The FASEB Journal express article 10.1096/fj.02-0104fje. Published online February 19, 2003.

Chamber-specific differentiation of Nkx2.5-positive cardiac precursor cells from murine embryonic stem cells

Kyoko Hidaka*, Jong-Kook Lee[‡], Hoe Suk Kim*, Chun Hwa Ihm*, Akio Iio*, Minetaro Ogawa[‡], Shin-Ichi Nishikawa[§], Itsuo Kodama[‡], and Takayuki Morisaki*[,†]

*Department of Bioscience, National Cardiovascular Center Research Institute and Department of Molecular Pathophysiology, Osaka University Graduate School of Pharmaceutical Sciences[†], Fujishiro-dai, Suita, 565-8565, Department of Circulation, Research Institute of Environmental Medicine, Nagoya University, Chikusa-ku, Nagoya, 464-8601[‡]; and [§]Department of Molecular Genetics, Kyoto University Graduate School of Medicine, Sakyo-ku, Kyoto 606-8507, Japan

Corresponding author: Takayuki Morisaki, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan. E-mail: morisaki@ri.ncvc.go.jp

ABSTRACT

Embryonic stem (ES) cells are a useful system to study cardiac differentiation *in vitro*. It has been difficult, however, to track the fates of chamber-specific cardiac lineages, since differentiation is induced within the embryoid body. We have established an *in vitro* culture system to track Nkx2.5(+) cell lineages during mouse ES cell differentiation by using green fluorescent protein (GFP) as a reporter. Nkx2.5/GFP(+) cardiomyocytes purified from embryoid bodies express sarcomeric tropomyosin and myosin heavy chain and heterogeneously express cardiac troponin I (cTnI), myosin light chain 2v (MLC2v) and atrial natriuretic peptide (ANP). After 4-week culture, GFP(+) cells exhibited electrophysiological characteristics specific to sinoatrial (SA) node, atrial, or ventricular type. Furthermore, we found that administration of 10^{-7} M retinoic acid (RA) to embryoid bodies increased the percentage of MLC2v(–)ANP(+) cells; this also increased the expression of atrial-specific genes in the Nkx2.5/GFP(+) fraction, in a time- and dose-dependent fashion. These results suggest that Nkx2.5(+) lineage cells possess the potential to differentiate into various cardiomyocyte cell types and that RA can modify the differentiation potential of Nkx2.5(+) cardiomyocytes at an early stage.

Key words: cardiomyocyte • green fluorescent protein • embryoid body • diversification • retinoic acid

Pluripotent embryonic stem (ES) cells can easily generate beating cardiac muscle by forming embryo-like cell aggregates called embryoid bodies (EBs) (1). ES cell-derived cardiomyocytes are considered one of the most effective sources of cells for cell transplantation therapy (2–5). Understanding their mechanisms of development is therefore important for accurately and safely controlling the direction of ES cell differentiation *ex vivo*. ES cell-derived cardiomyocytes express cardiac-specific genes and proteins in a developmentally controlled manner (6–10). For example, slow skeletal troponin I is expressed initially in ES cell-derived cardiomyocytes, whereas induction of cardiac troponin I (cTnI), occurs at later stages of cardiac development, which mimics the isoform transition that occurs during *in vivo* cardiogenesis (11). However, EBs at a given point in time contain cardiomyocytes in different

stages, which makes it difficult to characterize the differentiation of cardiac lineages. Cardiomyogenic lineage can be assessed by isolating cardiac progenitors from EBs and allowing them to differentiate *in vitro*. The identification and isolation of intermediate stages in EB-derived cardiomyocyte differentiation is necessary to elucidate the mechanism of cardiac development.

Another difficulty in studying ES cell-derived cardiomyocytes is that EBs contain cell types with different chamber specificities (8, 12). In *in vivo* cardiogenesis, diversification into chamber-specific cardiomyocytes is believed to occur at a very early stage in cardiac development (13). Even before the formation of the heart tube, chamber-specific gene expression has already begun, and several genes are expressed along the anterior-posterior axis of the cardiac mesoderm. Ventricular cardiomyocytes differentiate from the anterior side of the cardiac mesoderm, while atrial cardiomyocytes differentiate from the posterior side. The chamber-specific differentiation occurs in EB-derived cardiomyocytes despite the absence of anterior-posterior positional information. Sinoatrial (SA) node-, atrium-, and ventricle-like cells have been identified at a later stage of development in ES cell-derived cardiomyocytes by electrophysiological methods (8, 9). In mice, there are few immunological markers to identify chamber-specific cell lineages. Some markers, such as MLC2v, are specifically expressed in ventricular cardiomyocytes throughout development (14). Most atrial-specific protein genes, which include myosin light chain 1a (*Mlc1a*), myosin light chain 2a (*Mlc2a*), α-myosin heavy chain (*Mhca*), and atrial natriuretic protein (Anp), are also expressed in ventricles at early stages of embryonic development (14, 15). Nonetheless, it remains to be determined when chamber-specific diversification occurs in EBs.

Expression of transcription factor genes generally precedes that of structural genes. Several transcription factors are expressed in the heart primordium, where they play a pivotal role during heart development by controlling the expression of many cardiac muscle-specific genes (16). Among these, *Nkx2.5* is expressed throughout the course of development in the heart primordium, as well as in cardiomyocytes (17, 18). It plays a critical role in the transcriptional regulation of cardiac-specific genes by acting in synergy with other transcription factors (19–25). In mice, *Nkx2.5* gene expression begins in the cardiogenic mesoderm at embryonic day 7.5, several hours before the α-cardiac actin (*Actc1*) and β-myosin heavy chain (*Mhcb*) genes begin to be activated (17). Although it is also expressed in endoderm in the early stages of differentiation, Nkx2.5 is most strongly expressed in cardiomyocytes of the heart tube during cardiac development. In contrast to other transcription factors (such as Tbx5 and Irx4), which are non-uniformly expressed along the anterior-posterior axis of the linear heart tube, the *Nkx2.5* gene has a rather ubiquitous expression pattern in the heart tube (14). If Nkx2.5(+) cells include all cardiac precursor lineages during heart development, Nkx2.5 expression could be used to identify various types of cardiomyocyte precursors in a mixture of developing ES cells.

In this report, we have generated an ES cell line in which a GFP reporter gene has been knocked into one of the Nkx2.5 loci. We could obtain pure Nkx2.5/GFP(+) cells from d8 EBs, after the beginning of spontaneous contraction. We demonstrate that the Nkx2.5(+) cells derived from ES cells represent cardiac precursor cells and that they indeed diversify into SA node-, atrial-, or ventricular-like cardiomyocytes. These ES cell-derived cardiomyocytes exhibit immunologically different characteristics shortly after purification, suggesting that they are in various stages of differentiation and that they have already established chamber specificity at this early stage in EB development. Finally, we report the effects of retinoic acid (RA) on the chamber-specific differentiation of ES cells. Treatment with RA increases the percentage of MLC2v(–)ANP(+) cells and induces atrial-specific gene expression in the Nkx2.5(+) cell fraction.

MATERIALS AND METHODS

Vector construction and homologous recombination

Murine *Nkx2.5* genomic clones were isolated from a lambda FIXII strain 129/Sv genomic library (Stratagene, La Jolla, CA) by using mouse Nkx2.5 cDNA probes (18). An 11-kb fragment extending from the *Not* I site to the *Kpn* I site was cloned into pBlueScriptII SK+ (Stratagene), and an *Nco* I site was introduced at the initiation codon by site-directed PCR mutagenesis (Fig. 1A). A part of the coding sequence for the *Nkx2.5* gene (from the *Nco* I site to the *Sma* I site) was excised and replaced with a promoter-less *EGFP* gene (Clontech Laboratories Inc., Palo Alto, CA) and a puromycin resistance gene driven by the *Pgk1* gene promoter (26). The gene encoding the diphtheria toxin A fragment (27) was cloned into the *Kpn* I site for negative selection. Among puromycin-resistant ES cell colonies, the homologous recombination event was confirmed by RT-PCR and by Southern blotting using an external probe as shown in Fig. 1B.

Culture and differentiation of ES cells

The 129/Ola-derived ES cell lines used here are ht7 (provided by Hitoshi Niwa, University of Osaka, Japan) and its derivatives. These cell lines carry the hygromycin resistance gene in one of the *Oct-3/4* loci, which allows for selection of Oct-3/4-positive undifferentiated stem cells (28). The cells were grown routinely on gelatinized dishes without feeder cells by using culture medium containing Glasgow-modified Eagle's medium (GMEM, Sigma-Aldrich, St. Louis, MO), 1000 units/ml leukemia inhibitory factor (ESGRO™, Chemicon International Inc., Temecula, CA), 100 μg/ml hygromycin (Invitrogen Corp., Carlsbad, CA), 10% heat-inactivated fetal calf serum (FCS; Equitech-Bio Inc., Kerrville, TX), 1× non-essential amino acids (Invitrogen), 1 mM sodium pyruvate, 0.1 g/ml penicillin, 0.1 mg/ml streptomycin, and 0.1 mM 2-mercaptoethanol. Differentiation of ES cells was induced through formation of EBs as described previously (29). Briefly, EBs were generated by plating an ES cell suspension at a final concentration of 2.5×10^4 cells/ml in 20 μl (500 cells) of a differentiation medium (GMEM containing 10% FCS, 0.1 mg/ml penicillin, 0.1 mg/ml streptomycin, and 0.1 mM 2-mercaptoethanol) on the lid of bacterial dishes. After incubation in hanging drops for 2 days, 60 EBs were transferred into bacterial Petri dishes together with 10 ml of differentiation medium and cultured as floating EBs for *N* days (designated as d*N*) until dissociation for FACS analysis. We mainly used floating EBs or cells sorted from floating EBs for most of the analysis. For the experiments shown in Fig. 1C and Fig. 2A, EBs were transferred onto gelatin-coated dishes at d5 or d6 and were cultured further for *N* days (designated as d5+*N* or d6+*N*, respectively) to observe spontaneous contraction.

