Chapter 2
Embryonic Stem Cells and Neurogenesis

Robin L. Wesselschmidt

Abstract The discovery that embryonic stem cells (ESCs) can be induced to differentiate into pure populations of neural stem cells (NSC) with the capacity to become neurons, astrocytes, and oligodendrocytes has given scientists and clinicians access to a previously unavailable pool of cells that are key to our understanding of neurogenesis. Since the early 1980s, when mouse ESCs were first derived, there has been a steadily increasing interest in developing methods that permit directed differentiation of ESCs in to specific desired cell types. The derivation of human ESCs and experiments showing functional recovery in animal models has greatly increased interest in applying hESC technology to the field of regenerative medicine. As methods improve and processes are developed that permit the production of high purity populations of desired differentiated cell types, the promise of ESC technology is likely to be realized through their use to treat injury and diseases such as Parkinson's disease, stroke, and spinal cord injury. This chapter will review developments in ESC technologies as they relate to neurogenesis.

Introduction

The derivation of human embryonic stem cells and the discovery that they can be directed to differentiate into specified functional cell types has greatly accelerated research efforts aimed at developing robust scalable methods that can be used to derive medically relevant cell types and launched the field of regenerative medicine [1]. Basic biological studies aimed at understanding the molecular and cellular processes that drive differentiation have been underway for decades.

R. L. Wesselschmidt (✉)
Center for Applied Technology Development,
Beckman Research Institute, City of Hope, Duarte, CA, USA
e-mail: robinwesselschmidt@gmail.com

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Human and mouse teratocarcinoma cell lines [4–6] and mouse ESC lines [7, 8] have been crucial to early development efforts in this area. The derivation of hESC lines and subsequent discovery that somatic cells can be induced to become pluripotent stem cells (PSC) [9], cells that seem to be very similar to hESCs, has further fuelled efforts aimed at defining the mechanisms that direct the differentiation of pluirpotent stem cells toward specific desired differentiated cell populations. Since PSCs are capable of extended self-renewal, unlike somatic stem cells, they provide an excellent starting material for the development of cellular therapeutic agents and well-characterized cells for use in toxicology and drug screens [10, 11].

Efforts aimed at the development of reproducible processes amenable to scale-up production and banking of progenitor populations under current good manufacturing practice (cGMP) are underway for several diseases and injuries including stroke, diabetes, macular degeneration, heart disease, and spinal cord injury. The basic biological studies led by John McDonald in the late 1990's that were [12, 13] aimed at differentiating mouse ESCs into neural progenitors and their subsequent transplantation into an adult rat model of spinal cord injury were key in the subsequent development of human ESC-derived oligodendrocyte progenitors and to the eventual use of these cells in a clinical trial to treat humans with spinal cord injury [14]. The mESC studies of McDonald were refined and methods were developed by Keirstead et al [15, 16] that directed the differentiation of human ESC to homogeneous populations of oligodendrocyte progenitors.

When the first mouse ESC lines were isolated, the genomic tools that make it possible to efficiently dissect the molecular pathways that sustain self-renewal or drive differentiation were not available. Combining these genomic tools with genetic and pharmacological manipulation of the in vitro ESC culture system, researchers now have the tools to identify therapeutically relevant pathways and apply this knowledge to the development of improved differentiation methods that permit the production of highly purified progenitor populations using defined culture conditions that may eventually lead to the development of drugs or treatment regimes that permit the specific activation of endogenous stem cell pools or precise intervention with the required mixture of cells at the appropriate developmental stage required to repair damaged and aging tissues or organs.

This chapter will focus on studies of ESC neurogenesis published since the last review published in this series in 2005 [17]. The primary focus will be on the developments in the field of human ESCs and neurogenesis.

Modeling Differentiation In Vitro with Embryonic Stem Cells

Background

ESCs are derived from the inner cell mass of blastocyst stage embryos. ESCs were first derived from mice in 1981 [18, 19], non-human primates in 1995 [20], and from human blastocysts in 1998 [1]. The earliest reports of in vitro differentiation

of mESC were made by Wobus [7] and Doetschman [8]. They showed that mouse ESCs could spontaneously differentiate into cell types derived from all three germ layers upon lifting from the feeder cells, on in which they were and in many cases are still grown, to growth in suspension in medium containing fetal bovine serum (FBS). Upon culture in suspension as spherical cell clusters called embryoid bodies (EBs), ESCs spontaneously differentiate into mixed populations of progenitor cells of three germ layers. EB differentiation has been shown to proceed in a relatively ordered and predictable fashion that recapitulates very early embryogenesis, with initial establishment of extra-embryonic endoderm and primitive ectoderm followed by differentiation into populations of cells that represent all three germ layers of the early embryo [21].

The early differentiation protocols described by Wobus and Doetschman are dependent on unknown factors present in fetal bovine serum. The percentage and type of cells produced by the EB induction method varies with the batch of serum, concentration of ESCs, and density of EBs in the culture dish. By the early 1990s mESC culture medium was supplemented with leukemia inhibitory factor (LIF), which was found to promote mESC self-renewal and limit differentiation in FBS containing medium [22]. Original protocols for derivation and cultivation of hESCs called for media containing FBS, LIF, and co-culture with mouse embryonic fibroblast feeder cells, the system that had been so successful for maintaining mouse ESCs in a pluripotent state. However, it was difficult to maintain hESCs in an undifferentiated state under mESC culture conditions. Amit et al. developed a culture medium containing serum replacer instead of FBS and basic fibroblast growth factor (bFGF) instead of LIF, which quickly became the standard for maintaining hESCs [23]. Cultivation of hESCs is much more labor intensive than mESC culture, due primarily to the difficulty in subculture and passaging them. hESCs do not subculture well in the standard medium of the day and are often subcultured by manual microdissection of colonies, and not using methods that will permit single cell dissociation. The size of the cell clusters used to initiate EB formation has been shown to play a role in the number and type of differentiated cells obtained [24] and can lead to variable results when initiating differentiation. Efforts to avoid the use of EB to induce differentiation are part of many development programs. Efforts to develop methods that permit single cell passaging and subculture hESCs continue. The identification of Rho-associated kinase (ROCK) inhibitor [25] has greatly improved the survival of hESCs when passaged using enzymatic cell dissociation methods. When ESCs are induced to differentiate via an EB intermediate, the dominate cell types, which relies solely on the EB microenvironment and FBS, are mesenteric in origin such as skeletal myocytes [26], vascular endothelia [27, 28], cells of the hematopoietic cell lineage [29], and cardiomyocytes [30, 31]. Importantly, when hESC lines are induced to differentiate via the same EB induction method (suspension culture in the presence of FBS without bFGF), the same recognizable cardiomyocytes and blood islands are produced [32].

