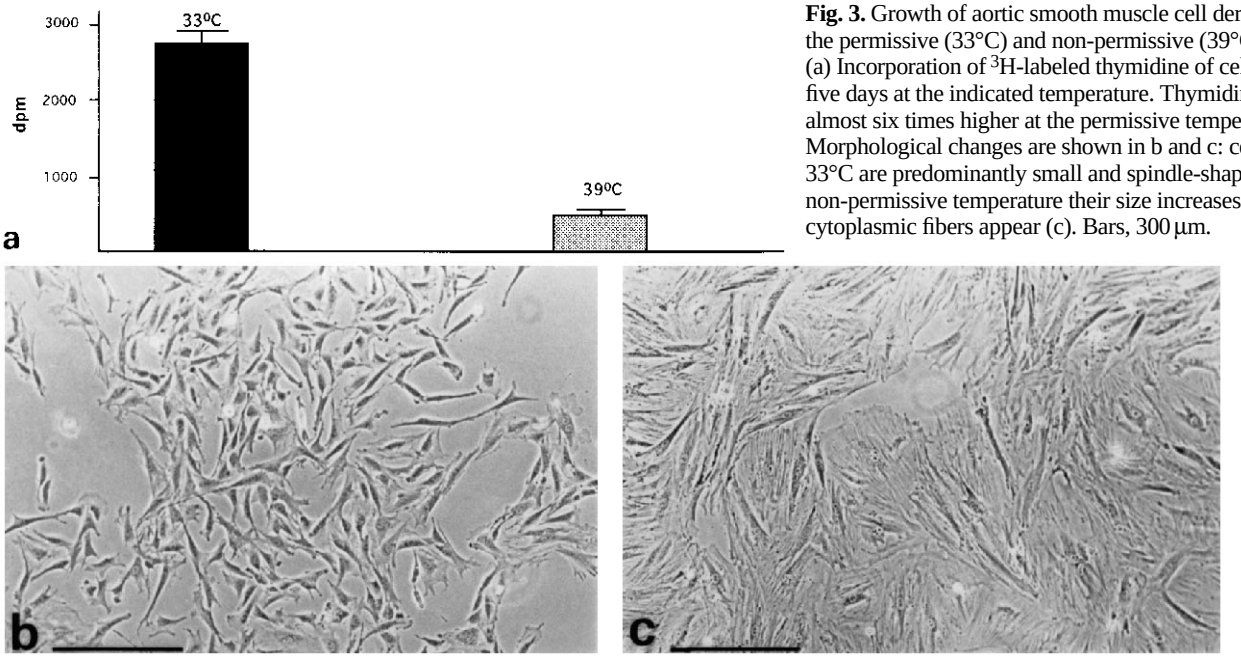

Fig. 3. Growth of aortic smooth muscle cell derived S7 cells at the permissive (33°C) and non-permissive (39°C) temperature. (a) Incorporation of ^{3}H-labeled thymidine of cells grown for five days at the indicated temperature. Thymidine uptake is almost six times higher at the permissive temperature. Morphological changes are shown in b and c: cells grown at 33°C are predominantly small and spindle-shaped (b); at the non-permissive temperature their size increases and abundant cytoplasmic fibers appear (c). Bars, 300 μm.

increased in distinct patterns depending on the origin of cells. $[Ca^{2+}]$ in aorta-derived S7 cells showed a transient 3-fold increase, peaking at about 20 seconds and after 1-3 minutes they returned to the resting levels (Fig. 5b). In heart-derived H10 cells grown at the non-permissive temperature intracellular $[Ca^{2+}]$ increased more than 10-fold over a period of 10-15 minutes (Fig. 5c) before reaching a plateau phase lasting several minutes (not shown). Normal $[Ca^{2+}]$ levels were not reached again before 30 minutes. This sustained elevation of intracellular Ca^{2+} concentration is unlikely due to a mere leakage from extracellular Ca^{2+}, since in our study addition of EGTA to the medium during measurement did not change this phenomenon (not shown). When grown at the non-permissive temperature only less than 10% of H10 and S7 cells failed to show a significant thrombin response.

Expression levels of thrombin receptor mRNA

A potential explanation for the different $[Ca^{2+}]$ response to thrombin would be differential expression of the thrombin receptor at permissive and non-permissive temperatures. Therefore we studied the effect of SV40 T antigen on thrombin receptor gene expression by northern blotting using a murine thrombin receptor cDNA probe. RNA was isolated from two different smooth muscle-derived cell lines (S5 and S7) and from heart-derived H10 cells grown at permissive or non-permissive temperatures. Thrombin receptor mRNA levels were almost unaffected by temperature shifts in smooth muscle cell lines (Fig. 6a). In H10 cells thrombin receptor mRNA levels were significantly lower at the non-permissive temperature (Fig. 6a). These results suggest that differences in thrombin receptor gene expression cannot account for the observed differences in intracellular $[Ca^{2+}]$ transient in response to thrombin.

