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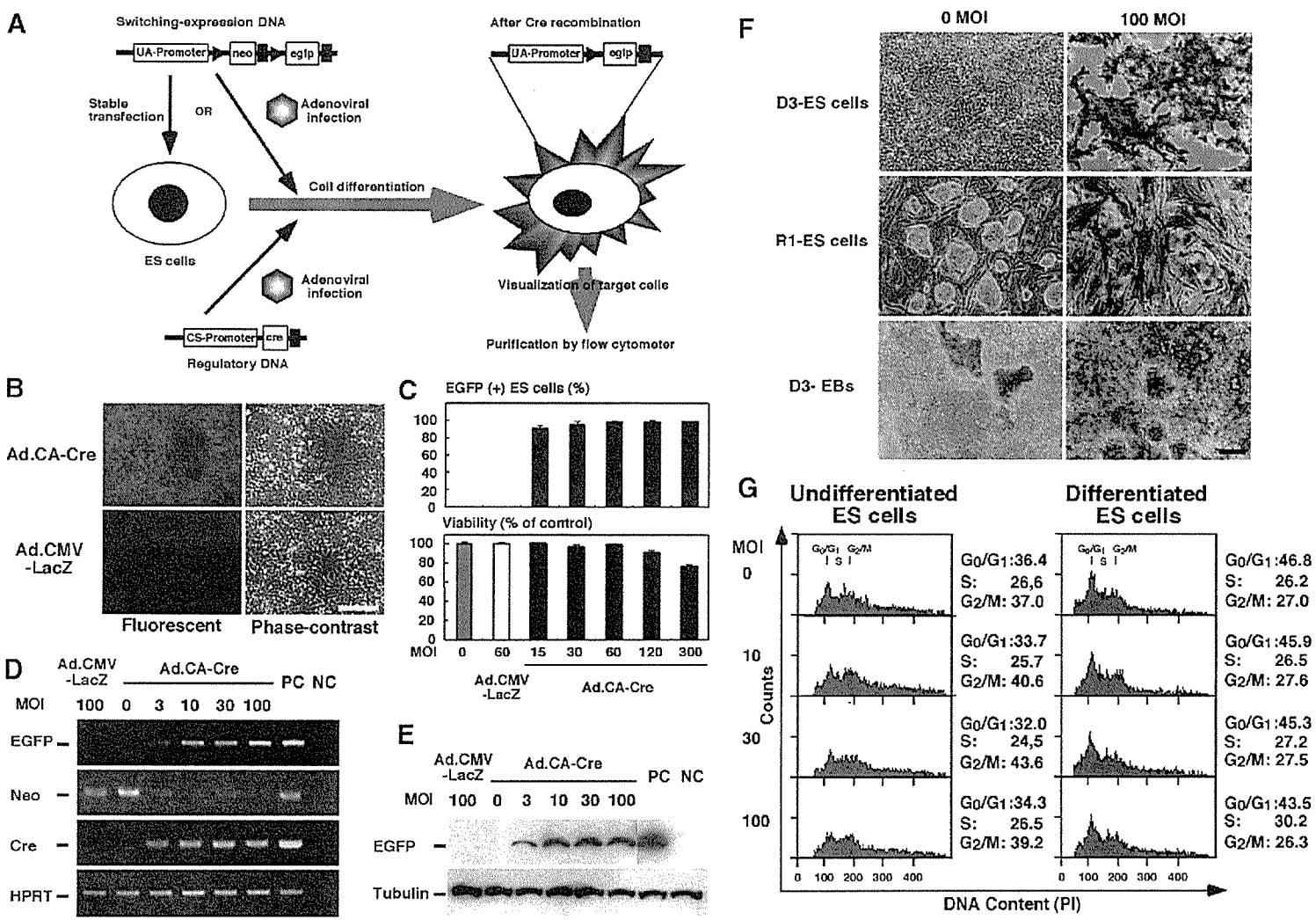

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Ad.CA-Cre infection even at a low m.o.i. (e.g., 10), while no background EGFP intensity was detectable following the infection of LE-ES cells with the control Ad.CMV-LacZ (Figs. 1B, 1C, 1D, and 1E).

We also examined the possible adverse effects of adenoviral vectors in ES cells (Figs. 1C and 2B) because previous studies indicated that cell cycle dysregulation or apoptosis could occur after adenoviral vector infection of certain somatic or germ cells [19–22]. ES cells were viable and intact at an m.o.i. of 60, which resulted in over 98% gene transduction efficiency and gene switching. Even at extremely high m.o.i. (300 or greater), only mild toxicity was observed. In addition, adenoviral vector infection of ES cells at m.o.i. of 10, 30, or 100 during the undifferentiated stage or during *in vitro* differentiation affected neither the populations of G_0/G_1, S, and G_2/M phases in the cell cycle (Fig. 1G) nor the efficiencies of cardiomyogenic differentiation and embryoid body (EB) formation (data not shown). Thus, adenoviral vector infection of ES cells at the proper m.o.i., e.g., an m.o.i. of 30, led to sufficiently high gene transduction efficiency without either cytotoxicity or cell cycle dysregulation and also efficiently induced subsequent transgene expression and the gene-switching reaction. We infected the LE-ES cells with the adenoviral vector at an m.o.i. of 30 during *in vitro* differentiation in the later experiments.

Visualization of Primary Cultured Cardiomyocytes

To explore the specificity and efficiency of our new method, we first infected primary cultured cardiomyocytes from 1-day-old neonatal mice with either Ad.Nkx2.5-Cre or Ad.αMHC-Cre, together with Ad.CA-LNEGFP (Fig. 2). Contracting cardiomyocytes were easily visualized by fluorescence microscopy under viable conditions (Fig. 2A and Supplemental Video 1), and all the visualized cells expressed endogenous Nkx2.5 or sarcomeric myosin heavy chain (sMHC) protein (Fig. 2B). In contrast, no primary fibroblasts were fluorescently visible following infection with either Ad.Nkx2.5-Cre or Ad.αMHC-Cre together with Ad.CA-LNEGFP (data not shown). On the other hand, many cardiomyocytes were EGFP-positive after infection with control Ad.CA-Cre together with Ad.CA-LNEGFP, while no cardiomyocytes were EGFP-positive after infection with Ad.CMV-LacZ and Ad.CA-LNEGFP. These findings indicate that the adenoviral vector system and our constructs efficiently and specifically identify target cells.

Expression of Endogenous Nkx2.5 and αMHC during *in Vitro* Differentiation of LE-ES Cells

While the LE-ES cells did not express Nkx2.5 or αMHC at an undifferentiated stage, we detected expression of Nkx2.5 and αMHC mRNA as early as 3 and 8 days, respectively,

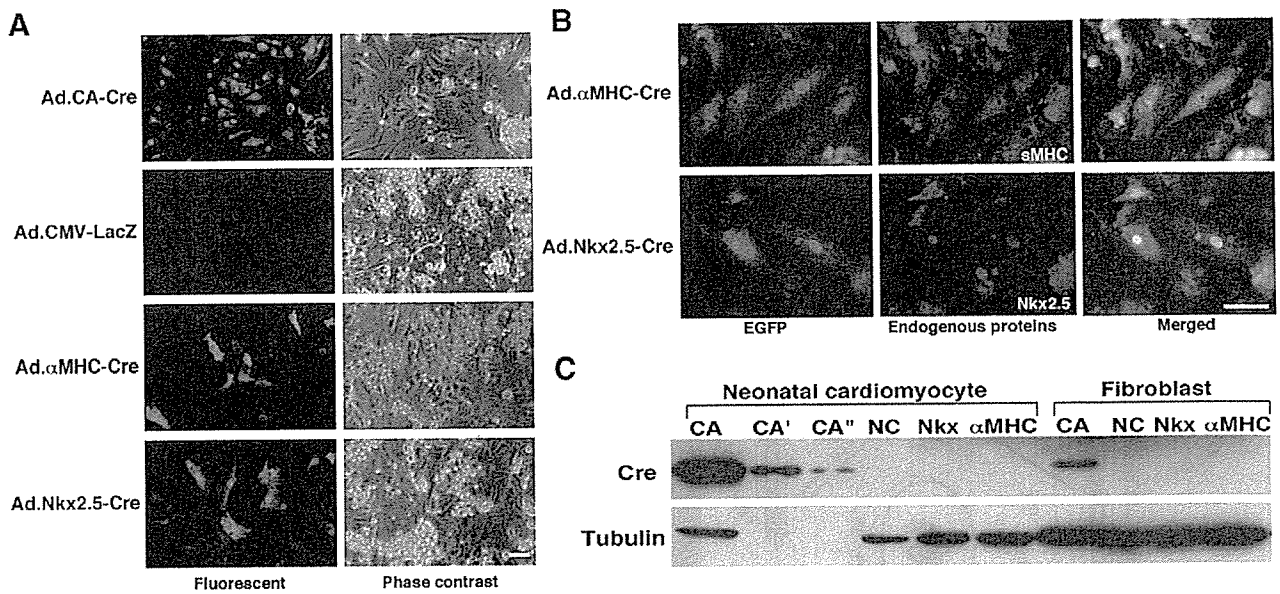

FIG. 2. Adenoviral conditional targeting in primary cultured cardiomyocytes. Primary cultured cardiomyocytes were (A) observed under viable conditions or (B) immunocytochemically stained 48 h after infection with a regulatory adenoviral vector and the switching-expression adenoviral vector, Ad.CA-LNEGFP. EGFP expression was clearly visualized under viable conditions using fluorescence microscopy (A) (video footage of this figure is available as Supplemental Video 1). Immunocytochemical staining for EGFP and endogenous sMHC or Nkx2.5 protein confirmed the specificity of this system. Scale bar, 20 μm (A, B). (C) Western blot analysis was performed using cell lysates from primary cultured cardiomyocytes or NIH3T3 mouse fibroblasts that were infected with the following adenoviral vectors at m.o.i. of 30 or 500, respectively: Ad.CA-Cre (CA, 5 μg protein loaded; CA', 0.5 μg protein; CA", 0.1 μg protein), Ad.Nkx2.5-Cre (Nkx, 5 μg protein), and Ad.αMHC-Cre (αMHC, 5 μg protein). NC, negative control (no adenoviral vector, 5 μg protein). Tubulin was detected using an anti-tubulin antibody as an internal control.