The basic finding that ESCs can differentiate in vitro into cellular derivatives of all of the three embryonic germ layers has led to the development of protocols aimed at the differentiation toward desired cell types. At this time, most of these

protocols do not produce highly pure populations of terminally differentiated cell types. A high-level process map for neurogenesis from ESCs would include (1) undifferentiated ESC culture and scale up, (2) neural induction, (3) specification or enrichment for the desired cell type, and (4) analysis of the desired cell type [33, 34]. Efforts to scale these processes using cGMP-compliant methodology, including those for cryopreservation of a stable functional final cell product suitable for use in a clinical setting are underway and if successful may allow us to realize the therapeutic potential of hESCs.

Undifferentiated Embryonic Stem Cell Culture

Mouse ESCs are commonly maintained in co-culture with a MEF cell layer in medium containing FBS supplemented with LIF, which promotes self-renewal and limits spontaneous differentiation [21, 22]. This combination of MEF/FBS/LIF was used to derive the first hESC lines [1, 2], but quickly abandoned for a somewhat more defined system that replaces FBS with Knockout Serum Replacer™ (KSR, Invitrogen Inc.) and uses FGF-2 instead of LIF to maintain pluripotency and prevent differentiation [23]. The feeder cell/KSR/bFGF culture system is recognized as the standard method for the propagation of undifferentiated hESCs at this time. It is proven to permit the maintenance hESCs in a diploid pluripotent state for over a year of continuous culture [35]. Efforts to eliminate feeder cells from the culture system has led to the development of a number of newer culture systems. The first feeder-free, serum-free culture system for undifferentiated hESCs shown to stably support long-term hESC growth uses MEF-conditioned medium (MEF-CM) and Matrigel™, an extracellular matrix isolated from mouse sarcoma cell line [36]. The elimination of feeder cells was a major step forward in simplifying hESC culture, but the medium and substrata in this system remains undefined and comprised animal-derived components. As the demand for the development of cells suitable for therapeutic applications increases so has the number of defined and nearly defined culture systems. A concerted effort by the International Stem Cell Initiative (ISCI), a leading international group of hESC scientists, was the systematic evaluation of a number of defined and semi-defined culture systems [37]. Of all the culture systems evaluated at the time, two commercially available systems, proved most reliable across all the teams at the time [38, 39]. These systems mTeSR™ (Stem Cell Technologies, Inc.) and StemPro™ (Life Technologies, Inc.) have been adopted by many laboratories and have been used in the scale-up of undifferentiated hESCs for the production of cell banks numbering several hundred vials containing 1–4 million cells per vial. New methods for propagation of undifferentiated human PSC lines, a key and often under appreciated step in the scale up production of differentiated cells, continue to be developed. Variably in the starting cell population can lead to mixed populations and variable and unpredictable results during scale up differentiation that complicates manufacturing continue to be developed. A method being employed to eliminate one of the variables in the culture is suspension, thereby eliminating the need for substrate all together [40–42].

Another consideration for scale up is the transition of it is worth considering when moving cells from one culture system to another and the affect these changes may have on the cells, the long-term stability under newer or modified culture conditions, and what genetic and epigenetic changes may occur as cells are adapted to different culture conditions. The method of undifferentiated culture may affect how efficiently hESCs differentiate toward specific lineages and may need to be tested empirically to find the best undifferentiated scale up methodology to prepare or condition the cells for differentiation. The growth surface matrix, basal media, growth factors, and inhibitors, and oxygen tension, that comprise the undifferentiated growth conditions may affect the efficiency of differentiation towards specific lineages [43]. The methods used to propagate undifferentiated hESCs, the medium, ECM, method for subculture, how many times, and how many systems the cells have been exposed as well as the number of population doublings (passage number) may lead to epigenetic and cytogenetic instability or may prevent efficient differentiation until adapted to the differentiation culture system that primes them to develop into the desired differentiated cell population. The difficulty in replicating published results, especially the efficiency of differentiation, may be due in part to the inadvertent conditioning of the undifferentiated cells.

Neural Induction

ESCs can be induced to differentiate into the three principal neural cell types: neurons, oligodendrocytes, and astrocytes through neural stem cell (NSC) intermediate (see Tables 2.1 and 2.2). Efforts to define the process of neurogenesis have led to the identification of cellular predecessors to NSCs that define distinct cell types on the path to the NSC. Primitive NSC (pNSC) [44] give rise to rosette NSC (R-NSC) [45] giving rise to neuroepithelial progenitor cells (NEP), which can be patterned by exposure to small molecules or growth factors to differentiate into specified predictable neural cell types [46, 47]. Challenges remain with regard to stable expansion of the neural progenitor populations capable of retaining a normal karyotype and remaining responsive to patterning cues with prolonged culture and expansion at the progenitor stage. The use of expression profiling and small molecule screens have helped identify pathways that permit more efficient production of specific cell types. Futhermore, the development of defined culture conditions and the use of small molecules are leading to more predictable methods that are amenable to scale-up under cGMP. ESC neurogenesis can be viewed as a step-wise process with identifiable stem/progenitor populations. This is nicely illustrated by the work of Swistowski and colleagues who have developed a four-step process for the production of dopaminergic neurons under xeno-free conditions [48] positioning this work for translation to cGMP manufacturing center in anticipation of clinical application of these cells for the treatment of Parkinson's disease. It is important to note that differentiation down the neural lineage does not

occur in the absence of the development of other cell types, there are other cell types in the cultures that must be eliminated in order to obtain pure neural populations. Increasing the efficiency of production of the desired population is a major effort and much attention is paid to this issue especially when considering scaling the process. Currently, it is common to achieve a 1- to 2-fold increase in cell number at the end of a differentiation process. So, depending on how many cells one is looking to produce an equal number of undifferentiated cells may be required at the initiation of differentiation.

ESCs have been shown to produce neurons of multiple phenotypes including GABAergic, glutamatergic, glycinergic, noradrenergic, and cholinergic neurons. Physiologic studies indicate that ESC-derived neurons develop functional synapses, exhibit spontaneous activity, and possess electrophysiologic properties remarkably similar to those of neurons in primary culture systems. ESC-derived neurons express proteins or gene products characteristic of primary neurons: those responsible for general neuronal functions such as β-III tubulin and neurofilament subunits: neuronal cell adhesion molecule (NCAM); transmitter synthesizing enzymes: glutamic acid decarboxylase (GAD), tyrosine hydroxylase (TH), choline acetyltransferase (ChAT); transmitter receptor subunits: glutamate receptor (GluR) γ-aminobutyric acid (GABA-R): and neurotransmitters: glutamate, GABA. Furthermore, ESC-derived neurons possess electrophysiological response to glutamate, GABA, and intracellular calcium flux, similar to primary cells (see Tables 2.1 and 2.2).

