

Transition in Cardiac Contractile Sensitivity to Calcium during the In Vitro Differentiation of Mouse Embryonic Stem Cells

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Abstract. Mouse embryonic stem (ES) cells differentiate in vitro into a variety of cell types including spontaneously contracting cardiac myocytes. We have utilized the ES cell differentiation culture system to study the development of the cardiac contractile apparatus in vitro. Difficulties associated with the cellular and developmental heterogeneity of this system have been overcome by establishing attached cultures of differentiating ES cells, and by the micro-dissection of the contracting cardiac myocytes from culture. The time of onset and duration of continuous contractile activity of the individual contracting myocytes was determined by daily visual inspection of the cultures. A functional assay was used to directly measure force production in ES cell-derived cardiac myocyte preparations. The forces produced during spontaneous contractions in the membrane intact preparation, and during activation by Ca^{2+} subsequent to chemical permeabilization of the surface membranes were determined in the same preparation. Results showed a transition in contractile sensitivity to Ca^{2+} in ES cell-derived cardiac myocytes

during development in vitro. Cardiac preparations isolated from culture following the initiation of spontaneous contractile activity showed marked sensitivity of the contractile apparatus to activation by Ca^{2+} . However, the Ca^{2+} sensitivity of tension development was significantly decreased in preparations isolated from culture following prolonged continuous contractile activity in vitro. The alteration in Ca^{2+} sensitivity obtained in vitro paralleled that observed during murine cardiac myocyte development in vivo. This provides functional evidence that ES cell-derived cardiac myocytes recapitulate cardiogenesis in vitro. Alterations in Ca^{2+} sensitivity could be important in optimizing the cardiac contractile response to variations in the myoplasmic Ca^{2+} transient during embryogenesis. The potential to stably transfect ES cells with cardiac regulatory genes, together with the availability of a functional assay using control and genetically modified ES cell-derived cardiac myocytes, will permit determination of the functional significance of altered cardiac gene expression during cardiogenesis in vitro.

THE heart is one of the first organs to develop during embryogenesis, with spontaneous contractions of the heart-tube appearing at about embryonic day 8 in the mouse (Sissman, 1970). Progress toward understanding the cellular and molecular mechanisms of cardiac gene expression and function during the early stages of cardiogenesis has been hindered due to difficulties in establishing an in vitro model system of cardiac myogenesis. Recent evidence indicates that the in vitro differentiation of mouse embryonic stem (ES)¹ cells provides a new model system of murine cardiac myogenesis (Doetschman et al., 1985; Robbins et al., 1992). ES cells, derived from the inner cell mass of the mouse preimplantation blastocyst, can either proliferate

indefinitely in an undifferentiated, totipotent state, or can be induced to differentiate by altering the culture conditions.

ES cell differentiation in vitro is somewhat programmed but highly disorganized, and a variety of embryonic structures are formed. A particularly striking feature of differentiating cultures of ES cells is the development of spontaneously contracting cardiac myocytes. Electron micrographs obtained from thin sections of the ES cell-derived contracting myocytes reveal myofilaments arranged in sarcomeres and intercalated discs, a structural marker of the cardiac phenotype (Doetschman et al., 1985). Cardiac gene expression has been demonstrated in this in vitro system including, cardiac troponins C and T (Pajek et al., 1993), phospholamban (Ganim, et al., 1992), cardiac alkali, and regulatory myosin light chains (Miller-Hance et al., 1993), and the α and β cardiac myosin heavy chain (MHC) genes (Sanchez et al., 1991). Moreover, there is evidence that the expression of several genes, including cardiac MHC (Sanchez et al., 1991) and tropomyosin (Muthuchamy et al., 1993) demonstrate a developmental pattern of expression that is consistent with

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1. *Abbreviations used in this paper:* CIRC, Ca^{2+} -induced release of Ca^{2+} ; ES, embryonic stem; LIF, leukemia inhibitory factor; MHC, myosin heavy chain; pc, postcoitum; TrIT, troponin T.

that observed during murine cardiac development in vivo. These findings provide evidence that the ES cell system may represent a model to study developmental aspects of cardiac gene expression (Sanchez et al., 1991; Muthuchamy et al., 1993). Electrophysiological studies have demonstrated voltage-dependent L-type Ca^{2+} channels and α_1 , β_1 , and β_2 adrenergic receptors in the spontaneously contracting myocytes in ES cell differentiation cultures (Wobus et al., 1991). Taken together these studies identify the spontaneously contracting myocytes in differentiation culture as cardiac myocytes, and further suggest that the ES cell differentiation culture system provides a model to study mammalian cardiac myocyte development in vitro.

A significant limitation in the usefulness of the ES cell differentiation culture system as a model of murine cardiogenesis resides in the inherent cellular and developmental complexity of this in vitro system (Doetschman et al., 1985; Robbins et al., 1992). Besides the formation of embryonic cardiac myocytes, which make up ~5% of cells in culture, other differentiated cell types and embryonic structures, including nerve cells, melanocytes, blood islands, and visceral yolk sac, are apparent during the in vitro differentiation of ES cells (Doetschman et al., 1985; Robbins et al., 1992). The relatively small number of cardiac myocytes in ES cell differentiation cultures presents a significant experimental obstacle toward performing detailed studies of the cellular and molecular mechanisms of cardiac gene activation and function.

In the present study this experimental difficulty has been overcome by using microdissection techniques to isolate spontaneously contracting cardiac myocytes from neighboring nonmuscle cells in attached cultures of differentiating ES cells. The attached culture format further allowed for the direct visual inspection of the differentiation cultures to determine the onset and duration of contractile activity of the myocytes prior to their isolation from culture. A functional assay was used to directly determine the spontaneous twitch contractile properties of the intact cardiac myocyte preparation. Following measurement of spontaneous twitch contractions, the cardiac myocyte preparation was treated chemically to permeabilize the surface membranes. This permitted direct control of the composition of the intracellular solution bathing the otherwise intact contractile apparatus. Free Ca^{2+} was controlled in the range 10^{-9} – $10^{-4.5}$ M in order to construct the steady-state isometric tension– Ca^{2+} relationship of the ES cell-derived cardiac myocytes. Results showed that early in development in vitro, the ES cell-derived cardiac myocytes demonstrated marked Ca^{2+} sensitivity of tension development; however, with continued development of the contracting myocytes in culture there was a significant decrease in the sensitivity of the contractile apparatus to activation by Ca^{2+} . This transition in the Ca^{2+} sensitivity of contraction paralleled that observed during murine cardiac myogenesis in vivo. This provides functional evidence in support of the hypothesis that ES cell-derived cardiac myocytes recapitulate murine cardiogenesis in vitro.

Materials and Methods

Differentiation Cultures of Mouse Embryonic Stem Cells

ES-D3 cells (Doetschman et al., 1985) were maintained in an undifferentiated

state by culturing on a monolayer of irradiated mouse embryonic fibroblasts (MEF; 4500 rad). Undifferentiated ES cell cultures were maintained at high densities on MEF feeder cells in DME supplemented with 15% fetal calf serum, 0.1 mM β -mercaptoethanol, and 2% leukemia inhibitory factor (LIF)-containing medium. LIF-containing medium was collected from confluent cultures of CHO cells stably transfected with a LIF expression plasmid (8/24 720 LIF-D[0.1]; Genetics Institute, Cambridge, MA). LIF has been demonstrated to inhibit the differentiation of cultured ES cells (Williams et al., 1988).

