Stem Cells and Neuronal Differentiation

Indrani Datta, Debanjana Majumdar, Kavina Ganapathy, and Ramesh R. Bhonde

Stem cells are being increasingly considered alternative and viable sources of treatment for debilitating nervous system disorders and neurodegenerative diseases. Stem cells specific to nervous tissue, i.e., neural stem cells (NSCs), exist in two neurogenic regions of the adult brain – subventricular zone (SVZ) in the lateral ventricle and the subgranular zone (SGZ) in hippocampal dentate gyrus [1–4]. The inaccessibility and unavailability of NSCs deep in the brain makes it a difficult proposition to use them in clinical applications. Different stem cells are thus being tested for their neuronal differentiation capability, as a cell source for generation of functional mature neurons and glial cells. The "gold standard" of stem cells are embryonic stem cells (ESCs) as they not only retain long-term self-renewal capacity but also exhibit pluripotency to all three germ lineages. Recent advances in technology have brought the advent of another pluripotent stem cells called "inducible pluripotent stem cells" (iPSc), derived through "reprogramming" of terminally differentiated cells by the addition of a select set of genes [5–7]. However, several limitations still exist for the use of iPScs in therapeutic applications, such as the use of viral vectors for transfer of genes, inclusion of oncogenes, and teratoma formation [5, 6, 8–10]. Stem cells may also be isolated from several tissue sources and these are termed as adult stem cells (ASCs). The first ASCs to be identified were the hematopoietic stem cells (HSCs) derived from bone marrow, but the second population of stem cells from bone marrow called mesenchymal stem cells (MSCs) gained prominence due to their unique properties [11–13]. MSCs are nontumorigenic and immunomodulatory in addition to possessing multilineage differentiation potential not only towards mesodermal lineage derivatives but also to phenotypes of other germ layer cells like neuronal, hepatocytes, and islet cells [14–17]. Although fetal and adult origin MSCs possess some common characteristics with respect to expression of mesenchymal markers and absence of hematopoietic and HLA-DR markers, their neuronal differentiation efficacy is still to be evaluated for consideration as suitable candidates for nervous system disorders and neurodegenerative diseases.

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Birth of a Neuron

The primary unit of the nervous system is neurons. Neurons are specialized cells of the nervous system consisting of axons and dendrites that

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receive, integrate, and transduce electrochemical signals. Neurons in the nervous system are highly polarized and form an ordered communication system with both neuronal and nonneuronal cells through synapses. The initial step in development of nervous system in vertebrates involves the segregation of ectoderm into epidermal and neural primordia. Initially, the neural plate in mammalian and avian embryos forms through apicobasal cell elongation of neuroepithelial cells and convergent extension [18–22]. This is followed by bending of the neural plate at localized regions termed hinge points – a single median hinge point and paired dorsolateral hinge points [23, 24]. The fusion of the neural folds forms the neural tube. In mammalian central nervous system, neurons are generated from the neuroepithelial cells near the lumen of the neural tube termed the ventricular zone. A vertical cleavage of neuroepithelial cells during symmetric division gives rise to two identical daughter cells that resemble the precursor cell, but a horizontal cleavage during asymmetric division produces basal daughter cells that retain contact with the basal surface and an apical daughter cell that loses contact with the lumen (Fig. 1). This apical daughter cell migrates away, and the time of this horizontal division is termed as the birthday of the neuron (apical daughter cell) and the basal cell remains in the proliferative zone. During this asymmetric division, there is a switch in the mitotic state generating one daughter cell (basal cell) mitotically active as a stem cell, while the other apical cell remains in the cell cycle for a number of divisions and is committed to generate neurons [25].

Differentiation and Specification of Neuroepithelial Cells

The nervous system consists of a diverse neural cell type population, derived from these multipotent neuroepithelial/precursor cells. The differentiation of the neural precursor cells from the neural tube across its rostrocaudal axis gives rise to the neurons and glia of the central nervous system encompassing the brain and spinal cord. The differentiation of the neural tube begins with the formation of three primary brain vesicles from which the brain develops – prosencephalon, mesencephalon, and rhombencephalon. The dorsal fusion of the neural plate occurs by the third week to form the neural tube, which by the end of the fourth week further extends across the rostrocaudal axis. This creates spatiotemporal differences within neuroepithelial cells at the

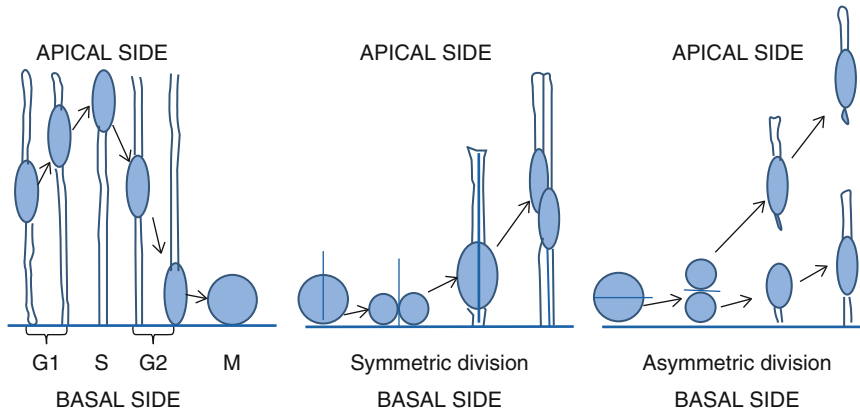

Fig. 1 Ventricular neuroepithelial cell nuclei undergo intracellular migration during cell cycle. A vertical cleavage (perpendicular to ventricular surface) gives rise to two daughter cells that sit side by side, both retaining apical connections. Both the daughter cells reenter the cell cycle. A horizontal (parallel) cleavage produces a basal daughter cell that retains contact with the basal surface and an apical daughter that loses contact with the lumen. The basal daughter stays in the epithelium, while the apical daughter migrates away (Adopted from Chenn and McConnell [25])

time of neural tube formation. The diversity in the cell fate specification of the neural precursors across the rostrocaudal axis is primarily dictated by the nature of environmental cues over time during development. The complex interplay of extrinsic microenvironment in the form of extracellular matrix and morphogens regulates intrinsic specifiers, often the master key transcription factors involved in patterning [26]. In addition, the developmental commitment with respect to the time of birthday for neural precursor cells is supported by the fact that neurogenesis precedes gliogenesis during the differentiation of neural tube. A developmental restriction with time in differentiation potential between early and late cortical progenitors is also noted. Late cortical progenitors can only produce upper layer neurons even in much younger microenvironment indicating that they lose the potency to produce earlier generated phenotypes [27]. The positional identities of the neural progenitors along the dorsoventral axis of the neural tube are through a gradient of signaling molecules secreted at the floor plate and roof plate (Fig. 2). The generation of functional specialized neuronal and glial cells involves a stepwise process starting with the specification of neuroepithelial cells to rapidly dividing transit-amplifying cells and then to migrating neuroblasts and glioblasts which in turn get specified to functional terminally differentiated cells under the influence of the adjacent microenvironment.

Isolation of Neural Stem Cells (NSCs)

Neural stem cells are multipotent cells possessing self-renewal capacity and differentiation capability towards cells of the central nervous system. During embryogenesis the germinal neuroepithelial cells along the neural tube compromises the NSC population which in adult brain gets restrained in two primary neurogenic areas, viz., the SVZ of the lateral ventricle and the SGZ in the dentate gyrus (Fig. 3; [2–4]). The SVZ of the adult brain harbors at least three distinct cell phenotypes: A, B, and C cells, besides the ependymal cells lining the lateral ventricle. Experimental evidence [28] suggests that the SVZ astrocytes (type B cells) represent quiescent stem cells that normally proliferate at a low rate and generate the migratory neuronal precursors (type A cells), through the generation of a third, intermediate cell type, the C cell (or D cell in the hippocampus), which has the characteristics of the classic fast-proliferating, transit-amplifying progenitor cells found in many self-renewing tissues [29, 30]. Adult neurogenesis is regulated by intrinsic specifiers and extrinsic modulators. The intrinsic programs include genetic and epigenetic factors essential for controlling NSC self-renewal and multipotency. The extrinsic factors include the niche where NSCs physically reside and primarily comprise periventricular astrocytes, ependymal cells, vasculature,

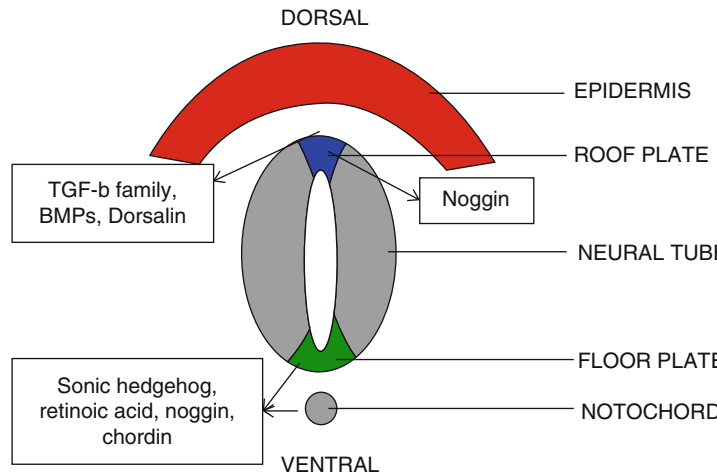

Fig. 2 Schematic representations showing the positional identities of the neural progenitors along the dorsoventral axis of the neural tube is through a gradient of signaling molecules secreted at the floor plate and roof plate

