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Stem Cells for Spinal Cord Injury: Strategies to Inform Differentiation and Transplantation

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Abstract

The complex pathology of spinal cord injury (SCI), involving a cascade of secondary events and the formation of inhibitory barriers, hampers regeneration across the lesion site and often results in irreversible loss of motor function. The limited regenerative capacity of endogenous cells after SCI has led to a focus on the development of cell therapies that can confer both neuroprotective and neuroregenerative benefits. Stem cells have emerged as a candidate cell source because of their ability to self-renew and differentiate into a multitude of specialized cell types. While ethical and safety concerns impeded the use of stem cells in the past, advances in isolation and differentiation methods have largely mitigated these issues. A confluence of work in stem cell biology, genetics, and developmental neurobiology has informed the directed differentiation of specific spinal cell types. After transplantation, these stem cell-derived populations can replace lost cells, provide trophic support, remyelinate surviving axons, and form relay circuits that contribute to functional recovery. Further refining stem cell differentiation and transplantation methods, including combinatorial strategies that involve biomaterial scaffolds and drug delivery, is critical as stem cell-based treatments enter clinical trials.

Keywords

Induced pluripotent stem cells; Embryonic stem cells; Neuronal differentiation; Biomaterial scaffolds; Transcriptional reprogramming; Combination therapy

1. Introduction

1.1 Epidemiology of Spinal Cord Injury

The consequences of spinal cord injury (SCI) extend beyond the initial trauma, disrupting a variety of normal sensorimotor behaviors and having far reaching psychological and economic impacts to patients and healthcare systems. SCI disables more than 270,000 people in the United States with 12,500 new cases annually and estimated lifetime medical costs exceeding several million dollars per individual depending on the severity of functional loss (DeVivo et al. 2011, Singh et al. 2014, National Spinal Cord Injury Statistical Center 2014). SCI patients have lower life expectancies, lower employment rates, and lower

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chances for successful marriage compared to uninjured peers, trends which have not changed significantly in the last 40 years and which transcend international borders (Singh et al. 2014, National Spinal Cord Injury Statistical Center 2014).

1.2 Pathophysiology of Spinal Cord Injury

Each traumatic SCI is unique and often occurs alongside multiple systemic injuries. This complicates both immediate and long-term management, thus making a one-treatment-fits-all strategy difficult. The primary mechanical trauma results in the immediate compression and disruption of axons and vasculature, triggering a secondary cascade of events including ischemia and excitotoxic chemical release, which exacerbate local cell death and significantly expand the injury site (Tator and Fehlings 1991). The rapid necrosis results in a cystic cavity that is infiltrated by inflammatory cells, microglia, fibroblasts and reactive astrocytes. These form a dense, fibrous glial scar that expresses a multitude of inhibitory chemical cues and serves as a physical and chemical barrier that prevents regeneration across the lesion (Schwab and Bartholdi 1996, Fawcett and Asher 1999, Silver and Miller 2004, Donnelly and Popovich 2008). Wallerian degeneration, chronic demyelination, and muscle atrophy persist months or even years after the injury, limiting the potential for functional recovery over time (Totoiu and Keirstead 2005).

1.3 Remodeling after Spinal Cord Injury

While spinal circuits were previously thought to be rigid with limited regenerative capacity after injury, research in the past few decades has uncovered remodeling in spared tissue that can result in spontaneous functional recovery. Regeneration of descending tracts from the brain is not necessarily or solely responsible for regained function; plasticity has been observed at various hierarchal levels in the central nervous system (CNS), including the cerebral cortex, limbic structures, corticospinal tracts (CST), propriospinal neurons (PNs), motor neurons (MNs) and segmental interneurons (INs) (Courtine et al. 2009, Isa and Nishimura 2014). Detour circuits formed by CST fibers, PNs, and local IN networks have been attributed to renewed innervation of intact central pattern generators (CPGs) (Flynn et al. 2011, Filli and Schwab 2015). Indeed, cell populations around the lesion are well placed to receive and respond to the host of pro-regenerative molecular cues that are upregulated after injury. These include molecules that are also expressed during development and signal intrinsic axon regeneration pathways as well as direct connectivity and synaptogenesis (Hollis 2015). Perhaps as a result of these cues, endogenous neurogenesis in ependymal cells, resulting in the proliferation of oligodendrocytes and astrocytes, has been observed in both rodents and primates after spinal cord injury (Johansson et al. 1999, McTigue et al. 2001, Yang et al. 2006, Barnabe-Heider et al. 2010).