Many of the basic methods and fundamental observations regarding ESCs and neurogenesis were developed using mESCs key discoveries and are listed in Table 2.1. The availability of hESCs and the relative ease with which human iPSCs can be generated have greatly expanded efforts aimed at in vitro differentiation and the drive to further refine and develop culture methods amenable to scale-up manufacturing (see Table 2.2).

Neural Induction via Embryoid Body Formation

ESCs can be reliably induced to differentiate by lifting them from adherent culture and allowing them to grow as small free-floating cellular aggregates, called embryoid bodies (EB), in the absence of inhibitors of differentiation, such as LIF or FGF-2. Early studies with mESC and teratocarcinoma cell lines showed that these spontaneously differentiating EBs contained cellular derivatives of all three germ layers [7, 8]. ESCs within the EBs spontaneously differentiate, based on intrinsic pathways and extrinsic signals from the culture medium and microenvironment that any particular cell may find itself within the EB. The EB method of inducing differentiation is effective for both mouse and human ESCs. However, the size and density of the starting EBs have been shown to influence the outcome of the differentiated cell population [24], leading to increased variability. EB formation is influenced by the homogeneity of the starting culture, generally a reduced number of EBs are obtained when the starting culture is contaminated

Table 2.1 Mouse embryonic stem cells and neurogenesis

Date	Observation	Cell line	RA induction	Neurons	Glia	Reference
1981	*Growth of whole murine EB's in vitro produced some neuron-like cells over a period of 6 weeks.* First derivation of ES cells from murine pre-implantation blastocyst. SQ injection of ES cells into athymic mice produced teratomas containing three germ layers when examined 6 weeks later	Murine ICRxSWR/J	No	Neuron-like cells in vitro	NE	[19]
1988	*First demonstration of definitive neuronal differentiation from murine ES cells* in vitro. NGF accelerated the generation of neurons from ES cells plated as whole EB's in vitro. One day after EB plating, 44% of EB's treated with NGF contained neuron-like cells, whereas only 8% of control EB's exhibited neuron-like cells. NGF treatment also eliminated presence of undifferentiated ES cells by eight DIV compared to persistent presence of undifferentiated ES cells in control treated cultures even at 9 DIV	Murine BLC 6 (129/Sv Gat)	No Whole EB's plated	Silver stain+	NE	[98]
1995	*Demonstration that RA has a strong action in inducing neural cell differentiation in ES cell aggregates.* Neuron-like cells comprised 38% of cells in 4−/4+ RA-induced cultures, and about 0% in 4−/4− cultures (two DIV after dissociation). *First demonstration of functional neurons derived from murine ES cells* in vitro. First demonstration of ES cel-derived astrocytes in culture. 4−/4+ RA-induced ES cells produced β-tubulin III and NFM+ neurons, and cell that expressed gene products for NFL, glutamate receptor subunits ($GluR_{1-4,6}$), Brn-3, GAD_{67}, GAD_{65} and GFAP. A noted absence of BF-1 expression suggested that RA may select for hindbrain neural phenotypes since BF-1 is expressed selectively in anterior regions of the CNS [54]. Neurons generated action potentials, express TTX sensitive Na+ channels, voltage gated K+ channels and Ca2+ channels, and were sensitivity to kainate, NMDA, GABA, or glycine	Murine D3 CCE	Yes 4−/4+ RA as EB's	β-tubulin III+, NFM+ RT-PCR: glutamate receptor subunits ($GluR_{1-4,6}$), Brn-3, GAD_{67}, GAD_{65} No BF-1, TH expression Physiologic properties: Responses to kainite, NMDA, GABA, glycine	GFAP+ RT-PCR: GFAP	[58]

(continued)

Table 2.1 (continued)

Date	Observation	Cell line	RA induction	Neurons	Glia	Reference
	First demonstration of ES cell-derived oligodendrocytes in cultures of RA- induced murine ES cells. Demonstrated nestin+ neural precursors in cultures of murine ES cells induced with RA (2+/2− as EBs then dissociated) that were capable of generating mature neurons (GABAergic and cholinergic) and glia (oligodendrocytes and astrocytes). O4+ cells were less than 1% of cultured cells. GFAP+ were 75% of cultured cells. MAP-2+ neurons were 25% of cultured cells. Voltage-dependent channels observed in voltage clamp studies	Murine CGR8 (129 Sv)	Yes 2+/2− RA as EBs	Neuron-like cells N-CAM+, Nestin+, GAD+, AChE activity+, MAP2+, MAP5+ NFH+, synaptophysin+ physiology: See detail	GFAP+, O4+	[99]
1995	*Demonstration of the value of ES cell-derived in vitro systems for the analysis of neuronal function and development on the cellular level*. RA induction for 2 days enhanced neuronal numbers but did not alter their phenotypic fate. Complex electrophysiological and immunocytological properties of post-mitotic neurons were evident and the sequence expression of voltage-gated and receptor operated ion channels paralleled previous studies in primary cultures of rat neurons [119].	Murine BLC6	Yes 4−/2+	Synaptophysin+, Synaptobrevins+, NF-L,M,H+, Synaptic vesicle protein2+, N-CAM+, GAD+ Physiology: Voltage-dependent (K+, Na+, Ca2+) and receptor-operated ($GABA_A$, glycine, AMPA, NMDA) ionic channels Ca2+-dependent GABA release	GFAP+	[100]
1996	*Demonstration of acquisition of neuronal polarity, synapse formation, and functional synaptic transmission in ES cell- derived neurons* in vitro. Within 14–21 DIV, RA-induced (4−/4+) ES cell- derived neurons formed excitatory synapses, mediated by glutamate receptors, or inhibitory synapses, mediated by receptors for GABA or glycine. Both NMDA and non-NMDA receptors contributed to the excitatory post-synaptic responses. Majority of synaptic connections were excitatory (∼80%) and the minority inhibitory. Only glycinergic inhibitory synapses were observed and no GABAergic synapses were found. The majority of ES cell- derived neurons displayed spontaneous activity	Murine D3	Yes 4+/4− RA as EBs	GAP-43+ axons MAP2+ dendrites Synaptophysin+, SV2+, Synapsin+, Physiology: See description	NE	[5]

(continued)

Table 2.1 (continued)