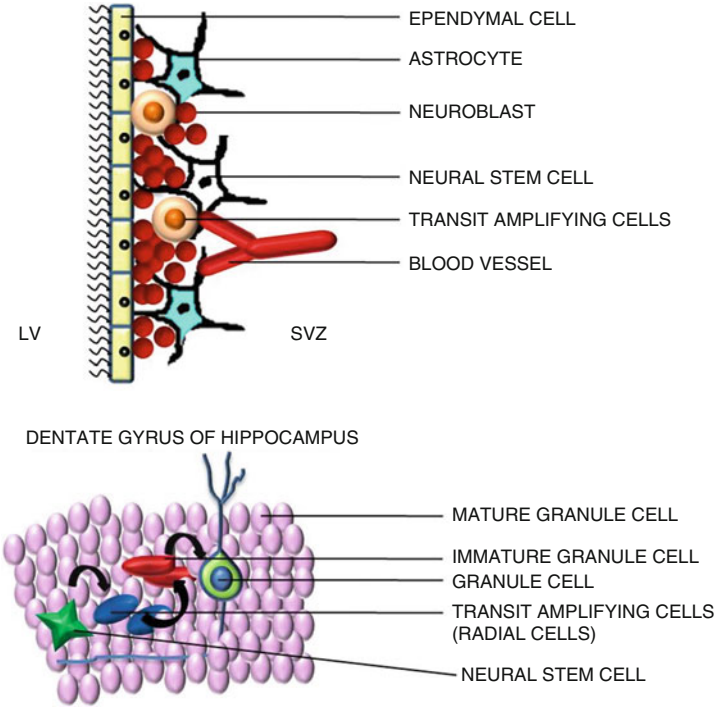

Fig. 3 Schematic representation of NSCs in the two primary neurogenic areas of the adult brain; viz., the subventricular zone (*SVZ*) of the lateral ventricle and the subgranular zone (*SGZ*) in the dentate gyrus

neurotransmitters, and the basal lamina. NSCs isolated from neurogenic areas of adult brain or embryonic CNS region are cultured in vitro by two methods – (1) two-dimensional culture in the form of monolayer on specific extracellular matrix and (2) three-dimensional suspension culture in the form of neurospheres. NSCs derived in serum- and feeder-free culture conditions as floating aggregates are known as neurospheres. Neurospheres have been extensively used for studying molecular mechanisms that regulate self-renewal and differentiation of NSCs. Although NSCs responsive to two mitogens bFGF and EGF have been reported, many labs use a combination of both [31–33]. In the presence of these mitogens, they undergo continuous symmetrical cell division (self-renewal) while retaining the differentiation potential to neurons, astrocytes, and oligodendrocytes. For mouse NSCs, several passages in the presence of these mitogens resulted in homogeneous morphology in culture that uniformly expressed nestin and Sox2. These cells are stem cells as they are clonogenic and maintain indefinitely the capacity to generate both neurons and astrocytes. It was further established that NSCs cultured in the absence of EGF and in the presence of exogenous Jagged1 showed enhanced neurogenic potential when put for differentiation [34], in turn suggesting that activation of Notch receptors on NSCs are pivotal for maintenance of undifferentiated state and differentiation potential. NSCs have also been isolated from brain tissue by fluorescent labeled sorting of cells through FACS for cell surface markers like CD133, CD24, or GFP expression driven by NSC-specific promoters such as nestin, Sox2, Sox1, and FGF1 [35, 36]. These NSCs once sorted were cultured in the presence of growth factors and in suspension culture form.

Differentiation of NSCs to Neuronal Cells

The derivation of NSCs from human fetal brain is characterized by classical bipolar morphology and other cellular morphologies. It also displays interkinetic nuclear migration along the cell process, a characteristic feature

depicted by neuroepithelial cells in vivo. There are reports of human fetal NSCs having been maintained in culture up to 35 passages retaining normal karyotype and differentiation ability. NSCs are immunopositive for set of neural precursor/radial glial markers such as nestin, vimentin, Sox2, brain lipid-binding protein (BLBP), RC2, GLAST, and 3CB2 [36–38]. Besides, the molecular markers include the *Pax* and *Hes* gene families, members of Notch and Wnt signaling pathways, RNA-binding proteins (musashi12), and cell surface markers CD24 and CD133 [39, 40]. Human NSCs are reported to express moderate levels of GFAP too. NSCs upon plating onto poly-ornithine/laminin substrate with removal of the mitogens show spontaneous differentiation to a mixed culture of differentiated mature neuronal (Tuj1+ and Map2ab+) and astroglial (GFAP+) cells. Neuronal maturation can be further achieved by exposing them to Neurobasal media with B27 along with neurotrophic factors BDNF and NGF [41]. Functional electrophysiological experiments suggest that the matured neurons indeed exhibit voltage-gated Na+, K+, and Ca2+ channels, similar to those observed in primary neurons [42]. Exposure of NSCs to BMP4 agonists has exhibited differentiation towards astrocytes [43, 44]. BMP2 in combination with CNTF facilitated generation of GFAP-positive astrocytes [45]. In addition, Glaser et al. [46] have reported that sequential exposure to bFGF, PDGF, and forskolin followed by thyroid hormone (T3) and ascorbic acid can yield differentiated oligodendrocytes (20 %) in culture expressing O4, CNPase, and myelin proteolipid protein. The differentiation potential of NSCs in vivo is generally obtained from transplantation studies into embryonic or neonatal brain or the subventricular zone of adult rodent brain. In such a neurogenic environment, neural progenitors have exhibited "site-specific" differentiation to neuronal cells. On the other hand, transplantation of multipotent NSCs into the injured brain has indicated that the host microenvironment has strong gliogenic signals imparted majorly by the proinflammatory cytokines (TFNα, IL6, and IFNγ) present in the vicinity [47–49]. Other cytokines like PDGF, SDF1, MCP1, and HGF also play a role in chemoattraction and migration of NSCs [50–52]. The number of neurons generated by transplanted NSCs is lower in the lesion area than in intact host striatum. They are localized mainly in partially injured or intact regions and do not repopulate neuron-depleted areas. NSCs transplanted in nonneurogenic areas during spinal cord injury have shown differentiation to astrocytes and oligodendrocytes and not neurons [53]. However, NSCs transplanted in Parkinson's disease in vivo model have shown increase in tyrosine hydroxylase-positive neurons in the midbrain area [54]. NSCs from neonatal rat upon transplantation in the lesion area of the brain of adult PD rat model have shown distinct neuronal differentiation indicating sufficient availability of intrinsic cues for dopaminergic traits in the lesioned brain [55]. Thus, transplantation of NSCs has indeed shown diverse response in acquisition of specific phenotypes depending on the recipient environment, i.e., whether in the control or injured brain and neurogenic or nonneurogenic region. Due to limitations in analyzing differentiation of NSCs in vivo, many groups of scientists have used the advantage of neurosphere assays to address the role of intrinsic gene function for self-renewal and differentiation of NSCs. Gene targeting and knockouts by homologous recombination in NSCs are explored to understand loss/gain of function of genes with respect to self-renewal and differentiation. Insertion of master key genes is also performed on NSCs for achieving terminally differentiated functional neurons.

Differentiation of Human Embryonic Stem Cells to Neuronal Cells

Due to the inaccessibility and serious ethical concerns surrounding the use of human neural stem cells, other pluripotent stem cells are tapped into for generation of human origin neural progenitors. Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) from

human skin fibroblast are presently considered the best resource for generation of human origin neural stem cells. In the last few years, there have been several reports on differentiation of hESCs and iPSCs to neural progenitors and specialized neuronal and glial cells too. The utility of pluripotent stem cells as banks for neural progenitors depends on the availability of standardized robust defined protocols of neuronal differentiation from these cells. Finally, the quality check for in vitro generated differentiated neural progenitors will be determined by their functional integration after transplantation in vivo.

ESCs are known to recapitulate embryonic developmental stages and so protocols of neuronal differentiation usually involve a multistep process. Indeed, the functional genomic screen of human stem cell differentiation revealed pathways involved in [56]. As per Spemann and Mangold [57], the Spemann organizer dictates the fate commitment of the neuroectodermal primordial cells through a set of crucial signaling factors like fibroblast growth factor (FGF), Wnt, Sonic Hedgehog (SHH), retinoic acid (RA), and bone morphogenetic proteins (BMP) inhibitors [58, 59]. Generation of neural progenitors from hESCs are initiated through two culture techniques – (1) embryoid body formation in serum-free condition and (2) presence of RA monolayer culture of ESCs on feeder-free specialized ECM-coated Petri dishes or coculture with stromal cell cultures. EBs usually mimic gastrulation processes and express markers from the three germ lineages [60, 61]. Embryoid body at specific day points are usually plated on suitable ECM with NSC media for the neuroectodermal outgrowth to occur. After eliminating the remnant of the EB, the outgrowth of neural progenitors can be propagated in the NSC media and defined ECM. This procedure is explained in detail in our published work that resulted in a good yield of neural progenitors from day 4 EBs under defined culture conditions [62] with more than 95 % population of cells expressing early neural markers like nestin, Sox2, musashi12, and negative for pluripotent marker Oct4. Initially, these cells organize into rosettes and express cell adhesion molecule N-cadherin [63]. A similar EB method of differentiation of hESCs to an enriched yield of NSCs was obtained in cGMP conditions by Swistowski et al. [64]. These NSCs could be maintained in xeno-free defined media for a prolonged period of time while retaining their ability to differentiate in vitro into functional dopaminergic neurons and upon in vivo transplantation could survive and give rise to differentiated dopaminergic neurons [65–67]. Transplantation of hESC-derived DA neurons has been found to attenuate locomotion deficits in PD rat model. Further analysis has also established that the increase in TH+ve cells in the midbrain is largely generated from grafted neural progenitors. Daadi et al. [68] have shown engraftment of hESC-derived midbrain dopaminergic neurons in a monkey model of Parkinson's disease. After transplantation the TH-expressing cells did not co-localize with GAD and maintained their DA-induced phenotype, extended neurite outgrowths, and expressed synaptic markers.

