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This Review is part of a thematic series on Stem Cells, which includes the following articles:

Differentiation of Pluripotent Embryonic Stem Cells Into Cardiomyocytes

Neural Stem Cells: An Overview
Mesenchymal Stem Cells
Derivation and Potential of Human ES Cells
Therapeutics and Use of Stem Cells
Stem Cells and Myocardial Infarction
Myocyte Death, Growth, and Regeneration in Cardiac Hypertrophy and Failure  

Toren Finkel, Roberto Bolli, Editors

Differentiation of Pluripotent Embryonic Stem Cells Into Cardiomyocytes

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Abstract—Embryonic stem (ES) cells have been established as permanent lines of undifferentiated pluripotent cells from early mouse embryos. ES cells provide a unique system for the genetic manipulation and the creation of knockout strains of mice through gene targeting. By cultivation in vitro as 3D aggregates called embryoid bodies, ES cells can differentiate into derivatives of all 3 primary germ layers, including cardiomyocytes. Protocols for the in vitro differentiation of ES cells into cardiomyocytes representing all specialized cell types of the heart, such as atrial-like, ventricular-like, sinus nodal–like, and Purkinje-like cells, have been established. During differentiation, cardiac-specific genes as well as proteins, receptors, and ion channels are expressed in a developmental continuum, which closely recapitulates the developmental pattern of early cardiogenesis. Exploitation of ES cell–derived cardiomyocytes has facilitated the analysis of early cardiac development and has permitted in vitro “gain-of-function” or “loss-of-function” genetic studies. Recently, human ES cell lines have been established that can be used to investigate cardiac development and the function of human heart cells and to determine the basic strategies of regenerative cell therapy. This review summarizes the current state of ES cell–derived cardiogenesis and provides an overview of how genomic strategies coupled with this in vitro differentiation system can be applied to cardiac research. (Circ Res. 2002;91:189-201.)

Key Words: embryonic stem ■ embryonic carcinoma ■ embryonic germ ■ in vitro differentiation ■ cardiomyocytes

Stem cell biology has been the subject of much recent discussion, but only the totipotent fertilized oocyte and blastomere cells of embryos at the 2- to 8-cell stage are capable of generating a fully viable organism. Stem cells from the embryo are derived from the inner cell mass (ICM), embryonic ectoderm, and primordial germ cells of the fetal genital ridge and represent pluripotent undifferentiated cells capable of proliferation, self-renewal, and the generation of large numbers of differentiated cell progeny; however, embryonic stem (ES) cells do not normally generate tissues of the trophoblast, precluding normal generation of a viable entity. As development proceeds and a stem cell becomes committed to a specific lineage or decreases its proliferative potential, it is usually described as a progenitor cell. Progen-
Three types of pluripotent stem cell lines have been established from mammalian embryos: embryonic carcinoma (EC), ES, and embryonic germ (EG) cells (Figure 1). The origin of ES cells from the preimplantation embryo (ICM or epiblast) is the defining feature that distinguishes these cells from other pluripotent embryonic cell lines. When returned to the embryonic environment by transfer into a host blastocyst or aggregation with blastomere-stage embryos, cultivated ES cells behave like normal embryonic cells. They can contribute to all tissues of the resulting chimeras, including germ cells. Thus, ES cells can be reincorporated into normal embryonic development and have the full potential to develop along all lineages of the embryo proper. EC cells are mainly feeder-cell independent, whereas mouse ES and EG cells require cultivation on feeder layers (mouse embryonic fibroblasts or STO cells) or the addition of a differentiation inhibitor factor, ie, leukemia inhibitory factor (LIF). Under these conditions, mouse ES and EG cells maintain a relatively normal and stable karyotype and have an unlimited ability to self-renew. Several groups have recently isolated and cultured human ES (hES) and EG cells. This has led to the suggestion that specific cell lineages derived from hES cells will be useful for therapeutic purposes.

Mouse EC, ES, and EG cell cultures have been used to generate cardiomyocytes in vitro. The morphology, structure, and function of these cardiomyocytes have been extensively studied, and the application of genetic and genomic approaches has led to recent insights into the program of cardiac differentiation. hES cells can also differentiate to cardiomyocytes in culture, opening the possibility for controlled in vitro studies of developing human heart cells. The aims of the present review are to describe the in vitro process of ES cell differentiation into cardiomyocytes, discuss recent results from genetic and gene manipulation studies, and describe the promise of postgenomic analyses in defining the molecular events responsible for cardiac differentiation.