1.4 Stem Cell Therapy

Although early surgical interventions focus on reducing the amount of damage done by secondary processes and stabilizing the spinal cord, most SCI treatments emphasize neuroprotection, neuroregeneration, and rehabilitation. Cell-based therapies have gained popularity as a research focus because they can provide multiple benefits, including replenishment of lost cell types, scaffolding for axon regeneration, and the delivery of immuno-modulatory, neurotrophic and anti-inhibitory factors. Neural progenitor cells,

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mesenchymal stromal cells (MSCs), olfactory ensheathing glia, Schwann cells, and various pluripotent stem cells are among those currently investigated for their utility after SCI. *In this review, we focus specifically on stem cells and strategies that have been used to differentiate and deliver them after SCI.*

2. Stem Cell Sources

2.1 Multipotent Stem Cells

Two types of multipotent stem cells, unspecialized cells capable of differentiating into a discrete population of specific cell types of the same germ layer, are primarily investigated for treatment after SCI: MSCs and neural stem cells (NSCs) (Figure 1). MSCs are appealing clinically because they can differentiate into many non- hematopoietic lineages, but are also easily isolated from patient bone marrow, thus allowing for autologous transplantation. However, several studies have demonstrated that their utility is confined to trophic and immunomodulatory effects after SCI in both animals and patients (Hofstetter et al. 2002, Parr et al. 2007, Tetzlaff et al. 2011, Urdzikova et al. 2014). High variability between studies, limited functional recovery, poor engraftment, and questionable neuronal differentiation *in vivo* limit the use of MSCs for cell replacement (Tetzlaff et al. 2011).

NSCs have been widely studied for transplantation after SCI because their maturation is restricted to glial and neuronal subtypes, thus reducing tumorgenicity while replenishing lost cells, aiding in remyelination and trophic factor secretion, and promoting axon regeneration. NSCs can be harvested from either adult or fetal spinal cord tissue and expanded as neurospheres in the presence of growth factors, including epidermal growth factor (EGF) and/or basic fibroblast growth factor (FGF2), prior to transplantation (Weiss et al. 1996, Shihabuddin et al. 1997, Uchida et al. 2000, Brewer and Torricelli 2007) (Figure 1). Fetal NSCs are generally heterogeneous, containing a mixture of neuronal and glial restricted progenitor cells, as well as self-renewing stem cells (Tetzlaff et al. 2011); in adults, ependymal cells along the central canal are NSCs that respond dramatically after SCI and constitute an endogenous source of stem cells to target (Weiss et al. 1996, Johansson et al. 1999, McTigue et al. 2001, Yang et al. 2006, Barnabe-Heider et al. 2010). Because NSCs can retain their positional identity through *in vitro* expansion, anatomical origin is an important consideration for cell replacement therapy and can be exploited to maximize integration into host spinal circuits (Hitoshi et al. 2002, Philippidou and Dasen 2013).

Functional recovery after NSC transplantation has been observed in a variety of animal models and can be enhanced by co-treatments with trophic factors (Tetzlaff et al. 2011). Though NSCs are capable of differentiating into all CNS types, both endogenous and transplanted NSCs in the spinal cord overwhelmingly become astrocytes and oligodendrocytes, with variable neuronal differentiation (Cao et al. 2001, Karimi-Abdolrezaee et al. 2006, Parr et al. 2008, Kriegstein and Alvarez-Buylla 2009, Barnabe-Heider et al. 2010). Furthermore, despite their many positive attributes, NSCs cannot be used for autologous transplantation and may be excluded from clinical use by contentions deriving them from fetal or post-mortem patient tissue. To circumvent this issue, many labs generate NSCs from pluripotent stem cells or directly reprogram them from somatic cells, such as fibroblasts.

