Avian Precardiac Endoderm/Mesoderm Induces Cardiac Myocyte Differentiation in Murine Embryonic Stem Cells

Diane Rudy-Reil, John Lough

Abstract—The ability to regenerate damaged myocardium with tissue derived from embryonic stem (ES) cells is currently undergoing extensive investigation. As a prerequisite to transplantation therapy, strategies must be developed to induce ES cells to the cardiac phenotype. Toward this end, cues from mechanisms of embryonic induction have been exploited, based on previous findings that anterior lateral endoderm (precardiac endoderm) from gastrulation-stage chick embryos potently induces cardiac myocyte differentiation in both precardiac and nonprecardiac mesoderm. Hypothesizing that avian precardiac endoderm acting as feeder/inducer cells would induce high percentage conversion of murine ES (mES) cells into cardiac myocytes, it was observed that the majority (≈65%) of cocultured ES cell-derived embryoid bodies (EBs) were enriched in cardiac myocytes and exhibited rhythmic contractions. By contrast, mouse EBs cultured alone, or on feeder layers of mouse embryonic fibroblasts or avian nonprecardiac posterior endoderm, contained only 7% to 16% cardiac myocytes while exhibiting a relatively low incidence (<10%) of beating. When mES cells were cocultured with a bilayer of explanted precardiac endoderm/mesoderm, the incidence of rhythmically contractile EBs increased to 100%. To verify that the rhythmically contractile cells were derived from murine ES cells, cell-free medium conditioned by avian precardiac endoderm/mesoderm was used to induce myocyte differentiation in a mES cell-line containing a nuclear LacZ reporter marker gene under control of the cardiac-specific α-myosin heavy chain promoter, resulting in rhythmic contractility in 92% of EBs in which the majority of cells (average = 86%) were identified as cardiac myocytes. The inductive efficacy of medium conditioned by avian precardiac endoderm/mesoderm may provide an opportunity to biochemically define factors that induce cardiac myocyte differentiation in ES cells. The full text of this article is available online at http://circres.ahajournals.org. (Circ Res. 2004;94:e107-e116.)

Key Words: cardiac myocyte differentiation ■ embryoid bodies ■ endoderm/mesoderm cell-free conditioned medium ■ cardiac induction ■ mouse embryonic stem cells ■ precardiac endoderm ■ precardiac mesoderm ■ pre-embryoid bodies

The potential of adult and embryonic stem (ES) cells to regenerate adult tissue has caused extraordinary interest in their therapeutic application. Adult stem cells, residing within niches of mature tissues and in bone marrow are, respectively, considered unipotent and multipotent with regard to their ability to form diverse cellular phenotypes. Many instances of adult stem cells’ ability to differentiate and become incorporated into adult tissues have been reported.1 By contrast, ES cells, which are derived from the inner cell mass of blastocyst-stage embryos, are considered pluripotent, because of their ability to differentiate into all tissues of the adult phenotype.1 Therapeutic use of ES cells will require the resolution of issues, including prevention of rejection, assurance that engrafted cells do not contain infectious or tumorigenic agents, and the ability to become functionally incorporated into target tissue.2 An additional requirement is the ability to induce and expand ES cells to transplantable numbers that have phenotypic identity with the target tissue to be engrafted. Toward this end, cues from mechanisms of embryonic induction should be of value in designing rationally based approaches.

The ability to regenerate damaged myocardium with new cardiac myocytes is a paramount objective. Although myocytes in the adult myocardium have been traditionally considered to neither divide nor regenerate, the existence of myocardial stem cells and their ability to incorporate into adult myocardium have recently been reported.3–4 Regarding ES cells, spontaneous cardiac myocyte differentiation has been observed in human and mouse lines.5–9 Although percentages of spontaneously differentiated cardiac myocytes are low, recent efforts to enrich cardiac myocytes are promising; for example, Xu et al obtained 70% cardiac myocyte enrichment by density gradient selection from spontaneously contracting human embryoid bodies (EBs).5