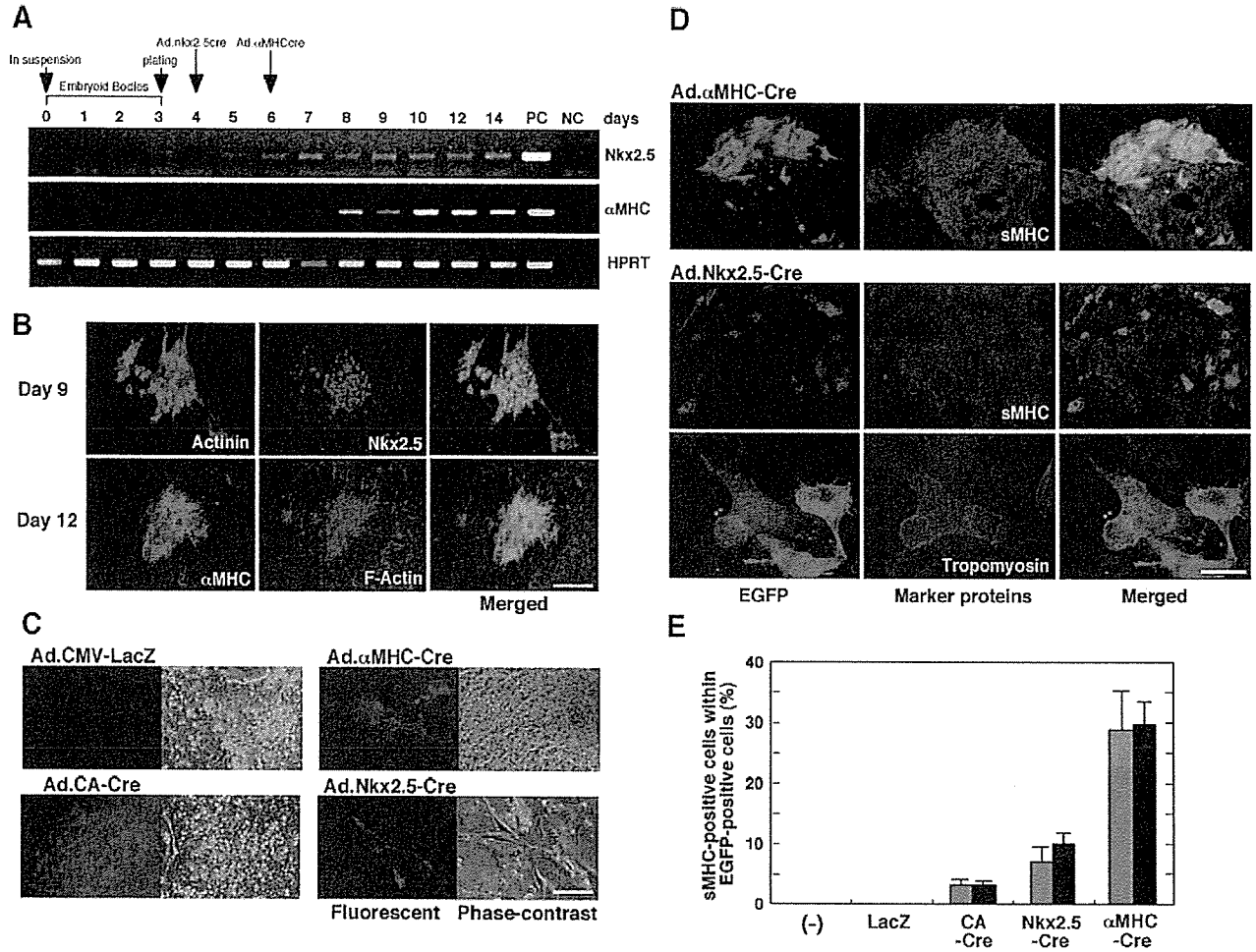

FIG. 3. Identification and *in vitro* sequential lineage analysis of the target ES cells. (A) The kinetics of mRNA expression of Nkx2.5 and αMHC during *in vitro* differentiation of LE-ES cells was analyzed by RT-PCR. PC, positive control (mouse heart tissue); NC, negative control (no template); HPRT, hypoxanthine–guanine phosphoribosyltransferase (an internal control). The experimental time line is shown at the top. (B) Immunocytochemistry demonstrates the cardiac differentiation of the LE-ES cells. (C) LE-ES cells were easily visualized under viable conditions using fluorescence microscopy 48 h following infection with a regulatory adenoviral vector. (D) Immunocytochemistry 48 h after infection with a regulatory adenoviral vector. (E) *In vitro* sequential lineage and quantitative analysis on days 10 (gray bars) and 14 (black bars). Scale bars, 50 μm (B, C), 20 μm (D).

after the formation of EBs; gene expression remained stable until day 14 (Fig. 3A). Cells were immunohistochemically positive for cardiomyocyte-specific sarcomeric proteins and endogenous Nkx2.5 or sMHC protein (Fig. 3B). The contraction of several clusters of LE-ES cells appeared on day 8 (data not shown). These findings all indicated cardiac differentiation of the LE-ES cells.

Distinct Visualization and *in Vitro* Sequential Lineage Analysis of ES Cell-Derived Target Cells

We infected the LE-ES cells with Ad.CMV-LacZ, Ad.CA-Cre, or Ad.Nkx2.5-Cre on day 4 or Ad.αMHC-Cre on day 6 (Fig. 3A). Some LE-ES cells were distinctly visible under viable conditions using fluorescence microscopy as early as 2 days following infection with Ad.Nkx2.5-Cre, Ad.αMHC-Cre, or Ad.CA-Cre, whereas no LE-ES cells could be visualized after infection with Ad.CMV-LacZ (Fig. 3C). The progeny marked by adenoviral vector infection were persistently visible under viable conditions by fluorescence microscopy through the termination of the experiment on day 14. This may be a new and useful experimental tool allowing *in vitro* sequential lineage analysis of ES cell-derived target cells.

The majority of the progeny cells marked by Ad. Nkx2.5-Cre or Ad.αMHC-Cre were seen in contracting clusters at a later stage, suggesting the subsequent cardiomyogenic differentiation of these progeny. To verify and quantify the cardiomyogenic differentiation, we costained adenoviral-infected LE-ES cells with an anti-EGFP antibody and an antibody to either of the sarcomeric

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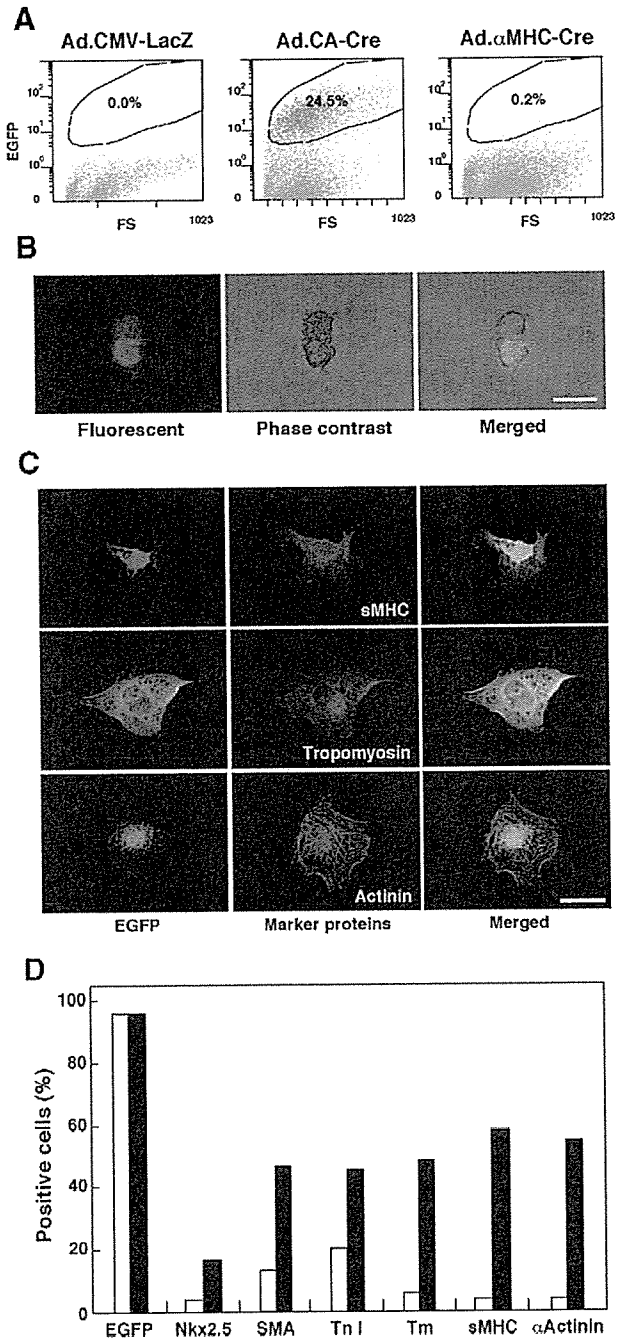
proteins, such as an anti-sMHC antibody, on days 10 and 14. We observed the majority of EGFP-positive LE-ES cells that had been infected with either Ad.Nkx2.5-Cre or Ad.αMHC-Cre within the clusters that expressed sarcomeric proteins (Fig. 3D). In contrast, the EGFP-positive cells marked by Ad.CA-Cre were scattered randomly, regardless of their expression of sarcomeric proteins. About 30% of the EGFP-positive cells that had been infected with Ad.αMHC-Cre expressed sMHC on days 10 and 14 (Fig. 3E). On the other hand, 7.0 and 9.8% of the EGFP-positive cells marked by Ad.Nkx2.5-Cre infection were stained with sMHC on days 10 and 14, respectively. In contrast, less than 3% of the EGFP-positive cells marked by control Ad.CA-Cre expressed sMHC, and EGFP-positive cells did not appear at all following infection with Ad.CMV-LacZ (the negative control) (Fig. 3E). Taken together, these findings suggest that our system of adenoviral conditional targeting specifically and distinctly detects target cells and that the LE-ES cells marked by Ad.Nkx2.5-Cre and Ad.αMHC-Cre have the potential to undergo subsequent cardiomyogenic differentiation.



Purification of Contractile Cardiomyocytes from ES Cells

We infected the LE-ES cells with Ad.αMHC-Cre on day 6 (Fig. 3A) and purified the cells by flow cytometry 2 days later, yielding viable cells that we subsequently analyzed (Figs. 4A and 4B). Immunocytochemical analysis showed that more than half of the purified cells expressed cardiomyocyte-specific sarcomeric proteins, such as sMHC (59.6%), sarcomeric tropomyosin (49.4%), troponin I (46.8%), and sarcomeric α-actinin (56.2%) (Figs. 4C and 4D). Notably, some of the purified and replated cells were stably contracting even when isolated as single cells (Fig. 4B and Supplemental Video 1). On the other hand, 17.3 and 47.8% of the purified cells expressed Nkx2.5 and SMA proteins, respectively. The present method using Ad.αMHC-Cre successfully isolated from ES cells a cell type with features consistent with those of differentiated cardiomyocytes, even when the purification was performed at the earliest time point (i.e., when αMHC was expressed only at a faint level).

Purification of Precontractile Cardiac Cells from ES Cells

The LE-ES cells that were infected with Ad.Nkx2.5-Cre and purified on days 4 and 6 (Fig. 3A) were unique (Fig. 5). mRNA expression patterns of some cardiomyocyte-specific molecules, such as cardiomyocyte-specific transcriptional factors (GATA-4 and MEF2c), atrial natriuretic peptide, and cardiac actin, were detected by RT-PCR

FIG. 4. Purification and characterization of ES cell-derived target cells following Ad.αMHC-Cre infection. (A) Flow-cytometric analysis and sorting of the LE-ES cells infected with either of the regulatory adenoviral vectors at an m.o.i. of 30 2 days before. (B) EGFP expression in the purified cells was distinctly visualized under viable conditions using fluorescence microscopy (video footage is available as Supplemental Video 1). (C) Immunocytochemistry using anti-EGFP antibody, together with antibodies for lineage markers, showed that cells purified after infection with Ad.αMHC-Cre exhibited features of mature cardiomyocytes. Scale bars, 20 μm (B, C). (D) Percentages of purified cells after Ad.αMHC-Cre infection that express lineage markers. The cells were infected with Ad.CA-Cre (control, open bars) or Ad.αMHC-Cre (closed bars) on day 6, purified and replated on day 8, and stained with each antibody 1 day later.

