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From Stem Cells to Oligodendrocytes: Prospects for Brain Therapy

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Abstract Multiple sclerosis is an autoimmune disease that destroys myelin-forming oligodendrocytes of the CNS. While the damage can be partially controlled using anti-inflammatory cytokines and steroids, endogenous repair is insufficient to replace lost cells. Until now cell replenishment (transplant therapy) has been viewed as unlikely to succeed due to allograft rejection in this sensitized immune environment. However, advances in stem cell biology give new hope for deriving patient-specific, autologous oligodendrocytes which may tip the balance to favor repair. The challenge will be to engineer these cells to respond to cues that can target their migration into lesions for brain and spinal cord repair.

Keywords Embryonic stem cell · Migration · Multiple sclerosis · Myelin · Oligodendrocyte · Transplant

Introduction

The inability of our central nervous system (CNS) to self repair exacerbates many neuro-degenerative conditions and extracts a tremendous toll on individuals and society. These range from those which affect the young including autoimmune pathology of multiple sclerosis (MS) and traumatic brain and spinal cord injury, to later onset diseases including Parkinson's and Alzheimer's. Individuals stricken face a long and difficult clinical management with treatment options that at best delay but do not stop progression. We are left with a pressing need for novel approaches to promote brain repair, and amongst those being pursued are cell replenishment and replacement strategies employing stem cells. This article will address the potential of stem cell therapeutics for regeneration in the demyelinating disease MS.

The lay public and legislative politic have become quite engaged in many debates over the promised miracles of stem cell regenerative medicine. Issues addressed almost daily range from theological to budgetary and unfortunately exaggerated claims of research progress. This noisy brouhaha tends to supplant the focus on largely untested principles of the potential for stem cells to fix that which is broken. Politicians speak of cures, patients grasp for incremental benefits, and biologists often seem left wondering whether this might be a repeat of the ruckus over gene therapy and cold fusion. Often lost in the debate is the clinical perspective of just what it is that stem cells are projected to fix. In neuronal degenerative diseases, for example, it is not entirely clear that the therapeutic objective is to replace lost neurons and their complex interconnections [1, 2]. MS in contrast presents a reasonable challenge for proof of principal, since the pathology of the chronic lesion deficit is constant and well defined [3] and the objectives for repair are clear [4]. Unlike neuronal degeneration or neurotrauma, stem cell therapeutics for MS has a defined agenda.

Brain Repair and Remyelination

Oligodendrocytes produce myelin sheaths which insulate neuronal axons. Myelin is critical for fast (saltatory) axonal

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conduction, for the survival of these axons [5, 6], and to suppress axon neogenesis and thus quench inappropriate rewiring of the brain and spinal cord [7, 8]. Decades of inquiry into MS have failed to identify cause [9, 10] but succeeded in defining a mechanism [11], its consequences on axon degeneration [12] and intervention therapy (interferon-beta immune suppression). While promising, this is not a cure, its efficacy remains controversial [13, 14], and endogenous repair is insufficient to overcome the damage [3].

In relapse remitting MS, regions with myelin loss (plaques) are self repaired (shadow plaques) by oligodendrocytes which are presumed to be generated de novo from an endogenous pool of precursors [15–17], termed 'adult' progenitor cells [18, 19]. While there can be substantial repair of local demyelination in experimental models [4], the local pools of these cells are insufficient for significant repair of MS lesions [20]. Recruitment of more distal progenitors into MS lesions appears to fail, as they migrate to the edge but not into demyelinated plaques [21], and the center of chronic lesions are completely devoid of oligodendrocytes [3]. Adult progenitors thus appear designed for local homeostatic maintenance rather than as a mobile defense for acute damage control. Thus in addition to immune suppression, effective MS therapy requires a mechanism to both supplement the precursor pool and target these cells into a lesion.

The myelin inhibitors that suppress inappropriate axon sprouting also interfere with repair after traumatic spinal cord injury (SCI). Compression of the cord results in severed projection axons and the loss of interneurons due to a breakdown of the blood-brain barrier and toxicity of released neurotransmitters and inflammatory cytokines [22]. Cellular hypertrophy (the glial scar) then contributes to an environment through which damaged axons cannot regrow [8]. A second wave of damage extends beyond this wound due to oligodendrocyte death and demyelination of adjacent, otherwise intact axons. Thus myelin in the acute wound prevents axonal regeneration while its subsequent loss in the sub-acute wound exacerbates the deficits.