As an alternative to using physical separation methods to enrich for spontaneously differentiating myocytes, the goal of directly inducing cardiac myocyte differentiation in ES cell
cultures is compelling. To address this task, we are using an approach based on the mechanisms of cardiac myocyte induction that occur in the embryo. As demonstrated by this and other laboratories, monolayer explants of gastrulation-stage anterior lateral endoderm from chick embryos, hereafter termed precardiac endoderm (preE), potently induce cardiac myocyte differentiation, including the specification of cardiac myocytes in nonprecardiac cells and the induction of terminal cardiac myocyte differentiation in precardiac mesoderm.\textsuperscript{10–12} In this study, we have addressed whether coculture of murine ES (mES) cells with a feeder/inducer layer of explanted chick preE and/or its associated tissues can induce cardiac myocyte differentiation. We report that EBs generated from mES cells cultured alone, or on a fibroblast feeder layer, exhibited a low incidence of spontaneous cardiac myocyte differentiation. Similarly, relatively little cardiac differentiation was noted in EBs generated from mES cells cocultured with nonprecardiac posterior endoderm (postE). However, when cocultured with preE, \( \approx 65\% \) of EBs exhibited expression of cardiac markers followed by rhythmic contractility. Most remarkably, coculture with combined precardiac endoderm and precardiac mesoderm, or replacement of this explant combination with cell-free medium conditioned by it, respectively, induced rhythmic contractility in 100% and 92% of EBs, the majority (\( \sim 86\% \)) of cells in which were cardiac myocytes.

**Materials and Methods**

**Cell Lines**

Two lines of mES cells were used in this study. Initial experiments used R1 ES cells.\textsuperscript{13} Then, to enable direct observation of cardiac gene expression in mES cells that were in coculture with avian explants, we used mES cells containing a single-copy Lac\( Z \) transgene knocked into the \( hprt \) locus under the control of the cardiac \( \alpha \)-myosin heavy chain promoter.\textsuperscript{14} \( \alpha \)-myosin heavy chain–Lac\( Z \) mES cells were prepared and generously provided by Dr Ravi Misra (Medical College of Wisconsin, Milwaukee, Wis).

**Media**

Cells and explants were cultured at 37°C in a 95% air/5% CO\( _2 \) humidified atmosphere. Components of the media used in these experiments are as follows: mouse embryonic fibroblast (MEF) feeder medium was prepared with DMEM (catalog no. 12100-046; Invitrogen, Carlsbad, Calif), 10% fetal bovine serum (FBS), 1% nonessential amino acids (NEAA), and 1% penicillin/streptomycin (PS).

MEF-conditioned medium was prepared as described by Xu et al.\textsuperscript{15} Briefly, mitotically inactivated MEFs were plated on 35-mm cell culture plates coated with 0.1% gelatin at a density of 55 000 cells/cm\( ^2 \). After 4 hours, MEF feeder medium was exchanged with ES cell growth medium consisting of 80% DMEM F-12, 20% KO basal medium with 200 \( \mu \)g/mL fibronectin (Boehringer Mannheim Corp, Indianapolis, Ind) at room temperature. Immediately before adding explants, excess fibronectin was replaced with 1.0 mL avian explant medium. Explants of anterior or posterior lateral plate endomesoderm, microdissected from Hamburger & Hamilton stage\textsuperscript{16} 5+/6 embryos as described previously, were exclusively used.\textsuperscript{17–21} Briefly, embryos were removed from chick eggs (Sunnyside Farms, Beaver Dam, Wis) and, with ventral side up, intact sheets of endomesoderm were gently teased from underlying ectoderm using sharp tungsten needles. When isolated endoderm or mesoderm was required, these were microdissected after a 1-minute digestion in ice-cold collagenase/dispose (1 mg/mL, Boehringer Mannheim Corp).\textsuperscript{22} Three or 4 identical explants were placed on the fibronectin-coated coverslip in each well and cultured in avian explant medium.

**Induction of Cardiac Differentiation**

Differentiation of mES cells was induced by implanting preEBs growing in suspension culture onto: (1) fibronectin-coated coverslips only (no cell control); (2) fibronectin-coated coverslips containing a feeder layer of MEFs (cell control); or (3) fibronectin-coated coverslips containing a feeder/inducer layer of avian explants. In the instance of cocultures that contained avian precardiac mesoderm, the mouse preEB was implanted sufficiently distal to the avian explant so that contracting avian cells could be unambiguously distinguished from contracting mouse cells at all times. And, in instances in which 2 preEBs were implanted rather than 1, these were positioned sufficiently remote to each other so that EB fusion was never observed. All cultures were maintained in avian explant medium (defined previously), which was replenished daily. The day of preEB plating was designated as day 0 of the cardiac induction phase and cultures were monitored for rhythmic contractility for up to 3 weeks. For experiments in which avian cell explants were replaced with explant-conditioned medium, the latter was prepared as described (but using a minimum of 8 explants) and replenished on a daily basis through induction day 7; after day 7, daily replenishment was made using fresh, nonconditioned avian explant medium. Rhythmic contractility was monitored for an additional 2 weeks.

