Perspectives Series: Molecular Medicine in Genetically Engineered Animals

In Vitro Differentiation of Murine Embryonic Stem Cells

New Approaches to Old Problems

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Introduction

The availability of embryonic stem (ES)[1] cell lines has revolutionized biology by enhancing our ability to manipulate the genome and by providing model systems to examine cellular differentiation. These lines, derived from the inner cell mass of preimplantation blastocysts (1, 2), exhibit three particularly useful properties. First, they are totipotent; ES cells maintained in culture remain competent to contribute to all tissues, including germ cells, after introduction into mouse blastocysts (3). Second, targeted mutations in ES cells may be created readily through homologous recombination (4, 5) and introduced into the mouse germ line to study their effects in vivo. Third, wild-type or mutant ES cells can differentiate in vitro to form a variety of cell types in a sequence that recapitulates the first stages of murine embryogenesis.

In vitro differentiation of ES cells offers a unique approach to examine events that occur during embryonic development and complements gene knockout studies in whole animals. Upon withdrawal of leukemia inhibitory factor and stromal contact, ES cells form embryoid bodies (EBs), spherical aggregates of differentiated cell types (Table I) which appear in a well-defined temporal pattern. Hematopoiesis within EBs has been studied most extensively (6–12), although vasculogenesis (6, 13, 14), myogenesis (6, 15–20), and development of neuronal-like cells (21) have also been observed.

ES cells give rise to multiple hematopoietic lineages in vitro

Erythrocytes, granulocytes, macrophage, mast cells, and megakaryocytes arise in developing EBs and are detected either by inspection of cytological preparations or identification of cell type–specific messenger RNAs ([6–11] and M. Weiss, unpublished data). B cell and γδ T cell lymphocyte precursors can be identified by characteristic cell surface markers and gene rearrangements in both EBs and populations of ES cells cultivated on stromal cell monolayers in the absence of leukemia inhibitory factor (10, 22, 23). Numerous hematopoietic cytokines are

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1. *Abbreviations used in this paper:* EB, embryoid bodies; ES, embryonic stem; KL, kit ligand.

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Table I. Cell Lineages Identified after In Vitro Differentiation of ES Cells

Cell lineage	Mode of identification	Notes
Mesoderm-derived		
Hematopoietic		
erythroid/myeloid	morphology; characteristic mRNAs and protein markers	pure myeloid colonies generated by replating EBs primitive and definitive erythroid progenitors recapitulate embryonic hematopoiesis
lymphoid	cell surface markers, T cell γδ receptor, Ig gene rearrangements	low level repopulation of B and T cell lineages after adoptive transfer of EB-derived progenitors
Vascular endothelial	morphology; characteristic cell surface markers; acetylated LDL uptake	associated with hematopoietic cells in blood islands of cystic EBs
Striated muscle		
myocardial	myosin and tropomyosin isoforms; morphology	spontaneous, rhythmic contractions observed in EB cultures ventricular chamber specification observed in EBs
skeletal	myosin and tropomyosin isoforms; myogenic bHLH proteins myoD and myogenin; morphology	temporal and spatial expression of different muscle markers correlates in developing EBs and embryos
Early embryonic mesoderm	brachyury mRNA expression	
Endoderm-derived		
Primitive endoderm (hypoblast)	collagen α IV mRNA expression	
Yolk sac visceral endoderm	α-fetoprotein and transferrin expression; cell surface markers	
Ectoderm derived		
Neuron-like	morphology, characteristic mRNAs and protein markers	neural differentiation enhanced by *trans*-retinoic acid

See text for references.

expressed within EBs; these probably support the blood cell formation which occurs independent of exogenous growth factors (except for those present in FCS) (7, 11, 24).

Examination of blood cell development has been facilitated by a "two-step" replating assay in which EBs are disaggregated into single cells and replated into methylcellulose cultures containing various cytokines. Precursor cells give rise to pure hematopoietic colonies that can be enumerated and analyzed (7, 11) (Fig. 1). This assay has been useful for defining the hematopoietic differentiation program in EBs and examining the developmental potential of ES cells containing targeted mutations in genes that are important for blood cell development (discussed below).