Attached cultures of differentiating ES cells were initiated by removal of the ES cells from feeders and the formation of ES cell aggregates in hanging drop cultures. The ES cells were removed from the feeder layer by trypsinization, centrifuged, and resuspended in differentiation media consisting of 20% FCS, 50 units/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin sulfate, and 0.1 mM β -mercaptoethanol in DME. 30- μl drops containing 300 ES cells were placed onto the undersurface of 100-mm tissue culture dish lids which were then placed over PBS. After 2 d, embryoid bodies, having formed as an aggregate of ES cells suspended in each hanging drop, were transferred to a suspension culture on a 100-mm bacterial dish and were cultured an additional 3 d before plating onto gelatin-coated glass cover slips (Fisher 22-mm circles No. 1) in six-well tissue culture dishes. The cardiac myocytes were identified by daily inspection of the cultures using light microscopy to document the number of days of continuous contractile activity of each contracting myocyte in differentiation culture. Another benefit of the attached ES cell cultures was that spontaneous contractile activity of cardiac myocytes was maintained in some instances for over 6 wk, making it feasible to explore alterations in contractile function during cardiac myocyte development in vitro. Day of differentiation culture refers to the time elapsed from the dissociation of the ES cells from MEF feeders.

Fetal and Adult Murine Cardiac Myocyte Preparations and Experimental Apparatus

Cardiac muscle was removed from adult female mice and from mouse fetuses (day 17 ± 1 postcoitum [pc]) and the ventricles were dissected from the atria and minced in relaxing solution (see below). Ventricular tissue was placed in a Waring blender and homogenized for ~6 s at slow speed. The resulting cell suspension was then centrifuged at 120 g for 1 min and the pellet was resuspended in 5–10 ml relaxing solution. The mechanical isolation procedure appears to permeabilize the myocytes since exposure of the cells to 0.2% Triton X-100 for up to 30 min does not alter maximum tension or the tension–pCa relation in these myocytes (Sweitzer and Moss, 1990; Metzger et al., 1993). However, since brief exposure to Triton X-100 improves resolution of the sarcomere pattern, cells were exposed to Triton X-100 for about 15 s before collecting mechanical data.

The attachment procedure was adapted from Sweitzer and Moss (1990) and involved pulling (Model P-30; Sutter Instr. Co., Novato, CA) borosilicate glass micropipettes to tip diameters of ~5 μm . The microelectrodes were inserted into an output tube of a force transducer (sensitivity, 5 μg ; 1–99% response time, 1 ms; resonant frequency, ~0.6 kHz, Model 403A; Cambridge Technology Inc., Watertown, MA) and an optical scanner (galvanometer) (Model 6350; Cambridge Technology Inc.). Sarcomere length was set at 2.20 μm by adjusting the overall length of the preparation. Tension and length signals were recorded, digitized, and stored on magnetic disks for subsequent analysis using an oscilloscope (Model 310; Nicolet Instr. Corp., Madison, WI).

An experimental chamber was constructed for attaching cardiac myocytes to the recording apparatus and for the activation and relaxation of the isolated preparation. The chamber, similar to those used previously (Metzger et al., 1989; Sweitzer and Moss, 1990) measured in cm, $15 \times 15 \times 1.5$, and was constructed out of stainless steel and aluminum. Four troughs were milled out of the stainless steel section (volume ~200 μl) to provide wells for attaching, relaxing, and activating the cardiac myocyte. The chamber was positioned on an anti-vibration table to isolate the experimental apparatus from building noise. The temperature of the experimental chamber was controlled to 15°C using thermoelectric modules coupled to a recirculating water bath heat-sink.

Micro-dissection and Attachment Procedure for Cardiac Myocytes Derived from Differentiating ES Cells

Micro-dissection of ES cell cardiac myocytes involved glass micropipettes for use as a dissecting tool to carefully separate the contracting myocytes from surrounding nonmuscle cells in differentiation culture (Fig. 1 B). The

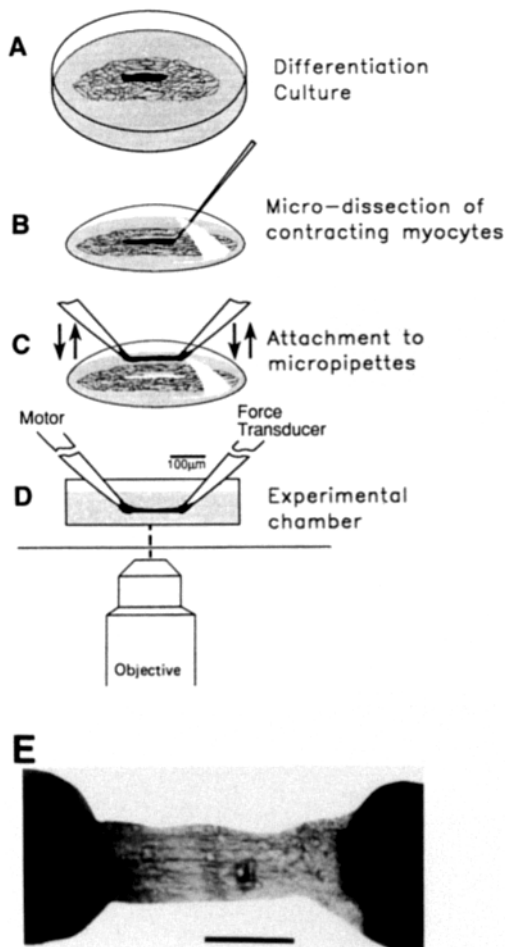


Figure 1. Micro-dissection and attachment procedure for ES cell-derived cardiac myocyte preparations. The mechanically isolated cardiac myocyte preparation is attached to a force transducer and motor using glass micropipettes which are coated with a polymeric isocyanate resin (A–C; see Materials and Methods). The length of the preparation is adjusted to the value determined before micro-dissection (D). E is a photomicrograph of an ES cell-derived cardiac myocyte preparation attached to the glass micropipettes. Bar, 150 μm .

isolated preparation was attached to a force transducer and motor (see above) using glass micropipettes (OD $\sim 5 \mu\text{m}$) which are coated with a polymeric isocyanate resin (Flexible Products, Inc., Joliet, IL) (Fig. 1 C). The end-to-end length of the preparation was adjusted to the value determined before micro-dissection from differentiation culture (Fig. 1 D). The attachment procedure and setting of preparation length were done on the stage of an inverted microscope. In some experiments the average sarcomere length of the isolated preparation was determined by analysis of electron-micrographs obtained from thin sections (see Results; Fig. 2).

ES cell-derived cardiac myocytes were chemically permeabilized by exposing the isolated preparation to relaxing solution (see below) which contained 0.2 to 0.5% Triton X-100. Test contractions were performed using maximum Ca^{2+} -activating solution (pCa 4.5; where $\text{pCa} = -\log[\text{Ca}^{2+}]$). Chemical permeabilization was continued until there was no further increase in isometric tension development upon activation at pCa 4.5.

Activating and Relaxing Solutions

Relaxing and activating solutions contained (in mM): EGTA, 7; free Mg^{2+} , 1; MgATP, 4; creatine phosphate, 14.5; imidazole, 20; and sufficient KCl to yield a total ionic strength of 180 mM. Solution pH was adjusted to 7.0 with KOH. Relaxing solution had a pCa of 9.0 while the pCa

of the solution for maximal activation was 4.5. The computer program of A. Fabiato (1988) was used to calculate the final concentrations of each metal, ligand, and metal–ligand complex, employing the stability constants listed by Godt and Lindley (1982). The apparent stability constant for Ca^{2+} -EGTA was corrected for ionic strength, pH, and experimental temperature (Fabiato, 1988). Experimental temperature was set at 15°C as the preparation viability decreases and sarcomere length nonuniformly increases more rapidly at higher temperatures.

Transmission Electron Microscopy

ES cell cardiac myocyte preparations were fixed using Karnovsky's fixation solution (3% glutaraldehyde and 3% formaldehyde in 0.1 M cacodylate buffer, pH 7.4) for 2 h at 4°C . Micropipettes were used to dissect the borders but not the ends of the preparation before fixation. Thus during fixation, the preparation remained attached by its ends to the gelatin-coated coverslip to preserve the overall end-to-end length. Following fixation, the ends of the preparation were gently teased away from the cover slip and the preparation was placed overnight in 0.1 M cacodylate rinsing buffer with 7.5% saccharose (4°C , pH 7.4). Postfixation was accomplished by immersion in 1% buffered osmium tetroxide for 1 h at room temperature. The preparation was then dehydrated in a graded ethanol series and propylene oxide, and incubated in a 1:3 mixture of propylene oxide and epoxy resin. After 12 h the ES cell-derived cardiac myocyte preparation was embedded in epoxy resin and polymerized for 3 d at 45°C and 1 d at 60°C . 50-nm-thick serial sections were cut with a diamond knife on an ultramicrotome (model MT-5000; Sorvall, Dupont Co., Newtown, CT). Ribbons of sections were mounted on copper grids, stained with aqueous uranyl acetate and lead citrate, and examined with a transmission electron microscope (model CM-10; Philips Electronic Instrs. Co., Mahwah, NJ) at 60 kV.