**Expansion of mES Cells and Formation of Pre-embryoid Bodies**

To expand mES cells, a MEF feeder layer was prepared by plating MEFs (50 000 cells/cm\( ^2 \)) on 0.1% gelatin-coated 35-mm-diameter culture plates 24 hours before the addition of mES cells. mES cells were expanded in MEF-conditioned medium supplemented with 4 ng/mL FGF2, which was refreshed daily. At \( \sim 60\% \) confluence, colonies were enzymatically dissociated using 200 U/mL collagenase IV at 37°C for 10 minutes.\textsuperscript{5} Cells were transferred to uncoated 35-mm dishes containing 2.5 mL MEF-conditioned medium plus FGF2 and grown in suspension with a daily exchange of medium for a maximum of 2 to 3 days. At this point, preEBs, defined as suspended spherical colonies measuring \( \sim 200 \mu \mathrm{m} \) in diameter, were selected for cardiac induction.
Immunohistochemistry
Cultures were fixed in 4% formaldehyde/phosphate-buffered saline for 45 minutes and permeabilized with 0.1% Triton X-100 for 45 minutes at 4°C. To minimize nonspecific binding of antibodies, cultures were preincubated in blocking buffer consisting of 2% donkey serum/1% BSA in phosphate-buffered saline for 1 hour at 4°C. To assess cardiac induction, EBs were incubated with monoclonal antibodies specific for sarcomeric myosin heavy chain (Developmental Studies Hybridoma Bank Antibody MF-20, Iowa City, Iowa) diluted 1:10 in blocking buffer. Alternatively, EBs were stained with monoclonal antibodies specific to βIIH3-tubulin type III (1:200; Chemicon Intl, Temecula, Calif), a neuronal marker. The secondary antibody was FITC-conjugated goat antimouse (1:400; ICN Pharmaceuticals, Inc, Aurora, Ohio). Antibody incubations were performed at 4°C overnight in a humidified chamber, followed by extensive washing with phosphate-buffered saline. Cells were mounted in Vectashield Mounting medium (Vector Laboratories, Burlingame, Calif) and observed on a Nikon Eclipse TE300 microscope.

Figure 1. Cardiogenesis on cue. Outline of protocol established to inhibit spontaneous differentiation of mES cells and induce cardiac myocyte differentiation.

Histochemistry
Cocultures were histochemically reacted to detect expression of β-galactosidase using standard techniques. Briefly, cells were fixed (0.2% glutaraldehyde/5 mmol/L EGTA/2 mmol/L MgCl2/0.1 mol/L phosphate buffer pH 7.3) for 10 minutes at room temperature, washed 3 times for 15 minutes with detergent solution (2 mmol/L MgCl2/0.01% sodium deoxycholate/0.02% Nonidet-P40/0.1 mol/L phosphate buffer, pH 7.3), and reacted using 1 mg/mL X-gal stain solution (2 mmol/L MgCl2/5 mmol/L potassium ferricyanide/5 mmol/L potassium ferrocyanide/0.1 mol/L phosphate buffer) overnight at 37°C. Cells were washed 3 times for 15 minutes with detergent solution and observed for blue reaction product indicative of β-myosin heavy chain promoter activation.

Results
Cardiogenesis on Cue
As shown in Figure 1, 3 sequential phases of mES cell culture were used in this protocol—expansion, suspension, and induction—the latter culminating in terminal cardiac differentiation. During expansion and suspension phases, the objective was to prevent spontaneous differentiation of mES cells, so that cardiac differentiation could be induced on cue during the induction phase. To accomplish this, mES cells were expanded on MEF feeder layers in MEF-conditioned medium, to not 60% confluence. This was followed by cellular dissociation and placement in suspension culture for a maximum of 2 to 3 days (in contradistinction to 7 days used in standard protocols), during which time ES cells
aggregated to form spheroids termed preEBs. It is noteworthy that no morphological evidence of differentiation, cardiac or noncardiac, was observed during the expansion or suspension phases.