MS and SCI are good examples of why therapeutic approaches to CNS repair are in desperate need of a paradigm shift. Regenerating tissues such as blood and epithelial surfaces employ a seed of multipotential cells in an undetermined "stem" state for self replenishment [23]. The best studied of these are stem cells in bone marrow, and hematopoietic reconstitution is the gold standard for Stem Cell Therapeutics [23]. The brain and spinal cord also contain uncommitted stem cells which can generate neurons and glia in vitro [24–27]. While these appear to serve as a resource to rejuvenate neural cells throughout life, their apparent inability to repair an acute wound may reflect the unique architecture of the brain which, unlike skin or blood, is perhaps too complex for extensive self repair. This then leads to intervention strategies such as grafting exogenous cells for replenishment. In preclinical models, transplanted stem and progenitor cells which generate oligodendrocytes promote repair and recovery in myelin mutant rodents [28–32], from demyelinating lesions [33, 34] and after SCI [35–38]. Stem cells have thus emerged as a great hope for therapeutic reconstitution and brain repair, and autologous histocompatible stem cells are the reagent of choice.

Stem Cell Therapeutics

The inner cell mass of pre-implantation blastocysts contains cells which can be clonally expanded in culture as embryonic stem (ES) cells [39–41]. Individually they are pluripotential, generating all three germ layer cells in culture and, when injected into a blastocyst, they fully particpate in organogenesis (chimerism) including germ cells to produce a monoclonal animal. ES technology was developed for mouse transgenics and has extended to a number of species including human ES cells [42, 43]. If, the logic goes, bone marrow stem cells can regenerate the hematopoietic system of a cancer patient, then ES cells hold the promise and potential to regenerate any organ system for any disease. Fueling the optimism for this strategy are estimates which suggest that restoring only a fraction (10%) of normal activity will promote functional recovery for many diseases, although this number remains an enigmatic estimate.

There are limitations to this logic for clinical therapy. First, cell replacement does not address the underlying disease, and for infectious or autoimmune diseases exogenous grafts may only 'feed the fire' with limited therapeutic benefit. Second, allograft donor cells are susceptible to immune rejection, and both the costs and complications of immune suppression overrides potential benefits for chronic progressive diseases. Third is the pressing concern of ES cell tumorigenicity [44]. Grafted ES cells generate teratocarcinomas [44, 45] and teratomas (embryoid bodies) which in themselves present an ethical dilemma [46]. While differentiated progeny of ES cells are not tumorigenic, there is an absolute need to assess the tumorigenic potential of a pre-differentiated ES culture in immune-compromised pre-clinical models before they can be considered clinically safe. Finally, a fourth problem is the ethics of blastocyst manipulations to generate ES cells. To avoid debates over when a fertilized egg becomes a person the field has aggressively explored ethically neutral alternative sources including multipotent 'adult' stem cells. After birth sources (umbilical cord, placenta) or amniotic fluid [47] may provide patient-specific stem cells which could be banked at birth for autologous grafting. Stromal mesenchyme may also provide multipotential cells [48], although their apparent neuroectoderm potential is under

intense debate [49]. Indeed the ability of any germ layer specific 'adult' stem cell to trans-differentiate remains controversial [23]. A promising alternative to trans-differentiation is the reprogramming of somatic cells by nuclear transfer [50, 51] or gene conversion [52] to generate 'induced' stem cells with properties similar to pluripotential ES cells [53, 54]. Thus there remains great hope that once such approaches are feasible the ethics of blastocyst manipulations will be moot, the limitations of allografts eliminated, and clinical stem cell therapeutics using autologous cells can be programmatic. There then will remain the challenge to program the differentiation of stem cells to efficiently generate tissue-specific cells for repair.

Stem Cells to Oligodendrocytes: Instruction Cues

A prerequisite for ES cell therapeutics is to instruct lineage differentiation prior to grafting. For ES-derived oligodendrocytes (OLs) this involves the sequential presentation of signaling cues which direct ES to OL development in vivo (Fig. 1). Since mature OLs are post-migratory, the focal cell for grafting is their immediate progenitor the oligodendrocyte precursor cell (OPC) (Fig. 1b). OPCs migrate through the complex architecture of the brain during early development, and when grafted into a neonatal recipient they can both migrate and differentiate into OLs [55, 56]. Whether the adult brain can also provide appropriate signals for the migration and maturation of ES-derived OPCs is largely unexplored. Thus the cues involved in developmental myelination (Fig. 1) are prime candidates for manipulating the adult CNS to favor ES-derived myelin regeneration and repair.