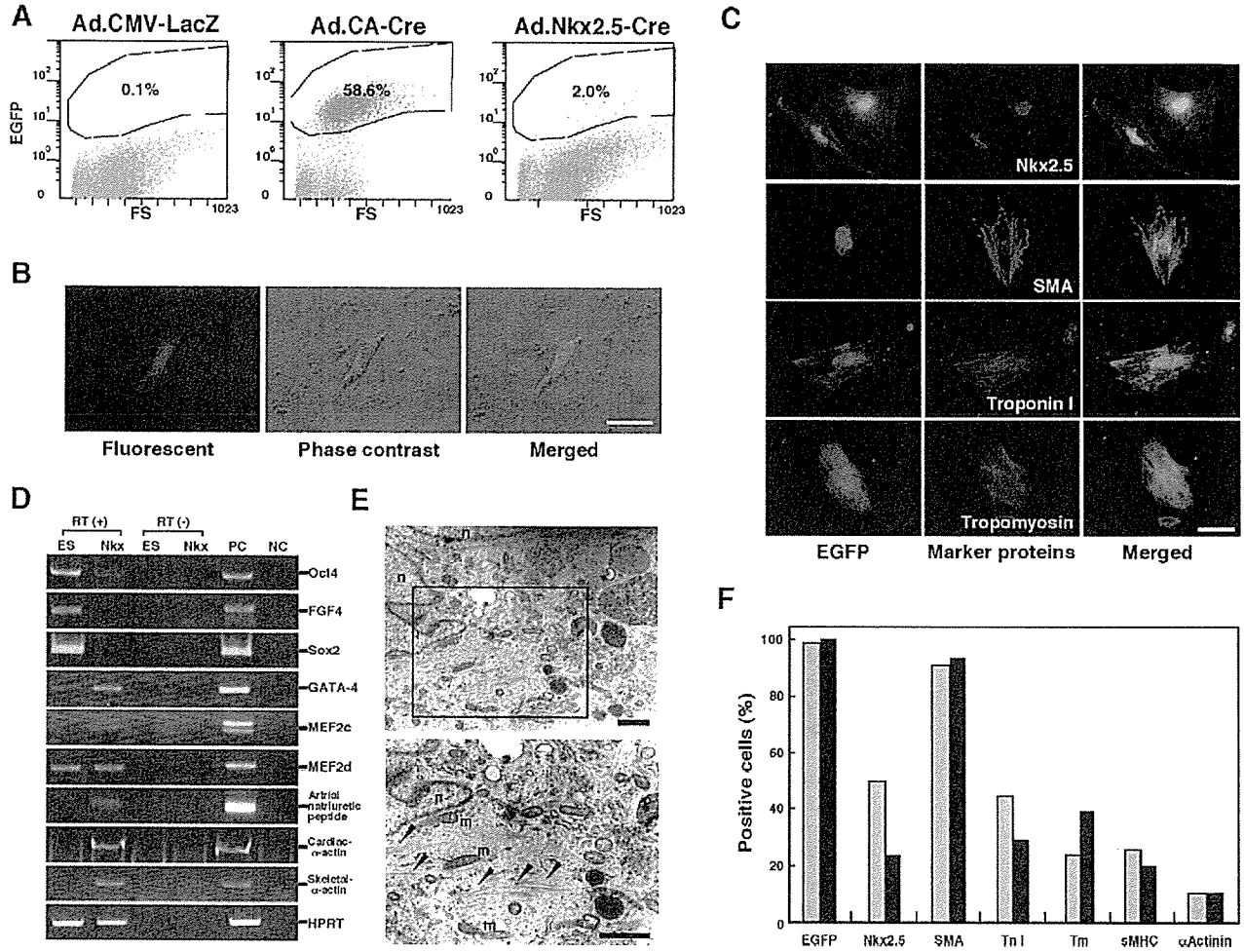


FIG. 5. Purification and characterization of ES cell-derived target cells following Ad.Nkx2.5-Cre infection. (A–C) The target cells were purified, replated, and stained in the same manner as described for Figs. 4A–4C, except that the timing of adenoviral vector infection was performed as described for Fig. 3A. (D) RT-PCR analysis demonstrated the expression of cardiomyocyte-specific molecules in these cells. (E) Electron microscopy revealed stress-fiber-like thin filaments (arrowheads) but no organized sarcomeres. The square region in the upper image is magnified in the lower image. n, nucleus; m, mitochondria. Scale bars, 20 μm (B, C), 1.0 μm (E). (F) Percentages of purified cells following Ad.Nkx2.5-Cre infection expressing lineage markers. The cells were infected with Ad.Nkx2.5-Cre on day 4, purified and replated on day 6, and stained with each antibody 1 day (gray bars) or 3 days (black bars) later.

analyses (Fig. 5D). Oct4 was expressed at a faint level, but neither fibroblast growth factor 4 nor Sox2 was expressed in these purified cells; all were highly expressed in ES cells at an undifferentiated stage (Fig. 5D), in accordance with previous findings [23].

Immunocytochemical and quantitative analysis showed that 50 and 23.5% of the purified cells expressed Nkx2.5 proteins 1 and 3 days after replating, respectively (i.e., 7 and 9 days after the initial induction of cardiomyogenic differentiation) (Figs. 3A, 5A, 5C, and 5F). Notably, over 90% of these cells expressed smooth muscle α-actin (SMA), while between 10 and 45% expressed sarcomeric proteins such as troponin I, sarcomeric tropomyosin, sMHC, and cardiac α-actin 1 and 3 days after replating (Figs. 5C and 5F).

Ultrastructural morphologic analyses indicated that the most distinct feature of these cells was the presence of thin filaments, which may support the SMA-positive immunocytochemistry findings (Figs. 5C and 5E). However, organized sarcomeres with myofibril formation or Z-line structures (i.e., the essential structures of mature cardiomyocytes) were not seen. None of the cells was contracting; thus, these purified cells demonstrated neither contractile elements nor contractile function.

The number of replated cells that were purified by either Ad.Nkx.2.5-Cre or Ad.αMHC-Cre was apparently unchanged. Bromodeoxyuridine uptake during the first 48 h of culture demonstrated that proliferating cells were 38.2, 10.2, or 7.4% of all the cells purified by Ad.CA-Cre,

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TABLE 1: The upregulated and downregulated genes in the purified ES cells that were marked by Ad.Nkx2.5-Cre infection

GenBank Accession No.	Gene description	Fold difference
Upregulated genes		
NM_007392.1	Smooth muscle α2 actin, aorta	6.6
BB067079	Wingless-related MMTV integration site 5A (Wnt5a)	6.4
X04653	Ly-6 alloantigen (Ly-6E.1)	6.4
U80011.1	Pituitary homeobox 2 isoform a (Pitx2)	6.2
M12233.1	Skeletal muscle α-actin	6
NM_021467.2	Skeletal troponin I (sTnI)	5.9
AV255689	Myocyte enhancer factor 2A (Mef2a)	5.5
NM_013601.1	Homeobox, msh-like 2 (Msx2)	5.4
L25602	Bone morphogenetic protein 2 (BMP2)	5.3
AW049938	Transforming growth factor, β2 (TGF-β2)	5.2
U43715	Heart and neural crest derivatives expressed transcript 2 (Hand2)	5
NM_025282.1	Myocyte enhancer factor 2C (Mef2c)	4.9
M15501	Cardiac α actin	4.8
X12972	Myosin alkali light chain, ventricular (MLC1v)	4.5
U21226	Heart and neural crest derivatives expressed transcript 1 (Hand1)	4.5
NM_019971.1	Platelet-derived growth factor, C polypeptide (PDGFc)	4.4
M29793	Cardiac troponin C (cTnC)	4.4
NM_007554.1	Bone morphogenetic protein 4 (BMP4)	4.2
AK020411.1	Bone morphogenetic protein 7 (BMP7)	3.7
NM_009523.1	Wingless-related MMTV integration site 4 (Wnt4)	3.5
AV161550	Lymphocyte antigen 6 complex, locus A (Ly-6A)	3.2
L07264.1	Heparin-binding EGF-like growth factor (HB-EGF)	3.1
NM_010741.1	Lymphocyte antigen 6 complex, locus C (Ly-6C)	3.1
L47552.1	Cardiac troponin T isoform A2b (cTnT)	3
Downregulated genes		
NM_013633.1	POU domain, class 5, transcription factor 1 (Pou5f1/Oct3/4)	−7.6
NM_011141.1	POU domain, class 3, transcription factor 1 (Pou3f1/Oct6)	−6
U31967.1	High-mobility-group box protein (Sox2)	−5.7
NM_009482.1	Undifferentiated embryonic cell transcription factor 1	−5.5
AB016516.1	Fibroblast growth factor 5 (FGF-5S)	−5.2
AK010332.1	Nanog homeobox	−5.2
NM_025274.1	Embryonal stem cell-specific gene 1 (Esg1)	−4.7
AA683849	Development pluripotency associate 5 (Dppa5)	−4.7
AK010743.1	Development pluripotency associate 2 (Dppa2)	−4.3
M12848	Myb proto-oncogene	−4.1
AV212609	ES cell-expressed Ras (Eras)	−3.8
X14849	Fibroblast growth factor 4 (FGF4)	−2.9

Ad.Nkx.2.5-Cre, or Ad.αMHC-Cre, respectively. The more significantly diminished growth potential of the purified ES cells that were marked by Ad.Nkx.2.5-Cre or Ad.αMHC-Cre (more so in the former than in the latter) relative to those marked by the control Ad.CA-Cre may indicate the procession of cardiomyogenic differentiation, since native cardiomyocytes in the body lose their growth potential after birth.

We investigated the transcriptional profiles by DNA microarray analysis between the purified (target) and the undifferentiated (control) ES cells to define more comprehensively their developmental features at the molecular level (Table 1). In summary, 460 genes were upregulated (signal log ratio >3), and 193 genes were downregulated (signal log ratio <−3) in the target cells. The majority of the downregulated genes were known, as they are expressed at high levels in immature and pluripotent cells, e.g., Sox2, Oct3/4, Oct6, Nanog, and FGF4. On the other hand, the upregulated genes were cardiomyocyte-specific transcriptional factors (Pitx2, MEF2a, Msx2, Mef2c, Hand1, and Hand2), sarcomeric genes (isoforms of α-actin and troponin), and growth factors that play essential roles in early heart development (TGF-β2, BMP2/4, Wnt-5a/4, PDGF, and HB-EGF). Interestingly, Ly-6 family genes, including Sca-1 (Ly6-6A/E), which were reported to be expressed in adult cardiac stem cells [24,25], were also upregulated in the purified cells. Several functionally unknown genes were identified in clusters of both the upregulated and the downregulated genes (data not shown).

Taken together, the purified cells seem to be precontractile and of the early cardiac lineage, and the purified cell population should uniquely express both immature and cardiac molecules.