**Mouse ES Cell–Derived Cardiomyocyte Differentiation Program**

In vitro differentiation of ES cells normally (except for neurogenesis) requires an initial aggregation step to form structures, termed embryoid bodies (EBs), which differentiate into a wide variety of specialized cell types, including cardiomyocytes (Figure 2). A number of parameters specifically influence the differentiation potency of ES cells to form cardiomyocytes in culture: (1) the starting number of cells in the EB, (2) media, FBS, growth factors, and additives, (3) ES cell lines, and (4) the time of EB plating. Within the developing EB, cardiomyocytes are located between an epithelial layer and a basal layer of mesenchymal cells. Cardiomyocytes are readily identifiable, because within 1 to 4 days after plating, they spontaneously contract. With continued differentiation, the number of spontaneously beating foci increases, and all the EBs may contain localized beating cells. The rate of contraction within each beating area rapidly increases with differentiation, followed by a decrease in average beating rate with maturation. Depending on the number of cells in the initial aggregation step, the change in beating rate and the presence of spontaneous contractions continue from several days to >1 month. Fully differentiated cardiomyocytes often stop contracting but can be maintained in culture for many weeks. Thus, developmental changes of cardiomyocytes can be correlated with the length of time in culture and can be readily divided into 3 stages of differentiation: early (pacemaker-like or primary myocardial–like cells), intermediate, and terminal (atrial–ventricular–nodal–His–Purkinje-like cells).

During early stages of differentiation, cardiomyocytes within EBs are typically small and round. The nascent myofibrils are sparse and irregularly organized or lacking, whereas others contain parallel bundles of myofibrils that show evidence of A and I bands. Adjacent cardiomyocytes often show different degrees of myofibrillar organization. With maturation, ES cell–derived cardiomyocytes become elongated with well-developed myofibrils and sarcomeres.
and they contain cell-cell junctions consistent with those observed in cardiomyocytes developing in the heart. Subsequently, densely packed well-organized bundles of myofibrils can be observed, and the sarcomeres have clearly defined A bands, I bands, and Z disks. Nascent intercalated disks, fascia adherens, desmosomes, and gap junctions have also been observed, and the spread of Lucifer yellow to adjacent cells after microinjection argues for functionally coupled gap junctions. Overall, the length, diameter, area, ultrastructure, and myofibrillar architecture of ES cell–derived cardiomyocytes and sarcomere lengths with differentiation are similar to those reported for neonatal rodent myocytes.

ES cell–derived cardiomyocytes express cardiac gene products in a developmentally controlled manner. As in early myocardial development, mRNAs encoding GATA-4 and Nkx2.5 transcription factors appear in EBs before mRNAs encoding atrial natriuretic factor (ANF), myosin light chain (MLC)-2v, α-myosin heavy chain (α-MHC), β-myosin heavy chain (β-MHC), Na⁺-Ca²⁺ exchanger, and phospholamban (Figure 3A). Sarcomeric proteins of ES cell–derived cardiomyocytes are also established developmentally in the following order: titin (Z disk), α-actinin, myomesin, titin (M band), MHC, α-actin, cardiac troponin T, and M protein (Figure 3B; see review21). Cardiomyocytes with characteristics of fetal/neonatal rodent cardiomyocytes express slow skeletal muscle troponin I isoforms and a greater proportion of β-MHC versus α-MHC, whereas cardiomyocytes that more rapidly contract preferentially express cardiac troponin I and α-MHC. Thus, the appearance of cardiac-associated gene products is a function of differentiation time, similar to that seen in normal myocardial development.

Spontaneously and rhythmically contracting cardiomyocytes can be isolated and studied in single cell assays (see Figure 2). Early ES cell–derived cardiomyocytes have electrophysiological characteristics typical of primary myocardium (Figures 3C and 3D), whereas terminally differentiated ES cell–derived cardiomyocytes have electrophysiological characteristics that are typical of those found in postnatal cardiomyocytes (see reviews15,23). Terminally differentiated

Figure 2. ES cell–derived cardiogenic differentiation (adapted from Wobus et al14). When allowed to form cell aggregates, EC, ES, and EG cells spontaneously differentiate into cells typical of all 3 primary germ layers (endoderm, ectoderm, and mesoderm). Undifferentiated ES cells on primary cultures of embryonic fibroblasts (feeder layers) are cultivated as EBs in hanging drops for 2 days and in suspension for 3 to 5 days before being plated onto gelatin-coated tissue culture dishes. The morphology of 2-, 5-, and 7-day-old EBs by scanning electron microscopy (bar=50 μm) is shown. After differentiation, individual properties of ES cell– or EC cell–derived cardiomyocytes can be studied and assayed by using a variety of techniques, including RT-PCR (eg, β-tubulin, α-MHC, and MLC-2v), immunofluorescence (eg, anti–troponin T antibody), and electrophysiology (eg, action potential of 7±7 day cardiomyocyte). ES-derived cells in vitro can also be used to study embryogenesis, abnormal development, functional genomics, pharmacotoxicology, and embryotoxicity of potential teratogens.
cells also show pharmacological and physiological properties of specialized myocardial cells, including atrial, ventricular, Purkinje, and pacemaker cells. T tubules are not prominent. Functionally, these cardiomyocytes show normal contractile sensitivity to calcium and exhibit many features of excitation-contraction coupling found in isolated fetal or neonatal cardiomyocytes.23,24 Terminal, unlike early, ES cell–derived cardiomyocytes are responsive to /-adrenergic stimulation.25 Mouse EC and EG cells also have a high capacity to form cardiomyocytes in vitro. After induction with dimethyl sulfoxide, P19 EC cells form EBs, which contain a high percentage of cardiomyocytes.8 These cardiomyocytes spontaneously contract, have characteristics typical of early and terminally differentiated cardiomyocytes, and contain transcripts that are temporally expressed.8,26 Similar results have been observed with mouse EG-1 cells.11 Thus, the differentiation of mouse ES, EC, or EG cells within EBs represents a highly regulated culture system in which gene expression, myofibrillar architecture, and cellular ultrastructure and function are controlled in a manner analogous to that found in vivo cardiac development. The rapid in vitro differentiation of ES cells to cardiomyocytes, together with contractile and electrophysiological assays, provides investigators with a valuable model system for studying cardiac developmental processes, particularly when coupled with genetic manipulations.