OLs are generated from neuroepithelial stem cells [60, 61] in the germinal zone of developing brain and spinal cord [62, 63]. They first emerge as transient amplifying glioblasts (OPCs) and their specification in vivo, and from ES cells in vitro, requires sequential instructive cues (Fig. 1a). The neural ectoderm is first specified by a set of cross-regulating inhibitory signaling pathways during gastrulation. Bone morphogenic protein (BMP) promotes epithelial differentiation from ectoderm via inhibitory (Id) regulators that block 'default' neurogenesis [64, 65]. Instructive cues including fibroblast growth factor (FGF) then promote neuroepithelial differentiation by inducing BMP antagonists (Noggin, Chordin, Follistatin) from Hensen's node (Spemanns' organizer in amphibians) to locally block BMP [66]. Dorsal-ventral specification cues including Sonic hedgehog (Shh) then instruct neural lineage determination [67, 68]. In the embryonic spinal cord OPCs are first generated in the ventral lateral domain pMN of the neural ectoderm [69–71]. This domain also generates motor neurons, and both lineages are specified by the ventralizing effects of Shh [72] via the basic helix-loop-helix factor Olig2 [73–75].

The inhibition of BMP signaling is also important for the differentiation of ES cells into neural stem cells in vitro [76]. Mouse ES cells self renew in the presence of leukemia inhibitory factor (LIF) and BMP or serum factors [64, 65]. Withdrawing these in non-adherent conditions promotes the aggregation of disorganized embryonic bodies with a mixture of differentiated cell types [77], and in adherent cultures generates a less complex mixture of largely neural progenitors. Protocols have been developed for the sequential instruction of ES cells into neural precursors [78] then

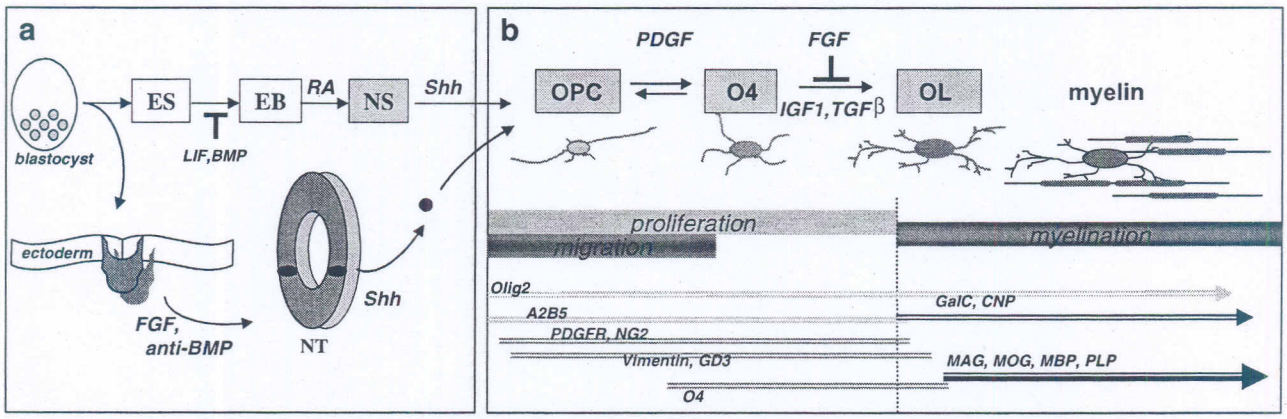

Fig. 1 OL development. **a** OPC specification; embryonic stem (*ES*) cells self-renew in leukemia inhibitory factor plus BMP or sera and sequentially generate *EB* (embryoid bodies) *NS* (neural stem) cells and OPCs via factors that first promote neural induction (FGF induced BMP antagonists) then *NT* (neural tube) ventralization (Shh). **b** OPC maturation in vitro, depicting the sequential emergence of antigens in migratory OPCs and postmitotic OLs. Maturation is driven by PDGF and inhibited by FGF. The sequential emergence of antigens marking this lineage is shown below. Monoclonal A2B5 detect gangliosides [57]; O4 sulfatide and glycolipids [58], and O1 galactocerebroside (*GalC*) [59]. Other markers include platelet-derived growth factor α-receptor (*PDGFR*), proteoglycan NG2, ganglioside GD3, cyclic nucleotide phosphohydrolase (*CNP*), myelin associated glycoprotein (*MAG*), myelin oligodendrocyte glycoprotein (*MOG*), myelin basic protein (*MBP*) and proteolipid protein (*PLP*)