Sorting and culture of GFP(+) cells

Embryoid bodies were dissociated with trypsin-EDTA (0.25% trypsin, 1 mM EDTA, Invitrogen) for 3 min at 37°C and light pipetting before the addition of differentiation medium to neutralize the trypsin. Cells were then resuspended into a smaller volume of medium and were pipetted to obtain single-cell suspensions. For detection of GFP, the single cells were resuspended in Hank's balanced salt solution containing 1% bovine serum albumin, and 2.5 μg/ml propidium iodide (PtdIns), and analyzed with FACSCalibur™ or FACSVantage™ SE (BD Biosciences, San Jose, CA). The sort gate for GFP(+) cells was established on the basis of the forward-scattered light (FSC), side-scattered light (SSC), and PtdIns and GFP fluorescence intensities of the control ES cells. Cells were sorted into culture medium and were re-analyzed to estimate sort purity. Typically, ~1–2 $\times 10^4$ GFP(+) cells were obtained from 1×10^7 cells derived from d8 EBs. The

GFP(+) cells were sorted from d8 or d10 EBs and further cultured on gelatin/laminin-coated dish for *N* days (designated as d8+*N* or d10+*N*, respectively).

Gene expression analysis by real-time RT-PCR

Total RNA was isolated from EBs by using TRIzol® Reagent (Invitrogen). RNA samples were treated with DNaseI (Promega Corp., Madison, WI) to eliminate genomic DNA contamination, and cDNA was synthesized by using SuperScript™ II reverse transcriptase (Invitrogen). For real-time PCR, the reaction was performed with a QuantiTect™ SYBR® Green PCR kit (QIAGEN, Valencia, CA) and the products were analyzed with an ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Levels of *Gapdh* transcript were determined by using TaqMan® Rodent GAPDH Control Reagents (Applied Biosystems) to normalize cDNA levels. The gene-specific primers used are indicated in Table 1.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde-PBS for 10 min at 4°C. They were washed three times with PBS and treated with a blocking buffer (0.1% Triton X-100, 2% skim milk in PBS) at room temperature for 1 h. After incubation with a primary antibody, cells were washed three times with the blocking buffer and incubated with a secondary antibody. The mouse monoclonal antibodies used are as follows: anti-myosin heavy chain antibody (MHC) F36.5B9 (Alexis Corp., San Diego, CA), anti-tropomyosin antibody CH1 (Sigma), anti-MLC2v antibody F109.3E1 (Alexis Corp.), and anti-cTnI antibody TI-1 (DSMZ, Braunschweig, Germany). Both anti-MHC and anti-tropomyosin antibodies recognize embryonic cardiomyocytes and skeletal myocytes, as well as neonatal atrial and ventricular cardiomyocytes. The rabbit polyclonal antibodies are as follows: anti-ANP (Protos Biotech Corp., New York, NY) and anti-chloramphenicol acetyltransferase (CAT) antibody (Sigma). The secondary antibodies used for immunofluorescence were Alexa546- or Alexa488-conjugated anti-mouse or anti-rabbit IgG (Molecular Probes Inc., Eugene, OR). Cells were observed in an Olympus IX70 or an Olympus BX51 microscope, and the image data were acquired with a SPOT CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI) or a Fluoview (Olympus Optical Co., LTD., Tokyo, Japan).

Electrophysiological studies

To examine the electrophysiological and pharmacological properties of cardiac cells derived from ES cells, we recorded action potentials by using a patch-clamp technique in the whole cell configuration at 37°C. Cells were cultured on gelatin-coated cover slips for 28 days after sorting with GMEM-supplemented 10% FBS. The cover slips containing cells were perfused with the normal Tyrode's solution composed of 143 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.25 mM $MgCl_2$, 0.25 mM NaH_2PO_4, 5 mM glucose, and 5 mM HEPES-NaOH, pH 7.4. The pipette solution contained 80 mM KCl, 60 mM KOH, 40 mM aspartate, 10 mM EGTA, 5 mM MgATP, 5 mM sodium creatine phosphate, 0.65 mM $CaCl_2$, and 5 mM HEPES-KOH (pH 7.3). Action potentials were elicited by applying a stimulus current at 1.0 Hz with a pulse-width of 1 ms. To examine the sensitivity to muscarinic stimulation, carbamyl choline (Sigma) was applied to quiescent cells. Data were sampled by a computer through a digitizer at 10 kHz (Digidata 1200, Axon Instruments, Burlingame, CA) and were analyzed by using pClamp software (Clampex and Clampfit, Axon Instruments).

RESULTS

Generation and characterization of the Nkx2.5GFP ES cell line

A targeting vector was constructed (Fig. 1A) in which a part of the first coding exon (from the initiation codon to the *Sma* I site) in the *Nkx2.5* gene was replaced with the *EGFP* coding sequence together with the *Pgk1* promoter-driven puromycin resistance gene. GFP fluorescence was observed in the beating areas of EBs derived from these clones but not in those of controls (Fig. 1C). As all ES clones tested (*n*=6) exhibited virtually the same level of GFP fluorescence, we used a single ES clone (designated "Nkx2.5GFP ES" in this report) for several experiments. The Nkx2.5GFP ES cell clone differentiated into beating EBs as frequently as the control ES cells (Fig. 2A). More than 50% of both control ES- and Nkx2.5GFP ES-derived EBs started beating at d5+2 (cultured as floating EBs for 5 days and as attached EBs for 2 days). More than 90% of EBs derived from both control and Nkx2.5GFP ES cells had beating foci at d5+4. Thus, cardiogenesis in Nkx2.5GFP ES cells was almost comparable with that of control ES cells. Next, we analyzed GFP(+) cells by flow cytometry at different EB developmental time points. A small fraction of GFP(+) cells was recognized by flow cytometry around d6, just before beating initiated, becoming more prominent at d7 (Fig. 2B).

To characterize the cardiac gene expression profiles of Nkx2.5GFP ES cells, we examined transcript levels of several marker genes at various stages by real time RT-PCR (Fig. 2C). *Flk1* gene expression was detected weakly at d3 and induced strongly at d4 (data not shown), indicating that mesoderm induction occurs at this stage (30). Following the induction of *Gata4* at d4, *Mef2C* and *Nkx2.5* were induced at d5 and *Tbx5* at d6. Some cardiac contractile protein genes (*Mlc2a*, *Mhcb*) were expressed already at d6, while others (*Mlc2v* and *Mhca*) were expressed from d7. The level of *Nkx2.5* transcript in Nkx2.5GFP ES cells was found to be almost half of that in control ES cells. The *Mlc2v* and *Anp* genes, shown genetically to be downstream targets of *Nkx2.5* (24), were also expressed slightly less in Nkx2.5GFP ES cells. However, the other transcripts (*Mef2C*, *Gata4*, *Tbx5*, *Mlc2a*, *Mhca*, and *Mhcb*) were expressed at levels comparable with those in control ES cells. These results together show that cardiogenesis in Nkx2.5GFP ES cells is comparable with that in control ES cells, although that some Nkx2.5-regulated genes are expressed less strongly.

Purification of Nkx2.5/GFP(+) cells from EBs

To further characterize Nkx2.5/GFP(+) cells, we purified GFP(+) cells from d8 EBs and analyzed them by immunostaining. The purity of the sorted GFP(+) cells was typically ~97% (Fig. 3A). The GFP(+) fraction was analyzed by immunostaining with anti-MHC or anti-tropomyosin antibody. As shown in Table 2, >98% of GFP(+) cells were stained positively for MHC and tropomyosin, compared with <1% of GFP(–) cells. We next isolated the GFP(+) fraction of d7 EBs and analyzed it by real-time RT-PCR. As shown in Fig. 3B, the GFP(+) fraction preferentially expressed genes for transcription factors active in the cardiac cell lineage, including *Gata4*, *Mef2C*, and *Tbx5*, at significantly higher levels than the GFP(–) fraction. The GFP(+) fraction also expressed genes encoding contractile proteins, including *Actc1*, *Mhcb*, *Mhca*, *Mlc2a*, and *Mlc2v*. We also detected *Mink*, which is known to be expressed in the cardiac conduction system (31), in the GFP(+) fraction. In contrast, *Gata1* and *Cdh5*, which are expressed in the hematopoietic and endothelial lineages, respectively (32), were detected preferentially in the GFP(–) fraction. We detected neither *myogenin* nor *MyoD* transcript (33) by real-time RT-PCR (cycle number=40) in floating EBs at any examined time points (d3-d10) (data not shown). These results indicate that almost all

Nkx2.5/GFP(+) cells represent developing cardiomyocytes, but not skeletal myocytes, and that this purification procedure is highly successful in enriching for EB-derived cardiomyocytes.

In vitro differentiation of purified Nkx2.5/GFP(+) cells

To follow the differentiation of Nkx2.5/GFP(+) cells, we sorted them at d8 from floating EBs and cultured them on a gelatin/laminin-coated dish for *N* days (designated as d8+*N*) in standard culture medium containing 10% fetal calf serum (FCS). While in culture, Nkx2.5/GFP(+) cells grew larger and migrated toward each other and appeared to grow slightly in cell number (Fig. 4A). After a few days, the Nkx2.5/GFP(+) cells formed colonies, which contracted synchronously and exhibited highly organized myofibrils (Fig. 4B). To examine the differentiation state of these cardiomyocytes, we stained them for cardiac troponin I (cTnI), a marker of late-stage cardiomyocytes in EBs (11). Some Nkx2.5/GFP(+) cells sorted from d8 EBs weakly expressed cTnI at d8+1 (36±11%). The population of cTnI(+) cells increased over time (42±7% at d8+2, 69±5% at d8+4), and the majority of GFP(+) cells strongly expressed cTnI at d8+8 (89±6%). These results suggest that Nkx2.5/GFP(+) cells harvested from d8 EB contain cardiomyocytes in different stages of differentiation, but that almost all of them can differentiate into cTnI-expressing cells in culture.