Genetic Manipulation of ES Cells

Mouse ES cells are amenable to genetic manipulation, and mutation/insertion events can be studied in EBs or in isolated cells after in vitro differentiation (see reviews21,27). Random insertion of expression vectors into the mammalian genome
permits “gain-of-function” studies, whereas targeting events are most often associated with “loss-of-function” studies. Random insertion of DNA into single sites (gene trapping) in the genome additionally provides a strategy for functional genomics that has led to the identification of genes important to cardiac development. Genetic manipulation and in vitro differentiation of ES cells permit modification of the mammalian genome without concern for adverse effects on overall embryonic development.

**Gain of Function**

The overexpression of specific gene products via pronuclear injection to create transgenic mice is an established routine protocol. Gain-of-function studies, using ES cells, have also been successfully applied to models of cardiomyogenic differentiation. Grepin et al.,28 for example, successfully applied this technique to examine the role of the zinc finger cardiac transcription factor GATA-4 in P19 EC cells. In the absence of cell aggregation, GATA-4 overexpression induced subtle cellular changes, including downregulation of embryonic markers Oct-4 and SSEA-1. With cell aggregation, GATA-4 overexpression led to an increased abundance of mRNAs for transcription factors (Nkx2.5, MLP, and Mhox), contractile proteins (cardiac troponin C and β-MHC), and peptide hormones (brain natriuretic peptide) and accelerated the expression of terminal differentiation markers. Thus, GATA-4 contributes to the differentiation of mesodermal cells, activates the cardiac gene program, and may be a nuclear target for inductive and/or differentiation signals in committed cardiomyocyte precursors.

In vertebrate embryos, signaling molecules, such as bone morphogenetic protein (BMP)-2 or BMP-4, recruit mesoderm into the cardiac lineage. To study the role of BMPs in cardiomyocyte differentiation, Monzen et al.29 developed a P19 clonal line (P19CL6noggin) that constitutively overexpressed the BMP antagonist noggin.29 Noggin overexpression blocked gene activation of cardiac transcription factors and contractile proteins, preventing differentiation to beating cardiomyocytes. Cardiomyocyte differentiation could be rescued by overexpression of BMP-2, by the addition of BMP proteins to the culture, or by overexpression of TAK1, a member of the mitogen-activated protein 3-kinase superfamily that transduces BMP signaling. Monzen et al also demonstrated that simultaneous overexpression of Nkx-2.5 and GATA-4, but not Nkx-2.5 or GATA-4 alone, could induce cardiomyocyte differentiation of P19CL6noggin cells. These data, using in vitro differentiation systems, established a precise molecular role of BMPs in cardiomyocyte development, which until that time had largely been undefined.

Thus, overexpression studies coupled with in vitro analysis of ES cell–derived cardiomyocytes can be used to elucidate the role of transcription factors and signaling molecules on cardiomyocyte differentiation in a manner complementary to that used to study transgenic mice. However, clonal variance, promoter dependence, and the apparent ability of some ES cells to silence the expression of ectopically expressed transgenes can adversely affect data interpretation of genetically modified ES cells, requiring the isolation and analysis of multiple cell lines. Because of these experimental limitations, gain-of-function studies have not been widely used to study cardiomyocyte differentiation paradigms in vitro. However, gain-of-function experiments have proven invaluable for the selection of lineage-specific cells.