into glia [32, 61, 79, 80]. First, ES cells in monolayer culture or dissociated embryoid bodies are cultured in serum-free conditions with retinoic acid (RA) [81], which interacts with FGF and Shh to pattern homeodomain and bHLH factor expression in the ventral spinal cord [82]. Neural differentiation is induced by autocrine signals such as FGF [83] and is enhanced by activated Notch [84]. Second, neural stem cells are amplified by mitogens including EGF [85] and FGF [86]. Third, gliogenesis is instructed by the ventralizing factor Shh [79, 87, 88]. Finally, the cultures are switched into media containing PDGF to promote OPC proliferation and survival [89, 90] and FGF to block terminal differentiation [91]. In addition to growth factors for induction and selection, genetic modifications have been devised to select OPCs and eliminate other cells in order to generate highly purified OPC populations from ES cells [79, 80]. These cultures thus approach the appropriate reagent for therapeutic intervention in demyelinating diseases.

The current explosion of studies on embryonic and adult stem cells in vitro is reminiscent of the expansion of tumor virology in the 1980's and neurobiology in the 1990's. Lessons learned from these prior studies are relevant to interpretations of stem cell fates in vitro and their therapeutic potential. General precautions associated with manipulating cells in vitro include the pitfalls of cell line cross contamination [92] and the concern that cells in culture can undergo alterations that can lead to malignant transformation. Unique concerns for stem cells include misinterpreting the stability of chemical induced phenotypes in vitro [49] and in vivo artifacts due to cell fusion [93]. Thus the stability of ES-derived OPCs is an important issue. OLs in vitro can revert to O4 progenitors [94], from O4 to O2A [95, 96], and from O2A into neural stem cells [97]. OPCs can also differentiate into other glial phenotypes in vitro [98, 99]. Such plasticity underscores the potential of culture conditions to influence fate [100]. Plasticity is not simply an artifact of culture, as neural fate can also be reprogrammed in vivo by surgical reposition at many stages of neural induction and patterning [66, 67, 101]. However the full repertoire of OPC fate potential in vitro is not evident in vivo, where their actual fates may be more restricted [102]. Thus it is important to determine whether in vitro derived ES progeny generated via distinct methods represent stable phenotypes with therapeutic benefit.

Stem Cell-Derived OLs for CNS Myelin Therapy

Pre-clinical studies on transplantation to rescue myelin pathology started with the work of Madeline Gumpel and colleagues some 25 years ago [103, 104]. Early studies used neural tissue grafts including CNS fragments or OLs isolated from newborns rodents [105–107]. These and subsequent studies demonstrated myelin sheath formation and the potential of exogenous tissue for remyelination in model systems including genetic dysmyelination (Shiverer mice, myelin deficient rats) and in experimental demyelination induced by chemicals (cuprizone, ethidium bromide), immune-mediated (experimental allergic encephalomyelitis), after viral attack (mouse hepatitis virus), and after traumatic CNS injury [34, 36, 37, 56, 108–111].

Studies to date have examined grafts of mitotic, mobile progenitor cells including OPCs isolated from neonatal rat brain [33, 55], an immortal rat OPC line CG4 [112], OPCs from human embryonic brain [113] and ES-derived OPCs from both mouse [32, 35] and human [36]. The ability of OPCs to both proliferate and migrate is key to their efficacy [29]. While it is impractical to consider xenograft neural tissue as a resource for clinical transplants, stem cells now provide new prospects for generating autologous OLs for remyelination. The potential of in vitro differentiated ES progeny still requires parallel comparisons of the efficacy of distinct ES-derived cell populations to generate OLs, manipulations to guarantee their clinical safety, and studies on how to manipulate the signaling environment in the adult brain to recapitulate the cues which direct OPC migration and OL maturation in development.