Date	Observation	Cell line	RA induction	Neurons	Glia	Reference
	First study to transplant murine ES cells into the CNS. Development of neurons after transplantation of RA-induced ES cells. RA induction enhances neuronal production and differentiation in culture	Murine D3 and E14TG2a	Yes 4+ RA Yes 4+	AChE+, GABA+, NSE+, Thy1.2+, III-β -tubulin+ GABA+, GAD+, NF+, III-β-tubulin+, A2B5+	NE GFAP+	[101]
	Demonstration that RA promotes neural and represses mesodermal gene expression in mouse ES cells in vitro. 4−/4+ RA treatment of EBs enhanced expression of NF-L, -M, GAD_{65}, GAD_{67}, Wnt-1, and MASH-1. In non-RA treated EBs (4−/4−), these genes were not expressed, with the exception of low levels of NF-L. RA downregulated expression of the mesodermal genes Brachyury, cardiac actin, and zeta-globin. During RA treatment, sequential neural gene activation was observed in the following order: Wnt-1, then MASH-1, then NFs, then GAD only with the appearance of mature neurons	Murine D3	Yes 4−/4+	RT-PCR: enhanced expression with RA treatment: NF-L, -M, GAD_{65}, GAD_{67}, Wnt-1, MASH-1	NE	[54]
	First demonstration that bFGF could be used to select for a highly enriched population of ES cell-derived NEPs. Nestin-immunoreactive cells could develop into glia and neurons (multiple neuronal phenotypes) in culture. Further demonstrated that ES cell-derived neurons could form synapses in vitro using TEM, and could respond physiologically to glutamate and GABA Using the bFGF, ITSFn induction system, over 95% cells were nestin+ and over 60% were MAP2+.	Murine J1 CJ7 R1 D3	No bFGF ITSFn Yes- RA in B27 suppl.	GABA+, Glutamate+ MAP2+, NF-M+, Synapsin-1+ No ChAT+ RT-PCR: GAD_{65}, AMPAR, NMDAR1, NMDAR2A,B,D Physiology: Responses to glutamate and GABA	GFAP+, O4+	[102]
1997	*Embryonic intraventricular transplantation of RA induced ES cells formed neurons, astrocytes, and oligodendrocytes that integrated within host tissues.* The differentiated transplanted cells temporally appeared in correlation with the normal postnatal development of each cell type. 4-day-old EBs were plated in culture for 5–12 days in ITSFn medium [46] prior to transplantation	Murine J1	No	NeuN+, MAP2+, TH+ Neuroepithelium formed in ventricles	CNPase+, Oligos, GFAP+	[103]

(continued)

Table 2.1 (continued)

Date	Observation	Cell line	RA induction	Neurons	Glia	Reference
	First demonstration of normal developmental characteristics of neuromuscular junctions from murine ES cells in vitro. Development of co-localization of agrin, synaptophysin and AChR in mixed muscle/neurons derived from ES cells. The temporal pattern of expression of striated muscle and neuronal markers closely matches similar expression in vivo during development	Murine D3 and BLC6	No 5D EBs	Cholinergic cells NF-L+ and synaptophysin (5D+2); NF-M, -H (5D+6); RT-PCR: NF-L,-M and synaptophysin (5D EB's); Neurocan (5D+2); tau (5D+6); NF-H (5D+18)	NE	[104]
1998	*First demonstration of genetic selection of lineage restricted neural progenitors from ES cells*. ES cells were used that contained a bifunctional selection marker/reporter gene βgeo integrated into the *Sox2* gene by homologous recombination. When induced with RA using the 4−/4+ protocol, approximately 50% of the dissociated cultured cells expressed β-galactosidase activity and Sox-2 immunoreactivity. Further addition of G418 resulted in cultures with over 90% expression of β-galactosidase activity and Sox-2 immunoreactivity. Of the selected cells, 46% were Pax6+, 35% were Pax3+, 24% were Mash1+, 14% were Math4A+, 30% were Delta1+, and 3% were Islet1+. Further differentiated produced mature neurons expressing GABA, glutamate, NF, and MAP's immunoreactivity. Therefore, genetic selection can be applied to select pure populations of neural restricted precursor cells, in this case NRPs	Murine E14TG2a CGR8 CCE-Sox2	Yes 4−/4+ RA	GABA+, Glutamate+ β-tubulin III+, MAPs+, Synapsin-1+, NFL+, NFH+, Nestin+	GFAP+	[74]

(continued)

Table 2.1 (continued)

Date	Observation	Cell line	RA induction	Neurons	Glia	Reference
1998	*RA induction favors differentiation of ventral CNS neurons.* RA exposure (2−/5+ protocol) generated neurons characteristic of the ventral CNS, somatic (Islet+) and cranial (Phox2b+) motoneurons and interneurons (islet -). RT-PCR = upregulated expression of GAD, TH, ChAT, *TrkB*, *TrkC*	Murine CCE	Yes 2−/5+ hanging drops	Somatic MNs and Interneurons Nestin+ NP's Pax6+, few Pax7+, Islet-1/2+, Phox2b+, Lim ½+, Lim3+, EN1+, peripherin+ NFM+, MAP-2+, RT-PCR : GAD, TH, ChAT. RA enhanced expression of these, particularly ChAT		[105]
	Demonstrated of a strict temporal differentiation profile for neuroglial cells in RA-induced ES cells in culture. The cell types first appearing on the following post-plating days: neurons [5], astrocytes and oligodendrocytes [9], microglia [16]. First demonstration of microglial differentiation from whole EBs. Therefore, EBs recapitulate the temporal order of neural cell development in the CNS	Murine BLC6 (129/Sv Gat mouse blastocyst)	Yes RA as EB's	NSE+, Synaptophysin+	GFAP+ astrocytes O4+ oligos C56+ microglia	[106]
1999	*First demonstration that murine ES cells could myelinate in the immature CNS. Development of procedure to enrich for GRPs from murine ES cells with potential for forming oligodendrocytes and astrocytes*. No RA used for induction. EB growth (4 days), then plated in ITSFn (5 days), then sequential propagation in (i) bFGF, (ii) bFGF and EGF, (iii) bFGF and PDGF. Demonstrated that the GRPs could myelinate axons in the developing nervous system of myelin- deficient mutant rats (spinal cord- 1-wk-old; intraventricular at E17).	Murine J1 Cj7	No	NE	A2B5+, precursors , O4+, CNPase+, GFAP+	[107]

(continued)

Table 2.1 (continued)