To induce cardiac differentiation, suspended preEBs were individually implanted adjacent to, or directly on, explanted avian precardiac cells. Because previous reports had indicated that the number of cells in a preEB correlates with efficiency of cardiac myocyte differentiation, it was considered that the size of suspended preEBs selected for induction may be crucial. Hence, selection was restricted to preEBs that were \( \sim 200 \mu \text{m} \) in diameter, which contained \( \sim 1000 \) cells. Adherence to this criterion resulted in optimal cardiac morphogenesis within 7 days after induction, as defined by the morphological development of relatively coherent vesicular structures containing numerous foci of rhythmically contractile cellular multilayers (Figure 2a); with increasing time in culture, differential rates of multilayering within areas of each EB resulted in a less uniform appearance. PreEBs smaller than \( 200 \mu \text{m} \) at the time of selection tended to dissociate during induction, exhibiting a noncontractile, neuronal phenotype (not shown). Although beating foci were sporadically observed in EBs generated from larger preEBs (\( \sim 400 \mu \text{m} \) diameter), these exhibited outgrowths of diffuse monolayer cells (Figure 2b) that contained noncardiac cell types (not shown). Smaller preEBs (\( \sim 200 \mu \text{m} \)) that were cultured in fresh, nonconditioned medium exhibited similar outgrowths that were more pronounced (Figure 2c).

The inductive effect of avian precardiac explants was evaluated in comparison with cells grown under control conditions that were developed to minimize spontaneous cardiac differentiation. The first was a "no cell" control in which suspended preEBs were directly plated onto fibronectin (Fn)-coated coverslips. The second was a "cell control" in which preEBs were implanted on a feeder layer of MEFs. Third, as another "cell" control, preEBs were implanted on a feeder layer of chick nonprecardiac postE. Control cultures were maintained in avian explant medium for up to 3 weeks, during which time enriched differentiation of neuronal cells was observed as detected by expression of \( \beta \)-tubulin type III. Figure 3 shows control explants that were immunostained for \( \beta \)-tubulin type III and cardiac (myosin heavy chain) markers at induction day 8; note that whereas neuronal differentiation occurred throughout the control EBs (Figure 3b and 3f), cardiac myocytes were relatively scarce, occurring in small foci (Figure 3d and 3h), thereby comprising a relatively small percentage of cells in the control EBs.

**Precardiac Endoderm Induces Cardiogenesis in PreEBs**

The effect of coculturing pre-EBs with precardiac endoderm (preE), which specifies and induces differentiation in precardiac mesoderm, is shown in Figure 4. The time of initiating coculture was designated as day 0 of the induction phase. After 4 days, cardiac differentiation in EBs was detected (Figure 4a), as indicated by strong expression of sarcomeric myosin heavy chain, which extended throughout these structures as shown in Figure 4b; the sarcomeric organization of myosin heavy chain is shown in Figure 4c. Within 7 days, foci of rhythmically contracting myocytes were seen that, consistent with the immunostaining pattern, were homogeneously dispersed throughout the EB and increased in size during the 3-week induction period (see online video available at http://circres.ahajournals.org). These phenomena depict a typical response to preE, which induced rhythmic beating in 65% of cocultured EBs during the 3-week induction period (as discussed below).

To ensure that the immunostained myosin heavy chain antigens in Figure 4 were in mES cells rather than in chick precardiac mesoderm that may have contaminated explanted endoderm, we used a mES cell line, \( \alpha \)-myosin heavy chain–lacZ cells, which were engineered to express nuclear LacZ at the onset of cardiac myocyte differentiation. Figure 5a shows a mouse-derived \( \alpha \)-myosin heavy chain–lacZ EB-induced with avian preE, which displayed rhythmic contractility by induction day 7. Similar to the myosin heavy chain staining pattern in Figure 4b, X-gal staining (blue), which was restricted to the nuclei of EB cells, extended throughout the EB. By contrast, control EBs grown on fibronectin only (no cell; Figure 5d) or in coculture with postE (Figure 5b) contained relatively few cardiac myocytes, which were present in clusters; typically, spontaneously appearing myocyte clusters were larger in EBs cultured on postE. Although the presence of contaminating chick myocytes cannot be ruled out, this experiment demonstrated that large numbers of differentiated cardiomyocytes in these cocultures were generated from mouse ES cells.
Inclusion of Precardiac Mesoderm With Endoderm Increases Incidence of Cardiogenesis in PreEBs