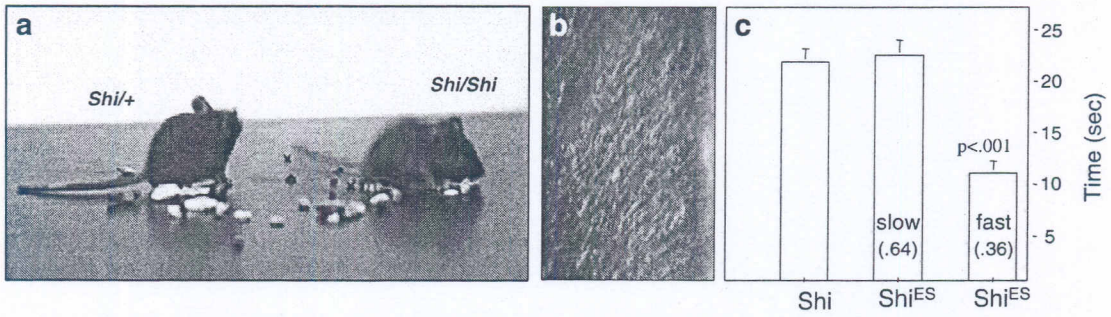

Fig. 2 ES-to-OL in Shiverer chimeras. **a** Adult Shi/+ (normal) and Shi/Shi (mutant) mice. **b** confocal of ES-derived MBP^+ OLs in Shi-ES chimeric mouse spinal cord. Shi/Shi mutants are devoid of MBP^+ cells. **c** Shi-ES chimeric mice were placed in 0.5 mm depth water tray and exit time recorded. Shi mutants lack balance and coordination, while 36% of Shi-ES chimeras showed improved locomotor function relative to age matched controls ($p<0.001$)

The original model for assessing the extent of myelination from exogenous grafts are dysmyelinating Shiver (shi) mutant mice. The Shi deletion spans the gene encoding myelin basic protein (MBP), a principal structural component of compact CNS myelin. Homozygous Shi mutants develop a pronounced tremor by 12 days age (Fig. 2a) with catonic seizures and a shortened life span. They are however both viable and fertile, and grafting of OPC into either neonates or young adults restores compact myelin. The Shi phenotype includes a dramatic motor impairment which can be partially rescued in transplanted neonates [31]. Similarly, when ES cells are injected into Shi blastocysts they generate wild-type (MBP^+) OLs in both brain and spinal cord (Fig. 2b), and ES-derived OLs in Shi-ES chimeras can improve motor function (Fig. 2c). Thus ES cells amplified in vitro retain the ability to generate mature, functional OLs and rescue myelin deficiency. This preclinical model has tremendous power to confirm the potential of any population of presumed stem cells to generate OPCs and OLs for brain therapy.

Engineering Stem Cells for Targeted Migration

Stem cell derived OPCs must overcome two significant challenges in order to repair demyelinated lesions in the adult brain. First they must survive and mature in an environment significantly different than they normally encounter during brain development, and second they must migrate in order to gain access to demyelinated lesions.

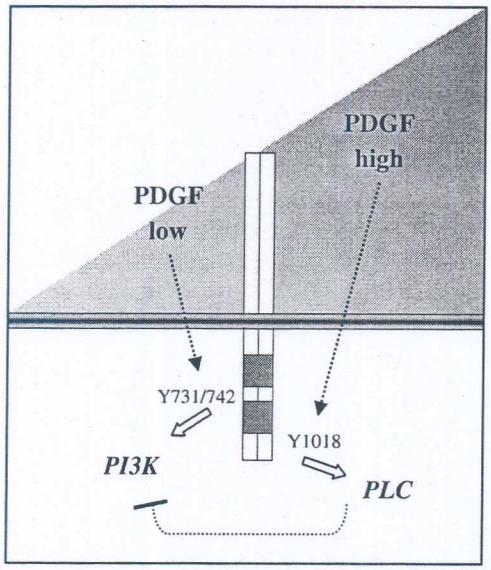

Fig. 3 The RTK Rheostat model for PDGF signaling. PDGFRα activates PI3-kinase (*PI3K*) at low and PLCγ at high ligand levels in OPCs. Since PI3K activation is necessary for OPC migration, the model suggests chemotactic migration represents pathway specific responses to distinct ligand levels

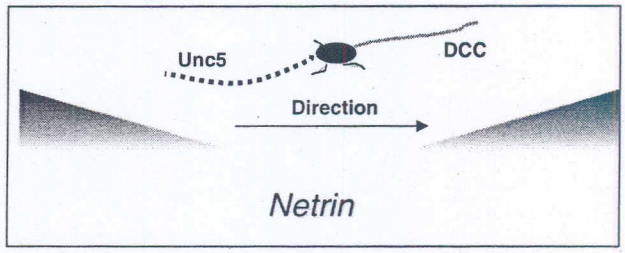

Fig. 4 Polarized Netrin receptors on OPCs. OPCs migrate away from focal Netrin sources such as the ventral spinal cord via repulsion cues from the receptor Unc5 on trailing processes, and toward Netrin such as in the optic chiasm via attraction cues from Neogenin and DCC on leading processes