Electrophysiological characterization of Nkx2.5/GFP(+) cells

To characterize the electrophysiological properties of Nkx2.5/GFP(+)cells, we examined action potential configurations under patch clamp. We found that 17 out of 20 cells (85%) showed continuous spontaneous beating at d10+7. At d10+28, 2 out of 15 cells (13%) showed spontaneous beating. Figure 5 shows representative tracing of action potentials of Nkx2.5/GFP(+) cells at d10+28. Two spontaneous beating cells demonstrated prominent pacemaker depolarization and slow upstroke phase, as characteristic of SA node cells. Thirteen cells were quiescent with resting membrane potential at –71 ± 2.5 mV, and action potential duration at 90% repolarization (APD_{90}) of 38.5 ± 9.5 ms. To further examine the chamber-specific characteristics of the quiescent cells, the effect of carbamyl choline (CCh, 10 μM), a muscarinic agonist, was tested. In 6 out of 13 quiescent cells (Fig. 5B), the resting membrane potential was hyperpolarized by 14±2.5 mV in response to CCh. In the remaining 7 cells, the action potential configuration was not affected (Fig. 5C). Carbamyl choline application to the spontaneously beating cells resulted in an appreciable hyperpolarization and a slowing of the beating rate. The CCh-sensitive and the CCh-insensitive quiescent cells were referred to atrial-type and ventricular-type, respectively. The proportion of atrial-type cells (40%) and that of ventricular-type cells (46%) were comparable (Fig. 5D). Nkx2.5/GFP(+) cells at earlier stages (d10+14, d10+21) also showed spontaneous beating (SA node-type) and quiescent (atrial-type or ventricular-type) action potentials (data not shown). These results indicate that Nkx2.5/GFP(+) cells have the potential to differentiate into various types of cardiac cells including SA node-type, atrial-type and ventricular-type cells.

Heterogeneity of purified Nkx2.5/GFP(+) cells at early stages

In chick, cardiomyocyte diversification has been identified by the expression of molecules specific to atrial or ventricular cell lineages (13), before the establishment of distinct heart chambers. To see whether early-stage EBs contain distinct precursor cell types, we examined the immunoreactivity of purified and cultured Nkx2.5/GFP(+) cells by using anti-MLC2v antibodies (Fig. 6, Fig. 7A) and found that ~20–30% of Nkx2.5/GFP(+) cells were MLC2v(+) at d8+4 (Fig. 7B).

In mice, there are no known atrial lineage-specific contractile proteins. It is possible that the MLC2v(–) cells represent undifferentiated ventricular cardiomyocytes. To further characterize these cells, we transfected them with a quail slow myosin heavy chain 3 (*SMyHC3*) promoter linked to a CAT reporter (34). The quail *SMyHC3* gene is expressed in the sinoatrial region of heart throughout development, which makes it a good marker for presumptive atrial cells (35). We confirmed that this promoter is atrial-specific in cultured mouse cardiomyocytes by examining atrial or ventricular primary cardiomyocytes (Fig. 6d–i). Using the same detection conditions, we detected the CAT protein in some MLC2v(–) cells in the Nkx2.5/GFP(+) fraction (Fig. 6c). Thus, the Nkx2.5/GFP(+) cell fraction contains both atrial- and ventricular-type cardiac precursors, and that they already show immunologically distinct characteristics shortly after sorting.

Effect of retinoic acid on cardiogenesis of EBs

Retinoic acid has been shown to affect cardiac diversification in EBs and embryos in a dose- and time-dependent manner (36). To examine the effect of RA, we treated EBs with RA or disulfiram, which is an inhibitor of retinaldehyde dehydrogenase (35), and evaluated cardiac differentiation by immunocytochemistry and RT-PCR. The Nkx2.5/GFP(+) fraction contained MLC2v(+)ANP(+), MLC2v(+)ANP(–) and MLC2v(–)ANP(+), MLC(–)ANP(–) cells (Fig. 7A), corresponding to the results of Miller-Hence *et al* (12). When we treated EBs with 10^{-7} M RA from d4–d8, the percentage of MLC2v(–)ANP(+) cells became significantly larger than that derived from untreated EBs (Fig. 7B); however, RA treatment from d4–d5 only did not affect this percentage. Treatment with RA at 10^{-8} M (d4–d8) also increased the percentage of MLC2v(–)ANP(+) cells, but only slightly. On the other hand, 10^{-6} M disulfiram slightly increased the percentage of MLC2v(+) cells, whereas 10^{-5} M disulfiram did not. Although the above treatments did not significantly affect the total percentage of GFP(+) cells, treatment of EBs with RA at 10^{-6} M (d4–d8) or 10^{-7} M (d3–d8) strongly inhibited production of GFP(+) cells (data not shown).

The transcription factor genes *Hrt1* and *Coup-tfII* are preferentially expressed in the anterior part of the heart tube during the early stages of heart development (37, 38). As shown in Fig. 7C, expression of both *Hrt1* and *Coup-tfII* was strongly induced by treatment of EBs with 10^{-7} M RA. Similarly, the levels of other atrial genes (although they are not exclusive to atrial cardiomyocytes), *Mhca* and *Mlc2a*, were higher in the GFP(+) cell fraction of 10^{-7} M RA-treated EBs. On the other hand, disulfiram (10^{-5} or 10^{-6} M) did not significantly affect the expression level of these genes in the Nkx2.5/GFP(+) fraction. Taken as a whole, these data suggest that exogenous 10^{-7} M RA preferentially induces atrial cardiomyocyte differentiation in EBs, but that blocking of endogenous RA production does not prominently affect the direction of diversification in our culture system.

DISCUSSION

Identification and purification of EB-derived cardiomyocytes

To identify and isolate cardiomyogenic cells that have differentiated in EBs, we have established an ES cell line carrying a GFP reporter gene knocked into the *Nkx2.5* locus. Similar approaches have been used to mark EB-derived cardiomyocytes via the *Mlc2v* or *Actc1* gene promoter (29, 39, 40), but we focused primarily on the fates of both atrial and ventricular cardiomyogenic lineages within EBs. Although the loss of one copy of *Nkx2.5* has recently been reported to affect cardiac morphogenesis (41), we found that *in vitro* cardiogenesis in EBs was not perturbed, consistent with other reports (42). The transcription of *Mlc2v* and *Anp* genes, however, was partially reduced

in Nkx2.5GFP ES cells, consistent with results obtained from analysis of *Nkx2.5* null mice (24). These results suggest that Nkx2.5 strictly regulates expression levels of these target genes but that a half dose of Nkx2.5 is sufficient to induce cardiac differentiation *in vitro*.

Because Nkx2.5 is also expressed in such non-cardiac tissues as the pharynx and stomach (43), it is possible that the GFP(+) cell population includes non-cardiac cells. We demonstrated that almost all GFP(+) cells purified from EBs stained with both anti-MHC and anti-tropomyosin antibodies, indicating that most GFP(+) cells we collected are developing cardiomyocytes. Reliable separation and collection of GFP(+) cells, however, was possible only when the GFP signal was sufficiently high (d7 or later), whereas the endogenous *Nkx2.5* gene could already be detected from d5. It will be necessary to further improve our method for marking Nkx2.5(+) cells in order to characterize them at earlier stages. Introducing extra copies of *Nkx2.5* enhancer-driven EGFP would help us to detect earlier stages of cardiomyocyte development.

Differentiation and diversification of purified Nkx2.5/GFP(+) cells

Cardiac TnI is a subunit of the thin filament-associated troponin-tropomyosin complex and plays a pivotal role in regulating cardiac muscle contraction (44). Isoform switching of troponin I is controlled developmentally during vertebrate heart development (45). In EBs, cardiac TnI is expressed only in a small proportion of beating foci at early stages of cardiac differentiation (foci contracting for less than 5 days), while it is expressed in most beating foci at later stages (contracting for more than 20 days) (11). Consistent with the report by Westfall *et al*, we observed that some Nkx2.5/GFP(+) cells weakly expressed cTnI just after plating, but many of them strongly expressed it after several days in culture. This suggests that Nkx2.5/GFP(+) cells can differentiate into cTnI(+) cells after isolation from EBs.

In the present study, most of Nkx2.5/GFP(+) cells at an early stage of culture (d10+7) were spontaneously beating as reported by Maltsev *et al* (7). The Nkx2.5/GFP(+) cells at an later stage (d10+28) showed variable action potential characteristics including SA node-type, atrial-type and ventricular-type. This means that the Nkx2.5/GFP(+) cell lineage may be pluripotent in differentiation, not only for working myocytes, but also for myocytes of the specialized conduction system. Compared with the cell sorting method using a ventricular specific marker, MLC2v, as reported by Muller *et al* (41) and Meyer *et al*. (42), our method using Nkx2.5 as a marker may be more useful for cell transplantation therapy in the heart with pacemaking dysfunction.

Signs of diversification are already apparent shortly after sorting; immunologically diverse cell types were stained differentially with MLC2v and ANP antibodies. Moreover, an atrial-specific promoter was active in some MLC2v(–) cells. These results suggest that the Nkx2.5/GFP(+) cell fraction contains both atrial- and ventricular-type cardiac precursors and that they already show immunologically distinct characteristics at early stages of EB development when their electrophysiological properties are not evident.

Retinoic acid modifies the diversification of cardiomyogenic cell lineages within EBs

Chamber-specific cardiomyocyte differentiation is closely related to positional information along the embryonic anterior-posterior axis. In the absence of a positional cue, other signals must regulate chamber-specific differentiation in EBs. Retinoic acid is a candidate regulator of chamber-specific differentiation along the anterior-posterior axis. In chick or mouse embryo,

excess administration of RA results in posteriorization of the heart tube (characterized by an enlarged atrium and reduced ventricle), whereas a lack of RA signaling results in its anteriorization (characterized by a smaller atrium and a large ventricle) (35, 46–49). We observed that administration of 10^{-7} M RA to EBs increased the percentage of MLC2v(–)ANP(+) cells, while inducing expression of atrial-specific genes as well. Thus, the effect of exogenous 10^{-7} M RA on EBs seems to be similar to that on chick or mouse embryos. Disulfiram, an inhibitor of retinaldehyde dehydrogenase, however, did not increase the percentage of MLC2v(+) prominently, nor decrease the expression of atrial genes. These results suggest that endogenous RA production may not be important in determining diversification in our culture system. Alternatively, endogenous or a low level of RA may be required for the development of both atrial and ventricular cardiomyocyte lineages. We observed that 10^{-5} M disulfiram reduced the level of both atrial and ventricular genes in the Nkx2.5/GFP(+) cell fraction. Wobus *et al* also reported that RA at 10^{-8} M (d5–d15) promoted ventricular myocyte differentiation (36).