Curve Fitting and Statistics

To derive values for the Hill coefficient (n) and the midpoint (termed pCa_{50} or K) from the tension–pCa relationship, data were fit using the Marquardt-Levenberg nonlinear least squares fitting algorithm using the Hill equation in the form: $P_r = [\text{Ca}^{2+}]^n / (K^n + [\text{Ca}^{2+}]^n)$ where P_r is tension as a fraction of maximum tension obtained at pCa 4.5 (P_0), K is the $[\text{Ca}^{2+}]$ that yields one-half maximum tension (e.g., pCa_{50}), and n is the Hill coefficient. Statistical analysis was by analysis of variance using the Bonferroni multiple comparison t test, $P < 0.05$.

Results

Differentiation cultures were initiated by removing ES-D3 cells from a monolayer of irradiated mouse embryonic fibroblasts and forming controlled ES cell aggregates in hanging drop cultures. After five days in suspension cultures, ES cell aggregates were plated onto gelatin coated glass coverslips. Once plated, the ES cell aggregate adheres to the coverslip surface and spreads out. Cell proliferation and differentiation continues, resulting in a number of distinct lineages, including spontaneously contracting cardiac myocytes. The establishment of attached cultures of differentiating ES cells was important as it permitted, by visual inspection of the cultures, the determination of the onset and duration of spontaneous contractile activity in specific cardiac myocytes as they developed in different regions of the culture over time. The attached culture format also made it feasible to isolate cardiac myocyte preparations with known contractile history (e.g., onset of contraction, duration of continuous contractile activity) from ES cell differentiation cultures by micro-dissection (Fig. 1). The earliest detected onset of spontaneous contractile activity was 8–10 d following removal of the ES cells from MEF feeders, similar to that obtained earlier (Robbins et al., 1992). The development of spontaneous contractions in culture is consistent with the early onset of spontaneous contraction of the primitive heart-tube during murine embryogenesis in vivo (Sissman, 1970). The duration of continuous spontaneous contractile activity

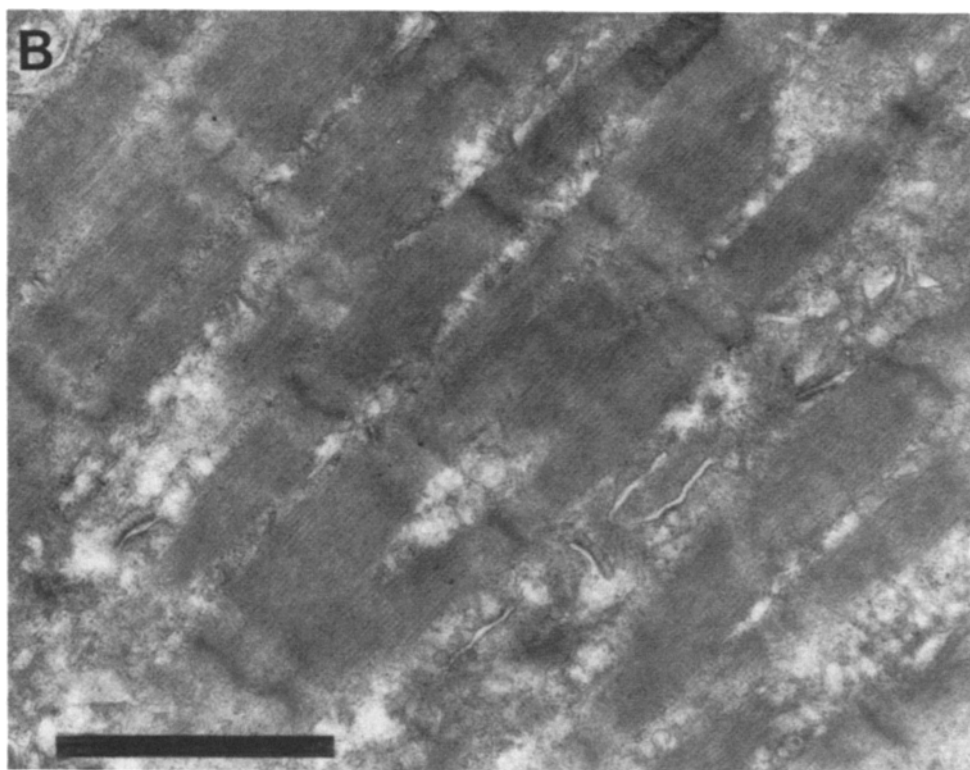


Figure 2. (A) Low magnification photomicrograph of an ES cell-derived cardiac myocyte preparation following microdissection from differentiation culture. Numerous parallel aligned cardiac myocytes are evident in this longitudinal thin section of the preparation. Toluidine blue stain. Bar, 25 μm . (B) Transmission electron photomicrograph demonstrating sarcomeric structure of an ES cell-derived cardiac myocyte. The average sarcomere length for this preparation is 2.01 μm . Bar, 2.0 μm .

in ES cell-derived cardiac myocytes was variable, averaging about one week, but in some cases continuing for greater than six weeks. This extended period of continuous contractile activity made it possible to explore developmental transitions in contractile phenotype in ES cell-derived cardiac myocytes. In summary, the attached ES cell culture format

overcomes the cellular and developmental complexities of the ES cell culture system by enabling the direct determination of the time of onset and duration of contractile activity of individual cardiac myocytes via daily inspection of the cultures using light microscopy. Further, the attached culture format made possible the micro-dissection of the individual

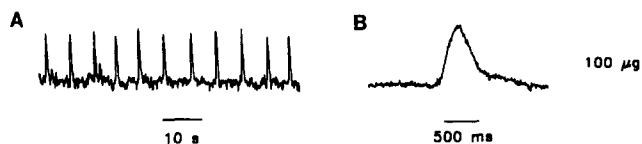


Figure 3. Slow (A) and fast (B) time-base records of spontaneous twitch tension obtained from an isolated ES cell-derived cardiac myocyte preparation. Peak twitch tension of this preparation was 75 μ g.

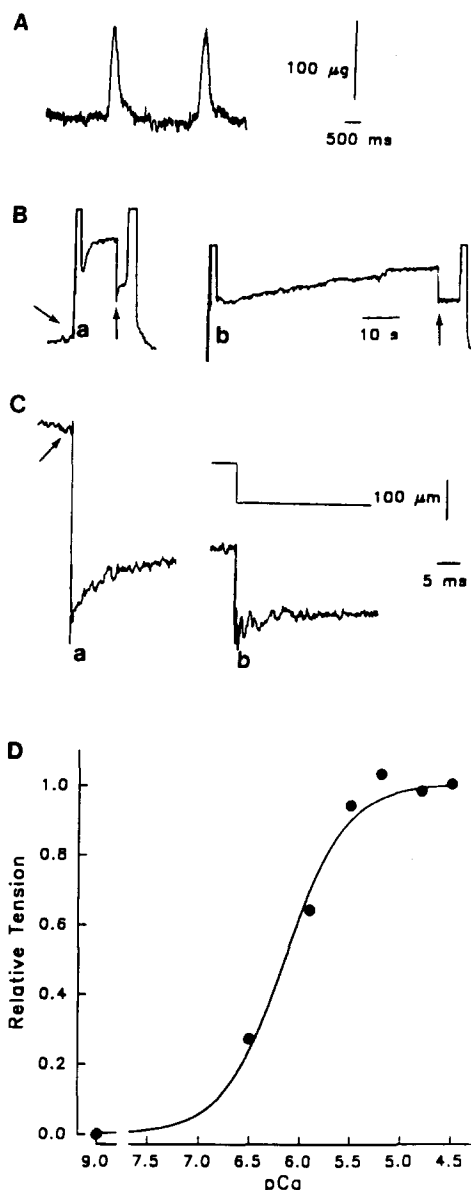
cardiac myocyte preparations with documented contractile histories from differentiation culture.