Discussion

The most valuable advantage of our method is the guarantee of the clear identification of target cells, including immature cells, independent of promoter strength. To this end, we combined adenoviral vectors and recombination systems with *in vitro* ES cell studies. In addition to high efficiencies of gene transduction, the adenoviral vector system provided stable transgene expression in ES cells. This is most likely due to the episomal nature of the transduced gene and protection by certain adenoviral proteins of the transgene from endogenous digestion and the efficient recombination-based gene-switching reaction, regardless of their undifferentiated or differentiated status. The simple addition of aliquots of regulatory and/or switching-expression adenoviruses to the culture media at any time, including during *in vitro* differentiation after the formation of EBs (when conventional gene transfer methods are inapplicable), allows the distinct identification of target cells. Obviously, the method could be applied to a wide variety of tissues and cell types simply by replacing the promoter in the regulatory adenoviral vector, although the clinical usefulness of these techniques should be carefully assessed in future studies. This method also creates a new route to elucidating the roles of certain genes in development, including the *in vitro* lineage analysis shown here.

While previous studies have suggested that adenoviral vectors induce cell cycle dysregulation or apoptosis in somatic or germ cells [19,22], such adverse effects have not been studied in depth in ES cells. For instance, Gordon *et al.* showed that four-cell embryos and blastocysts manifested toxicity after adenoviral gene transduction [21,22]. However, their doses were clearly too high (equivalent to over 1000 pfu/cell), and their purpose for using adenoviral vectors to generate transgenic animals may require stricter conditions and completely intact embryos. Our study clearly shows that adenoviral vector gene transduction within a proper range of m.o.i. ($\leq$60) has no significant adverse effects on cell cycle regulation, cell viability, or the efficiencies of cardiomyogenic differentiation and EB formation for our purpose: that is, *in vitro* developmental studies using ES cells. Nevertheless, the potential effects of adenoviral infection on the differentiation of ES cells into other tissue types and/or the expression of specific genes by DNA microarray analysis should be carefully examined for each individual purpose.

On the other hand, the imperfection of the current adenoviral vector with regard to its clinical use is not specific to this method, and a less immunogenic, gutted adenoviral vector may resolve the issue. In addition, this method may be less influenced by the specific problems of the current adenoviral vector than is *in vivo* gene therapy, because a smaller amount of adenovirus and transient expression of the Cre transgene during cell culture are sufficient for this method and because the remnants of the episomal adenoviral genome in cells are diluted by every cell division.

It was recently reported that cardiac cells were purified from ES cells by knocking the EGFP gene into one of the Nkx2.5 loci [7]. However, the knock-in strategy not only is labor intensive and time consuming, but also does not address the substantial issue of weak activities of native transcriptional regulatory elements. In fact, the expression levels of EGFP in the Nkx2.5-positive cells shown in the previous report were so low that the cells purified by the knock-in method exhibited features of differentiated cardiomyocytes [7]. Another biological and clinical hindrance of the knock-in method is the necessary destruction of a locus of the target gene; this condition not only renders clinical application difficult but also is accompanied by artifacts that may modulate the normal developmental process [26].

Another remarkable characteristic of the present strategy is that DNA microarray analyses efficiently identified known and unknown genes from the purified ES cells. The unknown highly upregulated or downregulated genes are thought to be involved in early heart development (and/or the target Nkx2.5 gene) or pluripotent (and/or undifferentiated) mechanisms, respectively, as were the known, identified genes. On the other hand, an unexpected and interesting finding of the immunocytochemical and quantitative analyses was the SMA expression in the majority of the purified precontractile cells. It was recently discovered that sequential activation of muscle α-actin isoforms occurs during cardiomyogenic development and that the SMA isoform is expressed early in cardiac development [27,28]. As development proceeds, SMA is sequentially replaced by skeletal and cardiac α-actin isoforms [28]. This notion was also supported by recent findings from a heart cell line derived from Nkx2.5-SV40 Tag transgenic mice [29,30], though such immortalized cell lines may be accompanied by potential problems such as features that are modified or different from normal cells. Thus, the precontractile cells purified by the present method may allow new insight into early cardiac development, and future analyses of the purified cells, including electrophysiological studies, may elucidate the overall role of SMA expression during early cardiac development.

In conclusion, we have developed a method that is efficient, feasible, and generally applicable to many tissues, and we isolated two distinct cardiac cell types from ES cells. This method contributes not only to the development of cell transplantation therapy using ES cells, but also to studies in developmental and stem cell biology.

Materials and Methods

Cell culture. Primary cultured mouse cardiomyocytes [17,31] and the mouse R1 and D3 ES cell lines [3,32] were maintained as previously

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described. To initiate cardiac differentiation, ES cells were cultured without leukemia inhibitory factor (LIF) in low-attachment petri dishes to generate EBs. After 3 days in suspension, the EBs were transferred onto and cultured in gelatin-coated culture dishes [3].

Recombinant adenoviral vectors. The switching-expression plasmids, pCMV-LNEGFP and pCA-LNEGFP, were constructed by subcloning *neo* cDNA and the flanked *loxP* sequences into the pIRES-EGFP plasmid (Clontech, Palo Alto, CA, USA). The mouse Nkx2.5 [10], the mouse αMHC [33], or the CA promoter was inserted into pHM.Δp-Cre, which had a multicloning site upstream of the Cre cDNA and the bovine growth hormone poly(A) signal. Each target DNA fragment was subcloned into the pAdHM4 plasmid. Recombinant adenoviral vectors were generated and gene transduction efficiencies were assessed as described previously [17,34,35].

Cell sorting. Flow-cytometric analysis and sorting of dissociated ES cells were carried out using an EPICS ALTRA Hypersort cell sorter (Beckman–Coulter, Inc., Miami, FL, USA). The viable purified target cells were plated onto culture dishes and cultured for 2 or 7 days before further analyses.

RT-PCR and DNA microarray analyses. RT-PCR and the primer sets were described previously [3]. DNA microarray analyses were performed using Affymetrix mouse oligo microchips (MGU74A and MOE430A), and the obtained data were analyzed using the Affymetrix Suite software and NetAffyx (Affymetrix Japan, Tokyo, Japan) in accordance with the manufacturer's instructions. In brief, 25 or 58 μg of total RNA was obtained from 6.0×10^5 cells purified by Ad.Nkx2.5-Cre on day 6 or from 1.0×10^6 undifferentiated ES cells, respectively. Subsequently, 5 μg of the total RNA from each group was reverse-transcribed, and the resulting cDNA was transcribed to biotin-labeled cRNA using the One-Cycle Target Labeling and Control Reagent (Affymetrix Japan).

Immunocytochemistry. Cells were immunocytochemically stained using antibodies against α-actinin (sarcomeric) (clone EA53; Sigma–Aldrich, Inc., St. Louis, MO, USA), tropomyosin (sarcomeric) (clone CH1; Sigma–Aldrich), troponin I (clone C5; Chemicon International, Inc., Temecula, CA, USA), SMA (clone 1A4; Sigma–Aldrich), Nkx2.5 (N-19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), MHC (MF20; Developmental Studies Hybridoma Bank, Iowa City, IA, USA), or GFP (Molecular Probes, Inc., Eugene, OR, USA) [3]. Rhodamine-labeled phalloidin was used to stain F-actin.

Immunoblotting. Cell lysates were prepared 48 h after adenoviral infection of primary cultured cardiomyocytes and NIH3T3 cells at multiplicities of infection of 30 and 500, respectively. Western blot analysis was performed using rabbit anti-Cre (Novagen, Inc., Madison, WI, USA) or rabbit anti-GFP (Molecular Probes) antibodies, horseradish peroxidase-conjugated anti-rabbit IgG, and chemiluminescent substrate (Pierce Chemical Co., Rockford, IL, USA) according to the manufacturers' protocols.

Transmission electron microscopy. After the purified ES cells were cultured for 2 days, they were prepared for ultrathin sections as previously described [31]. They were stained with uranyl acetate and lead citrate and observed with a scanning electron microscope (H-800; Hitachi High-Technologies, Co., Tokyo, Japan).

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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.ymthe.2006.05.010.

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Cell cycle-specific changes in hTERT promoter activity in normal and cancerous cells in adenoviral gene therapy: A promising implication of telomerase-dependent targeted cancer gene therapy

YOSHITERU MUROFUSHI[1*], SATOSHI NAGANO[1,4*], JUNICHI KAMIZONO[1,4], TOMOYUKI TAKAHASHI[1,2], HISAYOSHI FUJIWARA[5], SETSURO KOMIYA[4], TOYOJIRO MATSUISHI[1,3] and KEN-ICHIRO KOSAI[1,3]

[1]Division of Gene Therapy and Regenerative Medicine, Cognitive and Molecular Research Institute of Brain Diseases, Kurume University, Departments of [2]Advanced Therapeutics and Regenerative Medicine and [3]Pediatrics and Child Health, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011; [4]Department of Orthopaedic Surgery, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520; [5]Department of Cardiology, Respiratory and Nephrology, Regeneration & Advanced Medical Science, Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1194, Japan

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Abstract. Based on the finding that telomerase is reactivated solely in cancer cells, the human telomerase reverse transcriptase (hTERT) promoter has recently been used to target cancer cells by gene therapy. The recent, surprising observation that telomerase is physiologically activated even in normal somatic cells during S-phase has raised concerns as to the safety of this methodology. To clarify this issue, the present study carefully examined the changes in endogenous telomerase activities, hTERT mRNA expression, and hTERT promoter-based transgene expression in normal and cancer cells at synchronized phases of the cell cycle. Telomerase activity and hTERT expression were detected at variable, but relatively high, levels in all 12 cancer cell lines, while both were undetectable in the 11 normal cell lines. In HepG2 cancer cells, the highest levels of hTERT expression and telomerase activity, seen in the G_1/S- and S-phases, were 2-3-fold higher than the lowest levels of both, observed in G_0-phase and during asynchronization. No hTERT expression or telomerase activitiy could be detected in normal WI-38 fibroblasts at any phase of the cell cycle, including S-phase. Consequently, activity of the shorter hTERT promoter, which was transferred into HepG2 cancer cells via adenovirus transduction, was stronger than that of the longer hTERT promoter at all phases and that of two representatives of ubiquitously strong promoters, at both S-phase and asynchronization, but not at G_0-phase. In contrast, neither of hTERT promoters induced detectable transgene expressions in normal WI-38 cells at any cell cycle phase, including S-phase. These results, particularly the lack of problematic levels of S-phase-specific activation of hTERT promoters in normal cells, have promising implications for hTERT promoter-based targeted gene therapy of cancer.

