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Embryonic stem cells form an organized, functional cardiac conduction system in vitro

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White, Steven M., and William C. Claycomb. Embryonic stem cells form an organized, functional cardiac conduction system in vitro. Am J Physiol Heart Circ Physiol 288: H670–H679, 2005. First published October 7, 2004; doi:10.1152/ajpheart.00841.2004.—A functional pacemaking-conduction system is essential for maintaining normal cardiac function. However, no reproducible model system exists for studying the specialized cardiac pacemaking-conduction system in vitro. Although several molecular markers have been shown to delineate components of the cardiac conduction system in vivo, the functional characteristics of the cells expressing these markers remain unknown. The ability to accurately identify cells that function as cardiac pacemaking cells is crucial for being able to study their molecular phenotype. In differentiating murine embryonic stem cells, we demonstrate the development of an organized cardiac pacemaking-conduction system in vitro using the coexpression of the minK-lacZ transgene and the chicken GATA6 (cGATA6) enhancer. These markers identify clusters of pacemaking “nodes” that are functionally coupled with adjacent contracting regions. cGATA6-positive cell clusters spontaneously depolarize, emitting calcium signals to surrounding contracting regions. Physically separating cGATA6-positive cells from nearby contracting regions reduces the rate of spontaneous contraction or abolishes them altogether. cGATA6/minK copositive cells isolated from embryoid cells display characteristics of specialized pacemaking-conducting cardiac myocytes with regard to morphology, action potential waveform, and expression of a hyperpolarization-activated depolarizing current. Using the cGATA6 enhancer, we have isolated cells that exhibit electrophysiological and genetic properties of cardiac pacemaking myocytes. Using molecular markers, we have generated a novel model system that can be used to study the functional properties of an organized pacemaking-conducting system in vitro. Moreover, we have used a molecular marker to isolate a renewable population of cells that exhibit characteristics of cardiac pacemaking myocytes.

Embryoid bodies; pacemaking; electrophysiology; development; cardiac myocyte

SPECIALIZED CELLS of the cardiac pacemaking-conduction system initiate and synchronize atrial and ventricular contractions. Dysfunction of this intricate electrical system in the form of cardiac arrhythmias is a source of significant morbidity and mortality (9). Although several models have been used to study cardiac pacemaking and conducting cells in vitro, the molecular phenotype of these specialized myocytes remains uncharacterized (3, 4, 17, 20, 32, 39). Genetic markers have been shown to delineate components of the cardiac conduction system in vivo; however, the phenotype of cells expressing these markers remains unknown (36). Therefore, an in vitro model system in which differentiating pacemaking cells could be identified and studied as part of a functional tissue or as single cells would be invaluable for examining the molecular and cellular physiology of cardiac pacemaking myocytes.

Although several genetic markers have been used to identify components of the murine cardiac conduction system based on the location of their expression in the heart, it is unclear whether cells expressing these markers actually function as specialized cardiac pacemaking or conducting myocytes (18, 36, 38, 46). Kupershmidt et al. (30) used a gene-targeting approach to replace the coding region of minK, which encodes a β-subunit for the cardiac delayed rectifier potassium current (I_K), with lacZ. Expression of the minK-lacZ transgene has been detected as early as embryonic day 8.25 in mice and continues to be expressed in adults, where it is confined primarily to the more proximal cardiac conduction system [from the sinoatrial (SA) node through the interventricular bundles] (28, 30). There is additional evidence that minK is expressed in the Purkinje cells of the distal conduction system, indicating a more widespread distribution of expression than seen in the minK knockout mice (22, 49). Although adult minK knockout mice are more prone to atrial arrhythmias than wild-type animals, they exhibit no altered phenotype (30).

Another marker that has been used to identify more discrete components of the specialized cardiac conduction system is the proximal 1.5-kb promoter-enhancer region of the chicken GATA6 gene (cGATA6) (10). Using lacZ expression as a reporter of cGATA6 enhancer activity in transgenic mice, Davis et al. (10) demonstrated that cGATA6 is expressed in the cardiac primordia (before expression of minK and the formation of the heart tube) (10). Its expression in the adult mouse becomes restricted to regions of the heart that contain the SA and atrioventricular (AV) nodes as well as the AV bundle (bundle of His) (1, 10, 16). Subsequently, Davis et al. (10) created additional lines of transgenic mice in which the cGATA6 enhancer controlled expression of Cre recombinase. These mice were then mated with ROSA26 Cre reporter mice so that clonal populations of cGATA6-positive (lacZ positive) cells could be studied developmentally (10). Whereas the cGATA6 enhancer identified the same myocardial regions as the cGATA6-lacZ mice, additional discrete populations within the atria and ventricles were also marked (10). However, of all the reported markers of the cardiac conduction system, the cGATA6 enhancer exhibits the earliest and most restricted expression pattern (49).