Determination of Tension Generation in Isolated ES Cell-derived Cardiac Myocytes

To determine the tension generation of cardiac myocytes derived from ES cell differentiation culture, the contracting myocytes were micro-dissected and then attached to a capacitance-type force transducer and a galvanometer to provide fine control of the length of the preparation. This made it possible to directly determine the spontaneous twitch tension of the isolated preparation (Fig. 3). In some preparations, spontaneous contractions were maintained for over 5 h without significant alteration in the twitch tension amplitude or kinetics. This demonstrates that the isolated preparation was highly viable even following its removal from the cell-to-cell contacts present in differentiation culture.

To determine the sensitivity of the contractile apparatus to activation by Ca^{2+} the ES cell-derived cardiac myocyte preparation was chemically permeabilized with the nonionic detergent Triton X-100. This permitted access and control of the ionic contents of the intracellular compartment. Contraction was initiated by direct application of free Ca^{2+} which was buffered by EGTA, and the relationship between free $[\text{Ca}^{2+}]$ and steady-state isometric tension was determined. Fig. 4 shows isometric tension records obtained before and after chemical permeabilization of an ES cell-derived cardiac myocyte preparation. After permeabilization, maximum isometric contraction was initiated by exposure of the preparation to pCa 4.5-activating solution. The preparation was then relaxed and subsequently activated by solutions in which the pCa was varied between 9.0 and 4.5 to obtain the tension-pCa relationship (Fig. 4). In this preparation the pCa₅₀ value, defined as the $-\log[\text{Ca}^{2+}]$ where tension is one-half maximal, was 6.15, and the Hill coefficient, which characterizes the steepness of the sigmoidal tension-pCa relationship, was 1.5.

Figure 4. Records of isometric tension in a spontaneously contracting and subsequently chemically permeabilized cardiac myocyte preparation derived from the in vitro differentiation of ES cells. This preparation had been contracting 6 d before isolation from culture. (A) Spontaneous isometric twitch contractions. Contraction rate of 20 beats per min, 15°C. The vertical calibration bar is for all tension records shown in A-C. Preparation length was 360 μ m. (B) Slow time-base records of isometric tension obtained following permeabilization of the preparation shown in A with Triton X-100. (a) Maximum Ca^{2+} -activated isometric tension. At first arrow the



preparation was transferred from relaxing solution to maximum Ca^{2+} -activating solution (pCa 4.5; upward tension transients are due to the cardiac myocyte preparation crossing the solution-air interface). At the second arrow the length was rapidly released to obtain a tension baseline and the preparation was relaxed and length was re-extended. In b, the pCa of the activating solution was set at 6.5. The arrow denotes the time-point of step release in length. (C) Fast time-base records of isometric tension. These records were used to determine the active tension generated at each pCa since the tension baseline in the slow time-base tension records shown above is variable and due to small changes in surface tension surrounding the micropipettes upon transferring the solution bathing the preparation. In a the preparation was activated at pCa 4.5 and steady isometric tension developed, at which point the cardiac myocyte preparation was rapidly shortened (arrow) to obtain a tension baseline (record is from part B[a], above). Record b shows a fast time-base tension record (noisy trace) obtained at pCa 9.0 to determine resting tension which was then subtracted from the total tension value (i.e., a) to obtain the active tension generated by the preparation. Smooth trace in b is record of motor position. (D) Tension-pCa relationship in the ES cell-derived cardiac myocyte preparation shown in A-C. Tension values are normalized to the maximum tension value obtained in the preparation at pCa 4.5.

Table 1. Summary of the Dimensions, Sarcomere Length, and Maximum Ca^{2+} -activated Isometric Tension for the ES Cell-derived Cardiac Myocyte Preparation and the Fetal and Adult Cardiac Myocytes

	Length	Width	Sarcomere length	P_o
	μm	μm	μm	μg
ES cell-derived cardiac preparation	382 ± 64	66 ± 11	2.14 ± 0.09	69 ± 17
Fetal cardiac myocyte	84 ± 11	9 ± 1	2.20 ± 0.00	121 ± 56
Adult cardiac myocyte	73 ± 3	30 ± 9	2.20 ± 0.00	313 ± 187

Values are mean \pm SEM ($n = 4-8$). For the ES cell-derived cardiac preparation the length and width measurements were obtained from measurements of the multicellular preparation following isolation from differentiation culture. Sarcomere length of the ES cell-derived myocytes was determined using electron photomicrographs. There were $\sim 30-100$ cardiac myocytes in each ES cell-derived cardiac preparation studied (Fig. 2 A). The length of the ES cell-derived preparation was measured as the distance between the two micropipette attachment points. The length of fetal and adult cardiac myocytes refers to the distance between the two attachment points, not the overall end-to-end length of the isolated myocyte. Thus the overall end-to-end length of the fetal and adult cardiac myocytes was longer than the length calculated between the pipette attachment points. Sarcomere length was set at $2.2 \mu\text{m}$ by adjusting the overall length of the isolated myocyte. Fetal and adult cardiac myocyte width measurements are in general agreement with earlier studies (Zak, 1973). P_o is the maximum steady-state isometric tension obtained in chemically permeabilized preparations at pCa 4.5.

The ES cell-derived cardiac myocyte preparation does not have an overt striated phenotype that can be detected by conventional bright field microscopy. Due to the importance of sarcomere length on cardiac contractile performance (Allen and Kentish, 1985) it was important to determine the sarcomere length of the preparation used in this study. For sarcomere length determination the cardiac myocyte preparations were fixed in culture at the same overall end-to-end length used in the contractile studies (see Materials and Methods). The preparations were sectioned, stained, and sarcomere length determined directly from photomicrographs using transmission electron microscopy (Fig. 2). The average sarcomere length obtained from eight different preparations was $2.14 \pm 0.09 \mu\text{m}$, a value in good agreement with the sarcomere length required for maximal active tension development in mammalian cardiac myocytes (Table I).

Contractile Sensitivity to Ca^{2+} in Fetal Cardiac Myocytes Developing In Vivo

For comparison of the ES cell tension-pCa data, the Ca^{2+} sensitivity of tension was determined in cardiac myocytes isolated from the ventricles of fetal mice. Representative slow time-base records of isometric tension obtained from a fetal (d 18 pc) cardiac myocyte are shown in Fig. 5.

Comparison of the meaned tension-pCa relationships between cardiac myocytes developing in vivo and in vitro is shown in Fig. 6. The responsivity of the contractile apparatus to activation by Ca^{2+} in ES cell-derived cardiac myocytes was, in general, comparable to that of the fetal cardiac myocytes. The $p\text{Ca}_{50}$ values were 6.00 ± 0.07 and 5.95 ± 0.05 and Hill coefficients were 1.28 ± 0.18 and 2.03 ± 0.02 for cardiac myocytes developing in vitro and in vivo, respectively. Although the Hill coefficient was lower in the ES-

derived cardiac myocytes compared to the fetal cardiac myocytes, differences between these two groups did not reach statistical significance ($P = 0.07$).

However, in comparing the tension-pCa relationships obtained from ES cell-derived and fetal cardiac myocytes it was evident that at low concentrations of Ca^{2+} , steady-state isometric tension values were greater in the ES cell-derived cardiac myocytes (Fig. 6). The threshold for Ca^{2+} activation of the contractile apparatus was determined in each preparation by Hill plot transformation of the tension-pCa data and calculating the pCa at which $\log [P_r/(1-P_r)] = -2.5$ (in which P_r is the fraction of tension obtained by pCa 4.5) as detailed previously (Metzger and Moss, 1987). In this analysis, the threshold for Ca^{2+} activation of contraction occurred at significantly higher pCa (e.g., lower $[\text{Ca}^{2+}]$) in ES cell-derived cardiac myocytes compared to fetal cardiac myocytes (Fig. 6).