Electrophysiological characterization of S7 and H10 cells by whole-cell patch clamp experiments

Conditional expression of the SV40 T oncoprotein may affect ion channel function. To examine the properties of Na^+ and Ca^{2+} channels in S7 and H10 cells the whole-cell patch-clamp recording was performed under conditions where K^+ currents are suppressed. In S7 cells kept at 33°C a fast transient inward current was observed (Fig. 7a, open circle). This current was activated at around −50 mV and a peak was observed at around 0 mV. It was slightly suppressed by a treatment with 2 μM of nicardipine (Fig. 7a, filled circle), but was completely suppressed by replacing Na^+ in the extracellular solution with $Tris^+$ (not shown), suggesting that the fast current is a Na^+ current. In S7 cells at 39°C a sustained inward current, but no fast transient current was observed (Fig 7b, open circle). This current was slightly suppressed by replacing Na^+ in the extracellular solution with $Tris^+$ (not shown). It was, however, completely and reversibly suppressed by 10 mM Co^{2+}, indicative of a Ca^{2+} current. Interestingly, this current was significantly, although not completely, suppressed by 2 μM nicardipine (Fig 7b, closed circle). The nicardipine-insensitive component inactivated within 150 milliseconds, and the inactivation of the nicardipine-sensitive component was slow and subtle. Both nicardipine-insensitive and nicardipine-sensitive components showed activation thresholds at around −50 to −40 mV and a peak at around +10 mV. These results suggest that the nicardipine-sensitive sustained inward current has typical properties of the L-type Ca^{2+} current, while the nicardipine-insensitive current is more reminiscent of N-type Ca^{2+} currents (Hess, 1990). In S7 cells, the L-type Ca^{2+} current could be recorded as early as 8 hours after switching cells from 33°C to 39°C. This functional detection of L-type Ca^{2+} current could be reversed after growing cells at the permissive temperature (33°C) again.

In H10 cells kept at 33°C a relatively slow inactivating inward current was observed (Fig. 7c). This current was activated at around −50 mV to −40 mV and a peak was observed at around +10 mV to +20 mV. It was only slightly affected by replacing Na^+ in an extracellular solution with