Early embryonic hematopoiesis is recapitulated in EBs

Murine embryonic hematopoiesis is a dynamic process notable for the sequential emergence of distinct populations of blood cell progenitors (24a). In the first wave of visible hematopoietic activity (day 7.5 of gestation) primitive erythrocytes appear in the blood islands of visceral yolk sac. These cells are nucleated and express predominantly embryonic-type globins. By midgestation (day 10), hematopoiesis shifts to the fetal liver where enucleate definitive erythroid cells expressing adult-type globins, as well as other lineages, are produced. Around the time of birth, hematopoiesis shifts for the last time, to the bone marrow and spleen. Pluripotent hematopoietic stem cells, once thought to arise solely from the yolk sac, appear to have an intraembryonic origin as well (25–27).

Hematopoiesis in EBs also occurs in distinct waves. In whole EBs, expression of the embryonic globin gene, βH1, precedes that of the adult form, β major (9, 11, 28). The two-step replating assay demonstrates that separate populations of erythroid precursors appear in a characteristic temporal fashion (11). Primitive erythroid precursors which arise at day 4 or 5 of EB development and then rapidly disappear yield small colonies that synthesize embryonic globins. Definitive erythroid precursors appear later and generate larger colonies that contain adult globins. These two precursor populations have distinct growth factor requirements: the former are erythropoietin responsive while the latter require epo and kit ligand (KL, steel factor) for full development, consistent with diminished requirements for KL in primitive as compared with definitive hematopoiesis in vivo (29).

Vascular development within EBs

Indirect evidence suggests that a common hematopoietic and vascular cell precursor, the hemangioblast, gives rise to yolk sac blood islands, the earliest sites of visible hematopoiesis (30–36). Within these blood islands, clusters of hematopoietic cells are surrounded by endothelial cells which are believed to eventually establish the yolk sac vasculature (33, 37–40). The close relationship between hematopoietic and vascular development is underscored by marked deficiencies of both cell types in embryos lacking the receptor tyrosine kinase Flk-1 (41). This phenotype may reflect a defect in the putative hemangioblast, or alternatively, a requirement for endothelial cells to initiate or sustain normal hematopoiesis.