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2.2 Pluripotent Stem Cells

Pluripotent stem cells (PSCs) are characterized by their ability to replicate indefinitely while maintaining the ability to differentiate into specialized cell lineages from all three embryonic germ layers. Embryonic stem cells (ESCs) derived from the inner cell mass of pre-implantation blastocysts were the first isolated, differentiated, and proposed for use in cell therapy after SCI (Evans and Kaufman 1981, Thomson et al. 1998, McDonald et al. 1999) (Figure 1). In the absence of factors that maintain pluripotency, such as leukemia inhibitory factor (LIF), FGFs, or rho-associated protein kinase (ROCK) inhibitors (Smith et al. 1988, Williams et al. 1988, Watanabe et al. 2007), ESCs spontaneously differentiate; many strategies have been developed to preferentially induce differentiation along spinal neural lineages and even specify a positional identity along the neuraxis, discussed below. While several studies have shown benefits associated with ESC-derived neuronal transplants (Hatami et al. 2009, Johnson et al. 2010b), ethical concerns and potential side effects including teratoma formation caused by the presence of undifferentiated ESCs prevent clinical application.

Induced pluripotent stem cells (iPSCs) have become an appealing alternative to ESCs and allow for autologous transplantation of patient-derived cells. By reprogramming somatic cells with a defined set of transcription factors, the Yamanaka factors, adult mouse and human cells revert to a pluripotent state, thus alleviating ethical impediments caused by the destruction of embryos (Takahashi and Yamanaka 2006, Takahashi et al. 2007) (Figure 1). Differentiation strategies initially developed for ESCs have been translated to iPSCs. However, similar to ESCs, transplantation of iPSCs retains a high risk of immune rejection and tumorgenesis, the latter of which can be compounded by virally overexpressing oncogenic transcription factors for iPSC generation. Safer, non-viral methods have been developed, but spontaneous reversion to a pluripotent state is a unique concern for iPSC-derived cells and contributes to the tumorgenic potential (Saric et al. 2008, Chen et al. 2013). Considerations including the mechanism of reprogramming, the cell source, and the differentiation process are important to weigh in designing therapeutic strategies involving pluripotent stem cells (Khazaei et al. 2014).

3. Directing Neural Cell Fate

3.1 Cell-Cell Interactions

Neural induction is a default pathway for PSCs, but differentiation efficiency varies depending on both intrinsic cell line qualities, as well as the extracellular environment (Bain et al. 1995, Munoz-Sanjuan and Brivanlou 2002, Hu et al. 2010). Spatial cues play an important role in cell fate determination and can dramatically alter the efficiency of neural induction. Two dimensional (2D) adherent monolayer cultures or co-culture with feeder cells have been used with varying success, though the use of micropatterned surfaces or manual selection of spontaneous rosette structures can help direct neuronal morphogenesis (Zhang et al. 2001, Knight et al. 2015). Allowing cultures to grow in three dimensions (3D), instead of in 2D, generates environmental conditions that more closely mimic those found *in vivo*, and can consequently enhance neuronal differentiation. Non-adherent cultures that allow PSCs to aggregate into embryoid bodies (EBs) have traditionally been used, since EBs develop

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similarly to the pre-gastrulation embryo and are primed for application of patterning factors that can direct more specific cell types (Weitzer 2006). A problem with EBs is that they are heterogeneous and disorganized, which frequently results in variable neural induction. To improve differentiation consistency, several methods have been developed to control EB dimensions beyond static suspension culture, including the hanging drop method, cell encapsulation, microwell or microfabrication methods, and the use of bioreactors (Rungarunlert et al. 2009).

New 3D culture systems are now gaining traction. PSC-derived organoid cultures, evolved from EBs, have become appealing for *in vitro* disease modeling and as a source for complex cell populations (Lancaster and Knoblich 2014, Huch and Koo 2015). Several protocols exist to generate organoids for individual brain regions or heterogeneous cerebral organoids containing multiple regions (Lancaster and Knoblich 2014). The reliance on random structure formation can be avoided by using defined 3D scaffolds. Using Matrigel or a synthetic matrix separately, the Tanaka group was able to generate neuroepithelial cysts from single mouse ESCs that undergo classic dorsoventral patterning when in the presence of retinoic acid (RA)(Meinhardt et al. 2014). These studies demonstrate not only the feasibility of creating complex, organized spinal tissue, but also how bioengineered 3D scaffolds can be used to finely control the stem cell microenvironment. Such scaffolds can also be designed as transplantation vehicles for SCI treatment (Willerth and Sakiyama-Elbert 2008, Murphy and Atala 2014). We have done significant work optimizing neural differentiation within fibrin gels that have been modified for controlled release of growth factors or co-delivery of anti-inhibitory molecules. After transplantation, these combinatorial treatments are able to improve cell survival, differentiation, and neurite growth at the host-graft interface (Willerth et al. 2006, Willerth et al. 2007, Willerth et al. 2008, Johnson et al. 2010b, McCreedy et al. 2014b, Wilems et al. 2015).