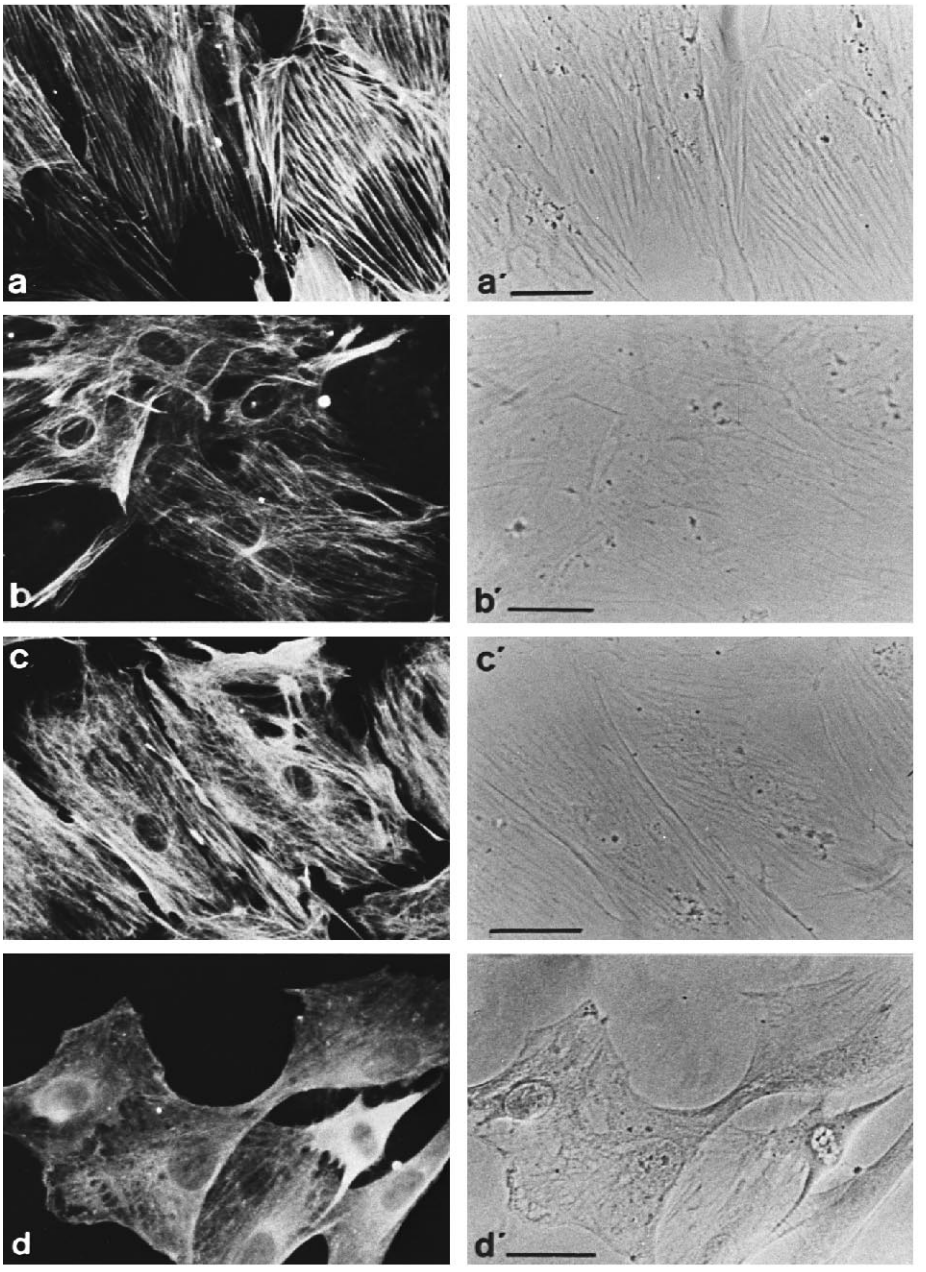

Fig. 4. Immunofluorescence microscopy (a-d) with corresponding phase contrast micrograph (a′-d′) of smooth muscle derived S7 cells using antibodies against cytoskeletal proteins. Cells were grown at the non-permissive temperature for five days. (a) All cells show a bright staining of fibers extending throughout the cytoplasm with a monoclonal antibody against the smooth muscle-specific isoform of α-actin. (b) Cells stained with an antibody against the muscle specific intermediate filament protein desmin. In a subset of cells desmin filaments spread from perinuclear bundles to the periphery of these cells. Other cells with no morphological visible differences are negative for desmin. A similar staining pattern with a somehow higher density of filaments is shown when a monoclonal antibody against vimentin was applied (c). As opposed to desmin, all cells are positive for vimentin. (d) A cluster of smooth muscle myosin-positive cells. Bars, 40 μm.

$Tris^+$ (not shown). This current was completely and reversibly suppressed by Co^{2+}, indicating that it is a Ca^{2+} current. Interestingly, this current was only partially suppressed by 2 μM of nicardipine, although it was completely suppressed by a higher concentration of nicardipine (30 μM). In H10 cells kept at 39°C a slowly inactivating inward current was observed (Fig. 7d). This current showed a similar current-voltage relationship as the one found in 33°C-cells. However, this current inactivated more slowly and was completely suppressed by Co^{2+} or nicardipine (2 μM), indicating L-type Ca^{2+} channel characteristics. A fast inactivating inward current was not observed in H10 cells at either temperature. The results of patch clamp recording of smooth muscle cells and heart tissue-derived cells are summarized in Table 2.

mRNA expression levels of the α_1-subunit of the dihydropyridine (DHP)-sensitive Ca^{2+} channel in S5, S7 and H10 cells

The observed changes in Ca^{2+} current at different temperatures may be due to changes in expression of the DHP-sensitive Ca^{2+} channel. To test this hypothesis northern blot analysis of RNA isolated from smooth muscle-derived S5 and S7 cells and heart-derived H10 cells using a cDNA encoding the DHP receptor revealed barely detectable levels of expression of the DHP receptor mRNA in smooth muscle-derived cells (S5 and S7) grown at the permissive temperature (33°C, see Fig. 6b). After temperature shift to 39°C expression of DHP receptor mRNA increased significantly in both cell lines associated with the appearance of an additional band of a larger transcript in S7 cells. In contrast, the levels of expression of DHP receptor