Correspondence to: Dr Ken-ichiro Kosai, Division of Gene Therapy and Regenerative Medicine, Cognitive and Molecular Research Institute of Brain Diseases, Kurume University, 67 Asahi-machi, Kurume 830-0011, Japan
E-mail: kosai@med.kurume-u.ac.jp

*Contributed equally

Abbreviations: hTERT, human telomerase reverse transcriptase; CRA, conditionally replicating adenovirus; HPRT, hypoxanthine guanine phosphoribosyl transferase; TRAP, telomeric repeat amplification protocol; RSV, Rous sarcoma virus long-terminal repeat; CMV promoter, cytomegalovirus immediate-early gene enhancer/promoter; MOI, multiplicity of infection

Key words: adenovirus, cancer gene therapy, cancer-specificity, hTERT, targeting cancer, promoter, telomerase, telomere

Introduction

Telomeres are the distal ends of human chromosomes composed of tandem repeats of the sequence TTAGGG. These sites may function to stabilize chromosomal ends and prevent chromosome degradation, end-to-end fusion, rearrangement, and loss (1-4). Telomeres in somatic cells undergo progressive shortening with each successive cell division; it has been hypothesized that the reduction in telomere length may function as an intrinsic clock involved in the onset of cellular senescence (1,2,5). In immortal cells, telomeres are resynthesized and maintained by telomerase, a specialized DNA polymerase responsible for replication of chromosomal ends (6,7). Human telomerase reverse transcriptase (hTERT), the catalytic subunit of human telomerase, is the major determinant of telomerase activity; ectopic expression of hTERT is sufficient to reconstitute telomerase activity in telomerase-negative cells

(1,2,8,9). Human telomerase activity and hTERT expression are detected in the majority (>90%) of human cancer cells, but are typically absent from normal cells (1,2,10,11). In conjunction with the fact that immortalization correlates well with a stabilization of telomere length, it has been proposed that human cancer cells achieve immortalization through illegitimate activation of telomerase expression (1,2,6).

One of the major challenges of cancer gene therapy is the restriction of transgene expression to cancer cells, because non-specific, extratumoral expression of therapeutic genes would result in the destruction of normal tissues (12,13). Tissue-specific promoters, such as the carcinoembryonic antigen promoter (14), have been used as a treatment for adenocarcinoma to achieve tumor-specific transgene expression. These tissue-specific promoters, however, have the disadvantage of targeting only limited cancer types. In addition, they may exhibit insufficient cancer specificity (leaky activation in normal cells) and/or weak activity, even in cancer cells. To increase the potential efficacy of gene therapy, hTERT promoters have been utilized for cancer gene therapy (13,15,16). Although the lengths of the promoter used differed between reports, hTERT promoter-based transgene regulation should be able to target a broad range of cancers with little effect on mature somatic cells. Recently, the hTERT promoter was also used to generate a conditionally replicating adenovirus (CRA); several studies have demonstrated that hTERT promoter-based CRA can selectively replicate in and kill a panel of cancer cells (17-20).

The majority of previous studies examining hTERT promoter-based cancer gene therapy focused on achieving efficacy in particular cancer models (13,15,16,21,22). The potential adverse effects in normal cells have not yet been thoroughly investigated, although some concerns have been implicated by the fact that telomerase activity is observed at low levels in certain normal cells, such as bone marrow and peripheral blood mononuclear cells (23,24). Relatively high levels of telomerase activity have been reported in hematopoietic cells, the basal layer of the epidermis, endometrial tissues during the menstrual cycle, fetal tissues, and the proliferative zone of intestinal crypts (1,24,25). Surprisingly and unexpectedly, endogenous telomerase activity and hTERT expression could also be detected in an S-phase-specific manner in common normal somatic fibroblasts, which were previously thought to lack both; the upregulated telomerase played a physiological role in the proliferation of normal cells (26). These results strongly suggest a need to evaluate carefully the potential adverse effects of such treatment, specifically determining if leaky expression of a transgene under the control of the hTERT promoter would occur in normal cycling cells to achieve harmful levels. This study carefully examined the changes in telomerase activity, hTERT expression, and hTERT promoter-based transgene expression in normal and cancer cells at specific phases of the cell cycle. The obtained results provide important general implications for hTERT promoter-based targeted cancer gene therapy.

Materials and methods

Cell culture. The human cancer cell lines MKN-1, -28, and -45 (gastric cancer), HCT-15, LoVo, and colo-205 (colon cancer), HepG2 and Hep3B (hepatoma), HeLa (cervical cancer), and HOS-MNNG, KHOS-NP, and SaOS-2 (osteosarcoma) were obtained and maintained as described previously (20). Normal human cell lines, WI-38 and IMR-90 (lung fibroblasts), were obtained from the RIKEN Cell Bank (Tsukuba, Japan), while MRC-5 (lung fibroblasts) and HUV-EC (human umbilical vein endothelial cells) were obtained from the Health Science Research Resources Bank (Osaka, Japan). Primary cultured human cells, NHDF (dermal fibroblasts), NHOst (osteoblasts), HMVEC-d (dermal-derived microvascular endothelial cells), HMEC (mammary epithelial cells), PrEC (prostate epithelial cells), HRE (renal epithelial cells), and SAEC (small airway epithelial cells) were obtained from Cambrex Bio Science Walkersville (Walkersville, MD, USA). All normal cells were maintained according to the manufacturer's protocol.

Reverse transcription-polymerase chain reaction analysis (RT-PCR). Extraction of total RNA from cells and semi-quantitative RT-PCR analysis of hTERT and hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA levels were performed as described previously (27,28). For nested RT-PCR analysis, cDNA was subjected to initial PCR amplification with 20 cycles of 94°C for 30 sec, 59°C for 60 sec and 74°C for 60 sec in the presence of Taq DNA polymerase (Promega, Madison, WI, USA) and primer set 1 (P1; sense, 5'-TTCCTGCACTGGCTGATGAGTGT-3', and antisense, 5'-CGCTCGGCCCTCTTTTCTCTG-3') (29). Subsequently, 1/25 of the amplified cDNA was subjected to a second PCR amplification of 35 cycles of 94°C for 45 sec, 60°C for 45 sec and 72°C for 90 sec with Taq DNA polymerase and primer set 2 (P2; sense, 5'-CCTGCTGGATTACATTAA AGCACTG-3', and antisense, 5'-AAGGGCATATCCAACA ACAA-3') (7).

Endogenous telomerase activity. Endogenous telomerase activity in cells was examined using the telomeric repeat amplification protocol (TRAP) with *T*elo *TAGGG* Telomerase PCR ELISA^{PLUS} kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol.

Cell cycle analysis. Cell cycle synchronization was induced as described (30), with the following modifications. Briefly, synchronization of cells in G_0-, S-, or G_2/M-phases was achieved by treatment with serum starvation (0.5%) for 72 h, 0.3 mM hydroxyurea (Sigma-Aldrich, St. Louis, MO, USA) for 32 h, or 0.4 μg/ml nocodazole (Sigma-Aldrich) for 20 h, respectively. Cells were synchronized in G_1/S phase by treatment with 5 mM thymidine (Sigma-Aldrich) for 20 h, followed by treatment with 5 μg/ml aphidicolin (Sigma-Aldrich) for 16 h (30). The percentage of cells entering each phase of the cell cycle was determined by flow cytometric analysis of propidium iodide-stained cells using a FACSCalibur cytometer (Becton Dickinson, San Jose, CA, USA) and ModFit software (Verity, Topsham, ME, USA).

Generation of adenoviral vectors. The short (260-bp; -181 to +79) and long (1454-bp; -1375 to +79) hTERT promoters [hTERT(S) and hTERT(L)] were isolated by *Mlu*I/*Bgl*II digestion from the pGL3-181 and pGL3-1375 plasmids (the kind gift of Dr S. Kyo, Kanazawa University, Kanazawa,

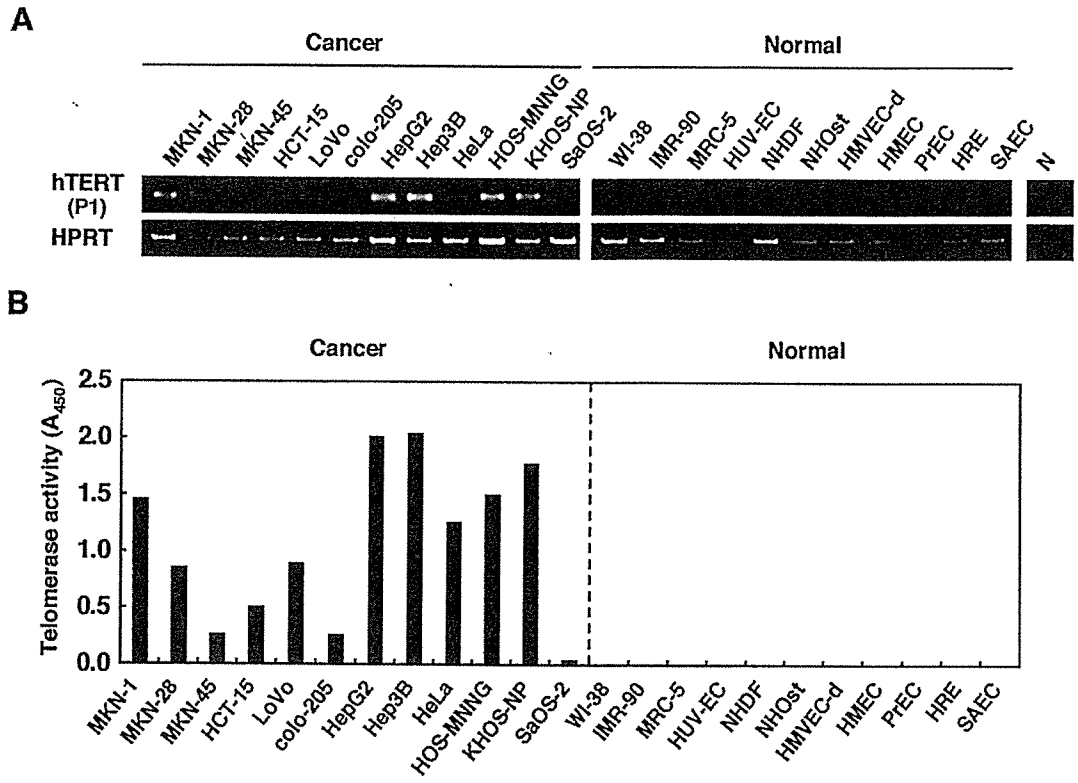

Figure 1. Endogenous hTERT expression and telomerase activity in a variety of cancer and normal cells. (A) Endogenous hTERT mRNA was detected by standard RT-PCR using the primer set 1 (P1) in all of 12 cancer cell lines, but was undetectable in 11 normal cells. Amplification of the HPRT gene served as an internal control. N, no template served as a negative control. (B) Endogenous telomerase activity was detected by TRAP assay at varying levels in all cancer cell lines, but could not be detected in any of the normal cells. Data are represented as the levels at A_{450}.

Japan), respectively (31). We generated E1-deleted replication-defective adenoviral vectors, Ad.hTERT(S)-LacZ, Ad.hTERT(L)-LacZ, Ad.RSV-LacZ, and Ad.CMV-LacZ, which express the LacZ gene under the control of the hTERT(S) promoter, the hTERT(L) promoter, the Rous sarcoma virus long-term repeat (RSV) promoter, and the cytomegalovirus immediate-early gene enhancer/promoter (CMV promoter), respectively (20,32). Adenoviral stocks were prepared and titered as described previously (33,34).

Promoter activity. HepG2 ($4x10^5$ cells/well) and WI-38 ($1.2x10^5$ cells/well) cells in 6-well plates were infected with each adenoviral vector at a multiplicity of infection (MOI) of 10 for 1 h and at MOI of 30 for 24 h, respectively, conditions which provided almost similar gene transduction efficiencies in both cell types (about 30%) without apparent cytotoxicity. The cells were synchronized at each phase of the cell cycle as described above and subsequently harvested. ß-galactosidase activity was measured using a ß-Galactosidase Enzyme Assay System (Promega) as described previously (14,20).