In summary, we have established an *in vitro* system to track the fate of Nkx2.5(+) cardiomyocytes derived from ES cells. They indeed differentiate and diversify into various types of cardiomyocytes, and exogenous RA can modify this behavior. These properties seem to be closely related to those of cardiomyocytes differentiated *in vivo*. Thus, this system is predicted to be useful to further understanding of the mechanism of cardiomyocyte differentiation and diversification at a cellular level and will provide us useful information to manipulate and control *in vitro* cardiac differentiation from multi-potent stem cells.

ACKNOWLEDGMENTS

We thank Dr. Hitoshi Niwa (Osaka University, Osaka) for the ES cell line, Dr. Issei Komuro (Chiba University, Chiba) for the Nkx2.5 cDNA and Dr. Frank Stockdale for the SMyHC3-CAT plasmid. We also thank Hiromi Terami, Akemi Ota, Yuko Saito and Masako Nakayama for their technical assistance. We are grateful to Yoshihiro Koyama (Nippon Becton Dickinson Co., Ltd.) for his assistance in cell sorting. This work was supported by research grants from the Ministry of Health, Labor and Welfare and from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

REFERENCES

1. Boheler, K. R., Czyz, J., Tweedie, D., Yang, H. T., Anisimov, S. V., and Wobus, A. M. (2002) Differentiation of pluripotent embryonic stem cells into cardiomyocytes. *Circ. Res.* **91,** 189–201

2. Kehat, I., Kenyagin-Karsenti, D., Snir, M., Segev, H., Amit, M., Gepstein, A., Livne, E., Binah, O., Itskovitz-Eldor, J., and Gepstein, L. (2001) Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J. Clin. Invest.* **108,** 407–414

3. Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M. (1998) Embryonic stem cell lines derived from human blastocysts. *Science* **282,** 1145–1147

4. Klug, M. G., Soonpaa, M. H., and Field, L. J. (1995) DNA synthesis and multinucleation in embryonic stem cell-derived cardiomyocytes. *Am. J. Physiol.* **269,** H1913–H1921

5. Min, J. Y., Yang, Y., Converso, K. L., Liu, L., Huang, Q., Morgan, J. P., and Xiao, Y. F. (2002) Transplantation of embryonic stem cells improves cardiac function in postinfarcted rats. *J. Appl. Physiol.* **92,** 288–296

6. Metzger, J. M., Lin, W. I., Johnston, R. A., Westfall, M. V., and Samuelson, L. C. (1995) Myosin heavy chain expression in contracting myocytes isolated during embryonic stem cell cardiogenesis. *Circ. Res.* **76,** 710–719

7. Maltsev, V. A., Wobus, A. M., Rohwedel, J., Bader, M., and Hescheler, J. (1994) Cardiomyocytes differentiated *in vitro* from embryonic stem cells developmentally express cardiac-specific genes and ionic currents. *Circ. Res.* **75,** 233–244

8. Hescheler, J., Fleischmann, B. K., Lentini, S., Maltsev, V. A., Rohwedel, J., Wobus, A. M., and Addicks, K. (1997) Embryonic stem cells: a model to study structural and functional properties in cardiomyogenesis. *Cardiovasc. Res.* **36,** 149–162

9. Maltsev, V. A., Rohwedel, J., Hescheler, J., and Wobus, A. M. (1993) Embryonic stem cells differentiate *in vitro* into cardiomyocytes representing sinusnodal, atrial and ventricular cell types. *Mech. Dev.* **44,** 41–50

10. Doevendans, P. A., Kubalak, S. W., An, R. H., Becker, D. K., Chien, K. R., and Kass, R. S. (2000) Differentiation of cardiomyocytes in floating embryoid bodies is comparable to fetal cardiomyocytes. *J. Mol. Cell. Cardiol.* **32,** 839–851

11. Westfall, M. V., Samuelson, L. C., and Metzger, J. M. (1996) Troponin I isoform expression is developmentally regulated in differentiating embryonic stem cell-derived cardiac myocytes. *Dev. Dyn.* **206,** 24–38

12. Miller-Hance, W. C., LaCorbiere, M., Fuller, S. J., Evans, S. M., Lyons, G., Schmidt, C., Robbins, J., and Chien, K. R. (1993) *In vitro* chamber specification during embryonic stem cell cardiogenesis. Expression of the ventricular myosin light chain-2 gene is independent of heart tube formation. *J. Biol. Chem.* **268,** 25244–25252

13. Yutzey, K. E., and Bader, D. (1995) Diversification of cardiomyogenic cell lineages during early heart development. *Circ. Res.* **77,** 216–219

14. Christoffels, V. M., Habets, P. E., Franco, D., Campione, M., de Jong, F., Lamers, W. H., Bao, Z. Z., Palmer, S., Biben, C., Harvey, R. P., *et al*. (2000) Chamber formation and morphogenesis in the developing mammalian heart. *Dev. Biol.* **223,** 266–278

15. Zammit, P. S., Kelly, R. G., Franco, D., Brown, N., Moorman, A. F., and Buckingham, M. E. (2000) Suppression of atrial myosin gene expression occurs independently in the left and right ventricles of the developing mouse heart. *Dev. Dyn.* **217,** 75–85

16. Srivastava, D., and Olson, E. N. (2000) A genetic blueprint for cardiac development. *Nature* **407,** 221–226

17. Lints, T. J., Parsons, L. M., Hartley, L., Lyons, I., and Harvey, R. P. (1993) *Nkx-2.5*: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. *Development* **119,** 419–431

18. Komuro, I., and Izumo, S. (1993) *Csx*: a murine homeobox-containing gene specifically expressed in the developing heart. *Proc. Natl. Acad. Sci. USA* **90,** 8145–8149

19. Bruneau, B. G., Bao, Z. Z., Tanaka, M., Schott, J. J., Izumo, S., Cepko, C. L., Seidman, J. G., and Seidman, C. E. (2000) Cardiac expression of the ventricle-specific homeobox gene *Irx4* is modulated by Nkx2-5 and dHand. *Dev. Biol.* **217,** 266–277

20. Durocher, D., Chen, C. Y., Ardati, A., Schwartz, R. J., and Nemer, M. (1996) The atrial natriuretic factor promoter is a downstream target for Nkx-2.5 in the myocardium. *Mol. Cell. Biol.* **16,** 4648–4655

21. Durocher, D., Charron, F., Warren, R., Schwartz, R. J., and Nemer, M. (1997) The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. *EMBO J.* **16,** 5687–5696

22. Hiroi, Y., Kudoh, S., Monzen, K., Ikeda, Y., Yazaki, Y., Nagai, R., and Komuro, I. (2001) Tbx5 associates with Nkx2-5 and synergistically promotes cardiomyocyte differentiation. *Nat. Genet.* **28,** 276–280

23. Molkentin, J. D., Antos, C., Mercer, B., Taigen, T., Miano, J. M., and Olson, E. N. (2000) Direct activation of a *GATA6* cardiac enhancer by Nkx2.5: evidence for a reinforcing regulatory network of Nkx2.5 and GATA transcription factors in the developing heart. *Dev. Biol.* **217,** 301–309

24. Tanaka, M., Chen, Z., Bartunkova, S., Yamasaki, N., and Izumo, S. (1999) The cardiac homeobox gene *Csx/Nkx2.5* lies genetically upstream of multiple genes essential for heart development. *Development* **126,** 1269–1280

25. Zou, Y., Evans, S., Chen, J., Kuo, H. C., Harvey, R. P., and Chien, K. R. (1997) CARP, a cardiac ankyrin repeat protein, is downstream in the *Nkx2-5* homeobox gene pathway. *Development* **124,** 793–804

26. McCarrick, J. W., Parnes, J. R., Seong, R. H., Solter, D., and Knowles, B. B. (1993) Positive-negative selection gene targeting with the diphtheria toxin A-chain gene in mouse embryonic stem cells. *Transgenic Res.* **2,** 183–190

27. Adra, C. N., Boer, P. H., and McBurney, M. W. (1987) Cloning and expression of the mouse *pgk-1* gene and the nucleotide sequence of its promoter. *Gene* **60,** 65–74

28. Niwa, H., Miyazaki, J., and Smith, A. G. (2000) Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat. Genet.* **24,** 372–376

29. Kolossov, E., Fleischmann, B. K., Liu, Q., Bloch, W., Viatchenko-Karpinski, S., Manzke, O., Ji, G. J., Bohlen, H., Addicks, K., and Hescheler, J. (1998) Functional characteristics of ES cell-derived cardiac precursor cells identified by tissue-specific expression of the green fluorescent protein. *J. Cell Biol.* **143,** 2045–2056

30. Kataoka, H., Takakura, N., Nishikawa, S., Tsuchida, K., Kodama, H., Kunisada, T., Risau, W., Kita, T., and Nishikawa, S. I. (1997) Expressions of *PDGF receptor alpha*, *c-Kit* and *Flk1* genes clustering in mouse chromosome 5 define distinct subsets of nascent mesodermal cells. *Dev. Growth Differ.* **39,** 729–740

31. Kupershmidt, S., Yang, T., Anderson, M. E., Wessels, A., Niswender, K. D., Magnuson, M. A., and Roden, D. M. (1999) Replacement by homologous recombination of the *minK* gene with *lacZ* reveals restriction of *minK* expression to the mouse cardiac conduction system. *Circ. Res.* **84,** 146–152

32. Fujimoto, T., Ogawa, M., Minegishi, N., Yoshida, H., Yokomizo, T., Yamamoto, M., and Nishikawa, S. (2001) Step-wise divergence of primitive and definitive haematopoietic and endothelial cell lineages during embryonic stem cell differentiation. *Genes Cells* **6,** 1113–1127