Besides dopaminergic neuronal differentiation, studies have also shown generation of different subtype neurons and glial cells of the CNS [69]. Treatment of these neuroepithelial cells by RA represses expression of anterior genes such as Otx2, Foxg1, and Pax6 and induces posterior Hox genes [70]. In later neuroepithelial cells (>15 days), this effect of RA is not seen. Neuroepithelial cells generated in absence of morphogens shows a dorsal fate expressing Pax6, Emx1, Ngn2, and Tbr1 but not ventral transcription factor Nkx2.1 indicating a predominant dorsal telencephalic fate [71]. This dorsal fate is determined by high levels of Wnt and low levels of SHH during hESC differentiation to telencephalic progenitors. The presence of SHH, one of the crucial morphogens for patterning midbrain DA neurons, represses the dorsal characteristics and induces ventral forebrain fate. Vazin et al. have depicted that SHH also generated GABAergic neurons [72]. Taking cues from development, it is also observed during in vitro differentiation of neural progenitors from hESCs that manipulating the timing of SHH exposure can give rise to distinct neuronal populations with specific transcriptional profiles and neurotransmitter

phenotypes [72–74]. hESC-derived ventral forebrain progenitors have been reported by few groups [75, 76] with highly enriched populations of NKX2.1:GFP-positive progenitors, including cells with telencephalic identity. Liu et al. [77] have shown that hESCs can be differentiated to NKX2.1(+)medial ganglionic eminence (MGE)-like progenitor cells which, after transplantation into the hippocampus of mice with mu P75-saporin depleted basal forebrain cholinergic neurons (BFCNs) and GABA neurons in the medial septum, produced BFCNs that synaptically connected with endogenous neurons and generated GABAergic neurons too. In the presence of low gradients of SHH and Wnts, the neuroepithelial cells exhibit phenotypes of LGE cells which express Gsx2 and low levels of Pax6 but not Nkx2.1 [78]. On removal of SHH, these LGE progenitor cells can differentiate to projection GABAergic neurons expressing GAD 65/67, DARPP32, Meis2, and Ctip2 [78]. Generation of cholinergic neurons from hESCs has been recently been reported by BMP9 and NGF treatment [79, 80]. Treatment of neuroepithelia with RA (0.1 μM) in a chemically defined media for 10–17 days suppresses anterior transcription factors Otx2 and Foxg1 and activates posterior transcription factors Hoxb4, Hoxc5, and Hoxc8. Further ventralization of the caudal neuroepithelia is brought about by SHH from day 14–21 resulting in Olig2-expressing motor neuron progenitors. These progenitors on removal of RA and SHH express Mnx1, Lhx3, and Isl1/2, markers for postmitotic motor neurons [81–83]. Transplantation of hESC-derived motor neurons into chick embryonic spinal cord and mouse spinal cord expressed Nkx6.1 and Mnx1, and the grafted cells survived after transplantation [83]. Neural progenitors derived from EBs under IGF-1/insulin signaling gave rise to retinal pigment epithelial (RPE) cells and upon transplantation into a rat model of retinal degeneration resulted in the formation of a donor-derived RPE monolayer that rescues photoreceptor cells [84]. Generation of neural progenitors from hESCs is also achieved by culturing the cells in a monolayer subjected to synergistic inhibition of glycogen synthase kinase3 (GSK3), transforming growth factor β (TGF-β), and Notch signaling pathways by small molecules [85]. The self-renewal of these neuroepithelial cells can be maintained in the presence of leukemia inhibitory factor, GSK3 inhibitor (CHIR99021), and TGF-β receptor inhibitor (SB431542). Further, they retain neurogenic potential and responsiveness to instructive neural patterning cues towards midbrain and hindbrain neuronal subtypes and exhibit in vivo integration. Unlike the neuronal differentiation reported, astroglial differentiation from hESCs is quite rare. This is primarily due to the lack of knowledge of astroglial fate commitment during embryonic development. Systemic analysis has indicated that astroglial progenitor markers have been detected after long-term culturing of the neural progenitors derived from hESCs specifically maintained in EGF-containing media. The glial progenitors express the markers NF1A, S100b, CD44, and GFAP [86]. Neural progenitors generated from hESCs cultured in presence of RA and SHH agonist purmorphamine (Pur) under defined culture conditions have shown differentiation to oligodendrocytes by expressing Olig2, Nkx2.2, and Sox10 [87, 88]. In vivo transplantation of these glial derivatives has not yet been tested.

Neuronal differentiation is achieved not only by regulation of external microenvironment but also through intrinsic regulators. MicroRNA expression profiling of NPs and neuronal progenies shows gain- and loss-of-function of miR-153, miR-324-5p/3p, and miR-181a/a that contribute to the shift of NPs from self-renewal to neuronal differentiation. Stappert et al. [89] have shown that miR-125b and miR-181a specifically promote the generation of neurons of dopaminergic fate, whereas miR-181a inhibits the development of this neurotransmitter subtype. Studies have also shown conditional induction of master key transcription factors along with extrinsic cues for faster generation of specialized neuronal cells.

Differentiation of Human-Induced Pluripotent Stem Cells (iPSCs) to Neuronal Cells

It was long believed that once programmed to commit to a specialized differentiated cell type, cells rarely undergo dramatic fate changes in vivo as a result of an irreversible loss of developmental potency. However, with the advent of nuclear transfer technology, the cloning of an animal from the nucleus of a terminally differentiated cell explicitly proved that epigenetic modifications to the genome acquired during development are reversible and that nuclei from even the most functionally specialized cells maintained the potential to generate an adult organism. Further, pathbreaking work by Yamanaka's research group showed that a combination of four transcription factors (Oct4, Sox2, Klf4, and cMyc) was sufficient to reprogram diverse somatic cell types in vitro to a pluripotent state [90]. These newly reprogrammed cells are known as induced pluripotent stem cells (iPSCs). Reprogramming to pluripotency showed a return to the developmental "ground state" mirroring the features of ESCs. Differentiation of these cells to a neuronal lineage too follows a similar combination of induction factors that are required to differentiate hESCs. The process of neuronal differentiation for iPSCs begins with the initiation of primitive neuroectoderm which is manifested by rosette formation. The developmental clock of the rosettes show onset of early neural markers OTX2, PAX6, Sox1, Nestin, NR2F1, NR2F2, and IRX2 followed by glial-like cells at the later day points [91]. Moreover, the cells that emerged from the rosettes during spontaneous differentiation were capable of differentiating into dopaminergic neurons in vitro and into mature-appearing pyramidal and serotonergic neurons weeks after being injected into the motor cortex of NOD-SCID mice. For some human iPSCs that do not differentiate efficiently to neural progenitors, inhibition of BMP by Noggin and SB31542 has been used to increase yield [92, 93]. hiPSCs were also differentiated by coculturing them with rat primary neuronal and glial cells and on matrigel-coated tissue culture dish with differentiation medium [94]. Distinct maturation properties were attained depending on the protocol used, and functional maturation was achieved the best when cultured along with primary neuronal culture. hiPSCs have been efficiently differentiated to region and transmitter-specific neuronal cells including glutamatergic, GABAergic, cholinergic, dopaminergic, and motor neurons as well as astrocytes and oligodendrocytes [95, 96]. The neural progenitors derived from hiPSCs upon transplantation into the fetal mouse brain migrated into various brain regions and showed in vivo differentiation into glutamatergic, GABAergic, and dopaminergic subtypes. Even differentiated Parkinson patient-derived iPSCs grew in the adult rodent brain and reduced motor asymmetry in Parkinsonian rats [97, 98]. A recent detailed gene expression microarray study indicated that expression of ion channels such as voltage-gated Ca^{2+}, Na^{+}, and K^{+} channels, ionotropic neurotransmitter receptors, and ionotropic purinergic receptors is distinctly upregulated in the differentiated progeny of iPSCs in comparison to the starting cell type [99]. Furthermore, electrophysiological recordings and morphological analysis showed that the grafted cells had attained neuronal integration and synaptic activity. Also, neuroepithelial cells derived from hiPSCs after grafting in stroke-damaged brain have shown improvement in recovery [100]. Transplantation of neuroepithelial cells obtained from hiPSCs has also shown improvement of neurological function in rats with experimental intracerebral hemorrhage [101], and differentiation of neural progenitors derived from hiPSCs in a transgenic rat model of ALS carrying a human mutated SOD1 (G93A) was reported by Popescu et al. [102]. In a recent study, successful differentiation of hESCs and hiPSCs to retinal ganglion cells in the presence of Notch inhibitor N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) has been reported [103]. Neural crest progenitors were also derived from iPSCs and compared with those from hESCs, followed by differentiation to functional