Results

Endogenous hTERT mRNA levels and telomerase activities in various human cancerous and normal cell types. Consistent with our previous results, hTERT mRNA expression was readily detected by standard semi-quantitative RT-PCR analysis in all of the examined cancer cells, which were derived from a variety of tissue origins; a more sensitive nested RT-PCR was not necessary for detection. The expression levels varied considerably among cell types (Fig. 1A); notably, both hepatoma cell lines (HepG2 and Hep3B) and two of the three osteosarcoma cell lines (HOS-MNNG and KHOS-NP) exhibited high expression levels of hTERT mRNA. Endogenous telomerase activity was detected by TRAP assays in all 12 cancer cell lines at varying levels; eleven cancer cell lines exhibited relatively high activity, while SaOS-2 cells, which have been reported as a telomerase-negative osteosarcoma (35,36), displayed only low levels of telomerase activity (Fig. 1B). The telomerase activity in each cancer cell line correlated well with the expression levels of hTERT mRNA. In contrast, neither hTERT mRNA expression nor telomerase activity could be detected in the 11 normal cell lines derived from a variety of tissues.

Thus, these findings support the widely accepted notion that hTERT is the telomerase catalytic subunit and is reactivated specifically in cancer cells (6,7,37), although the diversity of levels between individual cell lines is relatively large.

Expressions of endogenous hTERT mRNA in cancer and normal cells at each phase of cell cycle. Previous studies demonstrated that telomerase activity in cancer cells changed throughout the cell cycle (30,38). A recent study revealed that hTERT was also expressed in normal human fibroblasts, which were previously thought to lack hTERT expression and telomerase activity. This expression, however, was restricted to the S-phase (26). These results suggest that hTERT

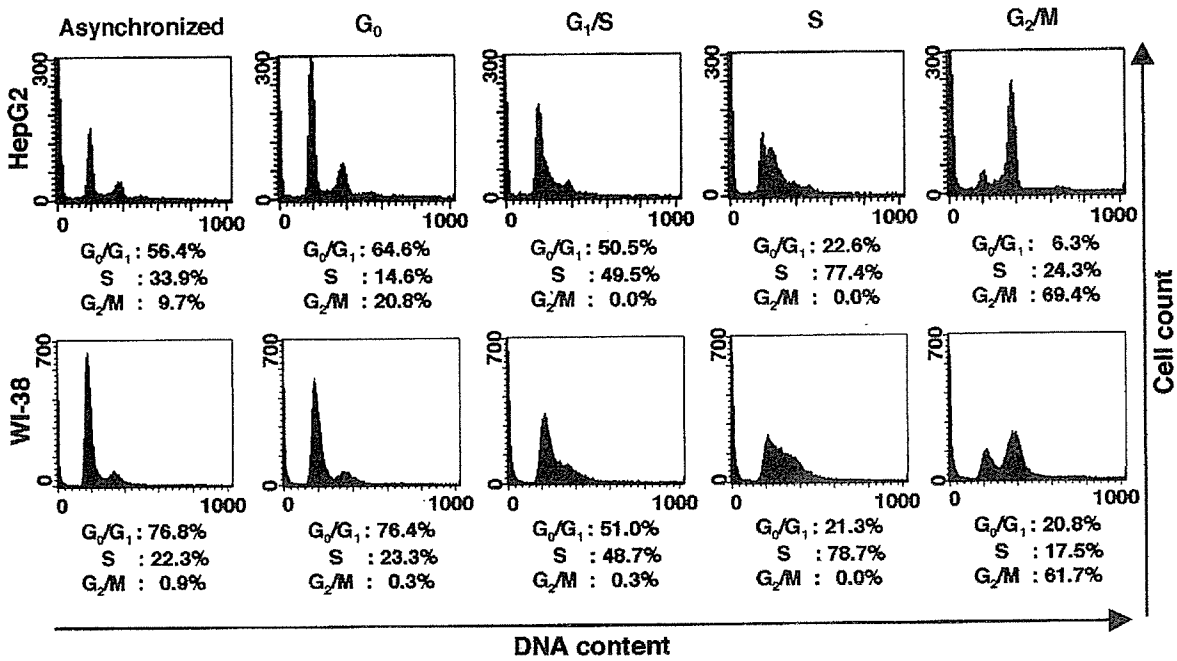

Figure 2. Synchronization of cancer and normal cells at specific phases of the cell cycle. HepG2 cancer and WI-38 normal cells were synchronized at G_0-, G_1/S-, S-, and G_2/M-phases by treatment with 0.5% serum starvation for 72 h, 5 mM thymidine for 20 h with 5 μg/ml aphidicolin for additional 16 h, 0.3 mM hydroxyurea for 32 h, and 0.4 μg/ml nocodazole for 20 h, respectively. The percentage of the cells entering the specific phase of the cell cycle was determined by flow cytometric analysis of propidium iodide-stained cells.

expression and/or telomerase activity might be detected in many normal cell types during S-phase of the cell cycle. We next compared the maximal levels of both hTERT expression and telomerase activity in normal cells with those in cancer cells. After synchronizing HepG2 hepatoma cells and WI-38 normal fibroblasts, we examined hTERT mRNA expression and telomerase activity. We chose the HepG2 and WI-38 cell lines for these experiments, because they have been widely used for both telomerase (26,30,37) and adenoviral gene therapy studies (20,34,39).

Through several pilot studies, we determined the optimal conditions for cell synchronization and adenoviral gene delivery/expression, as shown in Materials and methods. HepG2 and WI-38 cells were successfully synchronized and/or arrested at G_0-, G_1/S-, S-, or G_2/M-phases by treatment with serum starvation, thymidine/aphidicolin, hydroxyurea, or nocodazole, respectively. Assessment of DNA content by flow cytometry demonstrated that the percentages of cells in the desired phases of the cell cycle were high and similar between HepG2 and WI-38 cells (Fig. 2).

We examined the expression of endogenous hTERT mRNA in HepG2 and WI-38 cells by RT-PCR analysis at each phase of the cell cycle. HepG2 cancer cells exhibited high levels of hTERT mRNA at all phases of the cell cycle, including G_0-phase, as well as under conditions of asynchronization (Fig. 3A). In contrast, hTERT mRNA expression could not be detected in WI-38 normal fibroblasts at any phase of cell cycle, either by standard RT-PCR analysis or the more sensitive nested RT-PCR analysis (Fig. 3B), although a recent report noted that hTERT mRNA could be detected in WI-38 cells during S-phase by standard PCR using the same primers (P2) (26).

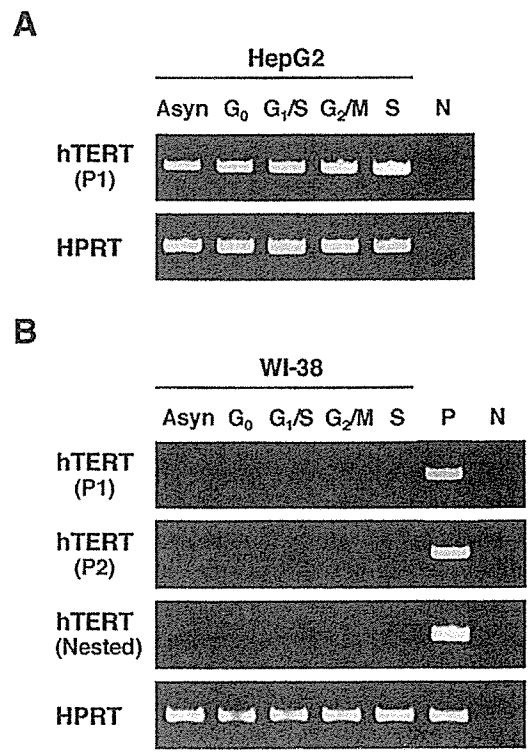

Figure 3. Endogenous hTERT mRNA expression at specific phases of the cell cycle in cancer and normal cells. HepG2 (A) and WI-38 (B) cells were synchronized at each phase of cell cycle, as described in Fig. 2. hTERT mRNA expression was then examined by RT-PCR. (A) hTERT mRNA expression was prominent in HepG2 cancer cells at all phases by standard RT-PCR analysis using the primer set 1 (P1). (B) In contrast, hTERT mRNA expression could not be detected by either standard RT-PCR using primer sets 1 or 2 (P2) or by nested RT-PCR. The HPRT gene was amplified as an internal control. Asyn, asynchronized cells; N, no template served as a negative control; P, template from HepG2 served as a positive control.

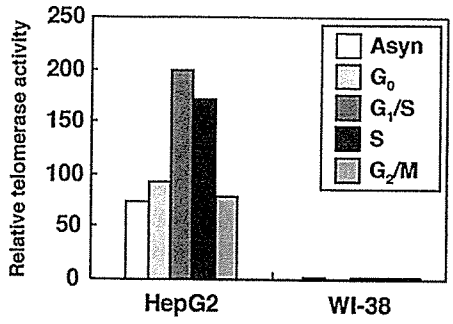

Figure 4. Endogenous telomerase activities at specific phases of the cell cycle in cancerous and normal cells. HepG2 and WI-38 cells were synchronized at each phase of the cell cycle, as described in Fig. 2. Telomerase activities were examined by TRAP assay. Data were represented as telomerase activity normalized to the supplied standard control.

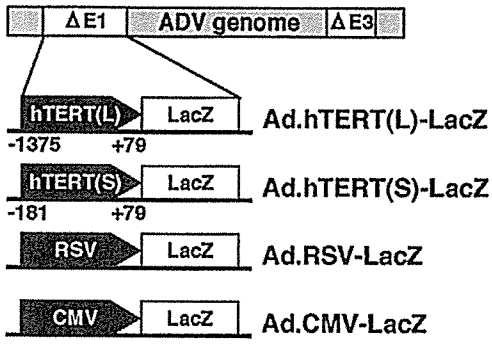

Figure 5. Schematic representation of replication-defective adenoviral vectors. A LacZ gene, downstream of the hTERT(S), hTERT(L), RSV, or CMV promoters was inserted into E1-deleted replication-defective adenoviral vectors.

Endogenous telomerase activities in cancer and normal cells at each phase of cell cycle. Next, we examined endogenous telomerase activity in HepG2 and WI-38 cells by TRAP assay at each phase of cell cycle. High levels of telomerase activity were observed in HepG2 hepatoma cells throughout the cell cycle, including in G_0-phase, although cell cycle-dependent changes of telomerase activity levels were prominent (Fig. 4). Telomerase activity at G_1/S- and S-phases were 2-3-fold higher than that seen under conditions of asynchronization. Despite the S-phase-specific upregulation (30,38), the fact that telomerase activities in G_0 and G_2/M (i.e., out of S-phase of cell cycle) remain relatively high is surprising. In contrast, no telomerase activity was detected in WI-38 normal fibroblasts at any phase of the cell cycle. These results suggest that both baseline hTERT expression and the resulting telomerase activity are significantly higher in cancer cells than the maximal levels seen in normal cells, despite the tight regulation of hTERT expression in both cell types in a cell cycle-dependent manner.