The use of specific growth conditions and genetic approaches can enrich the cardiomyocytes, but the percentage of differentiated cells belonging to any one phenotype in a heterogeneous population remains small. For cardiomyocyte differentiation, this percentage is often <3% to 5%. One of the major challenges has been how to isolate a pure population of ES cell–derived cardiomyocytes. Field’s group (Klug et al.9) first demonstrated that genetically selected cardiomyocytes could be isolated from differentiated ES cells. They prepared an ES cell line carrying a fusion gene composed of the cardiac α-MHC gene promoter and a neo8 cassette. With in vitro differentiation and selection with G418, 99.6% of the resistant cells were cardiomyocytes. We have had similar results with a pac8 resistance cassette and the MLC-2v promoter.21 ES cells expressing LacZ or a fluorophore have also been used to examine or isolate specific lineages of cardiomyocytes. Metzger et al.10 engineered a D3 ES cell line to regulate LacZ with the cardiac α-actin gene promoter, whereas Wobus et al.31 used the MLC-2v promoter. Separately, Kolossov et al.32 used the cardiac α-actin promoter to select ES-differentiated cells expressing the green fluorescent protein (EGFP). During early stages of in vitro differentiation, only cardiomyocytes expressed EGFP, which could be isolated by fluorescence-activated cell sorting. At later stages, noncardiomyocytes expressed EGFP. The ventricular-restricted MLC-2v promoter has also been used to direct enhanced cyan fluorescent protein and EGFP expression in ES cell–derived cardiomyocytes.33,34 The MLC-2v promoter constructs (0.25 and 2.1 kb) used in these studies were expressed exclusively in cardiac ventricular cells, making it possible to isolate and study relatively pure populations of ventricular cardiomyocytes from the heterogeneous mix of cells present in EBs. These ES cell clones represent a useful, but underused, system to study signaling pathways of myocardial lineage-specific cell proliferation and terminal differentiation. Importantly, purified populations of cardiomyocytes also may be suitable for regenerative cell therapies.19

**Loss of Function**

During early developmental stages, mammalian embryos can tolerate the addition or loss of embryonic cells. Techniques like homologous recombination that introduce targeted gene mutations have exploited this property to study protein function and developmental paradigms and, after transmission into the germ line, have been used to create hundreds of knockout (KO) animals. In principle, this technology may result in the inactivation of a specific gene function(s) that leads to (1) an abnormal phenotype, (2) no apparent phenotypic alteration, or (3) early embryonic lethality. In the latter case, ES cells containing mutations on chromosome pairs can be differentiated in vitro to generate cardiomyocytes.21,27 Use of this in vitro system has led to elucidation of the role of a number of signaling molecules/transcription factors, extracellular matrix (ECM) components, and calcium regulators on cardiac development.
**Signaling Molecules/Transcription Factors**

In vertebrate embryos, signaling molecules, such as BMPs, recruit mesoderm into the cardiac lineage. Similarly, LIF, a member of the interleukin-6 family of cytokines, is a growth and differentiation factor with pleiotropic activities that is involved in hematopoiesis, neuroectoderm, bone development, and acute inflammation. LIF is important for blastocyst implantation and stem cell self-renewal, and it is present in embryonic and neonatal cardiomyocytes. On the basis of the in vitro differentiation model with targeted cells, LIF has been found to affect commitment and differentiation of cardiomyocytes in a conflicting manner. In developing EBs between days 0 to 4, the presence of diffusible LIF inhibits mesoderm formation and commitment of cardiomyocytes. Differentiation of cardiomyocytes, but not commitment, is also severely hampered by the absence of LIF. The latter was shown in LIF-deficient and LIF receptor–deficient ES cells. Low concentrations of LIF in KO cells can also rescue the onset of cardiac differentiation, whereas higher doses can attenuate cardiomyocyte differentiation. Using these LIF-deficient cells, Bader et al subsequently found a role for parietal endoderm-derived factors in the differentiation of cardiomyocytes, underscoring not only how the use of the in vitro differentiation model, coupled with genetic manipulation, can elucidate the role of specific signaling molecules but also how new factors important for developmental processes can be identified.

Cripto-1 is a growth factor containing an epidermal growth factor (EGF)-like motif that is expressed in trophoblasts and in the ICM of mouse blastocysts, and it subsequently becomes restricted to developing myocardium. Mouse embryos deficient in Cripto-1 (tdgf) are embryonically lethal. In vivo, inactivation of Cripto-1 prevents the expression of cardiac transcripts for α-MHC, β-MHC, MLC-2a, MLC-2v, and ANF, suggesting that formation of precardiac mesoderm in vivo is defective. Loss of Cripto-1 in ES cells does not adversely affect differentiation of mesenchymal, endodermal, and ectodermal cells, and similar to the in vivo situation, inactivation results in an absence of MLC-2v, MLC-2a, and MHC gene expression. However, several critical cardiac transcription factor transcripts (Nkx2.5, GATA-4, and MEF2C) are present in the in vitro system, implying that Cripto-1 may act as a master gene regulator for progression of mesoderm to form functional myocardium.