33. Rohwedel, J., Maltsev, V., Bober, E., Arnold, H. H., Hescheler, J., and Wobus, A. M. (1994) Muscle cell differentiation of embryonic stem cells reflects myogenesis *in vivo*: developmentally regulated expression of myogenic determination genes and functional expression of ionic currents. *Dev. Biol.* **164,** 87–101

34. Wang, G. F., Nikovits, W., Schleinitz, M., and Stockdale, F. E. (1996) Atrial chamber-specific expression of the slow myosin heavy chain 3 gene in the embryonic heart. *J. Biol. Chem.* **271,** 19836–19845

35. Xavier-Neto, J., Neville, C. M., Shapiro, M. D., Houghton, L., Wang, G. F., Nikovits, W., Jr., Stockdale, F. E., and Rosenthal, N. (1999) A retinoic acid-inducible transgenic marker of sino-atrial development in the mouse heart. *Development* **126,** 2677–2687

36. Wobus, A. M., Kaomei, G., Shan, J., Wellner, M. C., Rohwedel, J., Ji, G., Fleischmann, B., Katus, H. A., Hescheler, J., and Franz, W. M. (1997) Retinoic acid accelerates embryonic stem cell-derived cardiac differentiation and enhances development of ventricular cardiomyocytes. *J. Mol. Cell. Cardiol.* **29,** 1525–1539

37. Nakagawa, O., Nakagawa, M., Richardson, J. A., Olson, E. N., and Srivastava, D. (1999) *HRT1*, *HRT2*, and *HRT3*: a new subclass of bHLH transcription factors marking specific cardiac, somitic, and pharyngeal arch segments. *Dev. Biol.* **216,** 72–84

38. Pereira, F. A., Qiu, Y., Zhou, G., Tsai, M. J., and Tsai, S. Y. (1999) The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development. *Genes Dev.* **13,** 1037–1049

39. Muller, M., Fleischmann, B. K., Selbert, S., Ji, G. J., Endl, E., Middeler, G., Muller, O. J., Schlenke, P., Frese, S., Wobus, A. M., *et al*. (2000) Selection of ventricular-like cardiomyocytes from ES cells *in vitro*. *FASEB J.* **14,** 2540–2548

40. Meyer, N., Jaconi, M., Landopoulou, A., Fort, P., and Puceat, M. (2000) A fluorescent reporter gene as a marker for ventricular specification in ES-derived cardiac cells. *FEBS Lett.* **478,** 151–158

41. Biben, C., Weber, R., Kesteven, S., Stanley, E., McDonald, L., Elliott, D. A., Barnett, L., Koentgen, F., Robb, L., Feneley, M., *et al*. (2000) Cardiac septal and valvular dysmorphogenesis in mice heterozygous for mutations in the homeobox gene *Nkx2-5*. *Circ. Res.* **87,** 888–895

42. Lyons, I., Parsons, L. M., Hartley, L., Li, R., Andrews, J. E., Robb, L., and Harvey, R. P. (1995) Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene *Nkx2-5*. *Genes Dev.* **9,** 1654–1666

43. Kasahara, H., Bartunkova, S., Schinke, M., Tanaka, M., and Izumo, S. (1998) Cardiac and extracardiac expression of Csx/Nkx2.5 homeodomain protein. *Circ. Res.* **82,** 936–946

44. Huang, X., Pi, Y., Lee, K. J., Henkel, A. S., Gregg, R. G., Powers, P. A., and Walker, J. W. (1999) Cardiac troponin I gene knockout: a mouse model of myocardial troponin I deficiency. *Circ. Res.* **84,** 1–8

45. Saggin, L., Gorza, L., Ausoni, S., and Schiaffino, S. (1989) Troponin I switching in the developing heart. *J. Biol. Chem.* **264,** 16299–16302

46. Chazaud, C., Chambon, P., and Dolle, P. (1999) Retinoic acid is required in the mouse embryo for left-right asymmetry determination and heart morphogenesis. *Development* **126,** 2589–2596

47. Niederreither, K., Vermot, J., Messaddeq, N., Schuhbaur, B., Chambon, P., and Dolle, P. (2001) Embryonic retinoic acid synthesis is essential for heart morphogenesis in the mouse. *Development* **128,** 1019–1031

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

49. Yutzey, K., Gannon, M., and Bader, D. (1995) Diversification of cardiomyogenic cell lineages *in vitro*. *Dev. Biol.* **170,** 531–541

Received March 18, 2002; accepted December 16, 2002.

Table 1

Primer List for Real-time PCR to detect differentiation of ES cells

Gene	Genbank	Sense sequence (5' to 3') Antisense sequence (5' to 3')
Actc1 *(α-cardiac actin)*	NM_009608	CCAGCCCAGCTGAATCC CCATTGTCACACACCAAAGC
Anp	XM_131840	CGTGCCCCGACCCACGCCAGCATGGGCTCC GGCTCCGAGGGCCAGCGAGCAGAGCCCTCA
Cdh5 *(VE-cadherin)*	NM_009868	AGACACCCCCAACATGCTAC GCAAACTCTCCTTGGAGCAC
Coup-tfII	NM_009697	AAGAGCTTCTTCAAGCGCAG CCTCTCTGTACAGCTTCCCG
Flk1	NM_010612	GGCGGTGGTGACAGTATCTT CTCGGTGATGTACACGATGC
Gata1	NM_008089	AGCATCAGCACTGGCCTACT AGGCCCAGCTAGCATAAGGT
Gata4	NM_008092	TCTCACTATGGGCACAGCAG GCGATGTCTGAGTGACAGGA
Hrt1	NM_010423	AGTGAGCTGGACGAGACCAT CTGGGTACCAGCCTTCTCAG
Mef2C	L13171	ACTGGGAAACCCCAATCTTC ATCAGACCGCCTGTGTTACC
Mhca (α-myosin heavy chain)	NM_010856	GAGATTTCTCCAACCCAG TCTGACTTTCGGAGGTACT
Mhcb (β-myosin heavy chain)	NM_080728	CTACAGGCCTGGGCTTACCT TCTCCTTCTCAGACTTCCGC
MinK	NM_008424	CACACACCAGGTTCCCTTG GCTGAGACTTACGAGCCAGG
Mlc2a (myosin light chain 2a)	NM_022879	TCAGCTGCATTGACCAGAAC AAGACGGTGAAGTTGATGGG
Mlc2v (myosin light chain 2v)	NM_010861	AAAGAGGCTCCAGGTCCAAT CCTCTCTGCTTGTGTGGTCA
MyoD	NM_010866	GCTCTGATGGCATGATGGAT GTGGAGATGCGCTCCACTAT
Myogenin	NM_031189	ATCCAGTACATTGAGCGCCT GCAAATGATCTCCTGGGTTG
Nkx2.5	NM_008700	CAAGTGCTCTCCTGCTTTCC GGCTTTGTCCAGCTCCACT
Tbx5	XM_132278	GGAGCCTGATTCCAAAGACA TTCAGCCACAGTTCACGTTC

Table 2

Immunostaining of cells from day 8 embryoid bodies before and after sorting by GFP expression

	Unsorted		Sorted	
	Control ES	Nkx2.5GFP ES	GFP(–) Fraction	GFP(+) Fraction
MHC	5.1% (±3.4%)	5.0% (±2.2%)	0.6% (±0.1%)	98.1% (±1.1%)
Tropomyosin	4.5% (±2.2%)	4.8% (±2.1%)	0.9% (±0.4%)	98.7% (±0.5%)

Embryoid bodies were dissociated on day 8. Cells in the unsorted and sorted fractions were plated on gelatin-coated dishes and stained with anti-MHC or anti-tropomyosin after overnight culture. More than 300 cells were counted. Values are given as mean ± SE from three independent experiments.

