Mini-Review

Mouse Embryonic Stem Cell Differentiation into Multiple Tissue Types

Sharlene Faulkes, PhD | Scientist • Chris Lannon, PhD | Scientist

SUMMARY

Mouse embryonic stem (ES) cells are permanent lines of undifferentiated pluripotent cells derived from early mouse embryos. They exhibit two important properties: the ability to self-renew and give rise to new pluripotent ES cells, and the ability to differentiate into all specialized cell types found in the adult mouse. Research in the 1980's and 1990's focused on combining techniques for manipulating the mouse genome with ES cell technology to produce chimeric and knock-out mice for evaluating the function of mammalian gene products *in vivo*. More recently, advances in controlling ES cell *in vitro* differentiation are providing insights into the mechanisms involved in embryonic development, tissue lineage specification and the generation of cells for potential therapeutic applications.

CHARACTERISTICS AND CULTURE OF MOUSE ES CELLS

ES cells are isolated from the inner cell mass of pre-implantation embryos or blastocysts at day 3.5 of mouse development. When mouse blastocysts are cultured on a feeder layer of mitotically inactivated embryonic fibroblasts, the outer layer of cells attach and undifferentiated cells from the inner cell mass (ICM) spontaneously form clumps that can be further passaged to yield ES cell lines.[1,2] These cells are considered pluripotent as they can be maintained indefinitely in the undifferentiated state in culture and have the ability to contribute to all of the tissues, including the germ cells, when injected back into mouse blastocysts.[3] Maintenance of ES cells *in vitro* is achieved by co-culture on irradiated mouse fibroblasts[2] or on gelatinized dishes with a differentiation inhibitory factor called Leukemia Inhibitory Factor (LIF).[4]

In addition to LIF, a number of other extrinsic and intrinsic regulators of self-renewal have been identified (for review, see Chambers and Smith).[5] It has recently been reported that Bone Morphogenetic Protein 4 (BMP-4) can replace the requirement of serum during ES cell line derivation and maintenance through suppression of neural differentiation.[6] The secreted Wnt proteins may also play a role in maintaining pluripotency, as Wnt signaling is down-regulated upon differentiation.[7,8]

The transcription factor Oct-4 (Oct-3, Oct-3/4, Pou5f1) is also required for regulation of cell fate in the early embryo; it is expressed in the ICM and down-regulated upon differentiation into trophoblast.[9] Induction of differentiation through withdrawal of LIF results in concomitant reduction in Oct-4 levels.[5] A second homeodomain-containing protein, Nanog, is expressed in the inner cells of a compacted morula and blastocyst (as well as early germ cells) and is down-regulated just prior to implantation. Nanog overexpression can maintain self-renewal of ES cells independently of the LIF/gp130/Stat3 pathway.[10,11] Other factors proposed to have a role in maintaining "stemness" include Sox2 (SRY-related HMG box 2) and FoxD3 (forkhead box D3).[5] Appropriate culture conditions and qualified reagents also play an integral part in retaining the pluripotency of ES cells.[12]

MOUSE ES *IN VITRO* DIFFERENTIATION INTO EMBRYOID BODIES

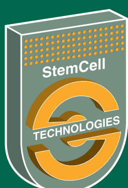
Upon withdrawal of LIF and stromal contact, mouse ES cells will spontaneously differentiate into complex, three dimensional cell aggregates called embryoid bodies (EBs). Differentiation within EBs occurs in a well-defined temporal manner with the initial formation of all three embryonic germ layers (ectoderm, mesoderm, endoderm) followed by further differentiation to terminally differentiated cell types, similar to *in vivo* embryogenesis.[12,13] Although a variety of cell types form during spontaneous differentiation, a heterogeneous mixture of cells are present and not all cell lineages (i.e. endoderm-derived) have been observed.