Experiments in which murine preEBs were cocultured beside chick precardiac mesoderm (preM) surprisingly revealed that the incidence of EB beating was similar to the response induced by preE (not shown). Based on this observation, we examined the possibility that preM augments the inductive effect of preE. Hence, explanted precardiac endoderm/mesoderm (preE/H11001M) was cultured alone for 24 hours, at which time a mouse \( \beta \)-myosin heavy chain–lacZ preEB was implanted to a distal area of the culture in a fashion enabling the unambiguous discrimination of contracting avian cells (precardiac mesoderm) from contracting mouse cells (EBs). As expected from previous experience,\(^{12,17} \) the precardiac mesoderm component of preE+M became rhythmically contractile during the 24-hour conditioning period. By induction day 7, 100% of mouse EBs, which remained both morphologically and spatially distinguishable from the contracting chick preE+M at all times, exhibited rhythmic beating (not shown). The murine origin and cardiac identity of the beating mouse EBs were verified by X-gal histochemistry (Figure 5c).

In some experiments, preEBs were deliberately implanted between the endoderm and precardiac mesoderm of the avian explant. In these cultures, boundaries of the mouse preEBs nonetheless remained fairly distinct, enabling the observation that their contractile activity was not synchronous with that of the avian cardiac mesoderm. Also, in some instances, individual differentiated mES cells appeared to become incorporated, in mosaic fashion, into the contractile multilayer of the chick mesoderm (Figure 5c).

Cell-Free Medium Conditioned by Explanted Endoderm/Mesoderm Mimics the Inductive Effect of Explanted Cells

As described, mouse preEBs were implanted distal to the avian explants. Because these cell populations remained spatially separated throughout the induction period, this suggested that direct physical contact between avian preE+M and mouse ES cells was not required for cardiac myocyte differentiation. To unequivocally verify that preEBs could be induced without direct contact between avian and mouse cells, the inductive effect of cell-free medium conditioned by explanted preE+M (ie, preE+M-cm) was evaluated. As tabulated in Figure 6b, explant-conditioned medium induced rhythmic contraction in nearly all (92%) preEBs; by contrast, only 7% of preEBs grown in fresh, nonconditioned medium exhibited rhythmic contractions. The chronology of differentiation induced by preE+M-cm was delayed when compared with induction by whole cells; for example, biochemical differentiation and onset of beating in preEBs were, respectively, observed, on average, 6 and 9 days after induction with preE+M-cm, in comparison with 4 and 7 days after induction...
with explants. A video showing a rhythmically contracting EB induced by preE+M-cm after 9 days of induction is included in the online data supplement.

Majority of Cells in Cardiogenic EBs Are Cardiac Myocytes

Appearance of the immunohistochemically-stained and X-gal-stained EBs, respectively, shown in Figures 4 and 5 indicated that induced EBs were enriched with cardiomyocytes. Moreover, direct observation revealed that beating foci always extended throughout the entirety of EBs (although rhythmically beating foci within an EB did not contract in synchrony; see online video).

To quantitate myocyte enrichment induced by preE+M-conditioned medium (preE+M-cm), induced and noninduced EBs at induction day 7 were immunostained for myosin heavy chain to identify cardiac myocytes (green) and counterstained with PI (red) to visualize all nuclei. Figure 7c through 7k show 3 random confocal sections taken through single EBs representing each of 3 experimental conditions—

1. fresh medium control (Fn only);
2. postE control; and
3. preE+M-cm—induced—demonstrating that differentiated cardiac myocytes were homogeneously distributed throughout EBs induced by preE+M-cm (Figure 7l through 7k). Five EBs cultured under each experimental condition were evaluated to quantitatively estimate the inductive effect of preE+M-cm. In each EB, all cells in the ten ×100 confocal microscopic fields illustrated in Figure 7a and 7b, comprising 1300 to 2000 cells per EB, were evaluated as nonmyocytes or myocytes; to identify myocytes, the convention was used that PI-stained nuclei must be clearly surrounded by myosin heavy chain-positive cytoplasm. This revealed averages of 86%, 16%, and 7% myocytes in EBs induced by cell-free preE+M-cm, postE, and fresh medium, respectively (Figure 7l). To verify the myocyte numbers induced by preE+M-cm, EBs were disaggregated and replated at nonconfluent density, followed by immunostaining and counting (Figure 7m); myocyte percentages in triplicate cultures (74±2.4% myocytes) were consistent with the myocyte percentages enumerated in the intact EBs. Because the latter determinations included cells that had been present in both the noncontractile monolayer and in the contractile EB multilayer before disaggregation, it is likely that cardiac myocytes represented an even higher percentage of cells in the latter.