Transition in Contractile Sensitivity to Ca^{2+} during Cardiac Development In Vivo and In Vitro

A possible explanation for differences in the threshold Ca^{2+} for activation of contraction between the ES cell-derived and fetal cardiac myocytes is that the ES myocytes could represent an earlier stage of cardiac development than the fetal myocytes. We therefore tested for developmental alterations in Ca^{2+} sensitivity of tension in ES cell-derived cardiac myocytes developing in vitro and in murine cardiac myocytes developing in vivo. Results showed that the Ca^{2+} sensitivity of tension decreases significantly during murine cardiac myocyte development (Figs. 5, 7, and 8). In fetal cardiac myocytes the threshold for Ca^{2+} activation of contraction was pCa 6.83 ± 0.07 (Fig. 6). In comparison, the threshold for Ca^{2+} activation of contraction in adult cardiac myocytes was pCa 6.30 ± 0.05 , a value significantly lower than the threshold Ca^{2+} obtained in fetal cardiac myocytes (Fig. 8). In addition, the $p\text{Ca}_{50}$ value decreased and the Hill coefficient increased such that the tension-pCa relationship was shifted to the right and became steeper in adult compared to fetal cardiac myocytes (Figs. 7 and 8).

The transition in the Ca^{2+} sensitivity of tension obtained during cardiac development in vivo is also apparent in the ES cell-derived cardiac myocytes developing in vitro. Comparisons in contractile sensitivity to Ca^{2+} activation were made between ES cell-derived cardiac myocytes that had been continuously contracting for less than one week in culture (e.g., Fig. 4) with those that had been continuously contracting for over five weeks in culture. To perform these experiments it was necessary to determine directly, by daily visual inspection of the differentiation cultures, the time of onset and the duration of continuous contractile activity of each of the spontaneously contracting cardiac myocyte preparations in differentiation culture. Results showed that the tension-pCa relationship was shifted to the right and became steeper in cardiac myocyte preparations isolated following prolonged contractile activity compared to myocytes isolated early upon initiation of contractility in vitro. The $p\text{Ca}_{50}$ and threshold for Ca^{2+} activation of contraction were significantly decreased, and the Hill coefficient was significantly increased in the long-term compared to the short-term contracting myocyte preparations (Figs. 7 and 8). It is important to note that in these studies the solutions used to activate and relax the contractile apparatus of the isolated myocytes had

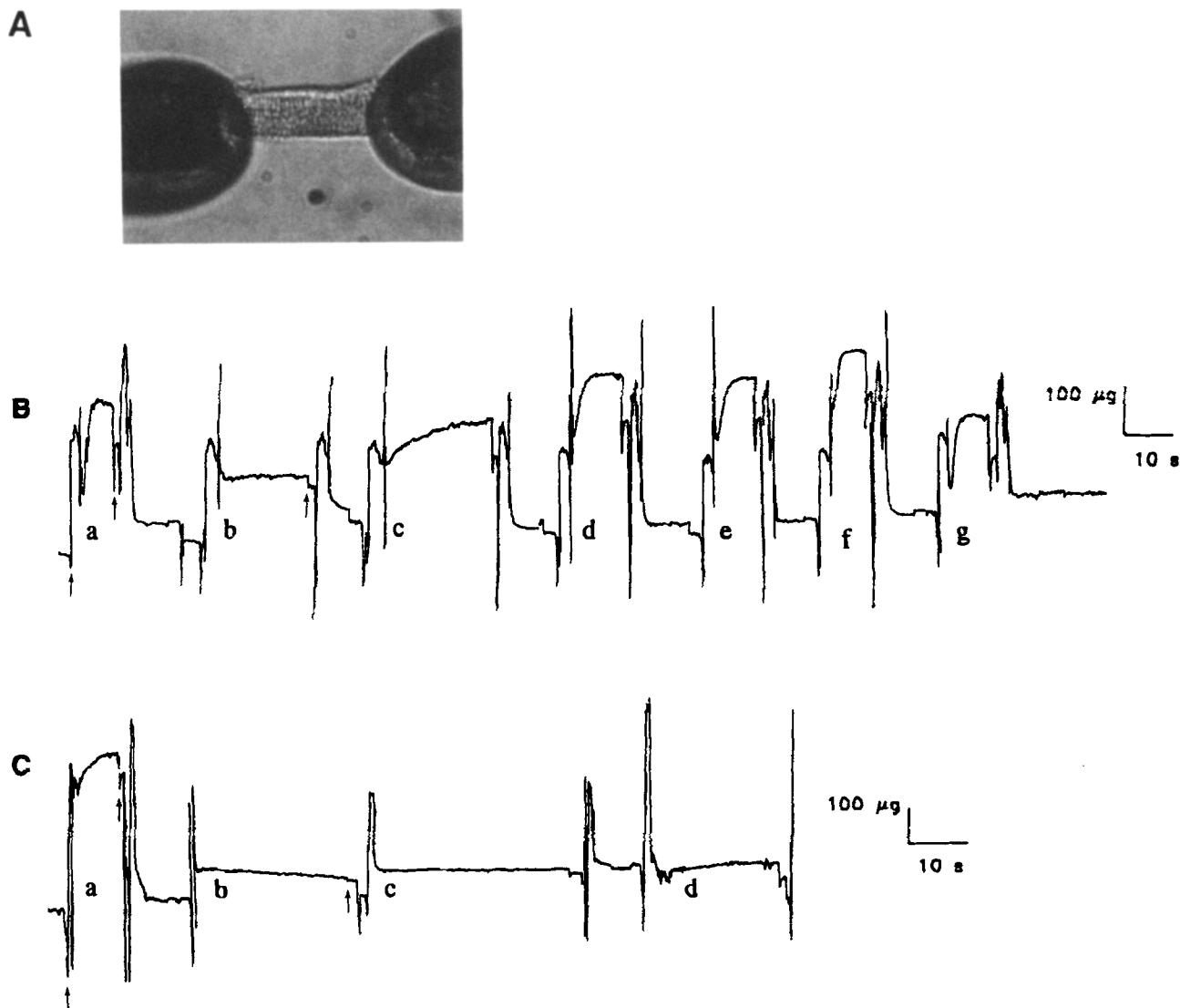


Figure 5. Records of isometric tension obtained from adult and fetal permeabilized cardiac myocytes during development in vivo. (A) Photomicrograph of an adult cardiac myocyte attached to micropipettes. End-to-end length (from attachment point to attachment point) was 68 μ m and sarcomere length was 2.2 μ m. (B) Slow time-base records of Ca^{2+} -activated isometric tension in a permeabilized adult cardiac myocyte. (a) At the first arrow the pCa of the solution bathing the cardiac myocyte was changed from 9.0 to 4.5. The upward tension transients are due to the cardiac myocyte preparation crossing the solution-air interface. At the second arrow length was rapidly released to obtain a tension baseline and the pCa was changed to 9.0 and length was re-extended. (b–g) The pCa of the activating solution was set at 6.0, 5.9, 5.7, 5.5, 5.0, and 4.5, respectively. The arrow denotes time-point of step release in length. (C) Slow time-base records of Ca^{2+} -activated isometric tension from a fetal cardiac myocyte (day 18 pc). (a) At the first arrow the pCa of the solution bathing the cardiac myocyte was changed from 9.0 to 4.5. At the second arrow myocyte length was rapidly released to obtain a tension baseline and the pCa was changed to 9.0 and length was re-extended. (b–d) The pCa of the activating solution was set at 6.4, 6.2, and 6.0, respectively. Sarcomere length was set at 2.2 μ m.

a pH of 7.0, and contained no added inorganic phosphate. It was important to control both pH and P_i because these factors have been shown to directly alter the Ca^{2+} sensitivity of tension development in cardiac muscle (Metzger et al., 1993). Thus, the basis of the observed shift in the tension–pCa relationship appears to be due to altered myofilament regulatory protein structure during myocyte development in vitro.