Date	Observation	Cell line	RA induction	Neurons	Glia	Reference
	Demonstration that BMP-4, a TGF-β superfamily member, inhibited RA-induced neural differentiation and enhanced mesodermal differentiation in murine ES cells. The effect of BMP-4 was restricted from D 5–8 of the 4−/4+ EB aggregation protocol. BMP-4 did not alter cell proliferation or death in EB's. As a baseline, ~25% of EB (4−/4+ stage at 5–6 days) cells were TUNEL+, similar to earlier observations [44]. Co-incubation with the anti-apoptotic molecule BAF (30–50 μM) reduced TUNEL+ by 35%, but did not alter neural differentiation. The effect of BMP-4 could be reversed by co-application of noggin, a BMP-4 antagonist	Murine D3	Yes 4−/4+	β-tubulin III+ (decreased five to tenfold by BMP-4) NeuN+, HNK-1+	GFAP+ (decreased by BMP-4)	[66]
1999	*Demonstration of murine ES cells as a source of late embryonic neural precursor cells*. NRPs (E-NCAM+) and GRPs (A2B5+/E-NCAM-) cells can be immunoderived from ES cells and can differentiate into postmitotic neurons and glia, respectively. ES cells grown as aggregates for 4 days, then plated on fibronectin-coated dishes in NEP basal medium E-NCAM+ cells expressed early neuronal markers upon differentiation (β-tubulin III+, MAP2+) but not GFAP glial markers. Immunopanned E-NCAM+ ES cell-derived precursor cells expressed differentiation markers for neurons upon differentiation (glutamate, GAD, glycine+). ES-cell -derived A2B5-immunoreactive cells differentiated into oligodendrocytes and two types of astrocytes [Type I (A2B5-/GFAP+), II (A2B5+/GFAP+) astrocytes]	Murine D3	Yes- RA used for long-term culture PDGF used for long-term glial induction	E-NCAM+ differentiated cells: Glutamate+, GAD+, Glycine+ RT-PCR: ChAT, GAD, Glutaminase A2B5+/E-NCAM- differentiated cells: (-) for markers of differentiated neurons	E-NCAM+ differentiated cells: no oligo or astrocytes A2B5+/E-NCAM- differentiated cells: Gal-C+ Olig Type I, II astrocytes	[108]

(continued)

Table 2.1 (continued)

Date	Observation	Cell line	RA induction	Neurons	Glia	Reference
	First demonstration that ES cell transplantation can be used to enhance recovery of lost function in adult rat model of SCI. Transplantation of dissociated 4−/4+ RA stimulated EBs into the spinal cord 9 days after contusion injury improved functional hindlimb spontaneous locomotion. ES cell-derived cells integrated and migrated 1 cm, and differentiated into neurons, oligodendrocytes and astrocytes	Murine D3 ROSA26	Yes 4−/4+	NeuN+, EMA+	APC CC-1+, GFAP+	[12]
2000	*First demonstration that ES cell-derived oligodendrocytes could (1) myelinate axons in culture, (2) myelinate axons in the injured mature nervous system (spinal cord), (3) myelinate axons in the adult myelin mutant shiverer mouse*. A simple and rapid method was developed to isolated and purify oligodendrocyte precursors, that involved an intermediate "oligosphere" step after dissociation of 4−/4+ RA treated EBs	Murine D3 ROSA26	Yes 4−/4+	β-tubulin III+, NF+	NG2+, 04+, 01+, MBP+, CNPase+ APC CC-1+	[13]
2002	*Demonstration that mESC can be induced to produce specific classes of neurons by treatment with factors* that are known to induce specific neuronal subclasses in vivo. Indicating that the system is amenable to production of specific neuronal subclasses in a predictable fashion although there is no discernable axis of development in the EB system	Murine MM13 W9.5 HBG3–(HB9eGFP transgene derived line)	Yes 2-/5 days+ RA/Shh	ChAT+, Isl1+, NeuN+	NE	[109]

(continued)

Table 2.1 (continued)

Date	Observation	Cell line	RA induction	Neurons	Glia	Reference
	Induced production of mid-brain domaminergic neurons by constitutively expressing the tissue transcription factor Nurr-1 in mouse ESC. Nurr-1 leads to induction of tyrosine hydroxylase (TH). Resulted in a four to fivefold increase in the proportion of DA neurons. Further increased the number of DA neurons by treatment with Shh, FGF8, and ascorbic acid. Showed that overexpression of transcription factors can drive differentiation down specific pathways	Murine D3 Nurr-1 tg ESC	No EB induction followed by serum-free treatment with bFGF (Okabe)	*β*-tubulin III+, TH+	NE	[110]
2003	*First demonstration that NT-derived mouse ESC can differentiate into neural subtypes and when transplanted into a mouse model of Parkinson's disease show some therapeutic benefit*. Indication of a potential therapeutic application for neurons differentiated from nuclear transfer-derived ESC lines	Murine CJ7 B5 BF1/lacZ-73 ntESC C15 CT2	No Co-culture with MS5 stromal derived feeder layers	*β*-Tubulin III+, MAP2+, TH+, DAT+	GFAP+, O4+, O1+, NG2+, MBP+, CNPase+	[111]

Abbreviations *AChE* acetylcholinesterase, *AMPAR* α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, *APC CC-1* adenomatous polyposis coli, CC-1 subtype antibody, *B21* defined media supplement (with RA), *BF-1* brain factor 1, *bFGF* fibroblast growth factor 2, *BNP-4* bone morphogenic protein 4, *ChAT* choline acetyl transferase, *CNPase* 2', 3'-cyclic nucleotide 3'-phosphodiesterase, *D* day, *DIV* days in vitro, *EB's* embryoid bodies, *EG* embryonic germ, *EGF* epidermal growth factor, *EMA* mouse specific antibody that preferentially recognizes neurons (123), *ES* embryonic stem, *FGF* fibroblast growth factor, *GABA* gamma amino butyric acid, *GAD* glutamic acid decarboxylase, *Gal-C* galactocerebroside, *GD3S, GD3* synthase, *GFAP* glial fibrillary acid protein, *GluR* glutamate receptor, *GRP's* glial restricted precursors, *ITSFn* chemically defined media [46], *MAP2* microtubule associated protein 2, *MBP* myelin basic protein, *MN* motor neuron, *N-CAM* neural cell adhesion molecule, *N/A* not applicable, *NE* not examined, *NEP* basal medium, neuroepithelial precursor basal media [108], *NeuN*, neuron-specific nuclear protein, *NF(L,M,H)* neurofilament (low, medium, and heavy forms), *NG2, NG2* chondroitin sulfate proteoglycan, *NGF* nerve growth factor, *NMDA* N-methyl-D-aspartate, *NP's* neural precursors, *NRP's* neuronal restricted precursors, *NSE* neuron specific enolase, *NT* nuclear transfer**,** *O4* pre-oligodendrocyte marker, *O1* mature oligodendrocyte marker, *Oligo* oligodendrocyte, *RA* retinoic acid, *RAR* retinoic acid receptor, *RT-PCR* reverse transcription polymerase chain reaction, *SCID* severe combined immunodeficiency, *SQ* subcutaneous, *TEM* transmission electron microscopy, *T3Rα* T3 receptor alpha, *TH* tyrosine hydroxylase, *Trk* tyrosine kinase, *TTX* tetrodotoxin, + positive reactivity, (−/−) double gene allele inactivation, 4−/4+ *RA* Induction protocol where EB's are exposed to RA only in the last 4 days of an 8 day protocol

with differentiated cells. While working at the laboratory scale it is relatively easy to maintain highly homogeneous cultures of undifferentiated ESCs, this becomes more challenging when scaling up cultures.