Schwann cells [104]. More than the therapeutic use of neural progenitors derived from hiPSCs, this in vitro cell-based technology is in the spotlight to reproduce cellular models of poorly understood diseases – such as Down syndrome, Friedreich's ataxia, Gaucher disease [105–107], amyotrophic lateral sclerosis [108], spinal muscular atrophy [109], Parkinson's disease [110, 111], schizophrenia [112], Huntington's disease [110], and Alzheimer's disease [113]. Furthermore, correction of genetic mutations in disease-specific iPS cells can rescue phenotypes in cultured cells [111, 114] or in mouse models of human diseases, such as sickle cell anemia [115]. The advent of iPSCs brought about an opportunity to study for the first time the cell biology and genetics of neurons derived from any individual. Furthermore, by recapitulating in vitro developmental steps for neuronal cells, it can provide indication for factors responsible for typical and atypical development. Fibroblasts of patients suffering from these diseases can be efficiently converted into iPSCs that are then differentiated into neurons to study the pathogenesis of these diseases (reviewed in [116–118]. Dimos et al. [108] derived iPSCs from an 82-year-old ALS patient, and these patient-specific iPSCs were efficiently differentiated even to motor neurons, the cell type destroyed in ALS. Cellular models are also made on neural progenitors generated from hiPSCs derived from fibroblasts of patients with central nervous system neuropathies [119, 120]. The studies reporting neuronal differentiation from pluripotent stem cells are listed in Table 1.

Direct Reprogramming of Fibroblasts to Induced NSCs (iNSCs) or Neural Progenitors (iNPs)

The primary limitation related to derivation of functional neuronal cells from hESCs and hiPSCs is the involvement of multiple steps, variability, and slow procedures. Generating neurons by differentiation of hESCs or iPSCs requires months of tissue culture procedures and renders large-scale studies difficult [90]. Moreover, the differentiation protocols are dependent on specific chemicals or growth factors such as pharmacological agents and bioactive proteins that may vary in consistency, thus introducing a further element of variability [120] and can form teratomas in vivo. This has led to the advent of direct lineage conversion or reprogramming to lineage-specific stem/progenitor cells of another germ layer in one step, bypassing the intermediate pluripotent stage, and these cells in the neuronal lineage are known as induced neurons (iN).

To reprogram fibroblasts to NSCs, two broad approaches have been used (Fig. 4). Thier et al. [121] used the same four factors (Oct4, Sox2, cMyc, and Klf4) applied for iPSC reprogramming but restricted Oct4 expression for the first 5 days using either protein transduction or mRNA transfection. This method tried to create a scenario in which reprogramming intermediates that have begun to acquire pluripotency are placed under the control of three factors (Sox2, cMyc, and Klf4). Sox2, which is known to strongly regulate neuroectodermal development with concomitant inhibition of mesendodermal development [122], in turn led to the acquisition of an NSC fate by the presumed pluripotent intermediates. Conceptually, this method is similar to that taken by Kim et al. to produce induced neural progenitors from fibroblasts [123] although well-controlled Oct4 expression in this study allowed the generation of tripotent induced neural stem cells (iNSCs). These iNSCs have extensive self-renewal capacity in comparison to the bipotent cells with limited passaging ability. Han et al. [124] and Lujan et al. [127] took a different approach for this direct conversion of fibroblasts. Each of these two groups started with a list of 11 candidate factors that resulted in generation of iNSCs or induced neural progenitor cells (iNPCs). The systematic elimination of factors narrowed the list down to the minimum combination of factors required. Han et al. identified four factors (Sox2, cMyc, Klf4, and Brn4/Pou3f4), whereas Lujan et al. arrived at a three-factor combination (Sox2, FoxG1, and Brn2). Both the nonuse of Oct4 and the inevitable role of Sox2 are noteworthy. In fact, Ring et al. [125] managed to generate iNSCs from mouse and human fibroblasts by

Table 1 List of neuronal differentiation studies from pluripotent stem cells

Source	Intermediate cell type	Markers	Target population	Morphogens/factors used	Markers	References
hESC	Neural progenitors	Nestin, Sox2, Musashi1, (-ve for OCT 4), Ki67	DA neuronal subtype	SHH, FGF8, polyornithin and fibronectin	TH, Nurr1, Map2ab	Datta et al. [62]
hESC	(Primitive anterior neuroepithelia)	Pax6, Sox2, N-cadherin	Neuro epithelial cells	RETINOIC ACID (RA)	Pax6, Otx2, Sox1, N-Cadherin, Hox	Pankratz et al. [63]
hESC	NSC	Nestin, Sox2, Glast, Vim and Blbp	DA neuronal subtype, Astrocytes	SHH, FGF8, cAMP, BDNF, GDNF, TGFß3, poly-l-ornithine, laminin	TH, LMX1a, VMAT, Girk2, β III Tubulin	Swistowski et al. [64]
hESC	Neuroepithelial cells	SOX1, SOX2, SOX3, Nestin, Musashi 1	Postmitotic neurons and astrocytes	FGF2, ± EGF	PSNCAM, A2B5, MAPII, GFAP, O4	Shin et al. [65]
mESC	Ventral midbrain cells		DA neuronal subtype	(FGF2, FGF 8, SHH) + Transfected with Wnt 5a	Nurr1, TH, β III Tubulin	Parish et al. [66]
hESC	Neural progenitors	Pax6, Sox1, Sox2, Nestin	neurons, astrocytes, GABAergic, dopaminergic, motor neurons and oligodendrocytes			Yan et al. [69]
hESC			GABAergic, dopaminergic cell types	Multivalent Shh conjugates		Vazin et al. [72]
hESC	Retinal progenitors	Six3, Crx, Rx, Pax6, Otx2, and Chx10	Retinal cells	SHH	S-Opsin	Amirpouret al. [73]
hPSCs	Neuroepithelial cells		Motoneuron progenitors	RA, SHH and purmorphamine	OLIG2	Hu et al. (2009)
hESC	Neural precursor_		Functional Motoneuron	FGF2, RA, SHH agonist	OLIG2, RALDH2, FOXP1	Patani et al. [74]
				FGF2, SHH agonist	OLIG2	
hESC	Neuroepithelial cells	Pax6, Sox1	Motoneuron	RA	HB9, HoxC8, ChAT and VAChT	Li et al. [81]
hESC	Neural progenitors		Glial progenitors	EGF	NF1A, S100b, CD44 and GFAP	Deneen et al. [86]

hESC	Neural progenitors		Oligodendrocytes	RA and purmorphamine	OLIG2, Nkx2.2 and Sox10	Jiang et al. [87] and Alsanie et al. [88]
hESC	Neural progenitors		DA cell type	miR-125b and miR-181a	NURR1, DAT, TH and GAD1	Stappert et al. [89]
hiPSCs/hESC's	Neural rosette	OTX2, PAX6, Sox1, Nestin, NR2F1, NR2F2, and IRX2	Dopaminergic neurons-in vitro and mature appearing pyramidal and serotonergic neurons in vivo		TH	Malchenko et al. [91]
hiPSCs	Neural progenitors	βIII tubulin, Ki67, Nestin, Pax6 and Sox2	Excitatory neurons	Cultured NP's on E18 rat primary cortical neurons	NeuN, MAP2, VGLUT and Synaptophysin	Verpelli et al. [94]
				Cultured NP's on rat primary glial cells	MAP2, synaptophysin and VGLUT	
				Cultured NP's on matrigel	βIII tubulin, MAP2	
hiPSC lines	Neuroepithelial cells	OTX2, LHX2, HOXB4, FOXG1	fore brain neurons, dopaminergic neurons, spinal motor neurons	Ornithine/laminin-coated coverslips + RA + SHH + FGF8 + BD NF + GDNF + IGF1	TBR1+, MAP2, CTIP2, VGLUT1, TH+	Zeng et al. [95]
miPSCs cell lines	Embryoid bodies to neuroepithelial like cells	Nestin, Sox2, and Brn2	Neurons, astrocytes, oligodendrocytes	Removal of FGF 2 from culture	βIII tubulin, GFAP, 04	Wernig et al. [98]
			Dopaminergic neurons	SHH, FGF8	βIII tubulin, TH, VMAT2, Enl, Pitx3, and Nurrl	
			Neurons and glia	in-vivo	GFAP, NeuN and β-III-tubulin	
			Glutamatergic neurons		EAAC1	
			GABAergic neurons		GAD67	
			Catecholaminergic neurons		TH	
hiPSCs/hESC's	Neural rosettes		Retinal ganglion cells	Notch inhibitor N-[N-(3, 5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT)	BRN3A, BRN3B, ATOH7/Math5, y-synuclein, Islet-1, and THY-1	Riazifar H et al. [103]