In summary, the transition in the shape and position of the tension–pCa relationship in ES cell-derived cardiac myocyte preparations during development in vitro was generally comparable with the alterations in Ca^{2+} sensitivity of tension

observed during murine cardiac development in vivo (Figs. 7 and 8).

Discussion

This study provides the first biophysical characterization of contractility in cardiac myocytes isolated from differentiation cultures of mouse embryonic stem cells. Analysis of the Ca^{2+} sensitivity of contraction in ES cell-derived cardiac myocytes demonstrated the following: (a) early during development in vitro the Ca^{2+} sensitivity of contraction of the isolated ES cell-derived cardiac myocytes was marked. The

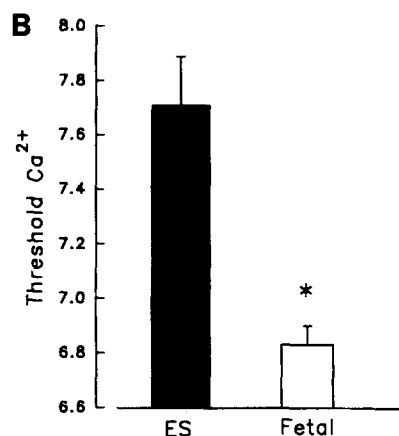
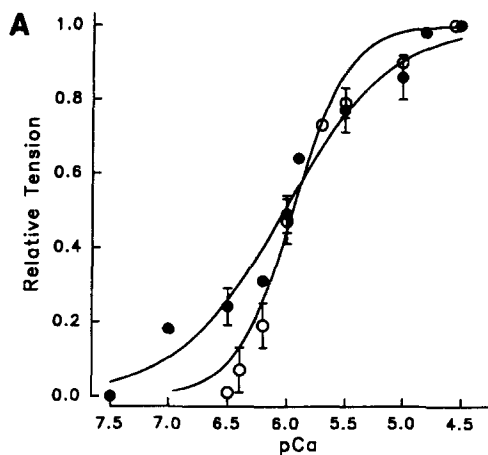


Figure 6. Tension-pCa relationships (*A*) and threshold for Ca^{2+} activation of tension (*B*) in fetal (open circles, open bar) and ES cell-derived (filled circles, filled bar) cardiac myocytes. For the tension-pCa plots the best fit line is shown (see Materials and Methods). Threshold for Ca^{2+} activation of tension was obtained from Hill transformation of the tension-pCa data by determining the pCa in each preparation at which $\log [P_r/(1-P_r)] = -2.5$ (see Materials and Methods). Values are mean \pm SEM, $n = 4-7$. Asterisk indicates significance, $P < 0.05$. In some instances error bars were covered by the symbol.

threshold Ca^{2+} concentration required for activation of contraction was significantly lower in the ES cell-derived cardiac myocytes compared to fetal cardiac myocytes; (*b*) during continued development in vitro there was a reduction in the sensitivity of the contractile apparatus to activation by Ca^{2+} ; and (*c*) the transition in the Ca^{2+} sensitivity of contraction obtained during cardiac development in vitro paralleled that obtained during murine cardiac development in vivo. These findings are consistent with the idea that cardiac myocytes derived from differentiation cultures of ES cells recapitulate murine cardiac myogenesis in vitro.

In general, the magnitude of the alteration in the shape and position of the tension-pCa relationship during cardiogenesis in vitro and in vivo was comparable. The pCa_{50} decreased by 0.31 and 0.35 pCa units, and the Hill coefficient increased by 1.26 and 1.87 Hill units during cardiac develop-

ment in vitro and in vivo, respectively. However, the magnitude of the developmental transition in the threshold for Ca^{2+} activation of tension generation was substantially greater in cardiac myocytes derived from the ES cell system (0.96 pCa units) compared to the murine system (0.53 pCa units). This suggests that a broader developmental range may be obtained in the ES cell system compared to the murine system, at least in terms of these functional data. Overall, these results demonstrate that the tension-pCa relationship is shifted to the right and becomes steeper during cardiogenesis in vitro and in vivo. The molecular basis of altered Ca^{2+} sensitivity of the contractile apparatus and its possible functional significance during embryogenesis is considered below.

This study is the first to show a developmental transition in the Ca^{2+} sensitivity of contraction during cardiac myo-

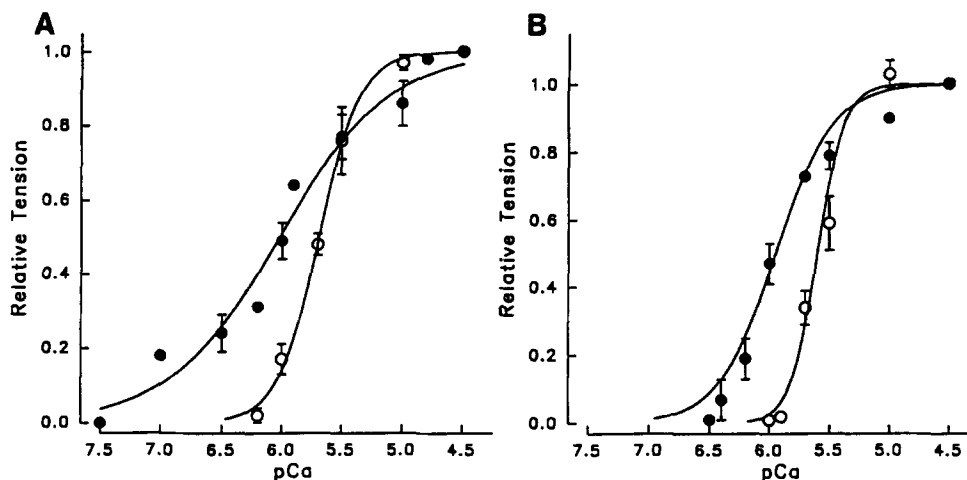


Figure 7. Summary of tension-pCa relationships in cardiac myocytes obtained during development in vitro from ES cells (*A*), and in vivo from mouse cardiac myocytes (*B*). (*A*) Filled circles are ES cell-derived cardiac myocyte preparations that had been contracting for an average of 6 ± 1 d in vitro, and open circles are preparations that had been contracting for an average of 37 ± 1 d in vitro. (*B*) Filled circles are fetal mouse cardiac myocytes (obtained on average at day 17 pc), and open circles are cardiac myocytes from adult mice. For each plot the best fit curve is shown (see Materials and Methods). Values are mean \pm SEM, average n of 6. In some instances error bars were covered by the symbol.

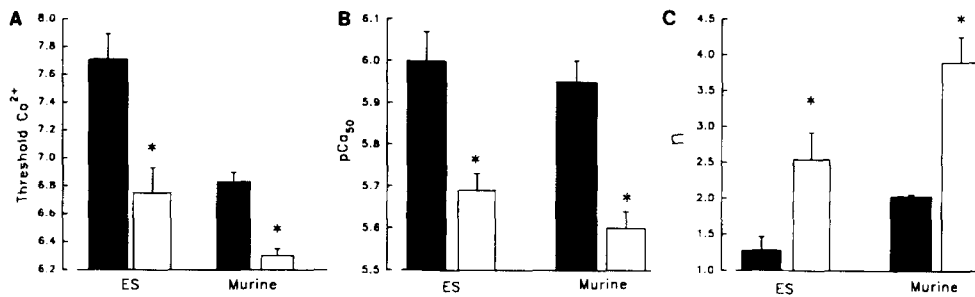


Figure 8. Alterations in the threshold Ca^{2+} for tension development (A), pCa_{50} (B), and Hill coefficient, n , (C) in cardiac myocytes during development in vivo and in vitro. For ES cell data, filled bars are for cardiac myocytes contracting for 6 ± 1 d, and open bars for cardiac myocytes contracting for 37 ± 1 d in vitro. For murine data, filled bars are fetal, and open bars are adult cardiac myocyte data. Asterisks indicate significance, $P < 0.05$. Values are mean \pm SEM, average n of 6.

cyte development in an in vitro culture system. The altered Ca^{2+} sensitivity of contraction obtained in ES cell-derived cardiac myocytes and murine cardiac myocytes during development in vitro and in vivo is in good general agreement with earlier reports which studied cardiac contractility during avian and mammalian development (Fabiato, 1982; McAuliffe et al., 1990; Godt et al., 1991). The general finding of these studies is that the sensitivity of the contractile apparatus to activation by Ca^{2+} decreases significantly during development of the heart.