Fig. 1

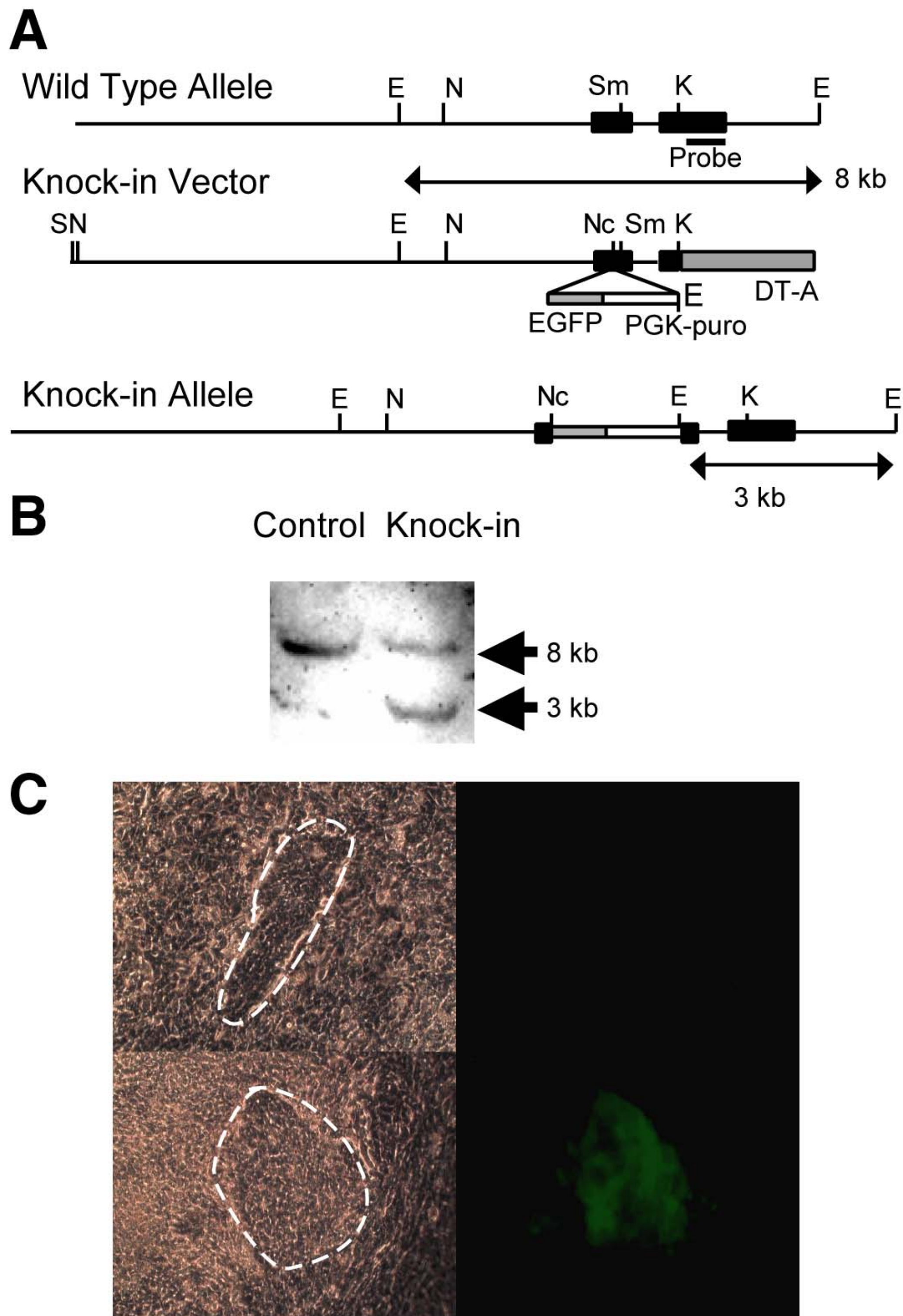

Figure 1. Generation of Nkx2.5GFP ES cells. A) Targeting vector and genomic organization of Nkx2.5GFP ES cells. The 3′ probe shown was used for Southern blot analysis. E, *Eco*RI; K, *Kpn*I; N, *Not*I; Nc, *Nco*I, S, *Sal*I; Sm, *Sma*I. **B)** Genotyping of ES cell clones. Genomic DNA was digested with *Eco*RI and analyzed by Southern blotting. Hybridization with a 3′ probe revealed the expected 8 kb and 3 kb fragments from the wild-type and targeted alleles, respectively. **C)** Spontaneous beating area of differentiated EBs. Embryoid bodies derived from a control ES cell line and an Nkx2.5GFP ES cell line were transferred onto gelatin-coated plates at d6 and cultured for an additional 2 days (d6+2). Spontaneously beating areas (inside of dotted line) had discernible fluorescence in the Nkx2.5GFP EBs (lower) but not the control EBs (upper). Bars, 100 μm.

Fig. 2

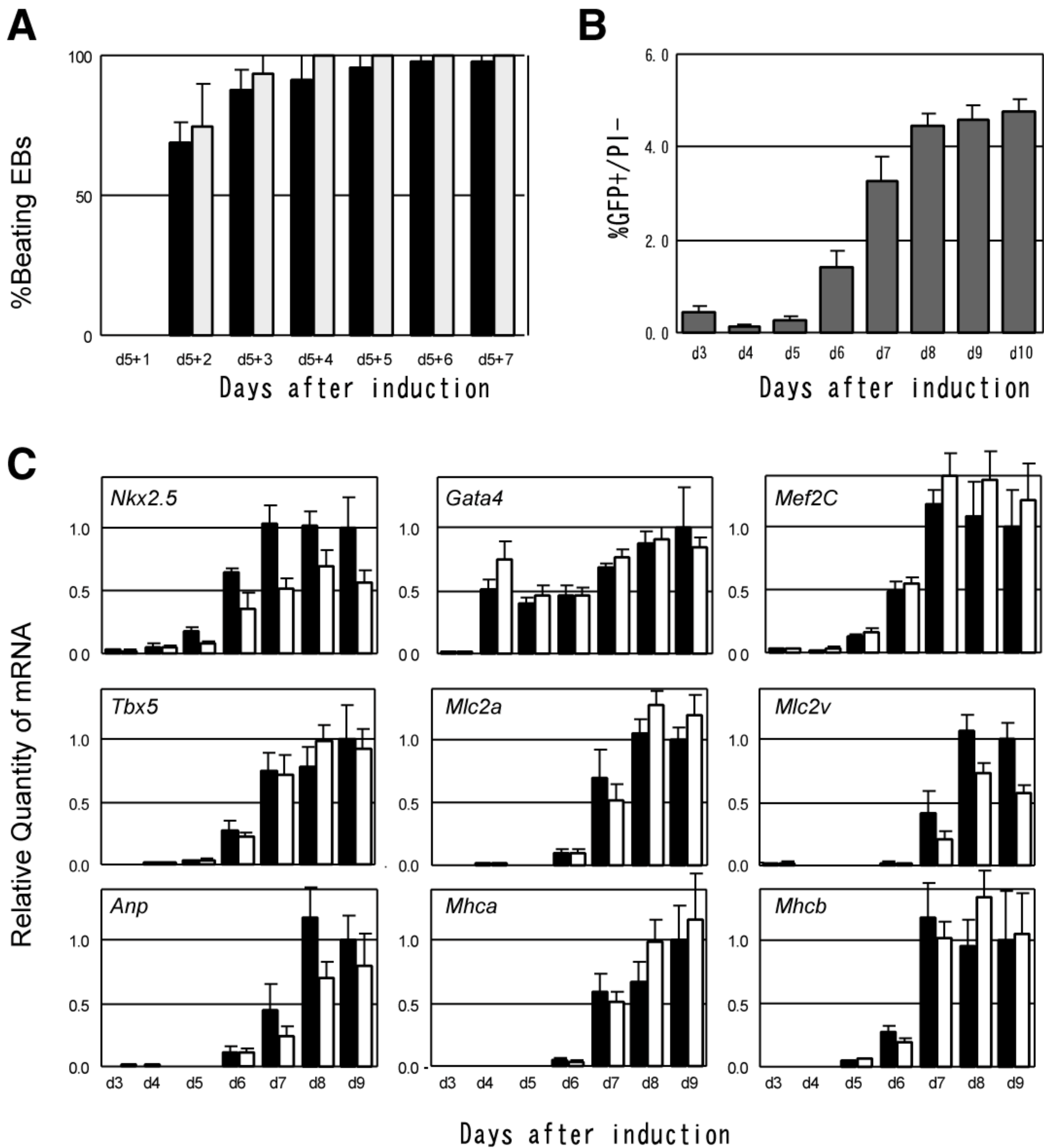

Figure 2. Cardiac differentiation and marker gene expression profiles of control and Nkx2.5GFP ES cell lines.
A) Cardiogenesis of EBs derived from control and Nkx2.5GFP ES cells. Embryoid bodies were transferred to a 24-well gelatin-coated dish at d5 and were cultured further for different *N* numbers of days. Total differentiation time is indicated as d5+*N*. The number of beating EBs (*n*=12) was counted daily. Values are given as mean ± SE from four independent experiments. **B)** Flow cytomeric analysis of developing EBs. Embryoid bodies were cultured in suspension until FACS analysis at different time points as indicated. The percentage of GFP(+) cells among all PI(–) live cells is indicated. Values are given as mean ± SE from three independent experiments. **C)** Real-time RT-PCR analysis of developing EBs. RNA was harvested from floating EBs at different time points as indicated. Real-time RT-PCR was performed by using gene-specific primers indicated in **Table 1**. Total levels of mRNA were normalized with *Gapdh*, and relative quantities of each gene-specific mRNA were determined as ratios to those of d8 control EBs. Values are given as mean ± SE from four independent experiments.

Fig. 3

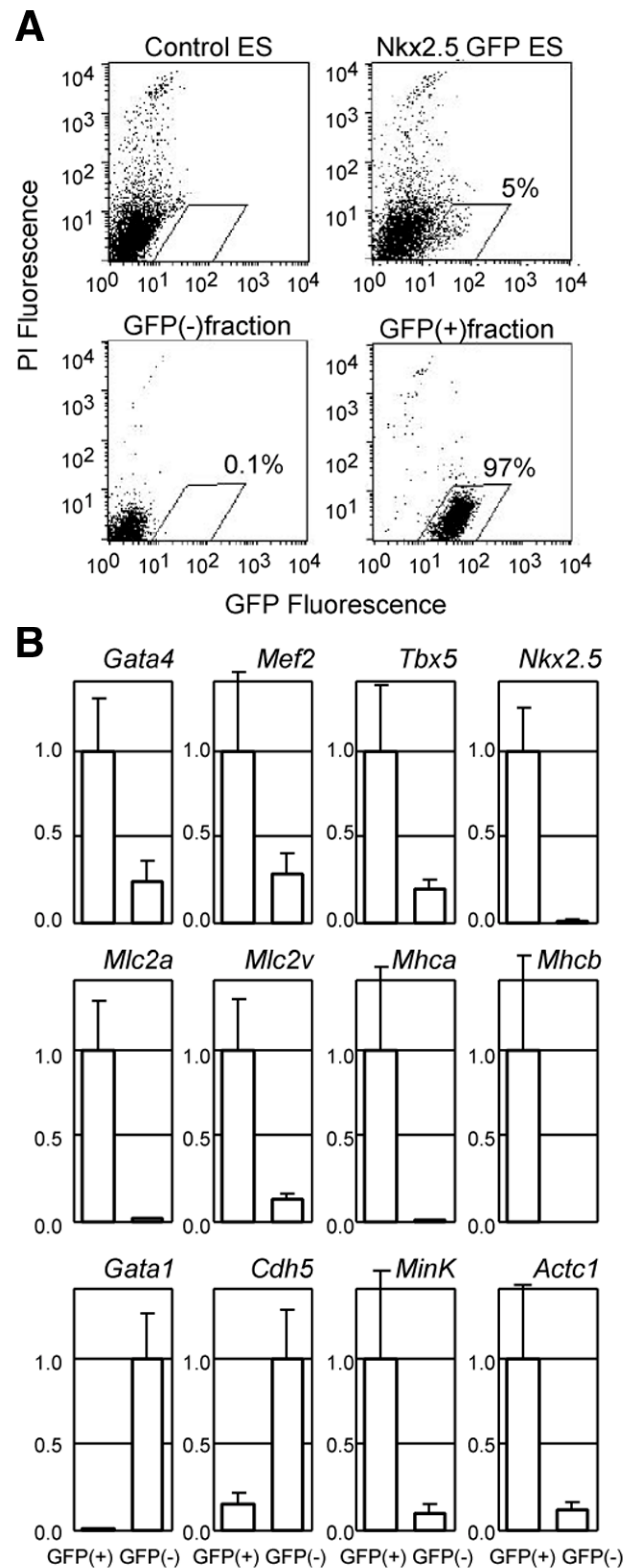

Figure 3. Purification of Nkx2.5/GFP(+) cells. A) Sorting of GFP(+) and (–) cell fractions. Embryoid bodies were cultured in suspension until sorting at d8. The unsorted (control ES and Nkx2.5GFP ES) and sorted fractions [GFP(+) and GFP(–)] were analyzed for sort purity. The numbers in each fraction indicate percentages of GFP(+) cells in an FSC/SSC-gated cell population. **B)** Real-time RT-PCR analysis of GFP(+) and (–) cell fractions. RNA was purified from each cell fraction at d7, and relative quantities of mRNA in the GFP(+) cell and GFP(–) cell fractions were compared with each other. Values are given as mean ± SE from three independent experiments.