ES *IN VITRO* DIFFERENTIATION INTO DIFFERENT TISSUE CELL TYPES

Protocols have been developed to enrich and further expand the repertoire of tissue cell types generated following *in vitro* differentiation of mouse ES cells. A summary is presented in Table 1 (page 191). An enrichment strategy, termed genetic selection has been used to express specific fate-determining genes (e.g., *HOXB4* transcription factor implicated in definitive hematopoiesis[14]) or drug resistance markers under the control of lineage-specific promoters[15-17] in ES cells to generate relatively homogenous populations of mature cell types. For example, Marchetti *et al.* used the vascular endothelium specific promoter Tie-1 linked to an antibiotic resistant gene to derive a purified population of endothelial cells from ES cells. These cells could be selectively expanded in culture and following transplantation were incorporated into the neovasculature of nude mice.[15]

Selection of desired cell types can also be achieved through cell sorting on the basis of one or more specific cell surface antigens. Yamashita *et al.* used this technique to isolate ES cell-derived Flk1^{+} hematopoietic progenitors by fluorescent activated cell sorting (FACS). These cells were shown to act as vascular precursor cells capable of differentiating *in vitro* and *in vivo* into both endothelial and mural cells of the vasculature.[18] Therefore, cell enrichment strategies also facilitate the characterization of precursor cells, providing new insights into the cellular origins of cells in vascular development.

Selective media and culture conditions are also widely used to select a desired cell population and have been instrumental in the enrichment of neural cell types.[19-21] Brustle *et al.* used this approach to generate a population of multipotent progenitor cells that express glial precursor markers. Mouse ES cells were cultured in a medium that favors survival of neural precursors followed by



the sequential addition of various growth factor combinations [fibroblast growth factor-2 (FGF-2), FGF-2 and epidermal growth factor (EGF), FGF-2 and platelet-derived growth factor (PDGF)], resulting in cells that could be maintained for several generations in culture. Upon growth factor withdrawal, these cells differentiated into either of two specific lineages, oligodendrocytes or astrocytes.[21] Specific combinations of cytokines have also been used to promote the growth of other mature cell types including mast cells[22], hematopoietic cells[23] and dendritic cells.[24] For instance, using a combination of granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-3, Fairchild and colleagues generated long-term, stable cultures of dendritic cells from differentiating ES cells.[24] Chemical inducers, such as the Vitamin A derivative-retinoic acid, have also been widely used to enhance neural differentiation during ES *in vitro* differentiation.[25,26]

Induction of endoderm-derived cells during ES *in vitro* differentiation and particularly, insulin-secreting cells have been more difficult to obtain. Soria *et al.* applied genetic selection using the human insulin promoter fused to an antibiotic resistance gene to select cells which initiate synthesis of insulin.[16] Lumelsky *et al.* demonstrated the formation of insulin producing pancreatic-like islet structures from ES cells based on methods known to promote the generation of neural cell types from ES cells.[27] However the use of nestin as a dependable marker for evaluating pancreatic development is somewhat controversial,[28] and further studies are required to conclusively show that nestin-derived cells can or cannot generate cells of the pancreatic lineage. Blyszczuk and coworkers have recently described a multistep process for differentiation of insulin-secreting cells without the selection and induction of nestin-positive cells.[29]

A recent report demonstrates the production of another cell type from the endodermal germ layer. Ali *et al.* have demonstrated that ES cells can differentiate into Type II alveolar epithelial cells, which are needed for proper gas exchange in the lungs.[30] These preliminary results are very promising and may provide a model system to evaluate the possibility of using ES-derived cells to regenerate damaged lung tissue.

Angiogenesis is the formation of new blood vessels by the sprouting, splitting, and remodeling of existing vessels, and is of importance in many areas of research, including the studies of solid tumor growth and metastasis in cancer biology, wound healing, inflammation-related diseases, and cardiology. An ES-based system has been established for the study of angiogenesis.[31] Feraud *et al.* plated undifferentiated mouse ES cells in semi-solid medium containing angiogenic cytokines. Three days later, the EBs have developed branching sprouts from their centers, indicative of sprouting angiogenesis.

Promotion of tissue-specific *in vitro* differentiation has been achieved by co-culture of ES cells on different stromal cell lines. Numerous studies have used the OP9 stromal cell line for the generation of lymphoid cells (B and NK cells).[32-35] The OP9 cell line is derived from the calvaria of op/op mice that have a spontaneous mutation in the macrophage colony-stimulating factor (M-CSF) gene. This is significant because M-CSF promotes macrophage proliferation and inhibits ES cell differentiation to lymphoid cells *in vitro*.[32] Kawasaki and colleagues have also identified a stromal cell line, Pa6, which expresses neural inducing activity (termed SDIA for Stromal-cell Derived Inducing Activity). Co-culture of Pa6 and ES cells results in efficient neural differentiation and derivation of a high proportion (30%) of transplantable, dopamine-producing neurons.[36]