Embryonic stem (ES) cells differentiated as embryoid bodies (EBs) have been used to develop numerous model systems for

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studying cardiac myocyte differentiation because they closely recapitulate developmental gene expression patterns in vitro (33, 47). When differentiated as clusters termed EBs, ES cells are capable of differentiating into any cell type in the body, including cells that constitute the specialized cardiac pacemaking-conduction system in vivo (33). We have used the cGATA6 and minK markers simultaneously in differentiating murine ES cells (EBs) to demonstrate the development of functional, organized cardiac pacemaking and conducting systems in vitro. Because the cGATA6 enhancer identifies cells that organize and function as pacemaking cells, we used this marker to isolate a population of cells that resemble nodal cardiac myocytes with regard to gene expression and electrophysiological properties.

**MATERIALS AND METHODS**

**Culture and genetic modifications of ES cells.** Murine J1 ES cells [kindly provided by the laboratory of Jaenisch (31)] were cultured as previously described (32). Briefly, undifferentiated ES cells were cultured in medium containing 10 U/ml leukemia inhibitory factor (Chemicon) in 10-cm² 0.1% gelatin-coated dishes and passaged every 48 h. Undifferentiated ES cells were transfected with linearized minK-lacZ targeting vector [kindly provided by Kupperschmidt et al. (30)] using LP2000 (Invitrogen) according to the manufacturer’s protocol. Transfected ES cells were cultured for 7 days in the presence of 300 µg/ml G418 (AG Scientific) and 20 µM ganciclovir (Sigma). The proximal 1.5-kb (−1.5/0.0) region of the chicken GATA6 promoter/enhancer (cGATA6) [kindly provided by Burch’s laboratory (10)] was inserted into the SalI and BambHI sites in the multiple cloning site of the promoterless enhanced red fluorescent protein (ERFP) vector (Clontech). After a second period of selection in the same concentrations of G418 and ganciclovir, the undifferentiated ES cells containing the minK-lacZ targeting vector were cotransfected with the linear cGATA6 −1.5/0.0-kb enhancer (cGATA6)-ERFP vector and the linear pcDNA3.1(+)-hygro vector (Invitrogen) using LP2000 and selected with 300 µg/ml hygromycin (AG Scientific) for 7 days.

**Differentiation of genetically modified ES cells as EBs.** Once undifferentiated ES cells containing both vector constructs (minK-lacZ and cGATA6-ERFP) were generated, they were differentiated using the “hanging-drop” method as previously described (32). Briefly, 20-µl drops containing 200 ES cells each in differentiation medium (growth medium without leukemia inhibitory factor) were placed on nontreated (tissue culture) petri dishes (Fisher), which were cultured for 3 days. After 3 days in suspension culture, ES cells were plated onto tissue culture dishes (10 cm²) coated with 0.1% gelatin where they continued to develop. On day 7 of differentiation, ES cells were dispersed into single cells by incubating the EBs in trypsin for 5 min followed by mechanical dissociation using a pipette. After 5 min of centrifugation (1,000 rpm), cells were suspended and plated onto 0.1% gelatin-coated dishes containing differentiation medium with 200 µg/ml G418 (Invitrogen). Each subsequent day, cells were washed multiple times with calcium/magnesium-free phosphate-buffered saline, and fresh medium containing 200 µg/ml G418 was added for a total of 7 days. After 7 days of selection, cells were cultured for 4–6 days in medium containing no G418. After this time, cells were passaged (using trypsin) and plated onto 0.1% gelatin-coated 35-mm dishes.

**Gene expression analysis.** Total RNA was isolated using the Qiagen RNeasy Kit and was reverse transcribed into cDNA using Superscript III (Invitrogen). Real-time PCR was performed with the ABI Prism 7000 System Detection Sequence (SDS) and software (Applied Biosystems) using SYBR Green (Applied Biosystems) as the detector. Primer sequences are provided in the supplemental table (see http://ajpheart.physiology.org/cgi/content/full/00841.2004/DC1). Gene expression data are shown as the cycle threshold (CT, the lower the number, the higher the gene expression).

**Electrophysiological recordings and data analysis.** On the day before the recording, EBs were dispersed and plated onto glass coverslips coated with 0.1% gelatin. On the following day, coverslips were transferred to a recording chamber mounted on an inverted microscope (Nikon Diaphot TMD) and superfused with extracellular recording solution. All experiments were conducted at room temperature (22–25°C). Whole cell voltage-clamp and current-clamp experiments were carried out using the standard gigaseal patch-clamp method (21). Recording electrodes were fabricated from 1.5-mm thin-walled borosilicate glass (no. 7052, Garner Glass; Clermont, CA) using a Flaming-Brown microelectrode puller (P-97, Sutter Imaging of EBs and single cells. Initially, EBs made from ES cells containing the minK-lacZ transgene were fixed and stained for β-galactosidase expression using the Stratagene β-galactosidase staining kit. For visualizing minK-positive cells, EBs were incubated the day of recording for 20 min at 37°C in medium containing 20 µM fluorescein digalactoside (FDG-C12) (Molecular Probes). Cells were then washed with phosphate-buffered saline and incubated for 1 h in differentiation medium before visualization. For fluorescence microscopy, cGATA6-positive cells (ERFP) were detected using a rhodamine filter, whereas minK-lacZ cells (green) were imaged using the FITC filter. All imaging (fluorescent and phase/contrast) was performed using a Nikon microscope along with MetaMorph Software (version 5.0 v, Advanced Scientific).