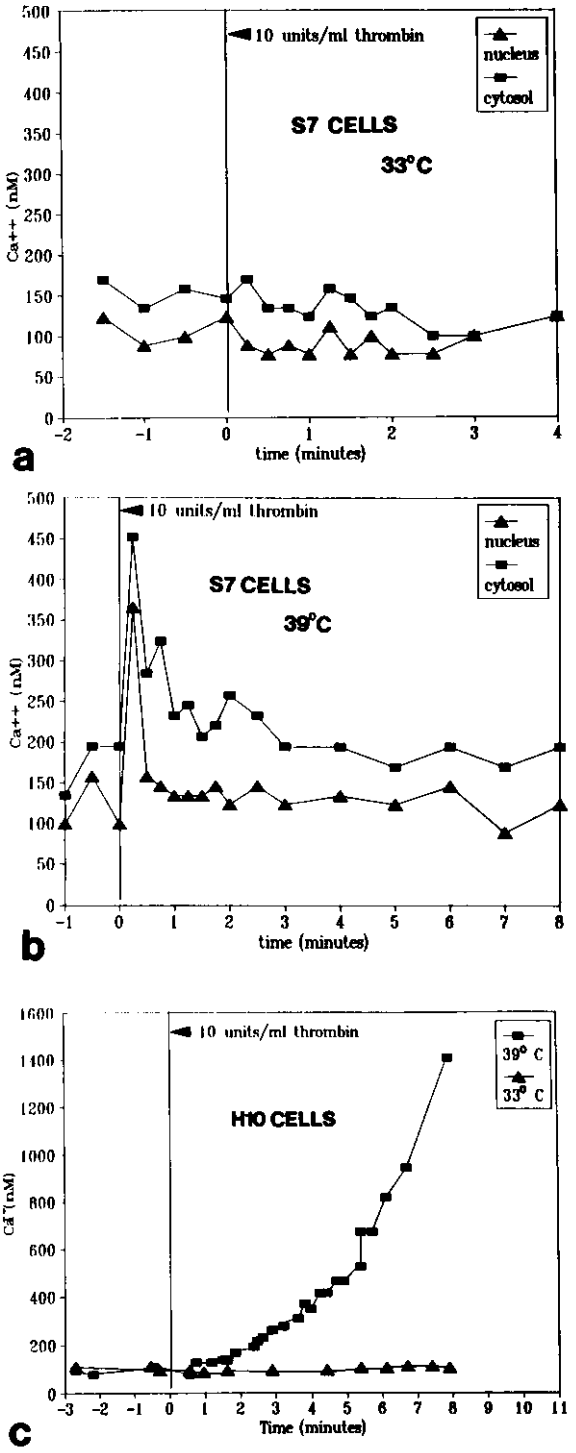

Fig. 5. Representative intracellular Ca^{2+}-concentration in fura 2-loaded single S7 (a,b) and H10 cells grown for five days at the indicated temperature before and after stimulation with 10 units/ml of thrombin. Cells grown at 33°C do not respond to thrombin (a,c). When cells were grown at 39°C smooth muscle derived S7 cells show a transient more than 2-fold increase in intracellular [Ca^{2+}] after thrombin application while heart-derived H10 cells respond with a sustained increase in intracellular [Ca^{2+}]. Note that [Ca^{2+}] in nuclear areas was always lower than in cytoplasmic regions.

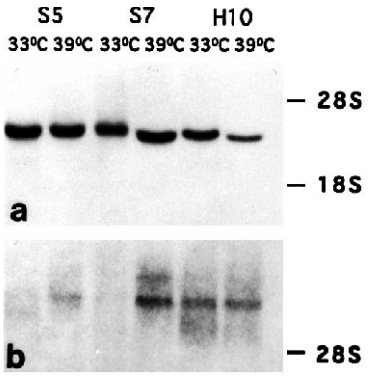

Fig. 6. Northern blot analysis of RNA isolated from smooth muscle-derived S5 and S7 cells and heart-derived H10 cells. RNA was harvested from cells grown at 33°C (permissive for SV40 T antigen) and 39°C (non-permissive temperature). (a) For hybridization a murine thrombin receptor cDNA was used. In smooth muscle-derived cells (S5 and S7) thrombin receptor mRNA levels are comparable at both temperatures. In H10 cells, however, thrombin receptor mRNA concentration decreases after the temperature switch to the non-permissive temperature. The subtle mobility differences of mRNAs in this experiment were also observed when ribosomal RNA was detected by ethidium bromide staining (not shown) and, therefore, are not likely to be due to different RNA sizes.
(b) Hybridization with a cDNA encoding the dihydropyridine (DHP) receptor. DHP receptor mRNA is hardly detectable in smooth muscle-derived cells (S5 and S7) grown at the permissive temperature (33°C). After a temperature shift to 39°C both S5 and S7 cells express higher levels with an additional band of a larger transcript detectable in S7 cells. H10 cells express substantial levels of the DHP receptor at either temperature. The positions of 18 S and 28 S ribosomal RNA are as indicated.

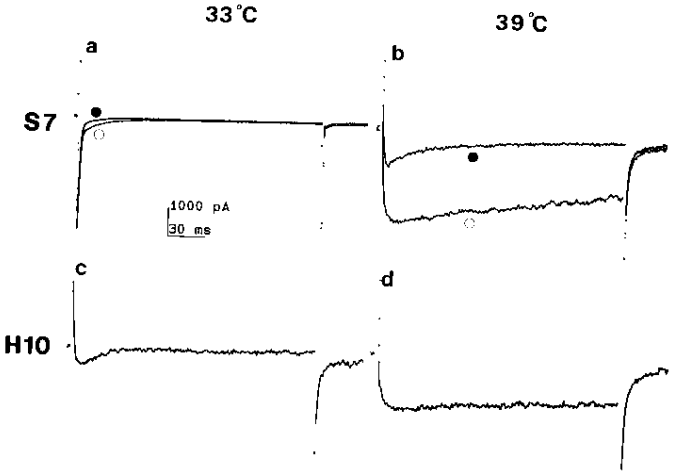

Fig. 7. Inward current recordings in S7 and H10 cells cultured at 33°C and 39°C. Cells were cultured at indicated temperatures for more than 24 hours and whole cell voltage-clamp recordings performed at room temperature as described in Materials and Methods. Holding potential was –80 mV. Inward currents were evoked by step depolarizations of 300 ms to +10 mV. In S7 cells (a and b) inward currents evoked in the absence (○) or presence (●) of 2 μM nicardipine in the same cell are shown.

Table 2. Relative size of inward currents at permissive and non-permissive temperatures

	Temperature (°C)	I_{Na}	I_{Ca} (nicardipine-sensitive)	I_{Ca} (nicardipine-less sensitive)
S7	33	+++	+	+
	39	+	+++	++
H10	33	–	+	+
	39	–	+++	++

–, not detectable; +, small; ++, medium; +++, large.

mRNA in H10 cells did not depend on the culture temperature (Fig. 6b).