An interesting finding of the present study was that the threshold for Ca^{2+} activation of contraction was significantly lower (i.e., higher pCa) in ES cell-derived cardiac myocytes that were isolated early upon initiation of contraction in vitro compared to fetal cardiac myocytes (Fig. 6). This is evidence that the thin filament is more sensitive to activation by Ca^{2+} in these ES cell-derived cardiac myocytes compared to fetal myocytes. Assuming that the sensitivity of the thin filament to activation by Ca^{2+} provides a functional measure of the stage of development of the myofilaments during cardiogenesis, and that the observed alterations in Ca^{2+} sensitivity of tension in ES cell-derived myocytes are strictly due to myofilament isoform transitions, then this result suggests that these ES cell-derived cardiac myocytes represent an earlier development stage than that of the fetal myocytes, which on average were obtained on day 17 pc. Due to technical limitations we were not able to obtain contractile data from fetal cardiac myocytes isolated at earlier stages of development in the mouse. It is now apparent that there are marked alterations in the expression of cardiac contractile genes during the very early stages of embryogenesis in the mouse (Lyons et al., 1990; Sanchez et al., 1991). The present results suggest that the ES cell in vitro differentiation culture system could provide a model to determine the functional significance of the transitions in cardiac gene expression during the very early stages of cardiogenesis.

The functional significance of altered Ca^{2+} sensitivity of the cardiac contractile apparatus during development is not known. One possibility is that the enhanced Ca^{2+} sensitivity of the cardiac myofilaments may permit direct activation of contractile apparatus by the *trans*-sarcolemmal Ca^{2+} current during the very early stages of cardiogenesis (Fabiato, 1982). There is evidence that Ca^{2+} -induced release of Ca^{2+} (CIRC) from the sarcoplasmic reticulum varies during devel-

opment of mammalian cardiac muscle. For example, Fabiato and Fabiato (1978) found no evidence of CIRC from the sarcoplasmic reticulum of rat ventricular myocytes obtained 2 d before birth; however, shortly after birth evidence of CIRC was obtained. There are several reports suggesting that the *trans*-sarcolemmal Ca^{2+} flux may be sufficient to directly activate the myofilaments during cardiogenesis (Fabiato, 1982; Nakanishi et al., 1988; Klitzner and Friedman, 1989). Activation of the myofilaments by the *trans*-sarcolemmal Ca^{2+} flux would permit tension generation in the primitive heart-tube at a time-point in cardiac development before the full development of the transverse-tubule-sarcoplasmic reticulum system. The early establishment of a rudimentary circulatory system would be essential for the delivery of oxygen and substrates to the cells and tissues of the rapidly developing organism during embryogenesis.

The molecular basis of altered Ca^{2+} sensitivity of contraction during cardiac development appears to involve developmentally regulated transitions in thin filament regulatory protein gene expression. For example, there are multiple isoforms of troponin T (TnT) expressed in the heart, and it has been shown that the phenotype of TnT demonstrates a developmental pattern of expression (Anderson et al., 1988). A correlation between the pattern of TnT expression and alterations in the Ca^{2+} sensitivity of contraction has been demonstrated in both cardiac and skeletal muscle (Schachat et al., 1987; Reiser et al., 1992; McAuliffe et al., 1990; Nassar et al., 1991). Interestingly, in avian fast skeletal muscle fibers alterations of TnT phenotype have been correlated to an increase in the Ca^{2+} sensitivity of tension during development in vivo (Reiser et al., 1988). In contrast, cardiac muscle Ca^{2+} sensitivity decreases during development (this study; McAuliffe et al., 1990; Godt et al., 1991; Nassar et al., 1991). Transitions in the phenotype of other thin filament regulatory proteins, including tropomyosin, may also play an important role in altering the Ca^{2+} sensitivity of the cardiac myofilaments during development. The finding that alterations in the Ca^{2+} sensitivity of contraction during ES cell differentiation in vitro parallels that during cardiac contractile development in vivo may therefore be related to developmental regulation of thin filament protein gene expression in the ES cell differentiation culture system. In this regard, there is recent evidence that during the in vivo differentiation of ES cells, cardiac TnT and tropomyosin gene

expression demonstrates a developmental pattern of expression similar to that observed during murine cardiogenesis in vivo (Muthuchamy et al., 1993; Pajek et al., 1993).

Earlier studies examining cardiac gene expression during the in vitro differentiation of ES cells were complicated due to the complex cellular and developmental heterogeneity evident in the differentiating ES cell aggregates, termed embryoid bodies, in suspension culture. An essential component of this study was the micro-dissection of ES cell-derived cardiac myocytes from culture for use in a functional assay to directly determine force production in the isolated preparation. By establishing attached cultures of differentiating ES cells it was possible to determine the contractile phenotype of specific cardiac myocytes as they developed in culture. The analysis of micro-dissected cardiac myocytes was critical for the determination of developmental alterations in contractility because the timing of the onset of spontaneous contractile activity varies considerably within different regions of the ES cell differentiation culture. By daily observation of the cultures using light microscopy it was possible to determine the onset and duration of spontaneous contractile activity in specific cardiac myocytes as they developed in differentiation culture. This experimental approach now makes it feasible to use the ES cell differentiation culture system to determine directly the mechanisms of cardiac gene expression and function during cardiac development in vitro.

There is a recent report indicating the possible development of multiple cardiac lineages during the in vitro differentiation of ES cells (Miller-Hance et al., 1993). In that study, differentiating ES cell aggregates were dispersed and individual myocytes were subsequently identified using myosin light chain-2V and atrial natriuretic factor (ANF) antibodies and indirect immunofluorescence microscopy. Variability in the expression of these cardiac markers suggested possible heterogeneity of the phenotype of the cardiac myocytes developing in culture. However, an important point to consider in interpreting these results resides in uncertainties of the exact developmental stage of each myocyte before determination of cellular phenotype. Given the marked developmental heterogeneity of the ES differentiation culture system it cannot be assumed that all of the myocytes were synchronized in terms of their stage of development. The precise classification of these cells as cardiac muscle progenitors, or atrial or ventricular myocytes is complicated due to the varied time course of cardiac gene specification during embryogenesis. For example, ANF is expressed in both atrial and ventricular myocytes during cardiogenesis and only becomes restricted to the atrial chambers after birth (Chien et al., 1993). The detection of multiple phenotypes in ES cell-derived myocytes (Miller-Hance et al., 1993), could indicate the presence of multiple cardiac lineages developing in vitro. Alternatively, the multiple phenotypes could arise from a single cardiac lineage. In this case the varied phenotype could be explained on the basis of differences in the development stage among the individual cells in culture. Presently, we favor the hypothesis that the ES cell-derived cardiac myocytes represent a model of early cardiac development, although alternative possibilities cannot be ruled out including the simultaneous development of atrial, ventricular, and/or cardiac progenitor cells in vitro.

The availability of a functional assay to determine contractile function in cardiac myocytes isolated during the in vitro

differentiation of ES cells, together with the capability to genetically manipulate undifferentiated ES cells using gain or loss of function experimental strategies, should make it possible to determine the functional significance of altered gene expression during cardiac development in this in vitro system. The use of the ES cell in vitro differentiation culture system to determine structure-function relationships of cardiac contractile and regulatory genes will provide two experimental advantages. First, the in vitro system will permit screening for those alterations in gene expression that are physiologically relevant. By performing cardiac contractile gene structure-function analysis in vitro there will be a reduced requirement for the establishment of transgenic lines of mice. Second, it is possible that the inactivation of certain genes may be lethal so that viable transgenic offspring will not be produced. Under these circumstances the availability of the in vitro approach may permit determination of the basis of the lethal gene mutation. The relative ease of genetic manipulation of ES cells, together with the ability of ES cells to rapidly differentiate in vitro, make the ES cell in vitro differentiation culture system well suited to determine contractile gene structure and function using loss or gain of function experimental strategies.