**Calcium imaging.** For imaging calcium fluorescence, EBs were loaded with 10 µM of the membrane-permeant acetoxyethyl ester derivative of the fluorescent calcium indicator Calcium Green (Molecular Probes) for 30 min at 37°C. The EBs were then washed and incubated for 1 h in differentiation medium before images were acquired. Cells were imaged on a Diaphot TMD (Nikon) inverted microscope using the ×20 objective. Images were captured using a digital camera (Roper Scientific) and analyzed with MetaMorph Software.

**Separation of cGATA6-positive cell clusters from spontaneously contracting regions.** Spontaneous contractions were counted by direct visualization under the microscope. To separate cGATA6-positive cells from spontaneously contracting regions, a scalpel fixed to a micromanipulator (Eppendorf) was lowered into the EBs between cGATA6-positive and contracting regions and pulled across the EBs. Spontaneous contractions were counted again after the separation.

**Isolation and culture of cGATA6-positive cells.** To create the selection vector, pcDNA3.1(+)neo was digested with BclI and religated. This resulted in the repositioning of the neomycin resistance gene (neo) immediately downstream of the multiple cloning site. The proximal 1.5-kb (−1.5/0.0) region of the multiple cloning site of the modified pcDNA3.1(+)neo vector (with the BclI fragment removed). In this newly formed vector, neo expression is controlled by the cGATA6 promoter. To enrich the population of cells containing the cGATA6-neo vector, linearized cGATA6-neo was cotransfected with linear pcDNA3.1(−)-hygro using LP2000 (Invitrogen). Transfected, undifferentiated ES cells were cultured for 7 days in ES growth medium containing 200 µg/ml hygromycin (AG Scientific) before being used for experiments.

J1 ES cells containing the cGATA6-neo vector were differentiated using a suspension protocol as previously described (27). Briefly, 3 × 10⁶ ES cells were placed into a nontreated (tissue culture) petri dish (Fisher), which contained differentiation medium, and cultured for 3 days. After 3 days in suspension culture, ES cells were plated onto tissue culture dishes (10 cm²) coated with 0.1% gelatin where they continued to develop. On day 7 of differentiation, ES cells were dispersed into single cells by incubating the EBs in trypsin for 5 min followed by mechanical dissociation using a pipette. After 5 min of centrifugation (1,000 rpm), cells were suspended and plated onto 0.1% gelatin-coated dishes containing differentiation medium with 200 µg/ml G418 (Invitrogen). Each subsequent day, cells were washed multiple times with calcium/magnesium-free phosphate-buffered saline, and fresh medium containing 200 µg/ml G418 was added for a total of 7 days. After 7 days of selection, cells were cultured for 4–6 days in medium containing no G418. After this time, cells were passaged (using trypsin) and plated onto 0.1% gelatin-coated 35-mm dishes.
Instruments; Novato, CA) and heat polished before use. Each of the pipettes had a tip resistance of 2–5 MΩ when filled with internal solution. Recordings were performed using an Axoclamp 2B patch-clamp amplifier (Axon Instruments; Union City, CA). Data were filtered at 2 kHz, and data were acquired using Clampex 8 software (Axon Instruments). Cells were identified as minK- or cGATA6-positive using either the FITC or the rhodamine dyes, respectively, during fluorescence microscopy. Action potentials were recorded from spontaneously depolarizing cells or were elicited by stimulation with 2.5-ms, 200-pA square-wave currents. Recordings were made ~1 min following establishment of the whole cell configuration. For current-clamp recordings, the extracellular bath solution contained (in mM) 140 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 glucose, and 5 HEPES at pH 7.4 (with NaOH). The intracellular pipette solution contained (in mM) 140 CsCl, 2 MgCl₂, 10 EGTA, 5 4-aminopyridine, and 10 HEPES at pH 7.3 (with KOH).

Voltage-gated calcium currents (I_{Ca}) were elicited in the whole cell configuration by holding cells at −80 mV for 500 ms and then applying 10-mV steps (500 ms) from −80 to +60 mV and returning to the holding potential of −80 mV. When I_{Ca} was recorded, the extracellular solution contained (in mM) 140 tetraethylammonium-chloride (TEA-Cl), 10 CaCl₂, 1 MgCl₂, 10 glucose, 5 4-aminopyridine, and 10 HEPES at pH 7.4 (with TEA-OH). The intracellular pipette solution contained (in mM) 140 CsCl, 2 MgCl₂, 10 EGTA, 5 Mg-ATP, and 10 HEPES at pH 7.3 (with CsOH).