DISCUSSION

In vitro immortalization of cells by oncogenes often induces failure to withdraw from the cell cycle, which, at least in certain cells, is a prerequisite to gain or to maintain a highly differentiated phenotype. In muscle cells expression of oncogenes was found to induce a less differentiated phenotype in various in vitro systems. Smooth muscle cells transformed with SV40 T antigen still show certain differentiation features of that cell type, such as heparin binding (Reilly, 1990), but their morphology, their expression pattern of the different isoforms of α-actin and their tendency to divide at high rates are reminiscent of a less differentiated state of this cell type found frequently in vascular atherosclerotic lesions (Legrand et al., 1991), the so called 'synthetic' state (Chamley-Campbell et al., 1981). In several skeletal muscle cells oncogene expression interfered with differentiation of these cells in vitro (see Introduction). However, oncogene expression and maintenance of a highly differentiated phenotype are not necessarily mutually exclusive. The expression of SV40 T antigen in C2C12 skeletal myoblasts delays but does not prevent terminal differentiation of these cells into myotubes (Iujvidin et al., 1990). In addition, primary cardiocytes transfected with SV40 T antigen still have the capacity for spontaneous contraction (Sen et al., 1988). SV40 T antigen also does not prevent spontaneous contraction of cardiocyte-derived AT-1 cells (Steinhelper et al., 1990), but confers muscle abnormalities in transgenic animals (De Leon et al., 1994).

To study the effects of oncogene expression on SMC- and cardiocyte differentiation we immortalized heart- and smooth muscle-derived cells from neonatal rats by retrovirus-mediated oncogene transduction of a temperature-sensitive (ts) mutant of SV40 T antigen. After shifting smooth muscle cells to the non-permissive temperature their morphology changed from a spindle-shaped fibroblast-like type to large flat cells growing in the 'hills and valley' pattern typical for vascular smooth muscle cells grown in primary culture (Chamley-Campbell et al., 1981). This change in morphology was accompanied by an increased frequency of desmin-positive cells in smooth muscle cells. Since desmin is an intermediate filament protein usually found only in muscle cells, but not in fibroblasts (Lazarides and Hubbard, 1976) this increase in desmin-positive cells together with the changed morphology and growth pattern indicates a more differentiated phenotype of S7 cells in culture conditions conferring low levels of SV40 T antigen. Differential gene expression in these cells at permissive and non-permissive temperatures could be used to identify factors involved in SMC phenotype modulation.

In heart-derived H10 cells an as yet unidentified isoform of sarcomeric myosin heavy chain reactive with monoclonal antibody MF20 (Bader et al., 1982) was found at the non-permissive temperature. Western blots using the same antibody were always negative. This may be due to the relatively high concentrations of the antigen necessary for detection by this antibody in western blots, particularly when murine cells are used (D. Bader, personal communication). S1 nuclease protection assays suggested that this myosin isoform is not either α- or β-cardiac myosin heavy chain (not shown). Recently a novel embryonic ventricular-specific cardiac myosin heavy chain (Bisaha and Bader, 1991) and two different atrial-specific myosin heavy chains were identified in the chick embryo (Yutzey et al., 1994; Oana et al., 1995). It is not known whether similar cardiac-specific embryonic myosin heavy chains exist in rodents.

Interestingly, the myosin heavy chain in H10 cells did not assemble into a regular network of filaments, as in primary cultures of neonatal cardiocytes, but was found in cytoplasmic patches suggesting the lack of a factor necessary for the assembly of those filaments. When we stained cardiocyte-derived AT-1 cells with antibody MF20 we found cells that did not reveal a filamentious organization of this protein but, like H10 cells, showed a dotted staining pattern. Since AT-1 cells are derived from an SV40 T antigen transgenic mouse (Steinhelper et al., 1990) this unusual myosin distribution in H10 and AT-1 cells maybe related to SV40 T antigen expression.

The lack of hallmarks of cardiac differentiation, such as intercellular junctions of the desmosome type, sarcomeric organization of myofilaments and spontaneous contraction, excludes a highly differentiated cardiac phenotype of these cells. However, the fact that the cardiac restricted troponin C promotor construct was active in H10 cells suggests a cardiac origin of this cell line. Therefore, H10 cells may serve as a 'cardiac background' to test the potential of certain factors to induce differentiation towards a more differentiated phenotype of the cardiac lineage. In contrast to other in vitro models for cardiac differentiation, namely embryonic carcinoma (EC) cells (for review, see McBurney, 1993) and embryonic stem (ES) cells (Doetschman et al., 1987) H10 cells may provide a background that is already committed to the cardiac lineage, while EC cells and ES cells are pluripotent and therefore less valuable to the separation of the commitment to cardiogenesis from the terminal differentiation of cardiocytes.