In liquid culture, wild-type ES cells form cystic embryoid

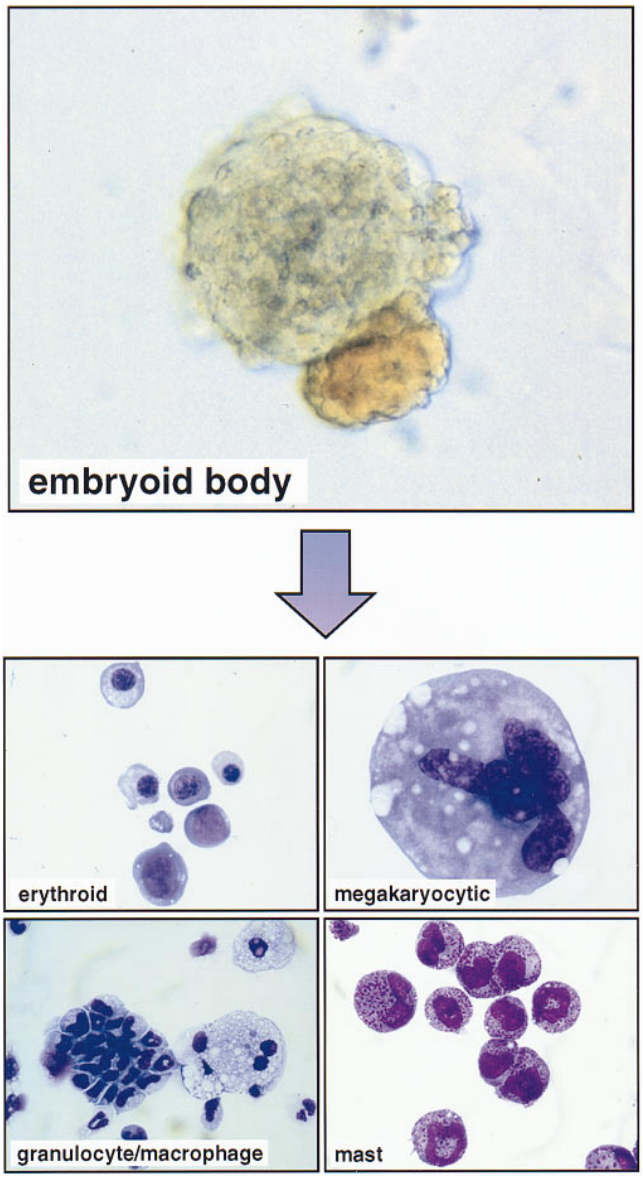

Figure 1. In vitro hematopoietic differentiation of ES cells. ES cells spontaneously differentiate in methylcellulose suspension cultures to form EBs, spherical structures that contain numerous differentiated cell types, including hematopoietic precursors. An EB with erythroid differentiation is displayed in the upper panel. Disaggregation of EBs into single cells and subsequent replating into methylcellulose medium containing cytokines results in the formation of pure hematopoietic colonies. Cytological preparations derived from several of these colonies are shown in the bottom panels.

bodies containing blood islands that are similar in structure to those of the embryonic yolk sac (6, 14). As such, EBs offer an experimental system to investigate the connections between early hematopoiesis and blood vessel development. In principle, it should be possible to isolate the elusive hemangioblast from EBs and demonstrate its bipotential nature by in vitro culture methods or reintroduction into the embryo.

In addition to initiating new blood vessel formation (vasculogenesis), EBs stimulate the growth of preexisting blood vessels (angiogenesis) in mice and on quail chorioallantoic membranes (13). Consistent with this observation, several angiogenic factors have been identified in differentiating ES cells (13).

ES cells form myocytes in vitro

In vitro myogenic differentiation in EBs is apparent with the initiation of spontaneous, rhythmic contractions after about 8 days in culture; structural features characteristic of myocardial cells are evident in such EBs (6). In addition, expression of cardiac-specific myosin heavy chain and tropomyosin genes are detectable at the onset of contractile activity (15, 16, 18). EBs contain distinct populations of cardiac myocytes including some that express a ventricle-specific form of myosin light chain (MLC-2V), suggesting that cardiac chamber specification may occur early in embryogenesis, before the formation of a heart tube, and independent of positional and hemodynamic stimuli (17).

Skeletal muscle cells also form in EB cultures as evidenced by the expression of specific myosin and tropomyosin isoforms, (17–19), functional nicotinic acetylcholine receptors (20), and characteristic staining with antibodies against myosin heavy chain (19). The noncardiac myogenic basic helix-loop-helix (bHLH) transcription factors myf5, myogenin, myoD, and myf6 are expressed in EBs in a sequence that reflects their temporal appearance in vivo (20). The observations that myogenic commitment and early muscle cell formation (both cardiac and noncardiac) occur in vitro, suggest that EBs will provide a useful model to examine the molecular events that govern these processes.

In vitro approaches to gene knockout studies

Methods are available to inactivate both alleles of a single gene in ES cells (42, 43). In vitro differentiation of resultant homozygous mutant ES cells can provide information to complement and extend gene knockout studies in animals. This approach has been particularly useful for defining the effects of targeted mutations in genes controlling blood cell development.

ES cells lacking the transcription factor GATA-1 fail to contribute to mature erythroid cells in mice (44). Consistent with these findings, in vitro differentiation of GATA-1$^-$ ES cells demonstrates a complete block to primitive erythropoiesis (45, 46). In contrast, GATA-1$^-$ EBs contain definitive erythroid precursors which give rise to colonies that initially appear normal but subsequently deteriorate due to developmental arrest and apoptosis of resident proerythroblasts. These studies demonstrate that GATA-1 is dispensable for early development of the definitive erythroid lineage but is required for survival of late, committed precursors (46, 47). Reintroduction of GATA-1 into the mutant ES cells restores erythropoiesis (45). Moreover, various mutant forms of GATA-1 and related proteins may be expressed in GATA-1$^-$ ES cells (48) to study their ability to rescue the mutant phenotype. These latter experiments illustrate how the in vitro system can be used to establish structure/function relationships among proteins involved in cellular differentiation by providing a physiological context which is superior to that of heterologous cell lines.

