Introduction

Stem cells are defined by their ability to self-renew and to differentiate into specific specialized cell types. Pluripotent stem cells such as embryonic stem cells are capable of differentiating into all cell types of the three germ layers. Self-renewal and differentiation potential are properties that make stem cells an attractive source for cell therapeutic efforts including the treatment of neurological diseases such as Parkinson's disease (PD). Parkinson’s disease is one of the most common neurological disorders and is characterized by the selective degeneration of dopamine (DA) neurons in the ventral midbrain. The midbrain region contains three groups of DA neurons, the retrorubral field (A8), the tegmental area of the ventral midbrain (VTA, A10) and the substantia nigra pars compacta (A9). Only the latter subgroup is primarily affected in PD and responsible for most of the motor dysfunction. Due to this rather selective loss of DA neurons in the substantia nigra, PD is considered a neurological disease amenable to cell replacement. Cell replacement therapy in PD has been attempted in several hundred patients worldwide using fetal human DA neurons. While promising results have been reported in several open label studies (e.g., 1, 2) placebo-controlled clinical trials using human fetal dopamine neurons have yielded modest clinical improvement at best. 3, 4 Furthermore, a subset of these patients displayed disabling graft-induced dyskinesias. There are many potential reasons for this relatively poor outcome as discussed in detail elsewhere. 2 However, the limited availability of donor tissue, the low percentage of DA neurons within fetal grafts and ethical concerns associated with the use of human fetal tissue suggest that alternative cell sources are required for successful clinical translation.

The currently most promising cell source for generating authentic midbrain dopamine neurons in vitro are embryonic stem cells (ESCs). The main advantage of a stem cell based strategy, in contrast to fetal tissue, is the availability of potentially unlimited sources of defined DA neurons at any stage of differentiation. While recent developments and novel differentiation protocols have brought the stem cell field closer to this goal, considerable challenges remain in translating these potential advantages of ESCs into safe and efficacious cell therapy.

Neural Development

The formation of the nervous system begins with neural induction, the process by which dorsal ectodermal cells of the gastrula-stage embryo are directed towards a neural identity in response to signals from the underlying mesoderm. These signals comprise Noggin, Chordin and Follistatin, which act on the overlying dorsal ectoderm by blocking BMP signaling, leading to the formation of the neural plate. 6, 4 Initially, the neural plate is thought to have an anterior character, as the inhibition...
of BMP signaling by BMP inhibitors induces the expression of anterior marker proteins, but not of posterior markers and is therefore referred to as the anterior ground state of the CNS. To develop a more posterior character, such as midbrain, hindbrain and spinal cord, this anterior ground state is modified by posteriorizing signals including retinoic acid (RA) and members of the fibroblast growth factor (FGF) and Wnt families.\(^9\)\(^13\)

Patterning cues for midbrain induction include fibroblast growth factor 8 (FGF8), a factor critical for the induction and maintenance of the midbrain-hindbrain organizer\(^14\) and sonic hedgehog (SHH),\(^15\) a ventralizing morphogen, secreted by the underlying notochord. The intersection of these two signals at the time of neural induction is essential in the formation of the ventral midbrain domain,\(^16\) where DA neurons are born.

**Derivation of Midbrain DA Neurons from Embryonic Stem Cells (ESCs)**

**General Properties and Differences between Mouse and Human ESCs**

Embryonic stem cells were first isolated from the inner cell mass (ICM) of a mouse blastocyst-stage embryo in the early 1980s.\(^17\)\(^18\) It has been shown that culture of the inner cell mass in the presence of mouse embryonic fibroblasts (MEFs) can result in clonal populations of cells with extensive proliferation capacity and pluripotent differentiation properties as demonstrated by their ability to generate chimeric mice. The ability of mouse ESCs to contribute to the germ-line of chimeric mice has been the basis of modern mouse genetics such as gene targeting in mice.\(^19\)\(^20\)

However, mouse ESCs cannot differentiate into trophectoderm under normal conditions. Mouse ESCs can be identified by a set of transcription factors characteristic of the pluripotent state including Oct4, Nanog and Sox2 or surface markers such as SSEA1. Mouse ESCs can be propagated on MEFs or under feeder-free conditions in the presence of leukemia inhibiting factor (LIF). More recent studies have shown that BMPs can substitute for serum-derived factors\(^21\) and that under certain conditions mouse ESCs can be propagated in the absence of any growth factors or feeders upon inhibition of FGF, Erk and GSK3 signaling (3i protocol).\(^22\) In 1998 the first successful isolation of human ESCs has been reported.\(^23\) Similar to mouse ESCs, human ESCs also express a set of characteristic surface markers including SSEA3 and SSEA4 and express a nearly identical set of transcription factors associated with pluripotency including Oct4, Sox2 and Nanog. Furthermore, both mouse and human ESCs can be propagated on MEFs. However, defined signals essential for human ESC self-renewal are distinct from those in mouse ESCs. For example, human ESCs are not dependent on LIF signals,\(^24\)\(^25\) but require FGF2 for maintaining an undifferentiated state.\(^26\) In contrast, in mouse ESC, the MEK pathway, which is activated by FGFs promotes differentiation.\(^27\)\(^28\) Similarly, TGFβ signals are important for human\(^29\) but not mouse ESC self-renewal. Furthermore, BMPs cause differentiation of human ESCs along extraembryonic lineages\(^30\) while promoting self-renewal in mouse ESCs.\(^31\) Recent studies in the mouse suggest that these surprising differences in growth requirements may reflect different developmental stages as mouse epiblast derived pluripotent stem cells mimic the growth conditions of human ESCs.\(^32\)\(^33\) One particular challenge for human ESC studies is the lack of an appropriate in vivo assay as the generation of chimeric mice, germ-line contribution or tetraploid complementation assays are not available given the ethical and biological constraints. While teratoma formation has been used as a surrogate assay,\(^34\) it is important to include functional assays in human ESC derivatives in vitro or upon transplantation in vivo such as in the case of ESC derived midbrain dopamine neurons.

**How to Define a Midbrain DA Neuron in Vitro**

The first step in defining midbrain dopamine neuron identity in vitro is the demonstration of neural and neuronal identity followed by co-expression of dopamine related biochemical markers such as tyrosine-hydroxylase (TH), aromatic acid decarboxylase (AADC), VMAT2 (vesicular monoamine transporter) and the high affinity dopamine transporter (DAT). In addition to positive biochemical markers, it is equally important to ascertain the absence of markers expressed in