Hyperpolarization-activated currents (I_{f}) were elicited in the whole cell configuration by holding cells at −40 mV for 50 ms followed by 10-mV steps (2 s) to −130 mV and returned to −40 mV (50 ms) after each step. After the I_{f} was recorded, cells were superfused with extracellular solution containing 10 mM cesium chloride. When I_{f} was measured, the extracellular solution was the same as that used for measuring action potentials (current clamp) except for the addition of 2 mM BaCl₂ and 0.5 mM 4-aminopyridine. The intracellular pipette solution contained (in mM) 10 NaCl, 130 K-aspartate, 2 Na₂ATP, 0.1 Na₃GTP, 2 MgCl₂, 1 EGTA, 10 HEPES, 10 tetraethylammonium chloride, at pH 7.3 (with KOH).

**RESULTS**

cGATA6- and minK-positive cell clusters form organized pacemaking-contracting units in EBs. Both the minK-lacZ transgene and the cGATA6 enhancer mark regions of the cardiac pacemaking and conducting system in vivo. We first examined the expression of the minK-lacZ transgene in EBs to determine whether cells expressing this marker were present in a localized or diffuse pattern. Approximately 100 EBs generated from ES cells containing the minK-lacZ targeting vector were fixed and stained for β-galactosidase expression on day 16 of differentiation. All of the EBs stained positively for expression of the minK-lacZ transgene. Cells expressing the minK-lacZ transgene were present in small clusters within EBs as shown in Fig. 1A.

To determine whether the minK-lacZ transgene and the cGATA6 enhancer identify the same cells, we generated EBs from ES cells containing both the minK-lacZ transgene and ERFP under transcriptional control of the cGATA6 enhancer. By incubating EBs with the fluorescent β-galactosidase substrate FDG, we are able to detect expression of both vectors simultaneously in live cells using fluorescent microscopy. In Fig. 1, B and C, minK expression is represented by green, whereas ERFP expression (red) marks cGATA6-positive cells within EBs. The cGATA6 enhancer is first expressed in these EBs at approximately day 5 of differentiation, whereas minK expression is not detected until day 8 (when spontaneous contractions are first observed). Although minK is expressed in discrete cell clusters, expression of cGATA6 is restricted to a subpopulation of minK-positive cells, recapitulating their expression patterns in vivo (10, 30). In fact, cells expressing minK extend from cGATA6-positive clusters and merge with nearby spontaneously contracting regions (Fig. 1, B–D and supplemental movie 1; http://ajpheart.physiology.org/cgi/
cGATA6-positive cell clusters are always separated from nearby spontaneously contracting regions. Although there is some heterogeneity with regard to the size and relative location of these cell clusters (as depicted in Fig. 1, B–C), the organization with respect to contracting regions is consistent. We examined the expression of minK and cGATA6 in more than 30 EBs, which were generated as hanging drops so that the developmental conditions for each EB were as standardized as possible. Approximately 90% of the EBs examined contained spontaneously contracting regions and expressed the minK-lacZ transgene and the cGATA6 enhancer in the arrangement depicted in Fig. 1, B and C. The cellular arrangement depicted by these two molecular markers is strikingly similar to that of the cardiac conduction system in vivo, where a pacemaking node of cells (cGATA6/minK positive) is bridged with working (contracting) myocardium by specialized, rapidly conducting myocytes (minK positive).

cGATA6-positive cells clusters initiate rhythmic calcium oscillations. Fluorescent calcium-sensitive dyes are useful for demonstrating functional coupling as well as excitation propagation in vitro (48). To determine whether cGATA6 actually identifies pacemaking or “nodal” structures, EBs were incubated with a calcium-sensitive fluorescent dye (Calcium Green) and imaged before and after the onset of spontaneous contractions. Calcium-dependent depolarizations generated by nodal (pacemaking) myocytes are propagated throughout the heart to control myocardial contractions (43). The cGATA6-positive clusters display a higher basal calcium concentration than surrounding cells. By day 6 of differentiation, before the onset of visible spontaneous contractions, rhythmic, spontaneous calcium oscillations are observed in cGATA6-positive cell clusters (Fig. 2, A–C, and supplemental movie 2; http://ajp-heart.physiology.org/cgi/content/full/00841.2004/DC1). After the onset of spontaneous contractions (day 10 of differentiation), calcium oscillations are observed emitting from cGATA6-positive clusters, extending into contractile regions (Fig. 2, D–F and supplemental movie 3). The rhythmic calcium oscillations emitted from cGATA6-positive cell clusters into nearby contracting regions persists at days 10 and 20 (last time point measured, data not shown) of differentiation.