Mice lacking the related transcription factor GATA-2 die of severe anemia during early embryogenesis. In vitro differentiation of GATA-2 null ES cells identifies blocks to both primitive and definitive hematopoiesis at the early progenitor or stem cell level and demonstrates that the KL-responsive de-

finitive lineages are most severely affected (49). Targeted mutations in the nuclear LIM domain protein rbtn-2 (Ttg-2) (50) and the transcription factor SCL/tal-1 (51) produce similar phenotypes in vivo: early embryonic death associated with complete absence of primitive erythropoiesis. Preliminary experiments indicate that ES cells lacking either SCL/tal-1 or rbtn-2 are unable to produce detectable blood cells in vitro (C. Porcher and S.H. Orkin, unpublished data; A. Warren, personal communication), suggesting a stringent requirement for these proteins at an early step in hematopoietic development.

In vitro differentiation has also been used to examine the functions of myogenic transcription factors. ES cells lacking myf-5 form skeletal muscle in vitro, a finding consistent with in vivo observations in myf-5 knockout mice. However, derepression of myoD in ES cell-derived myf-5^{-} myocytes indicates partial functional overlap and crossregulation among these bHLH proteins (19).

Enhanced experimental entry into early embryonic hematopoiesis

In vitro differentiation of murine ES cells generates relatively large numbers of early embryonic hematopoietic cells and their immediate precursors which can be isolated and manipulated for further study. The induction of hematopoietic progenitors from embryonic mesoderm, and their subsequent maturation, is influenced by cell-intrinsic properties, as well as by environmental cues. For example, bone morphogenetic protein-4 (BMP-4) induces ventral-type mesoderm formation, including blood cells, in Xenopus embryos (52, 53). Investigators have used ES cells to identify and characterize potential growth and differentiation factors which modulate murine embryonic blood cell development. Differentiation of ES cells in a serum-free system demonstrates that BMP-4, but not several other related TGF-β family members, induces hematopoietic cells from primitive mesoderm in EBs (54). NIH-3T3 cells produce a soluble factor that acts synergistically with known cytokines to enhance the production of hematopoietic CFU in ES cell–derived cultures (55); it is unknown whether this factor acts on prehematopoietic mesoderm or on committed hematopoietic cells. Thus, ES cells can be used to test candidate growth factors for initiation and support of embryonic hematopoiesis. EBs themselves also offer a potential source for new cytokines that stimulate primitive and/or definitive hematopoiesis.

Developmental relationships between different embryonic hematopoietic lineages may also be analyzed using EBs. For example, it is unclear whether primitive and definitive hematopoietic lineages arise from a common, committed hematopoietic stem cell, or independently from different areas of primitive mesoderm. If a bipotential cell capable of specifying both primitive and definitive hematopoiesis exists, it should be feasible to isolate and characterize this precursor from early EBs.

Problems for future research

The in vitro developmental potential of ES cells has not been fully explored. While many differentiated cell types form within EBs, their ability to arrange into three-dimensional structures resembling organs is limited. However, some cellular patterning does occur as evidenced by the formation of blood islands in cystic EBs (discussed above). Organization of cells into higher order structures is affected by mechanical and biophysical stresses (56) including those present in the uterine environment. External influences may alter in vitro differentiation of ES cells as well. For example, arrangement and polarization of epithelial cells is modulated by interactions with contact surfaces, extracellular matrix, and soluble factors (56, 57). Therefore, it may be possible to form rudimentary epithelial tissues by culturing EB-derived cells on various substrata.

Attempts to reconstitute the hematopoietic system of mice from ES cell–derived hematopoietic stem cells generated in vitro have been surprisingly unsuccessful; contribution has been limited to the lymphoid system in numerous transplantation assays (12, 58, 59). This difficulty may reflect fundamental differences between embryonic and adult hematopoietic stem cells. For example, hematopoietic stem cells derived from EBs might be unable to survive in an adult environment due to lack of appropriate growth factors or stromal interactions. Alternatively, these cells may require further maturation in situ to attain the capacity for long-term repopulation.

Conclusions

Mechanisms that dictate cell fate determination during development have been under active investigation for years. Historically, these problems have been addressed in more primitive species, in part because of experimental constraints imposed by the use of early mammalian embryos. These processes are now accessible through in vitro differentiation of murine ES cells which permits embryonic cells undergoing early commitment decisions to develop in an extrauterine milieu that is amenable to manipulation. This approach has proven most valuable in examining early hematopoiesis, but also offers promise for the study of other developmental pathways. In principle, in vitro differentiation of ES cells can eventually lead to clinical applications by generating cells or tissues suitable for transplantation.

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