3.2 Mimicking Development to Drive Differentiation

The traditional method of converting PSCs to CNS cells involves recapitulating the developmental environment of the neural tube via exposure to growth factors and morphogens (Figure 2). Induction efficiency and lineage specification can be adjusted by modulating the concentration and exposure duration of these factors. Early induction protocols called for the use of harvested or recombinant protein, but a more sophisticated understanding of the signaling, transcriptional, and epigenetic cues responsible for cell fate specification in recent years has enabled the use of small molecules to direct differentiation in lieu of proteins, which can be expensive and labile.

3.2.1 Neural Induction—Neural induction and the generation of nestin$^+$ NSCs is the first step towards differentiating specific spinal cell types from PSCs. While the spinal cord is a caudal CNS structure, the neural cells from which it is formed initially acquire a rostral character through a combination of transforming growth factor (TGF)-β, bone morphogenetic protein (BMP), FGF, and Wnt signaling (Wilson and Edlund 2001, Munoz-Sanjuan and Brivanlou 2002). Forming EBs results in spontaneous NSC production, but as previously discussed, the process can give variable neuronal yields (Bain et al. 1995). Taking cues from development, several groups have efficiently induced a neural fate by inhibiting

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TGF-β/Activin/Nodal and BMP signaling pathways *in vitro* using recombinant inhibitors or small molecule antagonists (Smith et al. 2008, Chambers et al. 2009, Patani et al. 2009, Chambers et al. 2012). EGF and FGF2 have been used to isolate NSCs from primary tissue, but can also promote survival and induction of NSCs from PSCs (Okabe et al. 1996, Shihabuddin et al. 1997, Reubinoff et al. 2001, Joannides et al. 2007, Chambers et al. 2012). The timing of growth factor exposure is important; after NSCs have developed, inhibition of FGF2 *in vitro* aids in the differentiation of NSCs into neurons and thus improves differentiation efficiency (Joannides et al. 2007, Chambers et al. 2012).

3.2.2 Caudalization with Retinoic Acid—Endogenous neural progenitors acquire a spinal identity in response to caudalizing signals, predominantly RA, which is involved in both anteroposterior and dorsoventral patterning of the neural tube (Durston et al. 1998, Muhr et al. 1999). RA inhibits FGF signaling and triggers differentiation in the neuroepithelium (Diez del Corral et al. 2003, Novitch et al. 2003). Treatment of ESCs with RA was among the first efforts to obtain spinal neurons; however, when used for prolonged durations, RA functions as a differentiation agent that significantly impacts neuronal subtype specification in the ventral spinal cord (Bain et al. 1995, Jessell 2000, Wilson and Maden 2005, Maden 2007). It is of note that RA serves many neuroprotective and neuroregenerative roles in the adult CNS and could have value for SCI therapy beyond its use in neural induction (Maden 2007).

3.2.3 Growth Factor-Mediated Patterning—A combination of factors released from mesodermal structures around the neural tube is responsible for neuronal differentiation: RA from the somites, sonic hedgehog (Shh) from the notochord, BMPs, EGFs, and Wnts from the ectoderm and roof plate, and FGFs from the posterior of the neural tube (Lee and Jessell 1999, Jessell 2000). While research parsing endogenous differentiation pathways has been ongoing for more than two decades, new genes and transcription factors are continually added to the current models as spinal neuron subtypes are being defined. The signaling involved in cell fate specification is synergistic, time-dependent, and concentration-dependent. The sensitivity of differentiation means slight changes to PSC induction protocols can significantly alter the PSC-derived neuronal population distribution, but also allows for optimization of specific subpopulations.

In the ventral spinal cord, RA and Shh work together to induce four classes of ventral interneuron progenitors (p0–p3), which give rise to various ventral interneurons, as well as a progenitor motor neuron (pMN) domain, which gives rise to motor neurons, astrocytes, and oligodendrocytes (Briscoe and Ericson 2001, Wilson and Maden 2005). These neuronal populations contribute to central pattern generator networks in the spinal cord that are critical for normal motor function and coordination (Arber 2012). A significant body of work by the Jessell lab has demonstrated the importance of Shh-dependent, differential homeobox transcription factor expression in generating the discrete ventral progenitor domains, as well as specification of neuronal subtypes (Briscoe and Ericson 2001). RA and Shh are necessary and sufficient to induce ventral spinal differentiation from PSCs; by modulating the relative concentrations and durations of signaling factors, they and others have been able to optimize induction protocols to enrich for specific ventral populations

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(Wichterle et al. 2002, Okada et al. 2004, Li et al. 2005, Kim et al. 2009, Dessaud et al. 2010, McCreedy et al. 2012, Brown et al. 2014, Xu and Sakiyama-Elbert 2015).