In summary, the findings of this study are consistent with the hypothesis that ES cell-derived cardiac myocytes recapitulate in vitro the developmentally regulated alteration in the Ca^{2+} sensitivity of tension characteristic of murine cardiogenesis in vivo. This result, together with earlier studies demonstrating cardiac-specific gene expression in the ES cell culture system (Robbins et al., 1992), are evidence that ES cell-derived cardiac myocytes recapitulate cardiogenesis in vitro. This in vitro model system of cardiac development will permit determination of the relationship between developmentally regulated cardiac gene expression and function during the early stages of mammalian cardiogenesis in vitro.

We thank Dr. Krystyna Pasyk, Ms. Heather Burrows, and Ms. Lisa Rankin for technical assistance; Drs. Larry Tobacman, Margaret Westfall, and Sally Camper for helpful comments on an earlier version of this manuscript; Dr. Thomas Doetschman for the ES-D3 cells; and Genetics Institute (Cambridge, MA) for the gift of the CHO/LIF cells.

Supported in part by the American Heart Association of Michigan. J. M. Metzger is an Established Investigator of the American Heart Association.

Received for publication 15 November 1993 and in revised form 25 March 1994.

References

- Allen, D. G., and J. C. Kentish. 1985. The cellular basis of the length-tension relation in cardiac muscle. *J. Mol. Cell. Cardiol.* 17:821-840.
- Anderson, P. A. W., G. E. Moore, and R. N. Nassar. 1988. Developmental changes in the expression of rabbit left ventricular troponin T. *Circ. Res.* 63:742-747.
- Chien, K. R., H. Zhu, K. U. Knowlton, W. Miller-Hance, M. van-Bilsen, T. X. O'Brien, and S. M. Evans. 1993. Transcriptional regulation during cardiac growth and development. *Ann. Rev. Physiol.* 55:77-95.
- Doetschman, T. C., H. Eistetter, M. Katz, W. Schmidt, and R. Kemler. 1985. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morph.* 87:27-45.
- Fabiato, A. 1982. Calcium release in skinned cardiac cells: variations with species, tissues, and development. *Fed. Proc.* 41:2238-2244.
- Fabiato, A. 1988. Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. *Methods Enzymol.* 157:378-417.
- Fabiato, A., and F. Fabiato. 1978. Calcium-induced release of calcium from the sarcoplasmic reticulum of skinned cells from adult human, dog, cat, rab-

- bit, rat, and frog hearts and from fetal and new-born rat ventricles. *New York Acad. Sci.* 307:491-522.
- Ganim, J. R., W. Luo, S. Ponniah, I. Grupp, H. W. Kim, D. G. Ferguson, V. Kadambi, J. C. Neumann, T. Doetschman, and E. G. Kranias. 1992. Mouse phospholamban gene expression during development *in vivo* and *in vitro*. *Circ. Res.* 71:1021-1030.
- Godt, R. E., and B. D. Lindley. 1982. Influence of temperature upon contractile activation and isometric force production in mechanically skinned muscle fibers of the frog. *J. Gen. Physiol.* 80:279-297.
- Godt, R. E., R. T. H. Fogaca, and T. M. Nosek. 1991. Changes in force and calcium sensitivity in the developing avian heart. *Can. J. Physiol. Pharmacol.* 69:1692-1697.
- Klitzner, T. S., and W. F. Friedman. 1989. A diminished role for the sarcoplasmic reticulum in newborn myocardial contraction: effects of ryanodine. *Pediatr. Res.* 26:98-101.
- Lyons, G. E., S. Schiaffino, D. Sassoon, P. Barton, and M. Buckingham. 1990. Developmental regulation of myosin gene expression in mouse cardiac muscle. *J. Cell Biol.* 111:2427-2436.
- McAuliffe, J. J., L. Gao, and R. J. Solaro. 1990. Changes in myofibrillar activation and troponin C Ca^{2+} binding associated with troponin T isoform switching in developing rabbit heart. *Circ. Res.* 66:1204-1216.
- Metzger, J. M., and R. L. Moss. 1987. Greater hydrogen ion-induced depression of tension and velocity in skinned single fibres of rat fast than slow muscles. *J. Physiol.* 393:727-742.
- Metzger, J. M., M. L. Greaser, and R. L. Moss. 1989. Variations in cross-bridge attachment rate and tension with phosphorylation of myosin in mammalian skinned skeletal muscle fibers. *J. Gen. Physiol.* 93:855-883.
- Metzger, J. M., M. Parmacek, E. Barr, K. Pasyk, W. Lin, K. Cochrane, L. Field, and J. Leiden. 1993. Skeletal troponin C reduces contractile sensitivity to acidosis in cardiac myocytes from transgenic mice. *Proc. Natl. Acad. Sci. USA.* 90:9036-9040.
- Miller-Hance, W. C., M. LaCorbiere, S. J. Fuller, S. M. Evans, G. Lyons, C. Schmidt, J. Robbins, and K. R. Chien. 1993. *In vitro* chamber specification during embryonic stem cell cardiogenesis. *J. Biol. Chem.* 268:25244-25252.
- Muthuchamy, M., L. Pajek, P. Howles, T. Doetschman, and D. F. Wiczeorek. 1993. Developmental analysis of tropomyosin gene expression in embryonic stem cells and mouse embryos. *Mol. Cell Biol.* 13:3311-3323.
- Nakanishi, T., M. Seguchi, and A. Takao. 1988. Development of the myocardial contractile system. *Experientia (Basel)*. 44:936-944.
- Nasser, R., N. N. Malouf, M. B. Kelly, A. E. Oakeley, and P. A. W. Anderson. 1991. Force-pCa relation and troponin T isoforms of rabbit myocardium. *Circ. Res.* 69:1470-1475.
- Pajek, L., M. Muthuchamy, P. Howles, T. Doetschman, and D. F. Wiczeorek. 1993. Analysis of contractile protein gene expression in developing embryoid bodies. *J. Cell Biochem. Suppl.* 17D:202.
- Reiser, P. J., M. L. Greaser, and R. L. Moss. 1992. Developmental changes in troponin T isoform expression and tension production in chicken single skeletal muscle fibers. *J. Physiol. (Camb.)*. 449:573-588.
- Robbins, J., T. Doetschman, W. K. Jones, and A. Sanchez. 1992. Embryonic stem cells as a model for cardiogenesis. *Trends Cardiovasc. Med.* 2:44-50.
- Sanchez, A., W. K. Jones, J. Gulick, T. Doetschman, and J. Robbins. 1991. Myosin heavy chain gene expression in mouse embryoid bodies. *J. Biol. Chem.* 266:22419-22426.
- Schachat, F. H., M. S. Diamond, and P. W. Brandt. 1987. Effect of different troponin T-tropomyosin combinations on thin filament activation. *J. Mol. Biol.* 198:551-554.
- Sissman, N. J. 1970. Developmental landmarks in cardiac morphogenesis: comparative chronology. *Am. J. Cardiol.* 25:141-148.
- Sweitzer, N. K., and R. L. Moss. 1990. The effect of altered temperature on Ca^{2+} -sensitive force in permeabilized myocardium and skeletal muscle. *J. Gen. Physiol.* 96:1221-1245.
- Williams, R. L., D. J. Hilton, S. Pease, T. A. Willson, C. L. Stewart, D. P. Gearing, E. F. Wagner, D. Metcalf, N. A. Nicola, and N. M. Gough. 1988. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature (Lond.)*. 336:684-687.
- Wobus, A. M., G. Wallukat, and J. Hescheler. 1991. Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca^{2+} channel blockers. *Differentiation*. 48:173-182.
- Zak, R. 1973. Cell proliferation during cardiac growth. *Am. J. Cardiol.* 31:211-219.