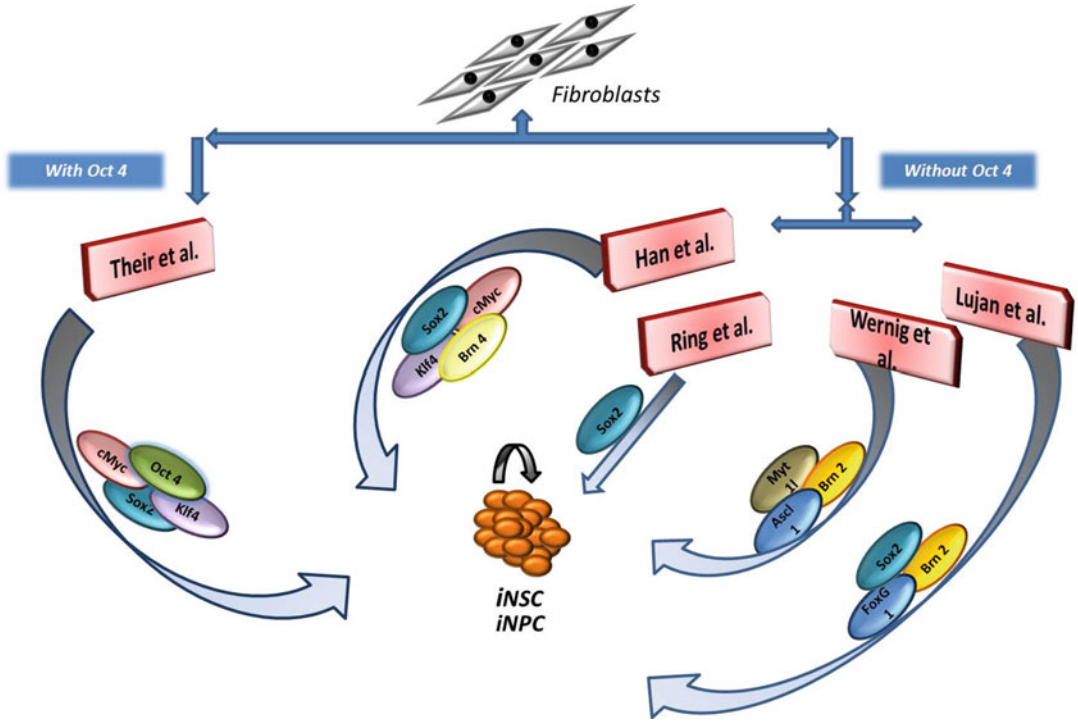

Fig. 4 Schematic representation of cell reprogramming approaches by introducing different sets of genes, adopted by different scientific research groups for deriving iNSCs or iNPCs from fibroblast cells

using the single factor Sox2 for reprogramming. In 2011, Pang et al. [126] reported a detailed method of reprogramming by screening a pool of 19 genes. Their study suggested that Ascl1 alone was sufficient to induce neuronal traits in fibroblasts, but the combination of *Ascl1*, *Brn2*, *and Myt1l* provided the best efficiency of reprogramming to iNSCs [126]. The iNSCs possess surprisingly robust self-renewal capability, with up to 130 passages reported in culture [124]. The iNSCs and iNPCs can undergo trilineage differentiation (neuron, astrocyte, and oligodendrocyte) in culture that when transplanted give rise to neuronal and glial progenies. This is consistent with their gene expression signatures that resemble endogenous NPCs. Lujan et al. reported successful myelination by oligodendrocytes differentiated from iNPCs. Further in the same year Caiazzo et al. reported the generation of differentiated functional dopaminergic neurons by direct conversion from mouse and human fibroblasts. Direct conversion in vivo for generation of iNSCs has also been reported recently [128, 129]. Transplanted human fibroblasts and human astrocytes engineered to express inducible forms of neural reprogramming genes converted into neurons when these genes were activated after transplantation [129]. Using a transgenic mouse model to specifically direct expression of reprogramming genes to parenchymal astrocytes residing in the striatum, it was shown that endogenous mouse astrocytes can be directly converted into neuronal nuclei (NeuN)-expressing neurons in situ. This provides evidence that direct neural conversion can take place in the adult rodent brain when using transplanted human cells or endogenous mouse cells as a starting point for neural conversion. Similarly, hiNPs transplanted to lateral ventricle were able to differentiate into NeuN-positive terminal neurons just by responding to induction of the surrounding environment. These differentiated terminal neurons were found in a variety of locations, displaying their migration ability in the brain [130]. Direct lineage

conversion is not only restricted to NSCs or neural progenitors but also for terminally differentiated specialized neuronal cells. A recent report [131] showed that two small molecules (forskolin and dorsomorphin) enable the transcription factor Neurogenin-2 (NGN2) to convert human fetal lung fibroblasts into cholinergic neurons with high purity (>90 %) and efficiency (up to 99 % of NGN2-expressing cells). These human induced cholinergic neurons (hiCN) show mature electrophysiological properties and exhibit motor neuron-like features, including morphology, gene expression, and the formation of functional neuromuscular junctions. NSCs exist in vivo in a highly regionalized manner and produce region-specific neuronal types, and it appears that iNSCs may also share this regional identity. Han et al. reported strong expression of ventral hindbrain markers in the iNSCs generated by Sox2, cMyc, Klf4, and Brn4/Pou3f4. Although the reason for this hindbrain signature is unclear, one can imagine that region-specific factors could be deliberately added to the reprogramming mix, directly inducing region-specific iNSCs that are both expandable and able to produce defined neuronal subtypes. Such a scenario could give iNSCs a potential advantage over iPSCs.

Differentiation of Mesenchymal Stem Cells

Mesenchymal stromal cells (MSCs) are multipotent somatic stem cells shown to reside within the connective tissues of most organs. These non-hematopoietic stem cells are tissue specific and more restricted than embryonic stem cells in terms of differentiation. MSCs are regarded as strong candidates for cell replacement therapies because of their ability to self-renew, differentiate to multilineage, migrate and home in on injury sites, and for being immunomodulatory [132–134]. MSCs were first derived from bone marrow by Friedenstein et al. in 1976 [135]. These cells were characterized based on plastic adherence, marker expression, and the ability to differentiate to adipogenic, chondrogenic, and osteogenic lineages [136]. Although BM-MSCs are preferred for therapies, the procedure for procurement of bone marrow is extremely invasive and painful for patients. Apart from the bone marrow, MSCs are also located in adult and fetal tissues (Fig. 5). There are an increasing number of reports describing their presence in adipose tissue [137–139]; periodontal tissues such as dental pulp, dental ligament, follicle, and papilla [137, 140]; peripheral blood [141]; umbilical cord Wharton's jelly, cord blood, and chorionic villi of the placenta [142–145]; amniotic fluid [146]; fetal liver [147]; and lung [148]. The stromal cell population isolated from these tissues of origin need to be characterized by the set of MSC criteria such as plastic adherence; expression of CD105, CD73, and CD90; negative expression of CD45, CD34, CD14 or CD11b, CD79 alpha or CD19, and HLA-DR surface molecules; and in vitro differentiation to osteoblasts, adipocytes, and chondroblasts [149]. These cells have received extensive attention in the field of tissue engineering and regenerative medicine due to their availability and multilineage potential.

MSCs being a connective tissue derivative obviously show differentiation potential towards mesoderm lineage. However, recent studies suggest that MSCs have the capacity to transdifferentiate to cells of the other two lineages too (Fig. 5), including islet cells, myoblasts, cardiomyocytes, hepatocytes, and neuronal cells [150–156]. A microSAGE analysis of 2,353 expressed genes in a single cell-derived colony of undifferentiated human mesenchymal stem cells reveals mRNAs of multiple cell lineages [157]. Although NSCs or neural progenitors have been successfully generated from ESCs [62, 74] and iPSCs [158, 159], differentiating MSCs to functional neuronal cells is considered to be more difficult as it is a more committed cell type.

In Vitro Neuronal Differentiation of MSCs

Neuronal Differentiation Using Extrinsic Cues

In vitro neuronal differentiation of MSCs or adult stem cells (ASCs) is performed primarily to estimate the neuronal plasticity of these

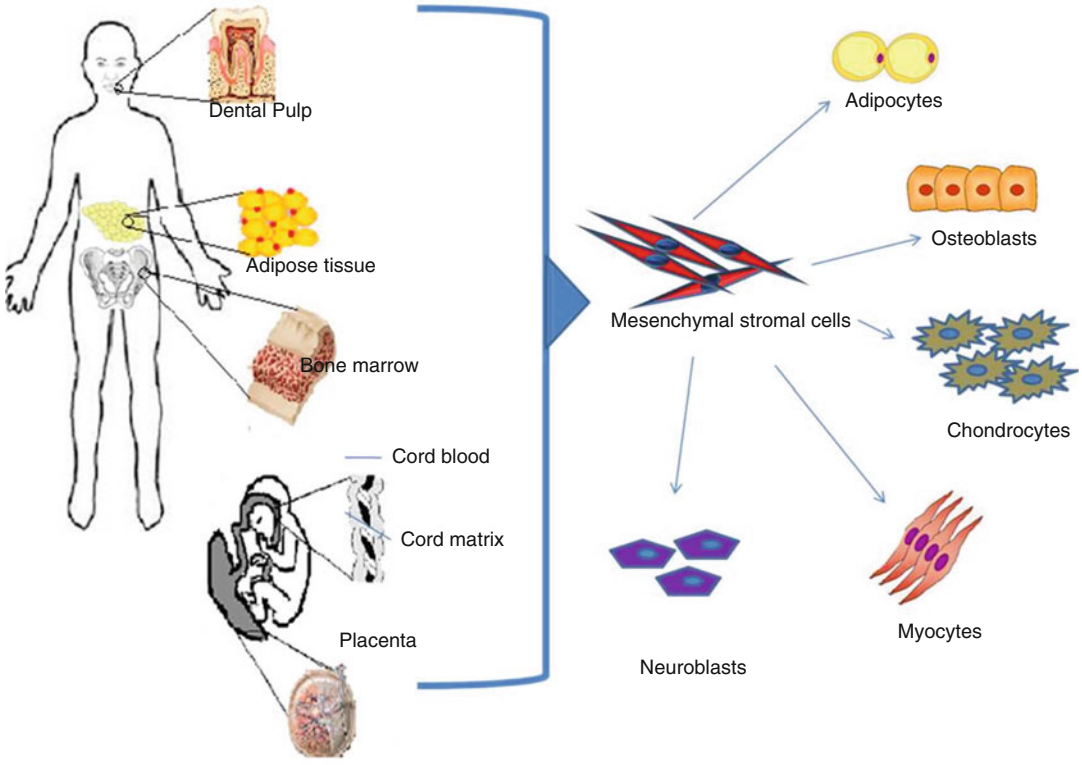

Fig. 5 Schematic representation of isolation of MSCs from adult and fetal tissues and their differentiation potential to different lineages