cGATA6-positive cell clusters control the rate of contraction in EBs. To determine whether cGATA6-positive clusters are functionally coupled with contracting regions, we performed experiments in which we physically separated the cGATA6-positive cells from nearby contracting regions. After cGATA6-positive cell clusters were identified near spontaneously contracting regions, a scalpel blade attached to a micromanipulator was lowered between the two regions and quickly pulled through the EB so that there was complete separation of the noncontracting, cGATA6-positive region from the nearby spontaneously contracting region without any discernable tissue destruction. In this set of experiments, we found that physical coupling between the two cell populations affects contraction rate (Fig. 3, A and B). Physically separating these two regions either reduces the spontaneous contraction rate from 56.5 ± 10 to 17 ± 7.5 contractions/min (n = 11, P < 0.01) or causes cessation of spontaneous contractions (Fig. 3C). To control for the effects of tissue destruction within the EBs, cuts were made on the opposite side of spontaneously contracting regions, away from the cGATA6 clusters. None of the control cuts caused a change in contraction rate.

cGATA6-minK copositive cells display characteristics of cardiac pacemaking myocytes. The cell clusters that function as pacemaking nodes in this model system express both the

![Image of calcium sparks emitted from cGATA6-positive cell clusters before the onset of spontaneous contractions.](http://ajp-heart.physiology.org/cgi/content/full/00841.2004/DC1)
cGATA6 enhancer as well as the minK-lacZ. Therefore, we performed experiments to examine the electrophysiological characteristics of cGATA6-minK copositive cells. Spontaneously contracting EBs (differentiation day 15) expressing both markers were dissociated into single cells and used for patch-clamp experiments. Isolated cells expressing both cGATA6 and minK exhibited two different morphologies. Cells displaying a “nodal” morphology (Fig. 4A) exhibit action potential waveforms characteristic of cardiac nodal cells with a prominent diastolic (phase 4) depolarization (Fig. 4C). Other cGATA6-minK cells with morphologies similar to an adult, contracting myocytes (Fig. 4B) display atrial-like action poten-

Fig. 3. Separating spontaneously contracting regions from cGATA6-positive cell clusters reduces spontaneous contraction frequency. A: representative region of an EB shows the spatial relationship between a cGATA6-positive cell cluster (red) and an adjacent spontaneously contracting region. B: an image of the same region (A) following physical separation of the cGATA6-positive cluster and the spontaneously contracting region, which resulted in the cessation of contractions. C: separating the cGATA6-positive cells from adjacent spontaneously contracting regions caused either a marked reduction or cessation of spontaneous contractions (*P < 0.05, n = 11).
tial waveforms (Fig. 4D). Approximately 10% of the cGATA6-minK copositive cells exhibit nodal characteristics with respect to their morphology and action potential waveform.

An important characteristic of a cardiac pacemaking myocyte is the expression of an inward, hyperpolarization-activated cation current (i.e., $I_f$). This current was initially termed the “funny” current because it carried an inward, depolarizing current at negative (resting) membrane potentials (13). Although most cardiac myocytes exhibit an $I_f$, cells in the SA and AV nodes express a much larger $I_f$ current (different current densities and kinetics), which causes them to spontaneously depolarize at a faster rate than other myocytes (11). With the use of the whole cell configuration of the patch-clamp technique, $I_f$ was elicited using hyperpolarizing steps in 10-mV increments from −40 mV to −130 mV from a holding potential of −40 mV. Remarkably, all GATA6-minK copositive cells exhibit a large cesium-sensitive $I_f$ (Fig. 4, E and F). The current (in pA) was normalized to cell size, which is represented by membrane capacitance (in pF). In 16 cells, the maximal $I_f$ density (at a membrane potential of −130 mV) was 58.4 ± 5.7 pA/pF. The reversal potential of this current was at −20 mV (data not shown), and it was significantly inhibited by 10 mM cesium chloride (8.4 ± 4.6 pA/pF, $P < 0.001$). Together, these properties demonstrate that this current is $I_f$ (12).

**Isolated cGATA6-positive cells exhibit electrophysiological properties and gene expression profile characteristic of cardiac pacemaking cells.** To isolate a pure population of cGATA6-positive cells, we created an ES cell population containing a vector in which expression of the neomycin resistance gene (neo) was controlled by the cGATA6 enhancer. This allowed us to select for a population of cells that were resistant to G418 (neomycin), indicating that the cGATA6 enhancer was active. On day 7 of differentiation, EBs made from ES cells containing the cGATA6-neo vector were dispersed into single cells and plated onto dishes with medium containing 200 μg/ml G418. Relative to the number of cells before selection, very few cells survived drug selection (Fig. 5A). Approximately 10–14 days after the completion of selection, colonies of cells with a similar morphology could be observed (Fig. 5B).