Expression of several other oncogenes such as c-H-ras, c-myc, v-erbB, EJ-ras, v-fms or polyoma middle T antigen in several stably transformed cell lines has been shown to modulate the functional expression of several types of ion channels including Na^+ and Ca^{2+} channels (Caffrey et al., 1987; Chen et al., 1988). Chen et al. (1988) reported that non-transformed 3T3 fibroblasts possess both L-type and T-type Ca^{2+} channels, but when transformed by polyoma middle T antigen lose T-type Ca^{2+} channels. They concluded from these results that oncogenes may regulate functional expression of T-type Ca^{2+} channels during cell growth, while expression of L-type channels may not be affected by oncogene expression.

In contrast, we found a regulation of DHP-sensitive voltage-gated Ca^{2+} channels, i.e. L-type channels, by SV40 T antigen in

both SMC and heart-derived cells. This different pattern of regulation of ionic channels by SV40 T antigen in our studies may be due to differences in the cell type, the type of oncogene and/or the site of oncogene integration. Chen et al. (1988) studied non-excitable 3T3 fibroblasts and Caffrey et al. (1987) used non-fusing BC3H1 skeletal muscle cells. In addition, Chen et al. (1988) compared the ionic currents between parental cells and stably transformed cells, while we compared cells of the same cell line expressing different levels of the oncoprotein. This may also be considered as a possible reason for these differences.

The size of dihydropyridine-sensitive Ca^{2+} currents detected by whole cell recordings in S7 cells correlated with the levels of expression of the dihydropyridine receptor as shown by northern blot analysis. This indicates that the change in size of dihydropyridine-sensitive Ca^{2+} current may be determined at the level of expression of the dihydropyridine receptor gene rather than modulation of the different channel protein submits by phosphorylation or other posttranslational modifications. The mechanism of the potential transcriptional regulation of the dihydropyridine receptor by SV40 T antigen or other oncoproteins, however, needs further examination.

In contrast, in H10 cells expression of the dihydropyridine receptor mRNA did not depend on SV40 T antigen expression, indicating that mechanisms other than transcriptional control of dihydropyridine receptor expression are responsible for the induction of Ca^{2+} currents at the non-permissive temperature. This difference between S7 and H10 cells is likely to be cell-type-specific rather than cell-line-specific. Results from experiments using other SMC-derived and heart-derived cell lines not presented in this paper support this hypothesis.

S7 and H10 cells may also be useful studying thrombin-induced signal transduction in muscle cells. In both cell types thrombin induced elevation of intracellular Ca^{2+} concentration was reduced or abolished by permissive culture conditions. The different kinetics of intracellular Ca^{2+} concentration in S7 and H10 cells after thrombin stimulation is most likely cell type-specific. A sustained elevated intracellular Ca^{2+} concentration in primary cardiocytes that persists longer than in SMC was previously reported (Steinberg et al., 1990).

The phenomenon of increased intracellular Ca^{2+} concentration after thrombin stimulation only in cells grown at the non-permissive culture conditions is not due to a transcriptional upregulation of thrombin receptor expression. In fact, the levels of thrombin receptor mRNA detected in H10 cells grown at non-permissive culture conditions were even lower than at the permissive temperature, suggesting a post-transcriptional mechanism regulating thrombin receptor signal transduction. Further studies are necessary to clarify whether posttranslational modifications of the thrombin receptor, such as phosphorylation or coupling mechanisms to other signaling molecules, are involved in thrombin receptor induced signal transduction. Likewise, future biochemical characterization of Ca^{2+} channel proteins at permissive and non-permissive temperatures may also shed some light on Ca^{2+} channel signaling in muscle cells.

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REFERENCES

Akaike, N., Kanaide, H., Kuga, T., Nakamura, M., Sadoshima, J.-I. and Tomoike, H. (1989). Low-voltage-activated calcium current in rat aorta rat smooth muscle cells in primary culture. *J. Physiol.* **416**, 141-160.

Bader, D., Masaki, T. and Fischman, D. (1982). Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. *J. Cell Biol.* **95**, 763-770.

Balk, S. D. (1980). Active proliferation of Rous sarcoma virus-infected, but not normal, chicken heart mesenchymal cells in culture medium of physiological composition. *Proc. Nat. Acad. Sci. USA* **77**, 6606-6610.

Bedard, P. A., Balk, S. D., Gunther, H. S., Morisi, A. and Erikson, R. L. (1987). Repression of quiescence-specific polypeptides in chicken heart mesenchymal cells transformed by Rous sarcoma virus. *Mol. Cell. Biol.* **7**, 1450-1458.

Beug, H., Leutz, A., Kahn, P. and Graf, T. (1984). Ts mutants of E26 leukemia virus allow transformed myeloblasts, but not erythroblasts to differentiate at the nonpermissive temperature. *Cell* **39**, 579-588.

Bisaha, J. G. and Bader, D. (1991). Identification and characterization of a ventricular-specific avian myosin heavy chain, VMHC1: expression in differentiating cardiac and skeletal muscle. *Dev. Biol.* **148**, 355-364.