cells under defined regulated environment. Studies on differentiation of MSCs to neuronal phenotype have typically followed two broad approaches – modulating the microenvironment by extrinsic cues and delivery of key transcription factors along with extrinsic cues to obtain better efficiency and yield [160]. The extrinsic cues used by researchers for the past decade portray a vast diversity – ranging from chemical inducers to embryonic growth factors and conditioned media from rodent primary neuronal and glial cultures. Woodbury et al. [161] were the first group to report neuronal differentiation from BM-MSCs. They obtained a fast transition of neuronal phenotype under the influence of chemical induction such as dimethyl sulfoxide (DMSO), butylated hydroxyanisole (BHA), and β-mercaptoethanol (BME). However, studies by Lu et al. [165] in 2004 indicated that pure chemical exposure of MSCs induces neuronal-like morphology but does not yield functional neurons and additionally leads to cell toxicity and cell shrinkage. Several other neuronal differentiation protocols involving 3-isobutyl-1-methylxanthine (IBMX), bFGF, dimethyl sulfoxide (DMSO), butylated hydroxyanisole (BHA), or epigenetic reprogramming by 5-azacytidine [163] reported rapid changes in morphology with rounded cell bodies, which were eventually reversible in the absence of the induction factors and showed drastic cell death with time [163]. Although 5-azacytidine treatments showed neuronal morphology by day 4, here too the decrease in cell density with time is noteworthy [163]. The cytoskeletal changes resulting in pseudo-neuronal morphology thus indicated cell toxicity. So, the use of embryonic morphogens and neurotrophic factors for neuronal differentiation of MSCs started gaining favor. Trzaska et al. depicted that BM-MSCs

exposed to midbrain cues (SHH and FGF8) were capable of differentiating them to dopaminergic neurons [164]. These ontogenically relevant cues were also used by Datta et al. (2011) to assess the neuronal plasticity of WJ-MSCs in comparison to BM-MSCs. The author's group [155, 156] in their study has shown phenotypical and functional characterization of BM-MSCs, WJ-MSCs, and DPSCs, not only in the presence of the morphogens but also when the differentiated cells were maintained in maintenance medium (absence of the morphogens). The MSCs from all the three sources spontaneously showed abundant expression of early neuronal markers such as nestin, musashi12, and A2B5 along with the mesenchymal markers. Upon induction, the upregulation of mature neuronal markers β-tubulin III and Map2ab was followed by a decrease in these early neuronal markers. Moreover, a distinct increase in dopaminergic-specific transcription factors (En1, Nurr1, Pitx3) and dopaminergic marker TH was observed. Functionally these cells could secrete dopamine constitutively and upon stimulation with ATP. The differentiated DPSCs though could secrete dopamine upon KCl stimulation too, in turn indicating the presence of purinergic receptors and potassium ion channels in the induced cells. In mature neuronal cells, the neurotransmitter is stored in vesicles within the cell and its release can be triggered by the influx of intracellular Ca^{2+}. The midbrain cues were thus capable of inducing the DPSCs to excitable cells, mimicking the physiology of neurotransmitter release of native neurons [156].

Studies have also targeted the signaling pathways for neural differentiation of MSCs by providing ligand molecules, molecular effectors, and inhibitors that are involved in upregulating transcription factors followed by gene expression. One such pathway frequently targeted for neuronal differentiation by many research groups is cyclic adenosine monophosphate (cAMP)-activated PKA (protein kinase A) pathway, which in the downstream mechanism phosphorylates CREB followed by regulation of different genes coded for cFOS, BDNF, and TH in turn aiding neural differentiation [166–168]. Based on this evidence, many studies have successfully induced neural differentiation of BM-MSCs and UCB-MSCs by using forskolin, dibutyryl-cAMP (db-cAMP), and 3-isobutyl-1-methylxanthine (IBMX). These studies determined the expression of neurofilament (NF), TH isoforms, and nuclear receptor related 1 (Nurr1) and also showed significant voltage-dependent ionic currents [169, 170]. However, the role of cAMP and the downstream effect in neural differentiation is not explicitly defined [171, 172]. Forskolin along with GDNF and embryonic midbrain morphogens were also used for neuronal induction of human exfoliated deciduous teeth (SHED) to differentiate to dopaminergic neurons [173]. Alexanian et al. [174] used the combination of small molecules that affect the regulation of chromatin structure and function and agents that favor neural differentiation to generate neural-like cells from human MSCs. The efficiency of neuronal differentiation and maturation was improved by two specific inhibitors of SMAD signaling (SMAD1/3 and SMAD3/5/8) that play an important role in neuronal differentiation of ESCs and were added to chromatin-modifying enzymes. Results demonstrated that human MSCs grown in these culture conditions exhibited higher expression of several mature neuronal genes, formed synapse-like structures, and exhibited electrophysiological properties of differentiating neural stem cells [174]. Recent studies have shown that the inhibition of histone deacetylases (HDACs) induces the differentiation of diverse cancer and stem cells which in turn suggests that HDAC inhibitors may be good candidates for neuronal induction of MSCs too. Jang et al. [175] investigated the effects of a HDAC inhibitor, valproic acid (VPA), for the neuronal differentiation of BM-MSCs. VPA-treated MSCs had significant increase in expression of neuro-progenitor marker nestin, musashi, CD133, and GFAP. VPA-pretreated MSCs upon differentiation with neuronal induction media (VPA-dMSCs) exhibited a cell body and dendritic morphology similar to neurons and neuronal-specific marker genes, including nestin, musashi, CD133, GFAP, NeuN, MAP-2, NF-M,

KCNH1, and KCNH5, but no functional study is reported. Some studies have also shown that MSCs cultured in the presence of rodent primary neuronal or glial culture-conditioned media or as coculture enhanced the expression of neuronal markers in the MSCs [177]. This could be due to the presence of potentially rich sources of neuronal differentiation promoting signals in the culture derivatives [178]. However, the use of rodent culture derivatives not only introduces undefined additives but is also xenogeneic.

Neuronal Differentiation Using a Defined Medium Composition

Researchers have also targeted enriching neuronal marker expression in MSCs by culturing them in suspension culture in NSC medium consisting of serum-free media with bFGF and EGF [176, 178]. MSCs, ADSCs, and DPSCs were capable of forming neurosphere-like structures and expressed neural progenitor markers in these conditions. Some studies have used the NSC media to prime the MSCs or ASCs towards neuronal lineage followed by ontogenic morphogens and growth factors as induction factors [179]. Besides specialized dopaminergic neuronal cells, MSCs have been differentiated to cholinergic cell types as well. The spindle-shaped or fibroblast-like WJ-MSCs changed into bulbous cells and positively expressed cholinergic neuronal markers, along with elevation of secretion of acetylcholine in the induced WJ-MSCs [180]. Transdifferentiation of BM-MSCs to cholinergic neurons was also demonstrated by Naghdi et al. [181] by the use of BME and NGF. This treatment with BME led to the generation of NF68-positive neuroblasts, which generated close to 80 % cholinergic marker-positive cells upon addition of NGF. When treated with neuronal induction medium consisting of brain-derived neurotrophic factor (BDNF), low-serum media and supplemented with hippocampal cholinergic neuro-stimulating peptide (HCNP) or rat denervated hippocampal extract (rDHE) or in combination, it enhanced the action of choline acetyltransferase (ChAT) [182]. A new multistep induction protocol has been reported by Darabi et al. [183] for the transdifferentiation of bone marrow stromal stem cells into GABAergic neuron-like cells. Rat BM-MSCs were pre-induced using β-mercaptoethanol (BME) and induced using retinoic acid (RA) and creatine. Immunostaining of neurofilament 200 kDa, neurofilament 160 kDa, nestin, fibronectin, gamma-aminobutyric acid (GABA), and glutamic acid decarboxylase (GAD) 65/67 was performed in the induced MSCs. Neuronal differentiation has been attained by adipose-derived stem cells too. Factors like bFGF, EGF, insulin, retinoic acid, and hydroxycortisone have been used for neuronal differentiation of AD-MSCs [184–186]. AD-MSCs expressed sodium current on treatment with bFGF and forskolin by increasing the intracellular cAMP levels, which was found to be useful in neural induction [171, 187]. A recent study has shown neuronal induction of ADSCs using BME, glial cell line-derived neurotrophic factor (GNDF), brain-derived neurotrophic factor (BDNF), retinoic acid (RA), 5-azacytidine, as well as their combinations [188]. NSC media consisting of EGF and bFGF along with IBMX and BDNF have also been used for ADSC neuronal induction [189]. A few studies have demonstrated the differentiation of MSCs towards glial cell type as well. A recent study has shown that norepinephrine, a neurotransmitter, when added in vitro can generate oligodendrocytes from the umbilical cord-derived multipotent progenitor cells in a three-dimensional environment [190]. Researchers have also shown that BM-MSCs and ADSCs can differentiate into Schwann-like cells (SLC) that have the potential to myelinate neuronal cells during regeneration [191].