Fig. 4

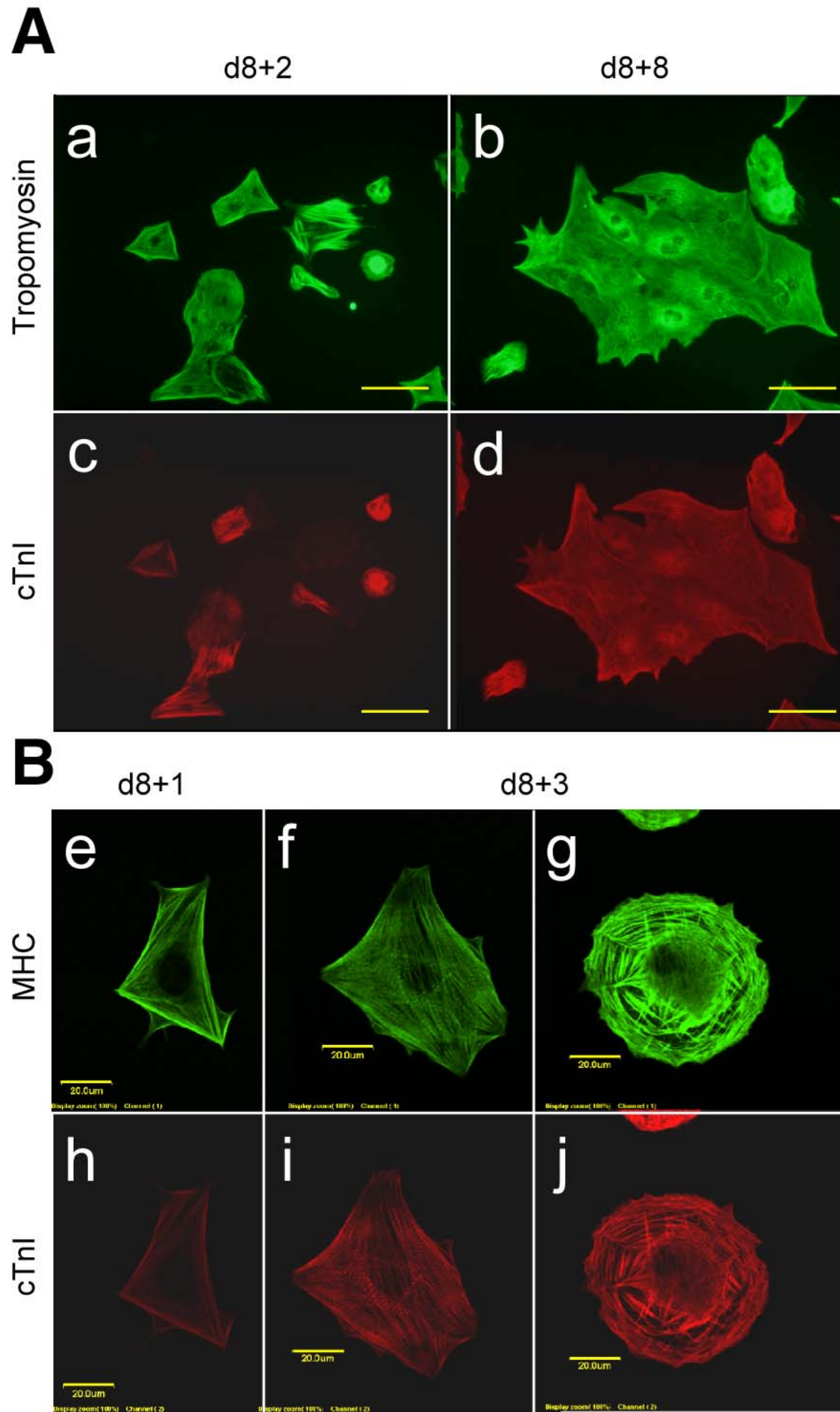

Figure 4. Differentiation of Nkx2.5/GFP(+) cells into cTnI(+) cells. GFP(+) cells were purified from floating EBs at d8 and cultured for one (d8+1, e, h), two (d8+2, a, c), three (d8+3, f–j), or eight days (d8+8, b, d). Cells were fixed and stained successively with anti-cTnI and anti-tropomyosin (**A**) or anti-cTnI and anti-MHC (**B**). Cells were observed with a fluorescent (**A**, bars, 100 µm) or a confocal (**B**, bars, 20 µm) microscope.

Fig. 5

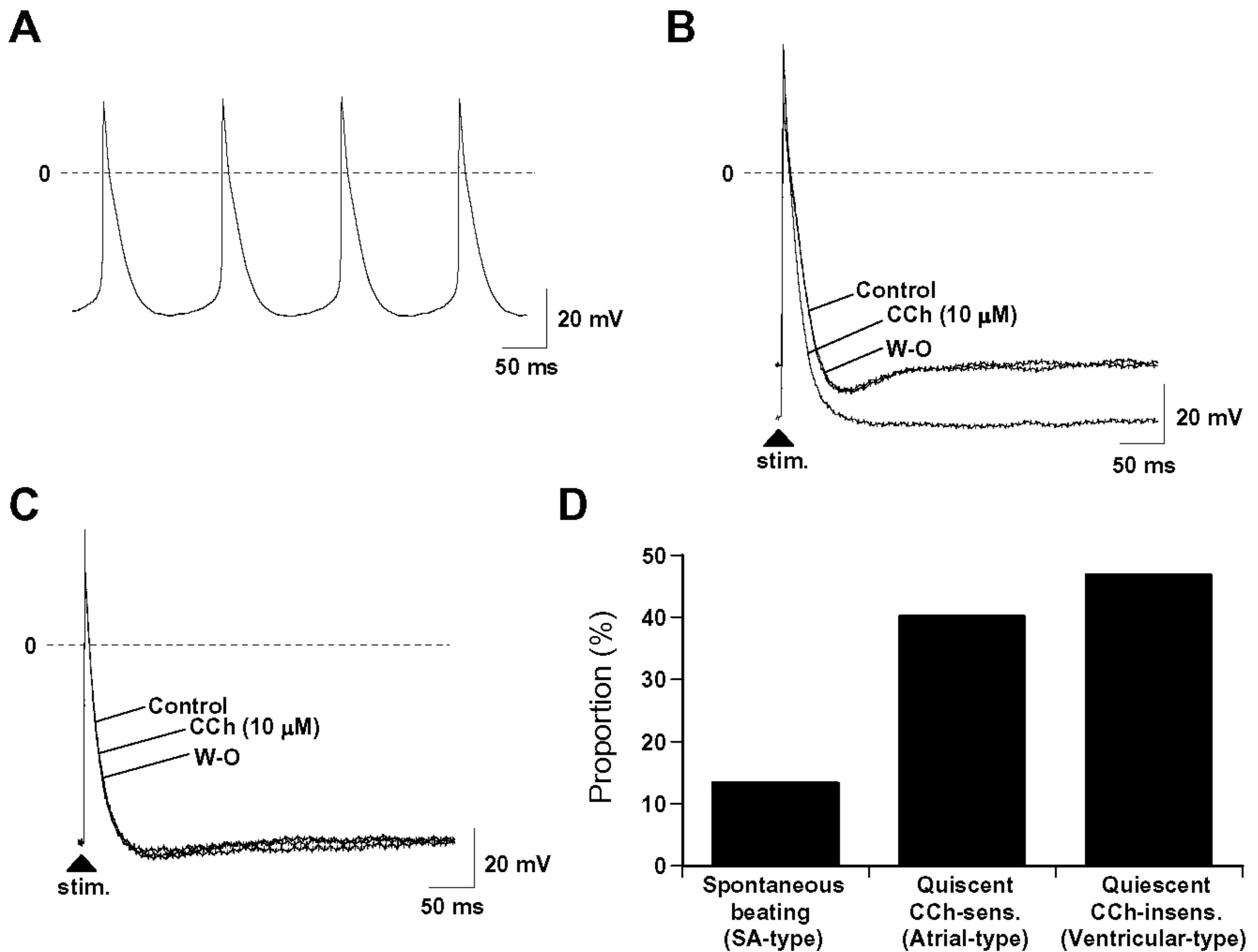

Figure 5. Electrophysiological properties of Nkx2.5/GFP(+) cells. Action potential waveforms and sensitivity to carbamyl choline (CCh: 10 µM) were examined. **A–C)** Representative traces of action potentials recorded in spontaneously beating (sinoatrial (SA) node-type) (**A**), quiescent CCh-sensitive (atrial-type) (**B**), and quiescent CCh-insensitive (ventricular-type) cells (**C**). Action potentials were recorded under 1.0 Hz stimulation. Carbamyl choline was applied to the bath solution for 5 min after recording at the control state. **D)** Proportions of SA node-type, atrial-type and ventricular type cells defined by automaticity and sensitivity to CCh (n=15).