Caffrey, J. M., Brown, A. M. and Schneider, M. D. (1987). Mitogens and oncogenes can block the induction of specific voltage-gated ion channels. *Science* **236**, 570-573.

Chamley-Campbell, J., Campbell, G. and Ross, R. (1981). Phenotype-dependent response of cultured aortic smooth muscle to serum mitogens. *J. Cell Biol.* **89**, 379-383.

Chen, C. and Okayama, H. (1987). High-efficiency of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**, 2745-2752.

Chen, C. F., Corbley, M. J., Roberts, T. M. and Hess, P. (1988). Voltage-sensitive calcium channels in normal and transformed 3T3 fibroblasts. *Science* **239**, 1024-1026.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

DeFeo, T., Briggs, M. and Morgan, K. (1987). Ca^{2+} signals obtained with multiple indicators in mammalian vascular muscle cells. *Am. J. Physiol.* **253**, H1456-1461.

De Leon, J. R., Federoff, H. J., Dickson, D. W., Vikstrom, K. L. and Fishman, G. I. (1994). Cardiac and skeletal myopathy in β-myosin heavy-chain simian virus 40 tsA58 transgenic mice. *Proc. Nat. Acad. Sci. USA* **91**, 519-523.

Doetschman, T. C., Eistetter, H., Katz, M., Schmidt, W. and Kemler, R. (1987). The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morph.* **87**, 27-45.

Endo, T. and Nadal-Ginard, B. (1986). Transcriptional and posttranscriptional control of c-myc during myogenesis: its mRNA remains inducible in differentiated cells and does not suppress the differentiated phenotype. *Mol. Cell. Biol.* **6**, 1412-1421.

Endo, T. and Nadal-Ginard, B. (1989). SV40 large T antigen induces reentry of terminally differentiated myotubes into the cell cycle. In *Cellular and Molecular Biology of Muscle Development* (ed. L. Kedes and F. Stockdale), pp. 95-104. Alan R. Liss, Inc. New York, New York.

Engelmann, G. L., Birchenall-Roberts, M. C., Ruscetti, F. W. and Samarel, A. M. (1993). Formation of fetal rat cardiac cell clones by retroviral transformation: Retention of select myocyte characteristics. *J. Mol. Cell. Cardiol.* **25**, 197-213.

Falcone, G., Tató, F. and Alemá, S. (1985). Distinctive effects of the viral oncogenes myc, erb, fps and src on the differentiation program of quail myogenic cells. *Proc. Nat. Acad. Sci. USA* **82**, 426-430.

Fiszman, M. Y. and Fuchs, P. (1975). Temperature-sensitive expression of differentiation in transformed myoblasts. *Nature* **254**, 429-431.

Frederiksen, K., Jat, P., Valtz, N., Levy, D. and McKay, R. (1988). Immortalization of precurser cells from the mammalian CNS. *Neuron* **1**, 439-448.

Hamill, O., Marty, A., Neher, E., Sakmann, B. and Sigworth, F. (1981). Improved patch-clamp technique for high resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* **391**, 85-100.

Hess, P. (1990). Calcium channels in vertebrate cells. *Annu. Rev. Neurosci.* **13**, 337-356.

Holtzer, H., Biehl, J., Yeoh, G., Megnathan, R. and Kaji, A. (1975). Effects of oncogenic viruses in muscle differentiation. *Proc. Nat. Acad. Sci. USA* **72**, 4051-4055.

Hunter, T. (1991). Cooperation between oncogenes. *Cell* **64**, 249-270.

Iujvidin, S., Fuchs, O., Nudel, U. and Yaffe, D. (1990). SV40 immortalizes myogenic cells: DNA synthesis and mitosis in differentiating myotubes. *Differentiation* **43**, 192-203.

Jahn, L., Fouquet, B., Rohe, K. and Franke W. W. (1987) Cytokeratins in certain endothelial and smooth muscle cells of two taxonomically distant vertebrate species, Xenopus laevis and man. *Differentiation* **36**, 234-254

Jahn, L. and Franke, W. (1989). High frequency of cytokeratin-producing smooth muscle cells in human atherosclerotic plaques. *Differentiation* **40**, 55-62.

Jat, P. and Sharp, P. (1989). Cell lines established by a temperature sensitive simian virus 40 large T antigen are growth restricted at the nonpermissive temperature. *Mol. Cell. Biol.* **9**, 1672-1681.

Kaneko, H. and Goshima, K. (1982). Selective killing of fibroblast-like cells in cultures of mouse heart cells by treatment with a Ca ionophore, A23187. *Exp. Cell Res.* **142**, 407-416.

Katz, E., Steinhelper, M. E., Delcarpio, J. B., D'Aud, A. I., Claycomb, W. C. and Field, L. J. (1992). Cardiomyocyte proliferation in mice expressing α-cardiac myosin heavy chain-SV40 T-antigen transgenes. *Am. J. Physiol.* **262**, H1867-H1876.

Lazard, D., Sastre, X., Frid, M. G., Glukhova, M. A., Thiery, J.-P. and Koteliansky, V. E. (1993). Expression of smooth muscle-specific proteins in myoepithelium annd stromal myofibroblasts of normal and malignant human breast tissue. *Proc. Nat. Acad. Sci. USA* **90**, 999-1003.