In vitro analyses of GATA-4 and HAND1-deficient ES cells have led to novel insights into the functional role of transcription factors during cardiomyogenesis. GATA-4, together with GATA-5 and GATA-6 transcripts, is present in the precardiac mesoderm and subsequently in the endocardial and myocardial layers of the heart tube and developing heart. Developing mice lacking GATA-4 die in utero because of myocardial defects, suggesting an absolute requirement of GATA-4 for heart development. In contrast, GATA-4–deficient ES cells exhibit only a slight inhibition of cardiac differentiation in vitro; differentiating cardiomyocytes retain the capacity to develop sarcomeres and junctional complexes. When injected into embryos at the 8-cell stage, GATA-4–deficient cells contribute to all primary cardiac tissues (endocardium, myocardium, and epicardium) and express cardiac-specific transcripts; therefore, GATA-4 alone is not essential for cardiomyocyte differentiation. Other GATA-binding proteins (GATA-5 and GATA-6) may promote cardiomyocyte differentiation or compensate for the loss of GATA-4, and the embryonic lethality may arise from the failure of the developing myocardium to fuse at the midline. Similarly, in ES cells, inactivation of HAND1, a member of the hand basic helix-loop-helix family expressed during initial stages of cardiogenesis, has no dramatic effects on cardiac differentiation. In vivo, heart development of HAND1 KO animals does not progress beyond the cardiac-looping stage and results in embryonic death between days 8.5 and 9.5. When included in chimeric mice, HAND1-null ES cells differentiate into beating cardiomyocytes that express cardiac-specific genes (Nkx2.5, α-actin, MLC-2v, and MLC-2a). Although these cells are underrepresented in regions of the developing heart, KO cells still contribute to heart formation. Therefore, HAND1 is not essential for the differentiation of ventricular cardiomyocytes, although other transcription factors may compensate for its loss.

**Cytoskeleton/ECM**

Proper embryonic development of cardiac muscle depends on the structural integrity of the early heart tube and interactions between ECM proteins and contractile proteins. Specifically, ECM proteins can regulate cell growth and differentiation via mechanically and biochemically regulated pathways using cell surface receptors and cytoskeletal proteins. Recent studies involving ES cells deficient in desmin, collagen, and β1-integrin indicate that these proteins, like transcription factors, are not always required for normal in vitro differentiation to cardiomyocytes. The intermediate filament and cytoskeletal protein desmin is muscle specific. According to in vitro studies with desmin-null ES cells, desmin is essential for skeletal and smooth muscle formation, but cardiomyocyte commitment and differentiation are largely unaffected by its absence. Reduced desmin expression in EBs heterozygous for the desmin mutation leads to a partial inhibition of cardiac muscle formation. These findings indicate that an intermediate filament protein like vimentin might compensate for the loss of desmin; however, the observation that only a reduced level of desmin in heterozygous-deficient ES cells interferes with early cardiac differentiation suggests that a certain threshold of desmin may abrogate any potential compensation. Collagen α1(I) KO animals die because of blood vessel rupture at day 13, but remarkably little is known about the hearts of these mice. By differentiating homozygous inactivated collagen α1(I) ES cells (R. Fassler, unpublished data, 1999) in vitro, Ding observed an increase in the number of beating cells and a decreased beating frequency in mutant cardiomyocytes at early stages. These differences were not observed at terminal stages. A delayed structural organization of collagen type 1 fibrils in mutant EBs and of myofibrils in collagen α1(I)–deficient cardiomyocytes was also seen, but only at early stages; however, the loss of collagen α1(I) did not influence α-MHC, β-MHC, ANF, or MLC-2v mRNA levels. The modest effect of lost collagen α1(I) function during early stages of differentiation and the absence of an...
abnormal cardiac phenotype in α2(I)-deficient animals may indicate a compensatory function of collagen fiber subtypes during cardiac differentiation.

Embryos lacking β1-integrin die shortly after implantation. Loss of β1-integrin in differentiating ES cells delays cardiac differentiation, delays expression of the cardiogenic specific gene α-MHC and β-MHC, and alters action potential parameters (Figures 4A and 4B). β1-Integrin-deficient cells transiently express ANF and MLC-2v (primarily in specialized cells of the atrium and ventricle) and show incomplete and disarranged sarcomeric architecture. Only cells exhibiting high-frequency pacemaker-like action potentials and arrhythmias survive. Altered cellular distribution of α--integrins and upregulation of β1-integrins correlate with growth and survival of embryonic cardiac pacemaker-like cells at early differentiation stages; however, integrins of the α family are unable to functionally compensate for the loss of β1-integrin during terminal differentiation. Therefore, β1-integrin is required for differentiation, cardiac specialization, and the maintenance of specialized cardiac phenotypes.

Intracellular Calcium

The in vitro differentiation model has only recently been used to examine the role of calcium-handling proteins on cardiomyocyte differentiation. Calreticulin affects many cellular functions, including the regulation of calcium homeostasis and the synthesis and transport of ion channels, surface receptors, integrins, and transporters, and it affects cell adhesion via interaction with cytoplasmic domains of integrin receptors. Loss of calreticulin in vivo impairs cardiac development and increases apoptosis of cardiomyocytes. Similarly, calreticulin-deficient ES cells in vitro show impaired adhesion and decreased cardiac differentiation. Specifically, the number of EBs exhibiting contractile activity was reduced in calreticulin-deficient cells. Because of the diversity of cellular functions, the mechanism of calreticulin on cardiac development remains open, and further in vitro analyses will be required.