Direct Conversion of MSCs to Neuronal Cell Types by Transduction

Apart from these studies, various other strategies have been employed to obtain neural cells types from different sources of MSCs. It has been shown that the microRNAs play an

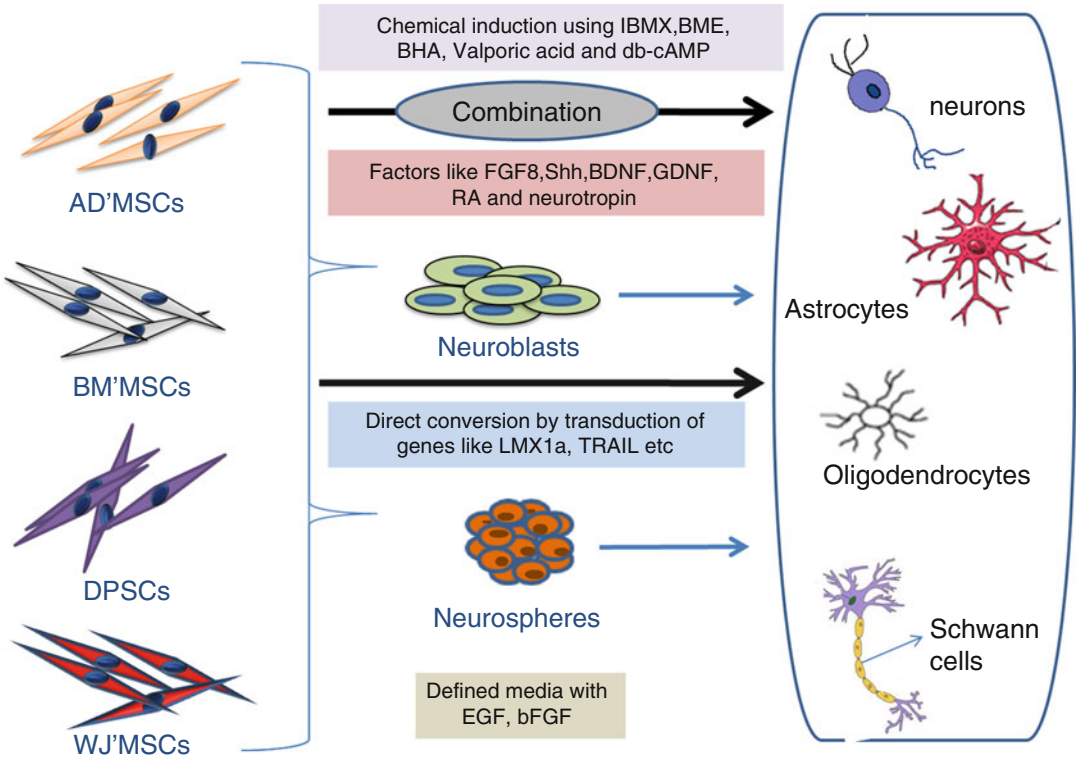

Fig. 6 Schematic representation of various in vitro neuronal induction strategies being reported in literature for obtaining differentiated neuronal and glial cells from adult and fetal tissue-derived stem cells

important role in the neuronal differentiation of WJ-MSCs [192]. These small RNAs of 18–24 nucleotides in length were involved in the regulation of gene expression and found to enhance motility and oxidative phosphorylation in neural cells derived from the WJ-MSCs. Studies aiming at the direct insertion of target genes for differentiation have also been reported. Transcription factor such as Neurogenin-1 was effective in converting MSCs into neuron-specific protein-expressing cells [193, 194]. The lentiviral delivery of transcription factor LMX1a showed enhancement of dopaminergic phenotype in differentiated human BM-MSCs [193, 194]. These neurons could synthesize higher level of the enzyme tyrosine hydroxylase (TH). It has been shown that the neuron-restrictive silence factors promote neural differentiation with enhanced electrophysiological properties [195]. AD-MSCs transfected with TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) showed therapeutic efficacy against brainstem gliomas, and these MSCs were able to differentiate into neural cell types, thus reducing the tumor volume in vivo [196]. The schematic representation depicting the various in vitro neuronal induction strategies adopted for transdifferentiation of adult and fetal tissue-derived stem cells to differentiated neuronal and glial cells is provided in (Fig. 6). The in vitro neuronal differentiation studies discussed over here is summarized in Table 2.

In vitro studies on neuronal differentiation clearly demonstrate that MSCs and adult tissue-derived stem cells possess a certain extent of neuronal plasticity and can respond to ontologically relevant embryonic cues. However, for transplantation in vivo, the microenvironment cannot be controlled as the embryonic developmental clock and most of the embryonic cues and morphogens would not be available. The in vitro neuronal plasticity of MSCs was indeed replicated in

Table 2 List of in vitro neuronal differentiation studies from mesenchymal stem cells isolated from different adult and fetal tissue sources

Mesenchymal stromal cells	Defined factors	Results	References
BM'MSCs	Dimethylesulfoxide, butylatedhydroxyanisol, β-mercaptoethanol	Neuronal like cells positive for Enolase, NeuN, Tau and Neurofilament-M.	Woodbury et al. [161]
BM'MSCs	Dimethylesulfoxide, butylatedhydroxyanisol, β-mercaptoethanol, high molarity sodium chloride, detergent	Neuronal like morphology due to cell shrinkage and toxicity, positive for NeuN and NF-M but described to be Non functional neurons.	Lu et al. [165]
BM'MSCs	Dimethylesulfoxide, butylatedhydroxyanisol, β-mercaptoethanol	Non functional Neuronal like cells positive for NF-200, S100β, (βIII-tubulin, NSE and MAP2. Negative for Na(+), K(+) currents and action potentials	Barnabe et al. [162]
DPSCs	bFGF, 5-azacytidine, db-cAMP, neurotrophin-3 and supplementary components	Neuronal like cells expressing Neurogenin-2, NSE, NF-M, GFAP and positive for active voltage-dependent channels.	Kiraly et al. [163]
BM'MSCs	Midbrain cues such as SHH and FGF8	DA like neurons positive for TH, Pitx3, nurr1, DAT and VMAT2and also expressed NeuN and beta III tubulin	Trzaska et al. (2007, 2011)
BM'MSCs, WJ'MSCs and DPSCs	SHH, FGF8 and bFGF	Assessed the early neuronal markers in naive MSCs from three sources, obtained neuronal like cells expressing EN1, Nurr1, Pitx3 and TH. Studies also showed the functional DA neurons through the dopamine release and increase of intracellular calcium	Datta et al. [155, 156]
BM'MSCs & UCB'MSCs	Forskolin, dibutyryl-cAMP (db-cAMP) and (IBMX)	Cells were positive for NF-M, TH isoforms and nuclear receptor related 1 (Nurr1) and also showed significant voltage dependent ionic currents	Wang et al. [169] and Lepski et al. [170]
DPSCs(SHED)	SHH, FGF8, Forskolin and GDNF	Dopaminergic cells positive for beta III tubulin nestin, TH and MAP2	Wand et al. [173]
BM'MSCs	Trichostatin A (TSA) RG-108, 8-BrcAMP 1 μM Rolipram	DA like neurons expressing Nurr1 and TH. Also determined the secretion of neurotrophins and dopamine	Alexanian et al. [174]
BM'MSCs	Combination of histone deacetylase and valproic acid	Neural progenitors positive for nestin, Musashi, CD133, and GFAP, NeuN, Map2, NF-M, KCNHl and KCNH5	Jeong et al. (2013)
BM'MSCs	bFGF and EGF	Neurospheres which was positive for nestin and musashi were co cultured with primary neurons and obtain specific neural cell type	Fu et al. [176]
WJ'MSCs	bFGF and EGF	Neurospheres positive for neural progenitor markers nestin, Sox2 and Pax6 transcription factors. When differentiated on fibronectin coated dishes these were able to generate neuron/glial - like cells which expressed Nfl, Map2 and GFAP	Balasubramanian et al. [178]

(continued)

Table 2 (continued)