Cardiac pacemaking cells exhibit characteristic electrophysiological properties, including expression of $I_{Ca}$, carried primar...
cGATA6-neo cells also express a large Ion channels and connexins 26–28; and * cycle thresholds into the following: ***** for cGATA6-neo cells express higher than Nkx2.5 and other genes that indicate a unique profile of these cells. Using real-time RT-PCR, we examined the expression of transcription factors (Table 1), which indicate a unique profile of these cells. In addition to expressing characteristic cardiac-specific markers, cGATA6-positive cells also express other genes that indicate these cells represent a primitive cardiac myocyte population. Whereas the selected cells express an anticipated high level of GATA6, they also express significant levels of the cardiac transcription factors Nkx2.5 and GATA4 (much more than MEF2c). Because cells of the cardiac conduction system are thought to display a partial skeletal muscle transcriptional profile (45), we examined MEF2D, which is expressed in the cGATA6-neo cells. However, the most highly expressed transcription factor of the ones that we examined is Msx2 (64-fold higher than Nkx2.5), which is expressed in the atrioventricular nodal region in chickens (8). Whereas Mx2 is involved in many processes such as limb development, it also has a significant role in tissue regeneration (7, 29, 37). The T-box transcription factors Tbx2 and Tbx3 are considered markers of primitive cardiac myocytes (35) with Tbx3 marking regions of the proximal conduction system in the adult heart (24). In the cGATA6-neo cells, Tbx3 is expressed at a high level (34-fold higher than Tbx2 and 9-fold higher than Tbx5).

cGATA6-neo cells express several cardiac structural and sarcomeric proteins. Of all the genes we examined (except for GAPDH), the most highly expressed was α-skeletal actin, which is expressed 12-fold higher than α-cardiac actin. Although β-myosin heavy chain (β-MHC) is expressed at a significant level, these cells express fivefold more α-MHC than β-MHC. With regard to the myosin light chain (MLC) isoforms, the atrial MLC-2a isoform is expressed at a high level while expression of the ventricular MLC-2v isoform cannot be detected (up to 40 PCR cycles). Additionally, the cGATA6-neo cells express a high level of desmin, which is a marker of striated myocytes, and a very low level of atrial natriuretic factor (ANF), which is considered to be an early marker of chamber (atrial or ventricular) working myocardium (25).

We examined the expression of 15 genes encoding ion channel subunits and connexins. Of the three connexin isoforms found in cardiac myocytes, cGATA6-neo cells express connexin 43 105-fold more than connexin 45, which is expressed 11-fold more than connexin 40. Expression of the T-type calcium channel subunit gene Cav1.3 is eightfold higher than the L-type calcium channel subunit Cav1.2. cGATA6-neo cells express significant levels of genes encoding the cardiac ryanodine receptor (Ryr2), the sodium–calcium exchanger (NCX1), the cardiac voltage-gated sodium channel (Scn5A), and minK. Expression of Kir3.1, which encodes the acetylcholine-gated potassium channel (KACH), is 42-fold higher than the expression of Kir2.1, which encodes the inward rectifier potassium channel. With regard to the gene isoforms encoding the hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels, which are involved in cardiac pacemaking, the HCN2 isoform is the most highly expressed. The relative expression of the four isoforms is HCN 2 ≥ HCN 3 > HCN 4 ≥ HCN 1 (low level of expression).

### DISCUSSION

All of the genetic markers of the cardiac conduction system currently available have been designated as such based on the location of their expression. None of the putative markers of the cardiac conduction system have been shown to identify cells that actually function as specialized cardiac pacemaking or conducting cells. Kupershmidt et al. (30) demonstrated that expression of the minK-lacZ transgene was colocalized with connexin 40 in cells of the interventricular bundle branches. Several markers have been shown to be localized to regions of the specialized cardiac conduction system based on patterns of β-galactosidase staining and action potential propagation (optical mapping) (30, 38). However, no one has isolated single cells expressing any of these markers to determine whether these cells display characteristics of cardiac pacemaking or conducting cells. We have used differentiating ES cells (as EBs) containing two markers of the cardiac conduction system to demonstrate the development of an organized pacemaking-

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**Table 1. Gene expression in cGATA6-neo cells**

<table>
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<tr>
<th>Reference</th>
<th>Relative Expression</th>
<th>Cycle Threshold</th>
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<td>2. Neo</td>
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<td>4. GATA6</td>
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<td>17. MLC-2v</td>
<td>&gt;&gt;&gt;</td>
<td>40</td>
</tr>
<tr>
<td>18. Desmin</td>
<td>★★</td>
<td>21.1</td>
</tr>
<tr>
<td>19. ANF</td>
<td>★★★</td>
<td>30.5</td>
</tr>
<tr>
<td>Ion channels and connexins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20. Connexin 40</td>
<td>★</td>
<td>28.9</td>
</tr>
<tr>
<td>21. Connexin 43</td>
<td>★★</td>
<td>18.7</td>
</tr>
<tr>
<td>22. Connexin 45</td>
<td>★★</td>
<td>25.4</td>
</tr>
<tr>
<td>23. Cav1.2</td>
<td>★★</td>
<td>27.4</td>
</tr>
<tr>
<td>24. Cav1.3</td>
<td>★★</td>
<td>24.4</td>
</tr>
<tr>
<td>25. Ryr2</td>
<td>★</td>
<td>28.9</td>
</tr>
<tr>
<td>26. Ncx1</td>
<td>★</td>
<td>28.0</td>
</tr>
<tr>
<td>27. Scn5a</td>
<td>★★</td>
<td>26.0</td>
</tr>
<tr>
<td>28. Kir2.1</td>
<td>★</td>
<td>28.7</td>
</tr>
<tr>
<td>29. KACH</td>
<td>★★</td>
<td>23.3</td>
</tr>
<tr>
<td>30. minK</td>
<td>★★</td>
<td>27.7</td>
</tr>
<tr>
<td>31. HCN-1</td>
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</tr>
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<td>32. HCN-2</td>
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<td>33. HCN-3</td>
<td>★★</td>
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</tr>
<tr>
<td>34. HCN-4</td>
<td>★</td>
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</table>