The cardiac ryanodine receptor (RyR2) serves as the major sarcoplasmic reticulum (SR) calcium-release channel to mediate a rapid rise of cytosolic free calcium. It is expressed early in developing myocardium, but a functional KO of this gene in mice leads to embryonic lethality at ~10 days post coitum (dpc) that is due to a potential calcium leak dysfunction. From KO ES cells, we have shown that RyR2 is a functionally active channel in early myocardial cells and that it modulates the beating rate. In the absence of RyR2, ES cells readily differentiate to cardiomyocytes, but the increase in the beating rate normally seen with time is markedly depressed (Figures 4C and 4D). KO myocytes show a slowing of the rate of spontaneous diastolic depolarization and an absence of calcium sparks. Therefore, loss of RyR2 does not prevent differentiation of ES cells to cardiomyocytes, but a functional channel is essential for the acceleration of diastolic depolarization that leads to an increase in beating rate that normally occurs with differentiation, potentially involving a calcium-release mechanism similar to the one found in adult pacemaker cells. Why then do RyR2-deficient (RyR2−/−) mice die at ~10 dpc, when loss of RyR2
neither prevents ES cells from differentiating into cardiomyocytes nor leads to cardiac myocyte cell death in vitro? With development, the heart rate increases to enhance cardiac output, which is required to meet the growing metabolic needs of the developing fetus. Coordinate contractions between the pacemaking and the outflow regions of the developing heart are also critical for continued embryonic development. Results from the RyR2/ES cell–derived cardiomyocytes suggest that a loss of SR calcium release by RyR2 prevents an increase in heart rate, resulting in inadequate blood perfusion, and embryonic death.

Genomic Analyses

Gene Trapping

The random insertion of exogenous DNA into single sites in the mammalian genome (gene trapping) provides a genome-wide strategy for functional genomics. This strategy is based on the introduction of gene-trap vectors (eg, a resistance gene [eg, neoR] and a promoterless lacZ reporter with a splice acceptor consensus sequence at its 5′ end) into ES cells.60,61 Resistant ES cell colonies are selected and expanded in vitro, and undifferentiated cells are injected into blastocysts to generate chimeric mice. Expression of the gene trap is assayed by β-galactosidase staining and is indicative of an insertion event within a transcriptional unit. In mice, gene-trap screens permit identification of genes that are expressed within specific tissues in a spatiotemporal pattern. One limitation of the in vivo gene-trap approach is the necessity to generate a large number of mice from ES cell clones to identify a limited number of developmental genes.

When performed in tandem with the developmental potential of ES cells to differentiate into distinct cell lineages, gene trapping facilitates the identification of genes expressed developmentally. In vitro differentiation of ES cells can also be used for the selection of tagged genes that are expressed in specific embryonic tissue.62 Elimination of ES cell clones either with mutational events in ubiquitously expressed housekeeping genes or with insertional events that do not activate the transgene precludes generation and examination of noninformative chimeric embryos. Thus, ES cell cultures provide a simple model system for studying the genetic pathways that regulate embryonic tissue development and permit high-throughput screening of clones for tissue-restricted gene-trap expression.63

Several groups have successfully used this system to identify novel retinoic acid responsive, neuronal, hematopoietic, and cardiovascular genes and to study mechanisms regulating specification and commitment of various cell and tissue types (Table).64,65 These studies underscore how gene-trapping techniques, in conjunction with in vitro and in vivo assays, can be used to identify important developmental genes associated with cardiac differentiation.
Principal steps of SAGE (adapted from Boheler et al. ES Cell – Derived Cardiomyocytes). A, To generate sequence tags, purified mRNA was used to generate double-stranded cDNA. By use of streptavidin-coated magnetic beads, the double-stranded cDNA was purified, followed by digestion with a type I restriction enzyme or anchoring enzyme (AE) that recognizes specific DNA recognition sequences (CATG for NalI). Magnetic beads were used to purify the fragments nearest the biotinylated primer; these fragments were equally divided and ligated to 2 unique linkers (A and B). Type II restriction enzymes or tagging enzymes (TEs) were used to generate SAGE tags 9 to 10 bp away from the recognition site. The SAGE tags were joined to form ditags and purified by PCR with a set of primers that recognize linkers A and B. Unique ditags were equally divided and ligated to form concatemers of purified ditags. The concatemers were cloned and sequenced. Individual tags can be extracted from the sequence by identifying CATG sequences. Each individual tag sequence was compared with GenBank databases to identify corresponding gene products. B, Comparison of normalized tag frequencies in P19 SAGE libraries, corresponding to undifferentiated P19 cells and 2 time points after EB formation in the presence of dimethyl sulfoxide (DMSO), is shown. The times chosen (days 3 and 0.5) reflect early steps in the differentiation process that ultimately yield high numbers of beating cardiomyocytes at day 3+4.0. On the left, undifferentiated and day-3+0.5 P19 cells contained a total of 115 935 tags. Of the 36 508 unique tags, these, 1388 increased and 3302 decreased by 2-fold at day 3+0.5. On the right is a comparison of day-3+0.5 P19 SAGE library time points. The libraries contained a total of 104 005 tags, of which 33 988 were unique. Among these, 1388 increased and 3302 decreased by >2-fold at day 3+3.0. C, Shown are selected gene transcripts and tag representation in the SAGE libraries. The 10-bp tag from selected transcripts identified in the SAGE library and the corresponding gene name are shown. The relative expressions of corresponding tags (in tags per million [tpm]) are shown, many of which were highly regulated in vitro differentiation.