Cell cycle-dependent transgene regulation in cancer and normal cells using the hTERT promoters carried in an adenoviral vector. Although several studies have identified hTERT promoters of different lengths, a recent report demonstrated that two hTERT promoters [hTERT(L): -1375 to +79 and hTERT(S): -181 to +79] had similar, potent activities in several cancer cell lines (31). We therefore constructed four E1-deleted replication-defective adenoviral vectors, Ad.hTERT(L)-LacZ, Ad.hTERT(S)-LacZ, Ad.RSV-LacZ, and Ad.CMV-LacZ, which expressed LacZ under the control of the hTERT(L), hTERT(S), RSV, and CMV promoters; the latter two, both strong promoters functioning in all cell types, served as positive controls (Fig. 5). We initially infected either HepG2 or WI-38 cells with each adenoviral vector. After either synchronizing the cells at G_0- or S-phase or leaving the cells without any synchronization, we measured ß-galactosidase activity, as described in the Materials and methods.

Highly S-phase-specific transgene expression was observed in HepG2 cancer cells after adenoviral delivery of transgenes controlled by either hTERT promoter; the activities of both hTERT promoters in HepG2 cells at S-phase were 5-7-fold higher than those seen at G_0-phase (Fig. 6). Unexpectedly, the activity of hTERT(S) was significantly higher than that of

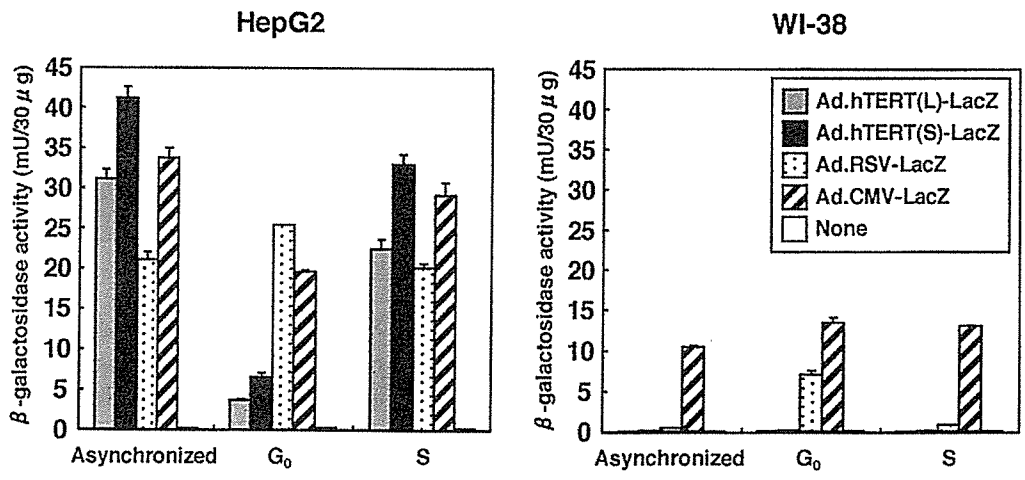

Figure 6. hTERT promoter activity at specific phases of the cell cycle in cancerous and normal cells. HepG2 and WI-38 cells were infected with either adenoviral vector shown in Fig. 5, then synchronized at each phase of the cell cycle as described in Fig. 3. We then measured ß-galactosidase activities; each bar represents the mean ± the standard error.

hTERT(L) in HepG2 cells under all conditions, including G_0- and S-phases. Notably, hTERT(S) activity at S-phase and under conditions of asynchronization, but not during G_0-phase, was higher than RSV and CMV promoter activities. In contrast, no hTERT(S) or hTERT(L) activity could be detected in WI-38 normal fibroblasts at any phase of the cell cycle or under conditions of asynchronization.

Discussion

Although the anti-cancer effects and cancer-selectivity of hTERT promoter-based cancer gene therapy look promising (13,15,16,21,22), the potential safety issues have remained concerning, because of both the potential telomerase activity in subsets of normal cells and the physiological upregulation of telomerase activity specifically during S-phase of the cell cycle, recently reported even in normal somatic cells, which were previously believed to lack both hTERT expression and telomerase activity (1,23-25). Through careful analysis of telomerase throughout the cell cycle, we elucidated the critical potential safety issues of hTERT promoter-based gene therapy. The results were promising, supporting the safe clinical applicability of hTERT promoter-based gene therapy. We observed high cancer-specificity of endogenous telomerase activity and hTERT expression (Fig. 1); the generality of this finding was verified using 12 cancerous and 11 normal cell types, including WI-38 normal fibroblasts previously used by Masutomi *et al* (26). In addition, endogenous hTERT expression, endogenous telomerase activity, and hTERT promoter activity in WI-38 normal fibroblasts were undetectable at all phases of the cell cycle, including S-phase. This result sharply contrasted the high levels of all three seen in HepG2 cancer cells even at G_0-phase. The discrepancy of our results with the previous study reporting detectable telomerase activity in normal cells may result from the different experimental systems and methods used; these methods included the release after synchronization in contrast to direct synchronization to obtain specific phases of the cell cycle, conventional transfection in contrast to adenoviral gene transduction, and the sensitive immunoprecipitation-TRAP as opposed to the standard TRAP assay in the previous and current studies, respectively (26).

The recently-proposed theory that S-phase-specific activation of telomerase is physiologically necessary for proliferation, but not the maintenance of telomere length, of normal cells remains a possibility (26). The levels of telomerase activity in normal cells, however, are significantly lower than those in cancer cells, being almost undetectable by the standard TRAP assay. The abnormally high telomerase activities in cancer cells may play a different, pathological role in the maintenance of the telomere length, leading to cancer cell immortalization (1,2,6). The differences in hTERT expression levels and hTERT promoter activities between cancer and normal cells should be much larger than the fluctuations seen in each cell subset throughout the cell cycle, regardless of parent cell type. Consequently, leaky transgene expression in normal cells remains close to undetectable levels, which likely does not approach a problematic level for the majority of hTERT promoter-based targeting cancer gene therapies.

Insufficient activity of tissue-specific promoters, as well as insufficient cancer-specificity has remained the critical problem with tissue-specific promoter-based targeting cancer gene therapy (40-42). These drawbacks significantly diminish the clinical utility of this technique, as weak anti-cancer effects often result in little or no benefit. The activity of hTERT(S) promoters were stronger than that of the RSV and CMV promoters, representative strong promoters, in HepG2 cancer cells under conditions of asynchronization and during S-phase, although it was weaker than the RSV and CMV promoters at G_0-phase. While stronger promoter activity than that of RSV and CMV does not guarantee an increased efficacy in all cancer gene therapy strategies, previous studies suggested that stronger therapeutic gene expression resulted in more beneficial outcomes using a variety of cancer gene therapy approaches (12,13). In previous studies, the RSV promoter was the best of three representative strong promoters for achieving optimal therapeutic expression levels of a suicide gene, providing maximal anti-cancer effects without conspicuous adverse side effects in the treatment of metastatic liver cancer (14). Therefore, a good quality for a useful cancer-specific promoter should be stronger activity than the RSV promoter in targeted cancer cells (14). Thus, stronger activity of the hTERT(S) promoter than those of RSV and CMV promoters is clearly beneficial, supporting the usefulness of hTERT(S) in cancer gene therapy. It would be interesting to compare hTERT(S) activity with therapeutic potential using specific therapeutic genes and cancer models in future studies that focus on potential clinical applications.

Previous studies have utilized several different hTERT promoter lengths of 1720-bp (-1543 to +77) (43,44), 457-bp (-378 to +79) (13,22), 204-bp (-239 to -36) (16,17), and 260-bp (-181 to +79) (15,21) for gene therapy strategies. Each hTERT promoter region worked well when examined individually, but these have not been compared with each other in gene therapy strategies. We therefore carefully evaluated the activities and cancer specificities of the longest [hTERT(L); -1375 to +79] and the shortest [hTERT(S); -181 to +79] hTERT promoters in specific phases of the cell cycle. These studies elucidated that hTERT(S) exerted stronger activity in cancer cells at all phases of the cell cycle and under conditions of asynchronization than the hTERT(L) promoter. The differential promoter activities may be explained by negative regulatory elements between -578 and -378, upstream of the transcriptional start site of the hTERT gene; a previous study suggested that MZF-2 (myeloid-specific zinc finger protein 2) bound to this site, potentially playing a role in the transcriptional repression of hTERT (45). The most important finding, however, for the application of gene therapy technology to clinical medicine is that neither hTERT(L) nor hTERT(S) exhibited any detectable promoter activity in normal cells at any phase of the cell cycle, including S-phase. Taken together, this study indicates that hTERT(S) will be effective and safe for future targeted cancer gene therapy, at least in combination with adenoviral gene therapy.

In conclusion, hTERT(S), the suitable hTERT promoter, carried by an adenoviral vector conferred strong transgene expression in a strictly cancer- and S-phase-specific manner. The levels of S-phase-specific hTERT promoter activity in

normal cells were virtually undetectable, which will likely not be problematic for targeted cancer gene therapy.

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Genetic Modification of Hepatocytes Towards Hepatocyte Transplantation and Liver Tissue Engineering

Hiroyuki Kuge,* Kazuo Ohashi,* Takashi Yokoyama,* Hiromichi Kanehiro,* Michiyoshi Hisanaga,* Fumikazu Koyama,* Ginny L. Bumgardner,† Ken-Ichiro Kosai,‡ and Yoshiyuki Nakajima*

*Department of Surgery, Nara Medical University, Nara, Japan
†Department of Surgery, Ohio State University Medical Center, Columbus, OH, USA
‡Division of Gene Therapy and Regenerative Medicine, Cognitive and Molecular Research Institute for Brain Diseases, Kurume University, Kurume, Japan

Cell-based therapies, including liver tissue engineering following hepatocyte transplantation, have therapeutic potential for several types of liver diseases. Modifications in the methodology to manipulate the donor hepatocytes in a more simple and timely manner prior to transplantation would enhance the therapeutic efficacy of this procedure. Conventional approach for vector-mediated gene transduction to the isolated hepatocytes has been performed under primary culture conditions that routinely require several days to complete. In our study, we have established a clinically feasible approach that requires only 1 h of infection time with an adenoviral vector system that results in an extremely efficient transduction efficiency (>80%). To optimize transduction efficiency and sustain normal cellular function, we determined that the isolated hepatocytes should be maintained in UW solution as a suspension medium and infected with adenoviral vectors (Ad) for no more than 1 h at a MOI of 1. To establish if the isolated hepatocytes could be used as a source for cell-based therapies, we transplanted the Ad-transduced hepatocytes into the liver or under the kidney capsule. When the cells were transplanted into the liver, Ad-transduced hepatocytes cultured in suspension conditions were found to have a significantly higher survival rate ($p < 0.01$) than Ad-transduced hepatocytes cultured under standard conditions. We also confirmed that these Ad-transduced hepatocytes have ability to survive long term and were able to engineer a biologically active hepatic tissue under the kidney capsule. Finally, we obtained high level of transduction into canine, porcine, and human isolated hepatocytes in a suspension solution mixed with Ad. In conclusion, the present studies demonstrate that isolated hepatocytes could be genetically modified using Ad when kept in a suspension solution. For this reason, this cell-modified technique could be used for the treatment of liver-targeted diseases and/or disorders.