Discussion

Previous work in this and other laboratories has demonstrated the potency of embryonic preE, but not postE, to induce terminal cardiac myocyte differentiation in comigrating precardiac mesoderm cells in the embryo. Because precardiac endoderm also induces cardiac differentiation in nonprecardiac embryonic tissues, we hypothesized that preE used as feeder/inducer cells would induce cardiac myogenesis in embryonic stem cells. As reported in this article, we have shown that preE, but not postE, induced cardiogenesis in most EBs. This finding is consistent with experiments indicating that a critical mass of parietal endoderm regulates cardiac myogenesis in EBs. Surprisingly, inclusion of precardiac mesoderm with endoderm (preE+M) strongly increased the incidence of cardiogenesis, causing all EBs to become rhythmically contractile. Experiments using cell-free preE+M-cm further demonstrated the cardiogenic potency of the secretory products of these explants while providing evidence that contact between inducing and responding cells was not required for cardiac differentiation and that all induced cells were derived from murine ES cells, rather than from precardiac mesoderm cells present in the explants.

Although the increased potency of preE+M, in comparison with preE alone to induce cardiogenesis in mES cells is unexplained, this result is consistent with recent findings indicating that stem cell differentiation is influenced by local environments. For example, it was reported that transplantation of human ES cells into chick embryo somites results in their differentiation into cells having epithelial and neural characteristics. And, more relevant to this article, coculture of hematopoietic progenitor or endothelial cells with embryonic cardiac tissue, or of ES cells with postmitotic cardiac myocytes, results in their differentiation to the cardiac phenotype.

In addition to its ability to induce terminal differentiation in precardiac mesoderm, anterior endoderm can induce cardiogenesis at ectopic sites in the embryo, suggestive of a role during the specification of cells to the cardiac lineage,
which occurs during early gastrulation.\textsuperscript{26} We previously speculated that at the onset of gastrulation, as ingressing epiblast cells diverge into endodermal and mesodermal lineages, the former specifies precardiac mesoderm in a subset of the latter.\textsuperscript{10} It is interesting to conjecture that these embryonic processes may be recapitulated during conversion of preEBs to EBs, and that the presence of signals from exogenous endoderm/mesoderm in these experiments expands the precardiac mesoderm subset by overriding other embryonic signals. In this regard, the morphology and content of cardiac myocytes in EBs induced by preE + M-cm are strikingly similar to explants of precardiac mesoderm induced by precardiac endoderm.\textsuperscript{17,27,28}

The observation of rhythmic contractility in virtually all EBs induced by preE + M or its conditioned medium is apparently unprecedented. Using a similar rationale based on induction with an endoderm cell line (END-2 cells), Mummery et al\textsuperscript{29} recently reported the occurrence of cardiac differentiation in 35\% of cocultures containing human embryonic stem cells (hESs), whereas Xu et al\textsuperscript{5} using an approach to myocyte enrichment that was not based on embryonic induction, observed cardiac differentiation in 70\% of hES cell colonies.\textsuperscript{5} Regarding the percentage of cells in each EB that differentiate into cardiac myocytes, Xu et al\textsuperscript{5} reported that 70\% enrichment could be obtained by selection from a Percoll gradient. Although Mummery et al\textsuperscript{29} did not specify the extent of myocyte enrichment induced by END-2 cells, it was reported that beating foci in human ES cell cultures contained 10 to 200 myocytes.\textsuperscript{29} Differences between the latter findings and those reported here may reflect differential responsiveness of mES and hES cells; hence, we are

Figure 5. Cardiac induction in EBs revealed by X-gal histochemistry. PreEBs from α-myosin heavy chain-lacZ mES cells were cocultured as indicated in the panels; after 7 days, cultures were histochemically reacted for β-galactosidase to detect α-myosin heavy chain gene expression. (a), Co-culture with precardiac endoderm (preE); (b), co-culture with posterior endoderm (postE); (c), co-culture with precardiac endoderm plus mesoderm (preE + M); (d), no cell control. All panels are at the same magnification; bars in (a) and (c) = 100 μm.