Some cell types such as olfactory ensheathing glia may not survive in a lesion [114], while other cells survive better within the hostile milieu of an injury [115]. The choice of transplantation site also depends on host pathology and graft cell migration properties. Peri-ventricular lesions may be accessible via ventricular routes, and direct intra-lesion transplants can facilitate local remyelination [35]. However, most demyelinated lesions in MS exist throughout white and grey matter and are not easily accessible by surgical approaches. Acute injury sites may release signal cues to attract transplanted cells, although this remains a significant hurdle for long-distance migration. Therefore the ability of transplanted cells to migrate into target lesions is critical for functional repair. Since the cellular environment affects the survival, migration and differentiation of transplanted cells, it follows that efficient repair may require modifying the signaling environment, the receptor expression profile of ES-derived OPCs, or both. Signaling cues that direct OPCs during development provide tangible targets for such interventions, and two such cues are PDGF and Netrin.

Platelet-derived growth factor (PDGF), the first characterized OPC mitogen [89], is produced by astrocytes and neurons in the developing CNS. PDGF is also strongly chemo-attractive [116] and thus a candidate to mobilize OPCs in adult brain. Transgenic studies support a role for PDGF in vivo, as mice lacking PDGF have fewer OPCs [117] while excess PDGF gives extra progenitors [118]. Harnessing PDGF for OPC traffic control in the adult brain requires an understanding of how it directs migration. For OPC motility this involves a strength of signaling mechanism to control phosphoinositol 3-kinase (PI3K) activation [119]. PDGF activation of PI3K is necessary for OPC motility, and signaling via PDGFRα activates PI3K at low but not high ligand strength (Fig. 3). Conversely, PDGF activates PLCγ at high but not low signal strength. This "rheostat" control mechanism thus appears to interpret ligand levels to stimulate OPC migration in a zone of low ligand level and inhibit migration in high ligand levels [119]. It follows that PDGF delivery into a lesion may not be chemo-attractive for transplanted

OPCs, which would be inhibited from entering a lesion with high ligand levels. However, ES-derived OPCs engineered to express 'PLCγ-uncoupled' PDGFRα transgenes could promote ES-derived OPC migration into a PDGF infused lesion.

A second signaling system that offers promise for ES-derived OPC navigation is Netrin, a laminin-related glycoprotein expressed by the ventral midline of developing CNS. Netrin controls dorsal-ventral migration and axon guidance [120] via transmembrane receptors Unc40 and Unc5, attracting axons that express Unc40 (vertebrate DCC, Neogenin) and repelling axons expressing Unc5 [121, 122]. A role for Netrin in OPC migration was identified in the rodent optic nerve where it is repulsive [123]. Subsequent studies suggest Netrin can either attract [124] or repel OPCs [125]. Both results are consistent with OPC migration into the optic nerve which, like axon pathfinding across the midline, involves migration first toward then away from a Netrin source.

How cells switch their response to Netrin from attraction to repulsion is not understood. For growth cones this may involve silencing of attraction after midline crossing by overriding signals [126], converting Netrin attraction to repulsion [122, 127], or contra lateral attraction [128]. For OPCs, asymmetric receptor expression (Fig. 4) may explain how Netrin dictates the polarity of OPC migration (Reilly et al., in prep). Netrin receptors have a polar distribution on OPCs in embryonic day 13.5 spinal cord, with Unc5b on the trailing and DCC/Ngn on the leading edge. Since these receptors have comparable affinity for Netrin (Kd=5nM) [129], OPCs migrating away from midline Netrin would have stronger Unc5 (repulsion) signals on lagging processes that could override a weaker DCC and Neogenin (attraction) signal on the leading processes. In contrast, as OPCs migrate towards a Netrin zone such as the optic chiasm, attraction signals on leading processes would override repulsion signals on lagging processes. This suggests receptor polarity dictates direction, with attraction as an anterior pole event and repulsion as a posterior pole event. This also suggests that manipulating these receptors on ES-derived OPCs may be an approach to direct their migration towards Netrin infused lesions in the injured brain. Thus the engineering of ES cells to generate OPCs expressing specific receptors holds great promise for controlling their migration and enhancing their contribution to recovery from demyelinating disease.

Summary

Stem cells offer great promise for clinical therapy, but many hurdles remain. Of particular note, the challenge of extending the phenomenal success and accomplishments of murine ES cell biology into human ES cells remains. Ethical limitations continue to surface, pragmatic issues of manipulating human ES cells in culture are unsolved, and the engineering of these cells by gene modifications for targeted migration is an essential next step.

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