Neural Induction in Adherent Culture

Adherent neural induction methods have been developed that allow efficient production of NEP without an EB intermediary. These monolayer cultures permit better monitoring of the process of neurogenesis and allow individual cells and colonies to be monitored if desired. Ying et al. developed an effective method for inducing neural differentiation using a transgenic mESC (*Sox1*-GFP) line [49, 50], to population of NEP that could withstand long-term culture while retaining their ability to differentiate into neurons and glia. Adherent neural induction of human ESCs has been described as well [34, 51–53], where NEP cells are propagated as a homogeneous monolayer culture expressing nestin, Sox1, Sox2, Sox3, NCAM, and Musashi-1 and Pax6.

Retinoic Acid

ESCs do not readily differentiate into neurons and glia in the absence of molecules or growth factors capable of inhibiting other lineages or promoting differentiation of the neural lineage. One of the most commonly used molecules for inducing neural differentiation of ESCs is retinoic acid (RA), a metabolite of vitamin A, which was shown to repress mesoderm differentiation and promote neurogenesis [54–56]. It has been used to modulate embryonal carcinoma cell (ECC) and ESC differentiation for decades and continues to play an important role as an inducer of neurogenesis in hESC and iPSC lines (see Table 2.2). In 1995, Bain et al. published an in vitro differentiation method that significantly increases the number of neural precursors produced by mESCs. Their method, the 4−/4+ method simply and reliably produces high numbers of neural progenitors in EBs, which when dissociated and plated further differentiate into neurons and glia [57]. Briefly, mESCs are cultured in suspension as EBs for 4 days, then RA is added to the culture medium and the EBs are cultured for an additional 4 days in the presence of RA. The "4−/4+" culture method has been used to neuralize differentiating mESCs and guided the development of methods used to produce enriched neuronal populations derived from hESCs [58, 59]. Under these culture conditions it is possible to derive all of the major neural lineages: neuroepithelial precursors (NEPs), glial-restricted precursors (GRPs), neurons, astrocytes, and oligodendrocytes from ESCs.

Table 2.2 Human embryonic stem cells and neurogenesis (selected publications)

Date	Observation	Cell line	NSC	Neurons	Glia	Reference
2000	*Derivation of hESC and observation that upon spontaneous differentiation in prolonged culture neural cell types observed*. hESC cultivation on MEF feeder cells without subculture—overgrowth in situ (4–7 weeks). Harvested neuroectodermal patches, cultured in serum-free culture medium in spheres, then replated, observed neural outgrowth	HES-1 HES-2	N-CAM + , nestin, vimentin, Pax-6 Neurosphere propagation	MAP2A+, Synaptophysin + β-III-tubulin+, GABA+	Not reported	[2]
2001	*Demonstration that hESC can give rise to neural progenitors/ NSCs are capable of differentiating into cells of all three neural lineages. Manual dissection of neural rosettes from spontaneous differentiation via overgrowth in situ- no EB intermediary*. hESC cultured on MEFs in FBS (3–4 weeks). NSCs cultured in DMEM/F12+B27, EGF, bFGF as neurospheres, further differentiation was induced by plating spheres on appropriate substrate. Or transplantation into neonatal mouse brain, where neurons and glia were observed. Oligodendrocyte precursors are rare	HES-1	N-CAM+, nestin+, vimentin+, PAX6+ neurosphere propagation	β-III-tubulin+, NF+, NSE+, NF-M+, MAP-2a,b+, Synaptophysin+, Serotonin+, TH+	GFAP+, O4+ rare	[112]
2001	*Demonstration that hESCs can produce neural progenitors/ NSCs capable of differentiating into cells of all three neural lineages. Enzymatic method used to isolate neural rosettes. Culture hESCs 4 days as EBs in medium containing 20% FBS, then plated and cultured in the presence of bFGF. Enzymatic removal of neural rosettes by selective adherence of neural rosettes to culture dish.. NSCs cultured as neurospheres in DMEM/F12+B27, bFGF. Further differentiation was induced by plating spheres on the appropriate substrate. Or transplantation into neonatal mouse brain. Oligodendrocyte precursors are rare*	H1 H9 H9.2	Nestin+ (96%) , Musashi-1+, PSA-,NCAM+	β-III-tubulin+, MAP2a,b+, TH+, NF+	GFAP+, O4+ rare In vivo	[113]

(continued)

Table 2.2 (continued)

Date	Observation	Cell line	NSC	Neurons	Glia	Reference
2001	*Demonstration that hESC could be biased to produce neural progenitors and neurons by treatment with RA. EB induction for 4 days in 20% FBS with 10uM all trans RA. Then plating and growth in EGF, FGF, PDGF-AA, then replating to allow differentiation into subsets of neural precursors and mature neurons. Enriched neural populations (60–90%) antibodies to NCAM and A2B5 and magnetic bead technology*	H1 H7 H9	NCAM+, Nestin+	β-III-tubulin+, MAP-2+, Synaptophysin+	A2B5+, Rare-GFAP+	[58]
2001	*Demonstration that hESC could respond to b-NGF and RA to increase neural numbers.* Differentiation induced by EB formation in KOSR containing medium. EBs treated with RA or β-NGF(100 ng/ml) significantly increased NF-H+ cells. Authors examined the instructional affects of 8 different growth factors on hESCs concluded that growth factor addition inhibits specific cell types and specific differentiation could be achieved using growth factor inhibitors (noggin or follistatin)	H9		NF-H+, dopamine receptor+, serotonin receptor+	Not examined	[114]
2004	*Demonstration that noggin (a BMP antagonist) could be used to promote neural differentiation of hESCs while inhibiting differentiation to other lineages*. hESCs cultured on MEFs in medium containing FBS. Neural progenitors/NSCs cultured as neural spheres in neural basal media +B27, N2, ITS-A, glutamine, + bFGF and EGF. Noggin treatment inhibited formation of extra-embryonic endoderm and mesoderm	HES2 HES3	Sox2+, Pax6+, nestin+	NF+, MAP2-a,b+		[68]
2004	*Neural progenitors transplanted into Parkinsonian Rat model do not cause tumors, did not differentiate in significant numbers to DA neurons, but provided behavioral improvement*. hESC for 8 days in FBS and noggin containing medium, then cultured for 6 additional days w/o noggin. Uncommitted neural progenitors transplanted to the striatum. Did not find significant in vivo differentiation into DA neurons in vivo. Suggested further in vitro induction required prior to transplantation	HES1	Neurosphere culture in EGF/bFGF >90% Nestin+ PSA-NCAM+, A2B2+	b-III-tubulin+, TH+		[69]