Key words: Hepatocyte transplantation; Gene modification; Ex vivo gene therapy; Adenoviral vector; Liver tissue engineering

INTRODUCTION

The use of primary or genetically modified primary hepatocytes offers new-generation therapeutic approaches, including hepatocyte transplantation, in the treatment of various types of liver diseases (8,12,13,35,48). Clinically, isolated hepatocytes have been transplanted into the livers of more than 60 patients to provide a temporal bridge until an orthotopic liver transplantation can be performed, in cases such as fulminant liver failure or as an improvement of hepatic metabolic deficiency (48). Recent clinical hepatocyte transplantation trials have reported encouraging results, providing significant therapeutic efficacy that persisted for a long-term period of time (15,29). In most of the cases, hepatocyte transplantation had been conducted by infusing cells into the liver through the portal vein or via the splenic circulation. However, one of the major limiting factors has been the low number of hepatocytes that can be efficiently infused into the liver without causing complications (i.e., 2–5% of the total number of viable hepatocytes of the host liver) (15,35,46). Moreover, researchers have shown that only a fraction (10–20%) of the transplanted hepatocytes into the liver is able to be engrafted in the liver parenchyma from the portal pedicles (19,28). In order to facilitate hepatocyte transplantation as a more clinically effective therapeutic approach, it would be important to increase the engraftment rate of the hepatocytes and to develop a

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Address correspondence to Kazuo Ohashi, M.D., Ph.D., Department of Surgery, Nara Medical University 840 Shijo-cho, Kashihara, Nara 634-8522, Japan. Tel: +81(744) 22-3051; Fax: +81(744) 24-6866; E-mail: kohashi@naramed-u.ac.jp

method to maintain hepatocytes at a more optimal functioning status.

Another innovative way to develop new therapies towards the treatment of liver diseases would be engineering new hepatic tissue using isolated hepatocytes, which would be transplanted into ectopic sites (16,26,35). To address this issue, a number of studies have been conducted to determine whether hepatocyte transplantation is possible in ectopic sites outside of the liver, but the results have been largely unsuccessful in terms of ineffective engraftment as well as poor survival rates. In our lab, we have targeted the space under the kidney capsule and other subcutaneous sites as possible ectopic delivery of the isolated hepatocytes, which have recently been shown to provide higher hepatocyte engraftment efficiencies and persistent survival rates (34,36,39–41). It is conceivable that additional modifications would enhance the likelihood of effective hepatic tissue engineering in these sites, and would allow for this application to be a viable therapeutic modality.

Genetic modification of the isolated hepatocytes followed by transplantation, called ex vivo gene therapy, could be a valuable approach to enhance hepatocyte engraftment and survivability. An important factor required for the ex vivo gene therapy is to achieve a high level of transduction efficiency without cellular function loss causing by toxicity issues. Among currently available viral vectors for hepatocyte ex vivo gene manipulation, many of the studies have used simple retroviral vectors (22,23). One clinical trial has been conducted by transplanting genetically modified hepatocytes using retroviral vectors (18). Unfortunately, the therapeutic efficacy of these experimental and clinical studies has been limited, due in large part to the extremely low transduction efficiency into the hepatocytes by the retroviral vectors. Another potential problem with retroviral vectors is the length of time that is required (i.e., several days) to produce the minimally effective transduction into the hepatocytes, but this extended period of time can result in hepatocyte dedifferentiation, leading to a functional loss and loss of engraftment ability (22,23,38). In marked contrast, recombinant adenoviral (Ad) vectors have shown remarkable transduction in the liver (mainly hepatocytes) when administered into an intravenous route (24). Predominant expression of a receptor for adenovirus, CAR, in the hepatocytes contributes to the high adenovirus affinity to the hepatocytes (30).

In the present study, we attempted to exploit the liver-targeting advantage displayed by the Ad vectors to effectively promote high transduction into the isolated hepatocytes in a short period after isolation (i.e., hours rather than days). Vector infection conditions in terms of suspension media selection, Ad vector dose, as well as incubation period were examined in the isolated hepatocytes to optimize transduction efficiencies. Subsequently, the genetically modified hepatocytes were transplanted into either the liver or under the kidney capsule in order to assess their engraftment and long-term survivability. This study demonstrated the potential of the Ad vector-manipulated hepatocytes to act as a cell source for transplantation or tissue engineering purposes in the treatment of liver diseases.

MATERIALS AND METHODS

Animals

Transgenic mice expressing human alpha-1 antitrypsin (hAAT) under the hepatocyte-specific promoter (hA1AT-FVB/N, H-2^q, 12–15 weeks old) (7) were used as a donor of hepatocytes for vector infection experiments and transplantation experiments. Wild type FVB/N mice (10–12 weeks old) were maintained at the Animal Center at Nara Medical University and used as the recipient mice for the hepatocyte transplantation. All animal procedures were conducted in accordance with the institutional guidelines set forth by Nara Medical University Animal Care Committee. Mice were placed in cages within a temperature-controlled room with a 12-h light/dark cycle and ad libitum access to food and water.

Hepatocyte Isolation and Purification

Hepatocytes were isolated from hAAT transgenic mice using a modified two-step collagenase perfusion method (5) with slight modification as previously described (7,34,38,40). Briefly, the liver was directly perfused using a catheter placed into the inferior vena cava with Hank's balanced salt solution (Sigma, St. Louis, MO) containing 0.09% EGTA followed by a second solution containing 0.03% collagenase type I (Sigma) and $CaCl_2$ (5 mM). Isolated cells were filtered through a nylon mesh membrane and hepatocytes were then purified by centrifuging at $50 \times g$ for 5 min. Cells were resuspended with DMEM medium (Sigma), and the cell viability was determined by trypan blue exclusion test. In our experiments, we only used isolated hepatocytes from a particular isolation if the viability of the cells exceeded 90%. In some experiments, hepatocytes were isolated from a piece of canine, porcine, and human liver pieces as described previously (15,29,34). Human liver samples were surgically obtained from a noncancerous portion of benign liver tumors. Written informed consent was given to the patient prior to the surgery. After isolation, the same purification steps as the mouse hepatocytes were followed.

Vector Preparation

Replication-defective recombinant adenoviral (Ad5) vector expressing either lacZ (Ad-CA-lacZ) or a non-

functional β-galactosidase-neomycin phosphotransferase (Ad-CA-null) driven by the CAG promoter (cytomegalovirus immediate-early enhancer, a modified chicken β-actin promoter) were generated using homologous recombination as previously described (21,25). These recombinant Ad5 vectors were propagated in 293 cells and recovered after 3 days by sonication (Bioruptor, Tosho Electric Co., Ltd. Kanagawa, Japan). The vector solutions were concentrated using Virakit according to the manufacturer's protocol (Virapur, LLC, San Diego, CA). Viral titers were determined by a plaque assay using 293 cells and the titer was expressed as plaque forming unit (PFU)/ml (21).

Adenoviral Vector Transduction to Primary Hepatocytes in Suspension

Isolated hAAT transgenic mouse hepatocytes were infected with Ad-CA-lacZ in suspension (at a density of 8×10^6 hepatocytes in 6 ml of suspension medium) in a 10-cm tissue culture polystyrene dish (Becton Dickinson Labware, Franklin Lakes, NJ) at 4°C. Adherence to the dish was avoided by shaking the dish gently at 20-min intervals. In order to optimize conditions, the following parameters were tested: vector dose [multiplicity of infection (MOI) from 0.04 to 100], duration of vector infection (4 min to 6 h), and different suspension media (UW solution, ViaSpan, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan; DMEM without FBS; or DMEM with 10% FBS). After the infection, hepatocytes were extensively washed with DMEM medium. The Ad vector-treated hepatocytes were cultured to either determine hepatocyte transduction efficiency or for transplantation purposes. For the determination of the transduction efficiency, cells were plated onto a culture dish (Primaria, Becton Dickinson). Eight hours later, hepatocyte plating efficiency was determined and the medium was changed with Williams E medium (WE) (Sigma). After a 24-h incubation period with WE, culture medium was collected and assayed for protein analysis of hAAT and albumin production to determine the hepatocyte function, and the hepatocytes were stained with X-gal to determine the transduction efficiency as described previously (32,38). Briefly, cultured hepatocytes were fixed with ice-cold 0.5% glutaraldehyde for 5 min and stained overnight using 5-bromo-4chloro-3indolyl-β-D-galactosidase (X-gal, Wako Pure Chemical Ltd, Osaka, Japan). Transduction efficiency was determined by counting at least 2000 hepatocytes and the data were expressed as a percentage of the X-gal-positive hepatocytes. Morphological structure of the hepatocytes was also determined. Hepatocyte gene transduction using Ad-CA-null was performed using the same protocol based on the optimized condition from the Ad-CA-lacZ experiment.

Hepatocyte Transplantation

Hepatocytes were prepared for transplantation under the kidney capsule space as previously described (34,36, 38,40). In brief, vector-treated or mock-treated hA1AT-FVB/N hepatocytes were resuspended with the mixture of serum-free DMEM and an equal volume of EHS-gel (Matrigel, BD Biosciences, Bedford, MA), which is an extracellular matrix component extracted from Engelbreth-Holm-Swarm cells, to a final ratio of 1.5×10^6 cells per 100 µl. A total of 3×10^6 hepatocytes were transplanted bilaterally under the kidney capsule space, respectively. Because EHS-gel can quickly polymerize into a three-dimensional gel at room temperature, all the procedures were done at 4°C. For hepatocyte transplantation into the liver, 1.5×10^6 hepatocytes were resuspended with 200 µl of serum-free DMEM and slowly infused directly into the portal vein for 5 min. All the surgical procedures were performed under inhalation anesthesia using isofluorane (Forane, Abbott Laboratories, Abbott Park, IL).

Enzyme-Linked Immunosorbent Assay (ELISA)

hAAT concentration in the culture medium and recipient mouse serum were measured by ELISA using a primary antibody against hAAT (DiaSorin, Stillwater, MN) and a secondary goat anti-hAAT-HRP antibody (Reseacrch Diagnostics Inc., Flander, NJ) as previously described (7,34,38,40). Albumin concentration in the culture medium was measured by ELISA using antibody against mouse albumin (Bethyl Laboratories, Montgomery, TX).

Histological Examination

The detection of the transplanted lacZ-transduced hepatocytes was performed using snap-frozen tissues in Tissue Tek O.C.T. compound (Sakura, Torrance, CA) (37). Sections (10 µm) were fixed with 0.5% glutaraldehyde and stained overnight for β-gal (X-gal, Wako Pure Chemical Ltd.). Cells were lightly counterstained with hematoxylin. For H&E staining and immunohistochemical staining against hAAT, the transplanted hepatocytes, including kidney tissues, were harvested and fixed in 10% buffered formalin. Specimens in paraffin-embedded blocks were sectioned at a thickness of 5 µm. Immunohistochemical staining for hAAT was performed using a rabbit anti-hAAT antibody (1:300, YLEM, Roma, Italy) and HRP-conjugated anti-rabbit antibody based on the Avidin-Biotin-Complex method (20,33,34) in the Vectastain ABC Elite Kit (Vector Laboratories, Inc., Burlingame, CA). Visualization of the immune complexes was performed by incubation with 3,3′-diaminobenzidine (DAB, Sigma). The sections were lightly counterstained with hematoxylin.