Figure 6. Precardiac endoderm + mesoderm, or medium conditioned by them, induce cardiogenesis in a high percentage of EBs. PreEBs were cocultured for up to 21 days with the indicated cells/explants (a) or with conditioned medium (b). As shown in (a), ~65\% of preEBs cocultured with chick precardiac endoderm (preE) became rhythmically contractile. Controls consisted of preEBs incubated on fibronectin only ("no cell" control), a feeder layer of mouse embryonic fibroblasts ("cell" control), or a feeder layer of avian nonprecardiac posterior endoderm (postE); see Figure 3. As shown in (b), cell-free medium conditioned by preE + M induced cardiogenesis in nearly all EBs, whereas fresh (nonconditioned) avian explant medium had relatively little cardiogenic efficacy.
Figure 7. The majority of cells in induced EBs are cardiac myocytes. To evaluate the percentage of cardiac myocytes in induced and uninduced EBs, intact EBs were fixed on induction day 7 and stained for myosin heavy chain and PI. As shown schematically in (a) and (b), cardiac myocytes in each EB were enumerated in 10 nonoverlapping confocal fields [5 x-y fields shown as the rectangles in (a), at 2 z-depths (dotted lines in b)] taken using the ×100 objective. To demonstrate the distribution of myocytes throughout EBs grown under each experimental condition, 3 images, each taken from the same EB, are shown in (c) through (k); bar = 20 μm. c through e, Three sections taken through a no cell (ie, fresh medium) control EB. (f) through (h), Sections through a postE control EB. (i) through (k), Sections through an EB induced by preE + M-cm; note the homogenous distribution of myocytes (green) in these sections (i) through (k) in contradistinction to the sporadic myocyte groups in (c) through (h). (l), Percentage of myosin heavy chain-stained cells in 5 EBs grown under each condition, revealing an average of 7.1%, 16.3%, and 85.6% myocytes in no cell, postE, and preE + M-cm EBs, respectively (P<0.001; t test); to verify this result, triplicate EBs disaggregated after 2 weeks of induction with preE + M-cm, and replated at nonconfluent density revealed an average of 74% myosin-positive cells (m; bar = 10 μm).
now evaluating whether hES cells similarly respond to avian preE+M-cm. In any event, it will be useful to ascertain whether the inductive potency of preE+M-cm can be mimicked by medium conditioned by END-2 and/or other cell lines derived from embryonic tissue, because the bulk availability of the latter would obviate the necessity of microdissecting preE+M bilayers (≈0.6×1.0 mm), each of which manufactures only ≈ 0.1 mL conditioned medium per day.

The protocol to induce mES cells to a cardiac phenotype (Figure 1) was designed to use culture conditions that could be seamlessly applied to human ES cells. Most significantly, LIF was omitted from, and FGF was added to, MEF-conditioned medium during the expansion and suspension phases to prevent spontaneous ES cell differentiation. Although LIF is routinely added for this purpose, high levels of this factor have been reported to inhibit cardiogenesis in mouse ES cells. Therefore, we speculate that the amount of LIF in MEF-conditioned medium was sufficient to prevent spontaneous differentiation without affecting myocyte differentiation. FGF, however, is known to promote cardiogenesis in embryonic tissue; most recently, Dell’Era et al demonstrated that FGF signaling is required for cardiac differentiation in mES cell cultures. Taken together, these findings raise the interesting possibility that the absence of LIF and presence of FGF during expansion and suspension may have “poised” ES cells to respond to the inductive influence of preE+M. This possibility is being addressed in current experiments to evaluate the ability of preE+M to induce cardiogenesis in EBs that had been cultured during the expansion and suspension phases with MEF-conditioned medium containing various combinations of FGF and LIF.

Finally, previous work has shown that although treatment of human ES cells with combinations of selected growth factors induces phenotypes having characteristics of specific embryonic germ layers, EBs enriched for single cell types were not generated. However, treatment of less primitive embryonic cells from gastrulation and postgastrulation stage embryos with specific growth factor combinations has been shown to induce specific cell types, including pituitary, neural crest, and cardiac myocytes. Together, these findings suggest that rationally defined growth factor cocktails, based on composition of the environment in which embryonic cells differentiate, may induce enriched cohorts of specific cell types from ES cells. The cardiogenic potency of preE+M, combined with the demonstration that preE+M can be replaced by its conditioned medium, provides a starting point for the biochemical identification of the secretory products of these cells, with the objective of defining a growth factor/cytokine cocktail that can induce cardiogenesis in ES cells on cue.

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References


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