(continued)

Table 2.2 (continued)

Date	Observation	Cell line	NSC	Neurons	Glia	Reference
2004	*Dopaminergic Neurons from hESCs after co-culture with PA6 cells*. After 3 weeks of co-culture with mouse stromal cell line PA6, 87% of hESC colonies contain post-mitotic TH+ cells and released dopamine and 3,4-dihydroxphe-hylacietic acid (DOPAC). When transplanted into 6-hydroxydopamine-treated animals, cells integrated into the rat striatum and some cells survive for at least 5 weeks	BG01	Sox1+, NCAM+	TH+, β-III-tubulin+, Dopamine and DPOAC release Nurr 1+, Ptx3, Lmx1b,	NA	[115]
2005	*Motoneuron specification of hESCs using RA and SHH*. Using chemically defined adherent culture system, showed that early neuroepithelial (NE) cells (Pax6+/Sox1-) can be patterned by RA and SHH to produce ~20% HB9+ spinal motoneurons	H1 H9	Nestin+, Pax6+/Sox1-	HB9+, ChAT+, β-III-tubulin+	GFAP rare	[116]
2005	*Enriched Oligodendrocyte progenitor populations derived from hESCs integrate and differentiate to produce oligodendrocytes and compact myelin in shiverer mouse model*. EB induction, EGF/FGF medium and timed exposure to RA. Highly enriched cultures "*Yellow sphere*" containing OPCs	H7	Pax6+ (<1%)	b-III-Tubulin (<10%)	Olig1+, Sox10+, A2B5+, NG2+, PDGFRα, GalC+, RIP O4	[16]
2005	*Functional recovery after transplant of hESC-OPCs into an adult rat model of spinal cord injury when transplated 7 days following injury, but not 10 months following injury. hESC-OPCs have therapeutic potential, but may only be effective during a limited period following injury.*	H7 and H7-GFP	Pax6+ (<1%)	b-III-Tubulin (<10%)	Olig1+, Sox10+, A2B5+, NG2+	[15]
2006	*Adherent differentiation of hESCs to multipotent neuroepithelial progenitors (NEP) using selective survival conditions*. Neural rosettes are in serum-free, feeder-free conditions. Maintained in Neural Basal media, B27, B-FGF in 5%O2. Propagated for 6 months without losing multipotent state. Maintained normal karyotype	BG01 BG02	Sox1, Sox2 , Sox3,Nestin, Musashi-1			[53]

(continued)

Table 2.2 (continued)

Date	Observation	Cell line	NSC	Neurons	Glia	Reference
2008	*Stroke Model: Functional recovery in ischemic rat model.* hESC-NSCs were cultivated as monolayer in defined media containing EFG, bFGF, and LIF cells that survived expressed neural stem cell markers. Transplanted NSCs migrated toward stroke-damaged host tissue, engrafted and improved the independent use of stroke-impaired forelimb	H9	nestin+, vimentin+	NF-M+, MBP+, MAP2+, β-III-tubulin+, TH+	GFAP+	[117]
2008	*Motoneurons produced at high efficiency in a chemically defined suspension culture using the small molecules purmorphamine and RA.* hESCs were restricted to ventral spinal progenitor fate (NKX2.2, IRz3, Pax7), Olig2+ progenitors and post-mitotic HB9+ cells are obtained at 96% of culture within 4 weeks without further purification or enrichment. (Purmorphamine activates SHH signaling.) *Reproducible and cost effective motoneuron production*	H9 H1	Pax6+/Sox1	HB9+ (96%)	NA	[118]
2008	*R-NSC- a functionally distinct NSC stage cells: neural rosette cells (R-NSCs) that can differentiate into both CNS and PNS.* Identified specific markers, maintenance promoted by activation of SHH/notch signaling. In vivo, neural overgrowth Forse1+, PLZF+, Dach1+, Sox2, nestin	H9 H1	Pre-NSC stage described			[45]
2009	*Rapid production of NEP in adherent culture using selective survival conditions in hESC medium +4 ng/ml bFGF on either gelatin or laminin.* NEPs could be expanded, cryopreserved, and differentiated	SA002 AS034	Sox1, Sox2, Sox3, nestin			[51]
2009	*Neural conversion by inhibiting SMAD signaling with Noggin and SB431542 in adherent culture.* No EB intermediary, hESC are exposed to SB43152 and noggin for 5 days in adherent feeder-free culture, then specified to neuronal fate. Method permits uniform conversion to NE (80%) and more rapid derivation of neuronal subtypes (19 days compared to 30–50 days)	H9 and iPSC lines	Pax6+, Nestin+	HB9+, β- III-tubulin+, TH+	NA	[119]

(continued)

Table 2.2 (continued)

Date	Observation	Cell line	NSC	Neurons	Glia	Reference
2009	*Dopaminergic neuron differentiation under xeno-free conditions, a 4-step, scalable process amenable to cGMP*. hESCs cultured in defined growth medium on a xeno-free substrate, NSC via EB formation and rosette isolation, DA differentiation FGF8 and SHH, removed and BDNF, GDNF, and TGF-b3, and dcAMP for 10–25 days	I6	Sox1, + Nestin+	β-III-tubulin+	GFAP+, GalC+	[48]
2011	*Astrocyte progenitors: Using chemically defined culture system to produce highly uniform populations of immature astrocytes*. Produced pure populations of astroglial progenitors that can be expanded to large quantities and regionally specified, functionally distinct astrocytes, by patterning the early neuroepithelial cells	H9 H7			>90% S100β+ and GFAP+	[46]
2011	*Neuronal progenitors: High purity in suspension with RA/FGF*. Use EB, w/o rosette collection. Novel media and manipulation of cells to achieve chemically defined, scalable conditions for high purity neuronal production. *Represents a population that can be further differentiated* in vitro *and* in vivo *to neuronal subtypes*	H7, CSC14, CSC14 -CL1	Nestin, Musashi-1	Doublecortin+, (91.2%) b-III-tubulin+	NG2- PDGF-a -	[120]
2011	Primitive Neural Stem Cells (pNSC): 7-day induction of hESC in chemically defined medium by inhibiting GSK3 and TGF-β and notch pathways. pNSCs can be maintained as long-term homogeneous self-renewing population of neuroepithelial cells in medium containing LIF/CHIR99021/SB431542. Oct4-/Sox2+/CD133+/ nestin/pax6/PLZF	H1, HUES9	Primitive NSC, Pre-R-NSC (Pre-NSC)			[44]