Transcriptome Analyses

From the foregoing discussion of in vitro differentiation assays, we have shown how cardiomyocyte differentiation is affected by signaling molecules and transcription factors, by interactions with ECM and structural proteins, and by molecules controlling calcium homeostasis. Although many growth regulators have been described, it is likely that more key genetic regulators of cardiac development remain to be identified, many of which may not be cardiac specific. Because in vitro differentiation of embryonic cells (gene expression, morphology, and function) proceeds as a developmental continuum, as described earlier, it is logical to assume that this model system must express key regulators (known and unknown) of cardiac development.

From sequencing of the human and mouse genomes, genomics has provided a number of tools (cDNA clones, sequences, and sequence diversity) that can be used to examine gene expression profiles in a global context. Molecular methods to analyze transcript abundance include sequencing of expressed sequence tags, subtractive hybridization, differential display, competitive polymerase chain reaction (PCR), cDNA or oligonucleotide microarrays (chips), and serial analysis of gene expression (SAGE) (see review66). Although microarrays (hybridization-based) or SAGE (sequencing-based) permit high-throughput transcriptome (identity and quantity of all mRNA transcripts) profiling, only SAGE currently has the potential for assaying the entire cellular transcriptome (Figure 5A).

We have recently used SAGE to examine P19 EC cells induced to differentiate to cardiomyocytes (Figure 5B).67 The SAGE catalogues from the P19 libraries contain a large number of gene products associated with muscle development, including transcription factors such as GATA-4, HAND1, M-twist, and Msx-1, and structural proteins, such as smooth muscle α-actin, which is normally present in mouse heart at 8.0 dpc. The latter was highly expressed in differentiated P19 cells at day 3+3. Tags specific to the cardiac troponin isoforms (T, C, and I) were expressed in only 1 to 6 copies/library in committed and differentiated P19 cells, but none was detectable in undifferentiated P19 cells (Figure 5C). Similarly, tags for myosin alkali light chain and the SR Ca2+-ATPase were detectable (1 to 3 tags/library) only in RNA isolated from differentiating cells.

In the present study, we identified a number of differentiation-responsive growth regulators that are also present in fetal and adult hearts. Growth regulators that had not previously been associated with cardiomyocyte differentiation include insulin-like growth factor–binding protein 5, a protein with diverse roles in development and an essential regulator of physiological functions; high-mobility group I protein C, which is involved in chromatin organization to mediate the effects of transcription factors, such as signal transducer(s) and activator(s) of transcription 3; podocalyxin,
a sialoglycoprotein involved in hematopoietic cell differentiation; pleiotrophin, a heparin-binding growth factor cytokine thought to modulate angiogenesis, neurogenesis, cell migration, and mesoderm-epithelial interactions; and Dlk-1, an inhibitor of adipogenesis that also regulates embryonic pancreas formation. Many other differentially regulated growth factors and transcription factors are present in the SAGE catalogues, but extensive analyses are required to determine any eventual role in cardiac development. The results of the present study primarily illustrate how transcriptome analyses coupled with in vitro differentiation can reduce the time necessary for the identification of “transcriptionally” regulated genes. The gene predictions made from this analysis provide a reference point to understand the in vitro differentiation process, the complexity of gene interactions, and the role of specific genes in promoting cardiomyocyte phenotypes.

**Pluripotent hES Cells**

In 1998, Thomson et al reported the derivation of ES cell lines from human blastocysts obtained from human eggs fertilized in vitro. hES cells have a number of characteristics that are similar to, but distinct from, mouse ES cells. hES cells require an embryonic fibroblast feeder-cell layer for culture, and proliferation depends on medium conditioned by mouse feeder cells. In contrast to the mouse, the feeder layers for hES cells cannot be replaced by LIF alone. Thus, the critical factors that maintain pluripotentiality of hES cells remain unknown. hES cells grow as compact flattened colonies with distinct cell borders and express a number of stage-specific embryonic antigens, SSEA-3 and SSEA-4, as well as TRA-1-60, TRA-1-81, and alkaline phosphatase. SSEA-1 is expressed only upon differentiation.

Similar to mouse cells, hES cells differentiate when they are removed from feeder layers and grown in suspension. EBs of hES cells are heterogeneous, and they can express markers specific to neuronal, hematopoietic, and cardiac origin. The differentiation of hES cells into EBs or into teratomas that form derivatives of all 3 germ layers after injection into severe combined immunodeficient beige mice remains spontaneous and uncontrolled; however, specific growth factors can affect this process. Activin-A and transforming growth factor-β1 mainly induce mesoderm; retinoic acid, EGF, BMP-4, and basic fibroblast growth factor (FGF) activate ectodermal and mesodermal markers; and nerve growth factor and hepatocyte growth factor promote differentiation into endoderm, ectoderm, and mesoderm. Cardiomyocytes are prominent in cultures supplemented with hepatocyte growth factor, EGF, basic FGF, and retinoic acid. None of these factors direct ES cell differentiation exclusively to a single cell type. These data indicate that hES cells have at least some important characteristics in common with mouse ES cells. In contrast, human EG cells do not differentiate into cardiomyocytes, because they represent partially differentiated (EB-derived) cell lines that are not as pluripotent as ES cells.