Average (n = 2 isolations) cycle thresholds are shown (lower numbers indicate a higher level of gene expression). Genes were grouped according to cycle thresholds into the following: ***** < 20; ★★★ 20–24; ★★ 24–26; ** 26–28; and * > 28. See text for definitions of abbreviations.
contracting system in vitro as well as to describe genetic and electrophysiological characteristics of cells identified by these markers.

The murine heart tube forms at approximately day 8 of embryonic development at which time slow, peristaltic contractions occur. Between 8 and 10 days of differentiation, rhythmic spontaneous contractions can be observed in murine EBs, indicating the presence of cardiac myocytes. In addition to the presence of contracting cardiac myocytes, specialized pacemaking and conducting cells are also present in developing EBs (32). The spontaneously contracting regions observed in differentiating EBs contain cells with electrophysiological characteristics of atrial, ventricular, and pacemaking-conducting myocytes (50). Cells with “nodal-like” action potentials have been found in single-cell dispersions of EBs (32), but there is no way to know before electrophysiological experiments are performed which cells in or near a contracting region might be specialized pacemaking or conducting cells. Whereas the development of electrical activity has been studied in EBs plated using multielectrode arrays, this method provides only field potentials in the regions of the surface electrodes and is not always capable of identifying the specific cells that initiate or conduct the action potentials (3, 20, 26). Although developmental gene expression patterns in EBs mimic the patterns observed in vivo, the number and location of various types of cells within EBs is considered to be random (23, 33). The presence of cardiac myocytes in developing EBs is classically confirmed by observing spontaneous contractions. Several groups have used microdissection to isolate spontaneously contracting regions and demonstrated the presence of cells resembling atrial, ventricular, and nodal myocytes based on their electrophysiological properties (14, 32, 52). However, the organization of these cell types within intact EBs has never been fully appreciated due to a lack of appropriate molecular markers.

Using two markers of the specialized cardiac pacemaker and conducting system simultaneously, we have demonstrated organized pacemaking-conducting-contracting units within EBs. Both the minK-lacZ transgene and the cGATA6 enhancer identify components of the proximal (SA node to the interven- tricular bundles) cardiac conduction system (10, 30). Although their expression patterns in vivo seem to partially overlap, the cGATA6 enhancer appears to identify more discrete populations of cells. In EBs, cGATA6-positive cell clusters could be identified at day 5 of differentiation, whereas minK-positive cells were observed at day 8, which is when spontaneous contractions are first observed in the EBs. cGATA6 cells are mostly present in fairly compact clusters, while cells expressing the minK-lacZ transgene are more diffuse. Approximately 90% of cGATA6-positive cells are also minK positive, and they seem to represent a subpopulation of the minK-positive cells.

In EBs, every spontaneously contracting region observed was associated with a cGATA6- and minK-positive cell cluster. Approximately 10% of the EBs observed did not contain cGATA6- and minK-positive cell clusters. These same EBs also contained no spontaneously contracting regions. Therefore, the colocalization of cGATA6- and minK-positive cell clusters is necessary for the development of spontaneous contractions in EBs. The cGATA6-positive cells were always separated from the spontaneously contracting regions by a “bridging” minK-positive region, which is similar to nodal organization in vivo with the presence of transitional myocytes (2). Although there is some heterogeneity in the arrangement of the marked cell clusters within the EBs (depicted in Fig. 2, A–C), they are always organized so that the cGATA6-positive cluster is connected to a nearby spontaneously contracting region by minK-positive cells.

Because the organization of the cells identified by the cGATA6 enhancer and the minK-lacZ transgene with respect to contracting regions are reminiscent of primitive cardiac conduction system, we designed experiments to test the functionality of such a system. Nodal cardiac myocytes spontaneously depolarize to generate electrical impulses that are propagated to “working” myocytes causing contractions (18). These spontaneous depolarizations are caused primarily by calcium influx (34). When EBs were incubated with a calcium-sensitive dye to image calcium fluxes, we were able to detect rhythmic, spontaneous calcium oscillations being emitted from cGATA6-positive cell clusters into the surrounding contracting regions. These spontaneous calcium oscillations were first observed in the cGATA6-positive clusters on day 6 of differentiation, before the onset of spontaneous contractions, and continued after the onset of spontaneous contractions. This indicates that nodal myocytes develop functional pacemaking properties before the onset of visible contractions. The ultimate test for the presence of functional pacemaking cells is to uncouple the pacemaking cells from the contracting cells and observe a change in contraction rate. By demonstrating that the rate of spontaneous contractions in EBs is dependent on physical coupling with cell clusters marked by the cGATA6 enhancer, we have shown that these cells function as pacemaking cells in a multicellular environment.