Abbreviations hESC human embryonic stem cell, *EB* embryoid body, *FBS* fetal bovine serum, *NE* neuroepithelial, *N-CAM* neural cell adhesion molecule, *PAX6, LIF* leukemia inhibitory factor, *EGF* epidermal growth factor, *bFGF* basic fibroblast growth factor, *PDGF IGF DMEM/F12, GF* growth factor, *SHH* sonic hedgehog, *GDNF* glial-derived neurotrophic factor, *IGF1* Insulin-like growth factor-1, *OPC* oligodendrocyte progenitor cell, *SCI* spinal cord injury, *RA* retinoic acid, *BMP* bone morphogenetic protein, *TH* tyrosine hydroxylase, *SHH* sonic hedgehog, *FGF* fibroblast growth factor, *ChAT* choline acetyltransferase, *CNS* central nervous system, *PNS* peripheral nervous system, *GSK3* glycogen synthase kinase 3, *TGF-β* transforming growth factor β, *BDNF* brain-derived neurotrophic factor, *PLZF* promyelocytic leukemia zinc finger

Noggin

Model organisms have provided the necessary foundation to identify pathways and modulating factors that drive differentiation during development and have been crucial to the development of the current in vitro differentiation protocols [60–65]. As knowledge of the stem cell niche and the growth factors that promote and inhibit differentiation are identified they are being applied to the development of protocols aimed at modulating the specific pathways that guide differentiation toward specified lineages [3]. Noggin is one such growth factor. Noggin has been shown to inhibit bone morphogenic proteins (BMP) and when added to ESC culture promotes neural differentiation by inhibiting mesoderm formation [66–68]. NSC progenitors derived from noggin treated hESCs have been transplanted into a rat model of Parkinson 's disease with functional improvement [69].

Enrichment and Characterization

Current methods to differentiate ESCs do not yield pure or homogeneous populations of differentiated target cells, with the possible exception of neuroepithelial cells [47]. Since nearly all current methods produce a heterogeneous population of differentiated cells, most methods include an enrichment step of one type or another to eliminate the undesired cells from the final cell product. These strategies often depend on the use of surface molecules that allow subpopulations to be eliminated based on differential expression of these surface proteins—some cells express the marker and others do not permitting the use of the marker to isolate the desired population. In some cases researchers have identified small molecules or proteins that have with differential sensitivity to cell types allowing elimination of unwanted cells via exposure to these compounds—drugs that are toxic to the unwanted population permit selection of the desired cell type.

Enrichment of specific cell types can be achieved through the use of fluorescence activated cell sorting (FACS) and immunopanning, which rely on differential expression of cell surface antigens. The identification of novel surface markers that specifically target subsets of mixed populations of neural cells has been described [70]. In this study a panel of 190 antibodies was used to immunophenotype and isolate specific populations of NSCs, neurons, and glial cells. NSCs follow the expression pattern CD184+/CD271−/CD44−/CD24+. Using the same panel of 190 antibodies and appropriate functional assays they identified a unique pattern for neurons and glial cells. Neurons were identified as CD184−/CD44−/CD15 low/CD44+ and gave rise to mature and subtype neurons that fired action potentials. A CD184+/CD44+ glial population gave rise to GFAP-expressing astrocytes.

Small molecule screens have been used to identify molecules that can be added to the culture medium and affect one type of cell but not others in the culture dish [71, 72]. Han and colleagues undertook a small molecule screen and identified a

small molecule that selectively eliminates NSCs, but not dopaminergic neurons from their cultures [73].

Transgenic and gene targeting technologies have been very useful in the development of protocols that drive differentiation toward specific lineages as well as permit the tracking cells in vivo following transplantation into animal models. Gene modified cell lines have been used to identify modulators of specific cell types and to determine the timing and dose of growth factors or small molecules during differentiation process development [74–80].

Genome-wide expression analysis is being used to define phenotypic classes of stem cells [81–83] and focused expression arrays have been developed to reveal pathways of neurogenesis [84]. Epigenetic analysis of hESCs and their differentiated progeny are starting to reveal unique epigenetic signatures of each class[85] and the proteome is being examined as well [86], new technologies allow ever more sensitive analysis that increases our understanding of these cells and permits the development of more refined methods for in vitro ESC neurogenesis.

The ESC System: Advantages, Limitations, and Potential

While the in vitro ESC system offers great promise in terms of scientific discovery and therapeutic potential, there are many hurdles to overcome before this potential is fully realized. Even as the first hESC-derived products have been approved for use in humans, many questions remain to be answered, many of which can only be answered upon careful monitoring of the transplant recipients [87–90].

Reliable and scalable xenogeneic-free culture media and methods are being developed [38, 39, 91] and the derivation of clinical-grade hESC lines [92] and iPSC lines [93] have been described. However, issues remain to be solved with long-term propagation and long-term preservation of hESC-derived cellular products. Genetic and epigenetic stability of both the undifferentiated cells and their and differentiated progeny needs to be determined. The percentage and type of cells in the ad-mixture that comprises the final cell product need to be determined during scale-up and production of the cell product. The reproducibility with which the ad-mixture is obtained during the process is also of interest and can only be addressed by conducting repetitive production runs at scale, a costly undertaking, but the only way to qualify the process and determine the overall robustness of the differentiation and manufacturing methodology. The identification and determination of the remaining undifferentiated hPSCs, if any, in the final cell product is a critical issue in the field, as is the determination of the minimal number of undifferentiated cells, that will induce a tumor in the diseased tissue or organ.

The methods originally developed to differentiate mouse ESCs have been optimized for and improved upon in the hESC system. When these in vitro differentiation methods are coupled with patient-derived iPSCs, an entirely new method to model human disease is created [94]. Proof of principle of this new

paradigm has been used in the mouse to correct sickle cell disease [95] and applied to an animal model of Parkinson's disease [96]. iPSCs, which can be produced relatively easily, but may have variable efficiency and propensity to become differentiated cell types [97] have greatly increased the genetic diversity of human PSC lines available for basic discovery and drug screening programs. iPSC research is in very early stages. Evaluating the long-term stability and determining their ultimate usefulness in the treatment of disease is ongoing. They offer a unique opportunity to apply both gene and cellular therapy to the regenerative medicine paradigm.

Whether the starting material is ESC or iPSC, the development of reliable and reproducible methods to direct their differentiation to a pure population of a specific cell type remains a major challenge in the field. Further, the development of effective enrichment methods that eliminate unwanted cells, especially undifferentiated PSCs, the development of qualified assays designed to assess the identity, purity, and potency of the final cell product remain a major challenge in the field if the goal is to apply these cells to the treatment of human injury and disease.

The in vitro ESC system combined with high-throughput screening and genome-wide analysis provides scientists with the means to identify the molecular mechanisms guiding mammalian development, the pathogenesis of disease, and to develop efficient methods that will allow the therapeutic application of these cells to regeneration and repair or perhaps allow us to gain sufficient understanding of human development that one day we might correct deficits and activate specific endogenous repair pathways without the need for cellular transplantation.

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