FUNCTIONAL STUDIES OF TRANSPLANTED MOUSE ES AND ES-DERIVED CELLS

Many studies show that transplanted ES and ES-derived cells are able to survive, integrate and, to some measurable extent, function within the host tissue. Bjorklund *et al.* transplanted small numbers of cells from disrupted day 4 EBs into a rat model of Parkinson's Disease, and obtained dopaminergic (DA) neurons which can reverse some symptoms of this disease.[37] However, although 56% of the animals showed surviving grafts containing DA neurons, 20% had lethal tumor-like masses called teratomas.[37] Another study also demonstrated "proof of concept" that ES cells can be directed *in vivo* to produce hepatocytes in response to endogenous signaling in adult animals.[37]

Before ES cells can be used therapeutically, methods must be developed to avoid formation of teratomas *in vivo*. The use of committed, terminally differentiated cells for transplantation, rather than ES cells themselves may reduce the incidence of tumors. For example, Yin *et al.* used a gene targeting approach to integrate the green fluorescent protein (GFP) gene into the locus of a fetal liver-specific protein, alpha-fetoprotein, in undifferentiated ES cells.[39] Following *in vitro* differentiation, GFP-positive cells were isolated by FACS and injected into haptoglobin-deficient mice. The authors detected low levels of haptoglobin suggesting that ES-derived cells are able to mediate liver specific functions *in vivo*. More importantly, in over 100 mice transplanted with the ES-derived differentiated hepatocytes, teratomas were not observed.[39] Other investigators using ES-derived oligodendrocytes[21,26,40] and DA neurons[36,41] report no evidence of teratomas, although long-term studies are needed to confirm these results. McKay and co-workers enhanced the generation of ES-derived DA neurons using growth factor manipulation and expression of the transcription factor nuclear receptor related-1 (Nurr1), which has a role in differentiation of midbrain precursors to DA neurons. Transplanted neural cells were shown to integrate and remain functional for up to 8 weeks, with no evidence of teratoma formation.[41]

Another important consideration for evaluating the efficacy of ES-derived cells is determination of the functional durability of the transplanted cells. One group found that a large proportion of mice receiving ES-derived insulin-producing cells developed hyperglycemia 12 weeks later, implying that the implanted cells had a limited *in vivo* life span.[16] Interestingly, Kyba *et al.* described an overexpression strategy with the transcription factor HOXB4, that has been implicated in self-renewal of definitive hematopoietic stem cells, combined with co-culture on the OP9 stromal layer to generate ES-derived hematopoietic stem cells. These cells were able to engraft lethally irradiated adults and contribute to long-term, multi-lineage hematopoiesis in primary and secondary recipients demonstrating long-term functionality of these cells.[14] This study is intriguing as it illustrates that ES-derived somatic stem cells, rather than terminally differentiated cells, may provide a suitable cell source for transplantation.

In summary, significant progress has been made in controlling the *in vitro* differentiation of ES cells into a wide variety of cell types. This provides a powerful model system to investigate molecular and cellular processes involved in lineage-specification and embryogenesis. Protocols based on the identification of genes and cell surface markers for somatic stem cells or lineage precursors may further improve the efficacy and safety of these strategies to provide functional cells for regenerative medicine.

TABLE 1 **Differentiated cell types derived from mouse embryonic stem cells *in vitro***

Embryonic Germ Layer	Cell Type	Reference(s)
Ectoderm (external layer)	neurons	19, 25
	dopaminergic neurons	20, 37, 41
	motor neurons	42
	oligodendrocytes	21, 26, 40, 43
	astrocytes	44
	epithelial cells	45
Mesoderm (middle layer)	adipocytes	46
	cardiomyocytes	47-50
	chondrocytes	51
	hematopoietic cells	23, 52 - 54
	hematopoietic stem cells	14
	dendritic cells	24
	mast cells	22
	B cells	32, 33, 35
	natural killer cells	33, 34
	lymphoid precursors	55
	endothelial cells	15, 18, 31
	osteoblasts	56
	striated muscle cells	57
	smooth muscle cells	18
	macrophages	58
Endoderm (internal layer)	pancreatic-like islets	27
	insulin-producing cells	16, 29
	alveolar epithelium	30
	hepatocytes	59

Embryonic stem cells differentiate *in vitro* into each of the three embryonic germ layers (ectoderm, mesoderm, endoderm) in a manner that recapitulates *in vivo* embryogenesis. These cells can subsequently be induced to differentiate into the majority of cell types of the adult mouse.

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