Lazarides, E. and Hubbard, B. (1976). Immunological characterization of the subunit of the 100Å filaments from muscle cells. *Proc. Nat. Acad. Sci. USA* **73**, 4344-4349.

Legrand, A., Greenspan, P., Nagpal, M. L., Nachtigal, S. A. and Nachtigal, M. (1991). Characterization of human vascular smooth muscle cells transformed by the early genetic region of SV40 virus. *Am. J. Pathol.* **139**, 629-460.

McBurney, M. W. (1993). P19 embryonal carcinoma cells. *Int. J. Dev. Biol.* **37**, 135-140.

Nachtigal, M., Greenspan, P., Terracio, L. and Fowler, S. D. (1987). Transformation of rabbit arterial smooth muscle cells with simian virus 40. *Arch. Virol.* **95**, 225-235.

Nachtigal, M., Legrand, A., Nagpal, M. L., Nachtigal, S. A. and Greenspan, P. (1990). Transformation of rabbit vascular smooth muscle cells by transfection with the early region of SV40 DNA. *Am. J. Pathol.* **136**, 297-306.

Oana, S., Matsuoka, R., Nakajiama, H., Hiratsuko, E., Furutani, Y., Takao, A and Momma, K. (1995). Molecular characterization of a novel atrial-specific myosin heavy-chain gene expressed in the chick embryo. *Eur. J. Cell Biol.* **67**, 42-49.

Olson, E. N., Spizz, G. and Tainsky, M. A. (1987). The oncogenic forms of N-ras or H-ras prevent skeletal myoblast differentiation. *Mol. Cell. Biol.* **7**, 2104-2111.

Papageourgiou, P. and Morgan, K. (1991). Intracellular free Ca^{2+} is elevated in hypertrophic aortic muscle from hypertensive rats. *Am. J. Physiol.* **260**, H507-H515.

Parmacek, M., Vora, A., Shen, T., Barr, E., Jung, F. and Leiden, J. (1992). Identification and chracterization of a cardiac-specific transcriptional regulatory element in the slow/cardiac troponin C gene. *Mol. Cell. Biol.* **12**, 1967-1976.

Perez-Reyes N, Halbert C. L., Smith P. P., Benditt E. P. and McDougall J. K. (1992). Immortalization of primary human smooth muscle cells. *Proc. Nat. Acad. Sci. USA* **89**, 1224-1228.

Reilly, C. F. (1990). Rat vascular smooth muscle cells immortalized with SV40 large T antigen possess defined smooth muscle cell characteristics including growth inhibition by heparin. *J. Cell. Physiol.* **142**, 342-351.

Sadoshima, J., Jahn, L., Takahashi, T., Kulik, T. J. and Izumo, S. (1992). Molecular characterization of the stretch-induced immediate-early gene expression and hypertrophy of cultured cardiac myocytes. *J. Biol. Chem.* **267**, 10551-10560.

Samarut, J. and Gazzolo, L. (1982). Target cells infected by avian erythroblastosis virus differentiate and become transformed. *Cell* **28**, 921-929.

Sartorelli, V., Hong, N., Bishopric, N. and Kedes, L. (1992). Myocardial activation of the human cardiac α-actin promotor by helix-loop-helix proteins. *Proc. Nat. Acad. Sci. USA* **89**, 4047-4051.

Sen, A., Dunnmon, P., Henderson, S. A., Gerard, R. D., and Chien, K. R. (1988). Terminally differentiated neonatal rat myocardial cells proliferate and maintain specific differentiated functions following expression of SV40 large T antigen. *J. Biol. Chem.* **263**, 19132-19136.

Steinberg, S. F., Robinson, R. B., Lieberman, H., Stern, D. M. and Rosen, M. R. (1991). Thrombin modulates phosphoinositide metabolism, cytosolic calcium, and impulse initiation in the heart. *Circ. Res.* **68**, 1216-1229.

Steinhelper, M. E., Lanson, N. A., Dresdner, K. P., Delcarpio, J. B., Wit, A. L., Claycomb, W. C. and Field, L. J. (1990). Proliferation in vivo and in culture of differentiated adult cardiomyocytes from transgenic mice. *Am. J. Physiol.* **259**, H1826-H1834.

Takahashi, T., Allan, P. D., Lacro, R. V., Marks, A. R., Dennis, A., R., Schoen, F. J., Grossman, W., Marsh, J. D. and Izumo, S. (1992). Expression of dihydropyridine receptor (Ca^{2+} channel) and calsequestrin genes in the myocardium of patients with end-stage heart failure. *J. Clin. Invest.* **90**, 927-935.

Vu, T.-K., Hung, D., Wheaton, V. and Coughlin, S. (1991). Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64**, 1057-1068.

Yutzey, K. T., Rhee, J. T. and Bader, D. (1994). Expression of the atrial-specific myosin heavy chain AMHC1 and the establishement of antero-posterior polarity in the developing chicken heart. *Development* **120**, 871-883.

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