Kehat et al have studied cardiomyocytes from hES cells. Cardiomyocytes were identified by the appearance of spontaneous contractions in EBs formed in suspension culture and plated. The median time to observe spontaneous contractions was 11 days after plating, and only 8.1% of the EBs contained beating areas, composed primarily of small mononuclear cells with round or rod-shaped morphology. As with mouse ES cells, adjacent myocytes displayed various degrees of myofibrillar organization, but in general, more highly organized sarcomeric structures were observed in EBs at later stages. The average beating rate was 94 ± 3 bpm, and electrophysiological measurements were typical of early cardiomyocytes. Importantly, these cells displayed chronotropic responses after administration of adrenergic and muscarinic agonists and expressed a number of cardiac-restricted gene products and transcription factors. Thus, the hES cell–derived cardiomyocytes exhibited structural and functional properties consistent with early-stage cardiac tissue.

hES cells provide a renewable resource for studying the mechanisms underlying human development. Establishment of the in vitro model of cardiomyocyte differentiation will, as with mouse ES cells, prove useful in the study of cardiac development and function. Specifically, questions related to human cardiac lineage commitment, formation of cardiac structures, development of regulated contractile function, and the molecular signals involved in the regulation of these events can now be addressed. Whether hES cells are amenable to genetic manipulation remains unresolved, but the description of these attributes represents the first steps in the study of human cardiomyocyte differentiation.

Although hES cells have broad implications for basic research, fundamental questions remain as to whether these cells will ever be useful in cell replacement therapy. Similar to that described by Odorico et al, 5 goals must be achieved before hES cells can be used in cardiomyocyte replacement therapies for humans. Investigators must (1) purify specific cell lineages (eg, ventricular, atrial, or pacemaker cells), (2) demonstrate that differentiation of cardiomyocytes function in a normal physiological way in vivo, (3) demonstrate the efficacy of transplantation in rodent and large animal models of heart disease, (4) ensure against the formation of ES–derived tumors, and (5) prevent immunological rejection. These goals are laden with obstacles that will ultimately require integration of molecular biology, cell biology, immunology, tissue engineering, transplantation biology, and clinical expertise.

**Conclusions and Perspectives**

In summary, studies of pluripotent mouse ES cells have led to in vitro models of cardiomyocyte differentiation. Morphological, electrophysiological, and molecular techniques indicate that the in vitro differentiation process recapitulates the developmental pattern of early cardiogenesis, and genetic studies have shown how signaling molecules, transcription factors, ECM components, and calcium-handling proteins affect this process. Genomic studies, coupled with in vitro differentiation, have also led to the identification of developmentally regulated genes (gene trapping) and to the identification of differentiation-responsive genes (SAGE). In conclusion, much of what we know about the differentiation of pluripotent ES cells to cardiomyocytes in vitro has been learned from studies with mouse ES cells. These cells have
been and will continue to be the subject of many future research endeavors, and much of what we learn will be applicable to the study of hES cells.

hES cells represent a renewable resource for studying the mechanisms underlying human development. With the cultivation of hES cells that can generate cardiomyocytes, the first steps toward the study of human cardiomyocyte development and gene replacement therapy have already been taken. However, it remains unclear whether hES cells will be as versatile as their mouse counterparts regarding self-renewal, genetic manipulation, or developmental capacity. Clinically, it is also unclear whether pure cell populations of hES cell–derived cardiomyocytes can be readily isolated in sufficient quantities for therapy or whether these cardiomyocytes can integrate and function appropriately in the heart after transplantation.

Finally, recent data indicate that adult stem and progenitor cells are able to produce differentiated cell types of other tissues or organs. The mechanisms resulting in transdifferentiation are not yet understood and may be influenced by epigenetic or genetic modifications, including spontaneous cell fusions. The identification of signaling molecules that regulate ES cell self-renewal, pluripotentiality, and differentiation to specific subtypes should promote our understanding of these processes in adult stem and progenitor cells. Thus, by continuing basic human and nonhuman embryonic and adult stem cell research and comparing gene expression patterns and phenotypes, the future application of human stem cells (ES, adult stem, and progenitor cells) in clinical settings may become viable. The aim will be to use adult pluripotent cell populations to generate replacement cells (cardiomyocytes) that avoid ethical issues and bypass problems of cellular rejection. Adult stem cells may even replace the need for long-term medical treatments or heart transplantation; however, we believe that continued research with both mouse and hES cells is critical to achieve this goal.

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References