Knowing that cells identified by the cGATA6 enhancer and the minK-lacZ transgene function as specialized pacemaking-conducting cells in vitro, we isolated single cells from the EBs to determine their electrophysiological properties. Based on action potential waveforms, cells can generally be classified as nodal (SA or AV), atrial, distal conducting (His-Purkinje), or ventricular. The differences in the action potential waveform shapes from various cardiac myocytes are due to the relative levels of expression of specific ionic currents (11). One common characteristic of cells of the specialized cardiac pacemaking and conducting system is the relatively high expression of \( I_f \) (42). Although cGATA6/minK copositive cells are heterogeneous with respect to their action potential waveforms (exhibiting both nodal and atrial), all of these cells express a significant cesium-sensitive \( I_f \).

Although the electrophysiological properties of cardiac pacemaking cells are fairly well established, the molecular phenotype of these cells remains unknown. We selected a population of cells that express the neomycin resistant gene (neo) under control of the cGATA6 enhancer to analyze the expression of a panel of cardiac genes encoding transcription factors, structural and sarcomeric proteins, ion channels, and gap junction proteins. Some of the results were expected for nodal, pacemaking cells, whereas others provide novel insight into the regulation of these unique cells. The cGATA6-neo cells express significant levels of \( Nkx2.5, GATA-4, GATA-6, \alpha-MHC, \) and \( \beta-MHC, \) and \( desmin, \) which confirm their identity as cardiac myocytes (15, 41). These cells express a high level of MLC-2a (an atrial-specific myosin light chain isoform) and...
no detectable MLC-2v (a ventricular-specific isoform). Because the SA and AV nodes are located in the right atrium, the MLC-2a expression supports their nodal phenotype.

Of the nine transcription factors analyzed, the two most highly expressed are Msx2 and GATA6. In the chicken heart, expression of Msx2, which is found in regenerating tissues, has been found only in portions of the specialized conduction system (7, 8, 29, 37). The fact that Msx2 is expressed at an extremely high level in the cGATA6-neo cells supports the idea that these are more primitive (less differentiated) myocytes. In Xenopus, increased expression of GATA6 delays myocardial development by maintaining cardiac myocytes in a primitive state and preventing the progression into differentiated atrial or ventricular myocytes (6). Another transcription factor expressed at a high level in the cGATA6-neo cells is Tbx3. Moorman and Christoffels (19, 25, 35) have shown that Tbx2 and Tbx3 bind Nkx2.5 and repress the transcription of ANF and connexin 43, which they consider to be markers of more differentiated (chamber) myocytes. Both ANF and connexin 43 are expressed at very low levels in cGATA6-neo cells. Importantly, Tbx3 becomes restricted to the SA and AV nodes and an internodal tract in adult mice, indicating that this transcription factor could also serve as a potential marker to identify cardiac pacemaking myocytes (24).

Although the expression of transcription factors and sarcomeric proteins correlate with what is known about cardiac nodal cells, the expression patterns of some of the membrane proteins is unexpected. Of the gap junction proteins expressed in the heart, connexin 45 is the isof orm most highly expressed in the SA and AV nodes (44). Although connexin 45 is expressed at a moderate level by the cGATA6-neo cells, connexin 43 (the most predominant cardiac isoform) is expressed at a much higher level. With regard to the HCN channel isoforms, which are responsible for If, HCN-2, and HCN-3 are more highly expressed in the cGATA6-neo cells than HCN-1 and HCN-4, which are the predominant nodal isoforms in vivo (40). Although these discrepancies in gene expression patterns between the cGATA6-neo cells and nodal myocytes in vivo can be attributed to the culture conditions as isolated cells, this is the first description of the gene expression profile exhibited by cGATA6-positive myocytes.

In summary, we demonstrate that coexpression of the two molecular markers cGATA6 and minK in differentiating ES cells reveal a functional cardiac conduction system in vitro. cGATA6-positive cell clusters act as pacemaking units that functionally couple with nearby contracting regions of EBs. This reproducible EB model system and the pacemaking-conducting cells identified by these markers will be an invaluable tool for studying the fundamental biology of cardiac pacemaking cells, designing targeted pharmaceutical agents, and developing novel cellular and tissue-engineered therapies. By isolating ES cell-derived cardiac myocytes with a pacemaking phenotype, we have generated a reproducible cell model system that can be used to probe the differentiation and molecular regulation of cardiac pacemaking cells.

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REFERENCES


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ES CELLS FORM A FUNCTIONAL CARDIAC CONDUCTION SYSTEM IN VITRO