Mesenchymal stromal cells	Defined factors	Results	References
DPSCs	EGF, FGF, ITS, retinoic acid	Neural progenitors positive for Nestin, beta 3 tubulin, EGF, FGF, ITS, retinoic acid Neuronal Nestin, PSA-NCAM,	Arthur et al. [179]
BM'MSCs	FGFβ, BDNF, EGF and NGF	Cholinergic neurons positive for GAP-43, NF-H, Neu-N	Naghdi et al. [181]
WJ'MSCs	BDNF, Low serum media, hippocampal cholinergic neuro-stimulating peptide or hippocampal extract and in combination	Functional cholinergic like cells were obtained with enhanced ChAT and secretion of Ach	Zhang et al. (2012)
BM'MSCs	β mercaptoethanol (BME), retinoic acid and creatine	GABAergic like cells positive for NF-200, NF-160, nestin, fibronectin, GABA and GAD65/67	Darabi et al. [183]
AD'MSCs	Valporicacid, insulin, hydroxyanisole, hydrocortisone, EGF, FGF.	Neuronal GFAP, Neu-N, nestin, IF-M	Schaffler and Buchler [185] and Safford et al. [184]
AD'MSCs	BDNF and retinoic acid	Neuronal cells positive for MAP 2, Neu-N, nestin, GalC, S 100, GFAP, TH	Anghileri et al. [186]
AD'MSCs	bFGF and EGF	Neuronal cells positive for Nestin, Sox2, vimentin, A2B5, GFAP, tuj1.	Lim et al. (2010)
AD'MSCs	bFGF, forskolin, ciliary neurotrophic factor, GDNF.	Neuronal cells positive for Map-2ab, NF-M, GFAP, GalC, O4, TH, DAT	Kim et al. [187] and Rooney et al. [171]
AD'MSCs	BME, GDNF, BDNF, RA and 5-azacytidine and combinations	Neural cells expressed nestin, BIITub & ENO2. *In vivo* brain promoted their migration from the transplantation site to the recipient cerebral parenchyma.	Pavlova et al. [188]
AD'MSCs	1st step-EGF, bFGF with IBMX, BDNF; 2nd step-BDNF	Both induced Neural cells were positive for GFAP and TUJ1 markers but 1st step method showed higher expression then 2nd step method	Ying et al. [189]
WJ'MSCs	Defined medium containing the neurotransmitter norepinephrine (NE)	Under two-dimensional conditions, differentiated into oligodendrocyte precursors. In a three-dimensional environment, the MLPCs differentiated into committed oligodendrocytes that expressed myelin basic protein	Hedvika et al. [190]
AD'MSCs	Glial growth factors (GGF-2, bFGF, PDGF and forskolin	Spindle-like morphology similar to Schwann cells, expressed the glial markers, GFAP, S100 and p75, When co-cultured with NG108-15 motor neuron-like cells, induced neurite growth in NG108-15 cells	Mantovani et al. [191]
AD'MSCs	β-mercaptoethanol, all-trans-RA, and mixture of forskolin, bFGF, PDGF and heregulin	Schwaan like cells express S100 and GFAP. Enhance neurite outgrowth in co-culture with sensory neurons	Jiang et al. [87]

(continued)

Table 2 (continued)

Mesenchymal stromal cells	Defined factors	Results	References
WJ'MSCs	B27 supplement, RA, and bFGF and carried out miRNA analysis	Gene Ontology database showed that 136 genes were associated with cell motility, energy production, oxidative phosphorylation and actin cytoskeleton organization, indicating that miR-34a plays a critical role in cell migration	Chang et al. [192]
BM'MSCS	LMX1a transduction	LMX1a protein was concentrated in the cells' nuclei and specific dopaminergic developmental genes were upregulated, expressed higher levels of tyrosine hydroxylase, and secreted significantly higher level of dopamine	Brazilay et al. [193]
AD'MSCs	TRAIL transduction	Type of cells assessed Astrocyte, oligodendrocyte positive forTuj 1, GFAP, CNPase, adiponectin, sialoprotein. Efficacy was tested *In vivo* and *In vitro*	Choi et al. [196]

in vivo embryonic brain. Muñoz-Elias et al. [197] for the first time had demonstrated that both rat and human BM-MSCs assume neuronal functions in vivo in an embryonic CNS microenvironment. The transplanted adult MSCs not only survived and migrated in embryonic day 15.5 (E15.5) rat ventricles in utero but also differentiated in a regionally and temporally specific manner. Transplantation of MSCs during the gliogenic clock in neonatal mouse brains showed that BM-MSCs could migrate throughout the forebrain and cerebellum and differentiate into astrocytes after injection [198]. The in vivo differentiation of MSCs to neuronal cells thus gets restricted from embryonic to neonatal and then for adult CNS. The analysis of postmortem brain samples from females who had received bone marrow transplants from male donors showed that marrow cells can enter the brain and generate new neurons just as rodent cells do [199]. The underlying diseases of the patients were lymphocytic leukemia and genetic deficiency of the immune system, and they survived between 1 and 9 months after transplant. This was one of the first indicative studies suggesting the neuronal plasticity of marrow cells and probable neurogenic environment of the adult CNS. Most studies reported have targeted the transplantation of undifferentiated MSCs in the disease or injury model, and some studies have transplanted these MSCs after priming the cells with neuronal induction cues or growth factors. DPSCs upon transplantation in neurogenic area of the CNS, hippocampus, of mice underwent proliferation and maturation, forming NPCs and neurons [200]. Transplantation of these cells in a nonneurogenic area of the CNS shows survival, engraftment, and improvement of behavioral scores of the disease rodent model but rarely shows differentiation to mature neuronal cell type. An early study in 1998 [201] showed engraftment and migration of human BM-MSCs implanted in the brains of albino rats – similar to astrocyte grafts. Human SHED derived stem cells and WJ-MSCs transplanted post-neural induction with SHH, FGF8 have differentiated into dopaminergic neurons in vivo and further elevated the dopamine content [179, 176, 173]. Regeneration by neural-induced human BM-MSCs in rat models of Parkinson's disease has also been reported [202–204]. MSCs treated with stromal derived factor-1 (SDF-1) increased the release of dopamine and also helped in preserving the TH-positive cells [205] in Parkinsonian rat model. hMSC treatment had a protective effect on progressive loss of dopaminergic

neurons induced by MG-132 in vivo through differentiation and trophic effect [206]. Human umbilical cord MSC-derived neuron-like cells have shown rescue of memory deficits and reduced amyloid-beta deposition in an AβPP/PS1 transgenic mouse model [207]. In addition, neuroectodermally converted BM-MSCs led to decrease of Aβ peptides by regulation of two genes F-spondin and neprilysin [208]. All these data suggest that MSCs induced by recently developed methodologies could be a potential source of cells to replace damaged neurons and glia in injured spinal cord and/or to promote cell survival and axonal growth of host tissue. Protection of dopaminergic neurons against the neurotoxic effects and motor deficits was obtained too by transplantation of undifferentiated ADSCs, WJ-MSCs, and BM-MSCs in MPTP-induced rats [209–212] indicating the paracrine neurotrophic effect of the cells rather than the graft cells differentiating in the adult CNS. Transplantation of GLP-1 transected hMSCs in the right ventricle of double transgenic mice mutant expressing APP and presenilin-1 showed a reduction in Aβ40/42 positively stained plaques, and the number of reactive astrocytes measured in the dentate gyrus of the hippocampus also decreased [213] again representing its paracrine effect. Contribution towards reduction in ischemic damage has been reported in ischemic stroke mouse model after transplantation of BM-MSCs and ADSCs [214, 215]. Bang et al. [216] have reported that autologous BM-MSC transplantation shows improvement of Barthel index and Rankin score in stroke patients. A long-term follow-up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke showed clinical improvement (Lee et al. 2010). Presently several clinical studies have established the safety of transplantation of autologous BM-MSCs. Four patients showed a significant slowing down of the linear decline of the forced vital capacity and of the ALS-FRS score [217]. The effect of a combination of autologous undifferentiated and neural-induced bone marrow mesenchymal stem cells (MSCs) on behavioral improvement in rats after inducing spinal cord injury has also been examined. In all treatment groups (differentiated, undifferentiated, and mix), there was less cavitation than lesion sites in the control group. The Basso-Beattie-Bresnahan (BBB) score was significantly higher in rats transplanted with a combination of cells and in rats transplanted with neural-induced MSCs alone than in undifferentiated and control rats [218]. Put together, the data obtained from the in vivo transplantation of MSCs in rodent models and clinical trials suggests that MSCs can promote endogenous reparative mechanisms that may prove applicable and beneficial for neurodegenerative disease treatment.

Conclusion

Regeneration of neurons in CNS has always remained as an enigma mainly because the neurogenic area that exists in the adult brain is deep-seated, and with currently available techniques, it is quite unlikely to engineer any modulation and be of further use in degenerative cases. This also limits the clinical use of hNSCs for any nervous system disorder. Important for effective differentiation of neuronal and glial subtypes is the patterning of primitive neuroepithelial cells. Taking inspiration from the ontogenic cues, directed differentiation has been performed from pluripotent stem cells both by modulating the extrinsic signals and changing the intrinsic master key transcription factors. Indeed, hESC and hiPSC neuronal differentiation quite efficiently mimics the developmental clock, and functional specialized neurons have been obtained by several scientific groups across the globe. However, immunological issues along with the risk of teratoma formation limit the therapeutic use of pluripotent stem cell-derived NSCs. MSCs score precisely on these points over pluripotent stem cells, and thus studies to determine the neurogenic plasticity of MSCs have been very much in the spotlight. A lot of experimental work on MSCs suggests that they bring about neuro-rescue in a multipronged way. While there are several research publications both in vitro and in vivo models, the exact mechanism of neuroprotection has not yet been

understood. Moreover, the functional integration of these differentiated neuronal cells with the host brain neurons under both normal and disease CNS environments needs to be further elucidated to make the use of MSCs as candidates for cell replacement therapy a clinical reality.

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