Fig. 6

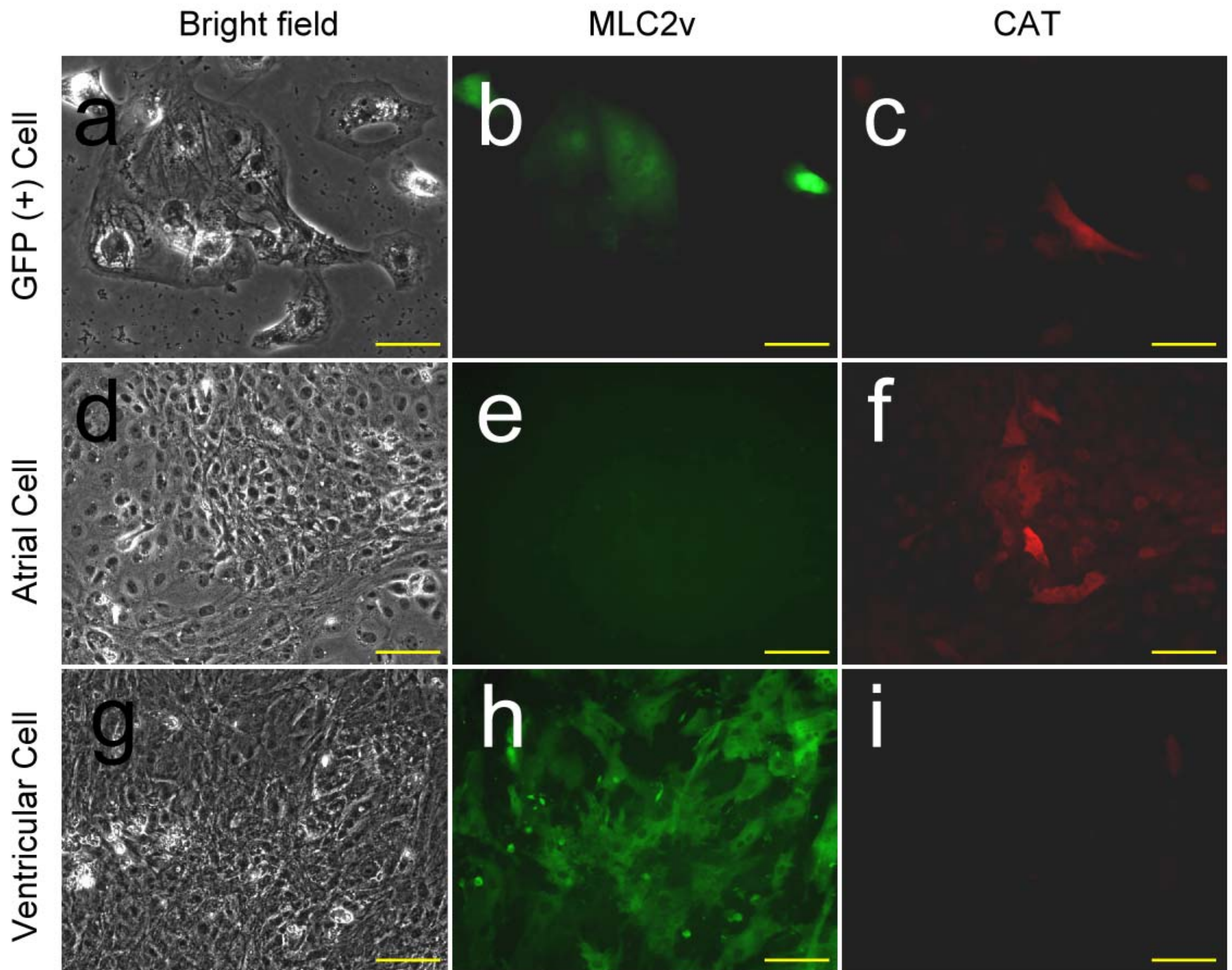

Figure 6. Expression of an atrial-specific promoter and a ventricular marker in Nkx2.5/GFP(+) cells. GFP(+) cells sorted from d8 EBs (**a–c**), atrial (**d–f**), and ventricular (**g–i**) cardiomyocytes prepared from E13.5 embryos were cultured for 2 days before transfection with SMyHC3-CAT. Cells were stained two days after transfection with anti-MLC2v (**b–h**) and anti-CAT (**c–f**) antibodies. Bars, 100 µm.

Fig. 7

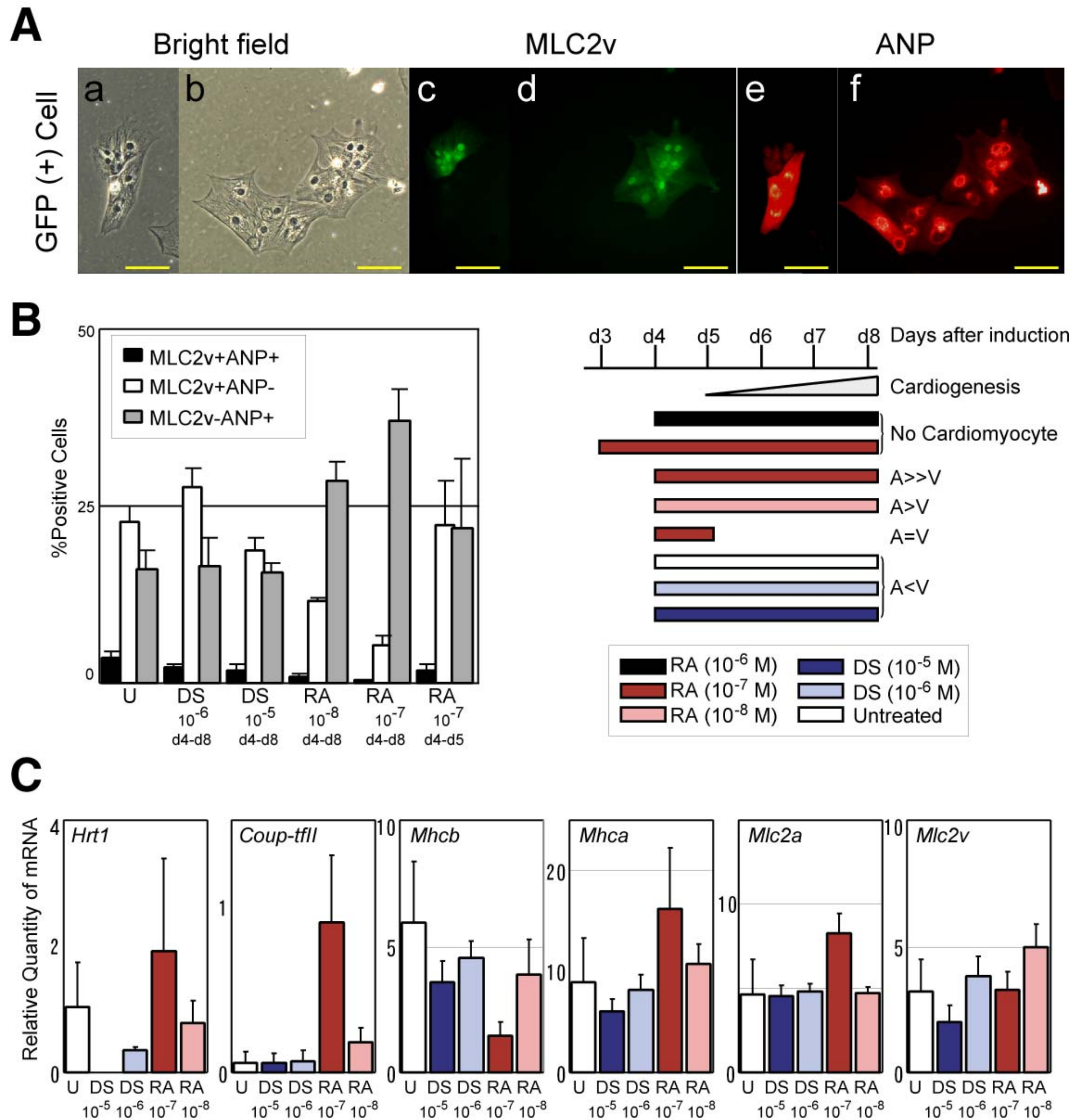

Figure 7. Effect of RA on cardiac diversification of EBs. A) Differentiation of Nkx2.5/GFP(+) cells into ANP- and/or MLC2v-positive cells. GFP(+) cells sorted from d8 EBs were cultured for 4 days and stained with anti-MLC2v (**c**, **d**) and anti-ANP (**e**, **f**) antibodies. Bars, 100 μm. **B)** Retinoic acid modifies the percentage of MLC2v(–)ANP(+) cells. Embryoid bodies were treated with RA or disulfiram (DS) at various concentrations for different periods as shown. Nkx2.5/GFP(+) cells were sorted at d8 and analyzed at d8+4. Values are given as mean ± SE from three independent experiments. The results are summarized at left. Untreated (U) EBs or 10^{-6} M DS-treated EBs gave rise to slightly more MLC2v(+) than MLC2v(–)ANP(+) cells (A<V). Treatment of EBs with 10^{-7} M RA (d4–d8) greatly increased the percentage of MLC2v(–) ANP(+) cells (A>>V). This effect was weaker with 10^{-8} M RA (A>V). Treatment with 10^{-7} M RA (d4–d5) or 10^{-5} M DS gave rise to MLC2v(+) cells as frequently as MLC2v(–)ANP(+) (A=V). Treatment with 10^{-6} M RA (d4–d8) or 10^{-7} M RA (d3–d8) inhibited generation of GFP(+) cells (data not shown). **C)** Retinoic acid increases the expression of atrial-specific genes. Embryoid bodies were treated with RA or DS from d4 to d8. Nkx2.5/GFP(+) cells were sorted at d8 for real-time RT-PCR. Relative amounts of the PCR products were determined as ratios to those of a control sample (unsorted d8 EBs). Values are given as mean ± SE from three independent experiments.