Little work has been done to develop PSC-derived populations from the dorsal spinal cord, in part because the molecular mechanisms involved in diversification are not as well established as those in the ventral cord (Lee and Jessell 1999, Helms and Johnson 2003). Dorsal interneurons are primarily associated with sensory circuits, though dI3 and dI6 interneurons have been implicated in motor coordination (Dyck et al. 2012, Bui et al. 2013). There are six classes of early-born dorsal interneurons (dI1–dI6) as well as two late-born ones (dIL^{A,} dIL^{B}). These domains can be further subdivided by their dependence on BMP signaling from the roof-plate for development: dependent Class A (dI1–dI3) and independent Class B (dI4–dI6, dIL$^{A/B}$). Similar to Shh signaling in the ventral spinal cord, gradients of BMP activity contribute to the formation of discrete homeobox transcription factor expression domains that give rise to Class A progenitor cells (Timmer et al. 2002). Treatment of EBs with RA alone induces differentiation of dorsal Class B interneurons and V0 and V1 interneurons, which are less dependent on roof plate and floor plate signaling for development (Kim et al. 2009). Wnt, FGF, and EGF signaling are important for initial neural crest development, but their roles in dorsal interneuron diversification are less well understood (Lee and Jessell 1999, Lee et al. 2000, Muller et al. 2002, Helms and Johnson 2003). At least one group has shown that a combination of these developmental factors directs dorsal interneuron differentiation in mouse embryonic stem cells (Murashov et al. 2005). Others have bypassed neural induction entirely and used transcriptional programming to obtain sensory neurons from fibroblasts (Blanchard et al. 2015).

3.2.4 Refining Inductions for Subtype Specification—There are many subpopulations of neurons and glia beyond the cardinal classes defined during spinal cord development. While spinal oligodendrocytes and astrocytes arise from the pMN domain, which requires RA/Shh signaling, they also occur elsewhere in Shh independent ways and can require the addition of FGFs, BMPs, tumor necrosis factors (TNF), interleukins (ILs), platelet-derived growth factor (PDGF) and/or ciliary neurotrophic factor (CNTF) signaling to direct their differentiation *in vitro* and *in vivo* (Benveniste et al. 2005, Nistor et al. 2005, Krencik and Zhang 2011, Roybon et al. 2013, Douvaras and Fossati 2015, Goldman and Kuypers 2015). Many interneuron populations have not yet been classified by unique transcription factor profiles, and the populations that have been characterized are certain to be even more subdivided. As additional spinal subtypes and their differentiation mechanisms are identified, it will be important to translate those to optimize PSC induction protocols. For example, Notch signaling acts as a transcriptional switch between inhibitory and excitatory interneuron subpopulations in both dIL and V2 interneurons, and possibly contributes to diversification elsewhere in the spinal cord (Mizuguchi et al. 2006, Del Barrio et al. 2007). By adding a Notch inhibitor to an RA/Shh induction, V2a interneurons can be selectively enriched in PSC-derived cultures (Brown et al. 2014).

There has recently been interest in developing PSC-derived populations that not only comprise of defined neuronal subtypes, but also have a defined positional identity, as these are important for migration and integration into host motor circuits (Philippidou and Dasen 2013). Much of this work has been done in motor neurons, which have genetically defined

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subtypes that are anatomically and functionally distinct. Subtle changes to the induction can alter Hox signaling and thus positional identity, including the type of signaling agent used (inhibitor versus recombinant protein), the exposure time and sequence of induction factors, and the addition factors that stimulate FGF and Wnt/β-catenin signaling (Maden 2007, Patani et al. 2009, Peljto et al. 2010, Patani et al. 2011, Davis-Dusenbery et al. 2014, Lippmann et al. 2015). Stem cell therapies that match host cell populations as closely as possible will provide the best opportunity for success.

3.3 Transcriptional Reprogramming

Driving differentiation of PSCs in a chemically defined environment has limitations, especially in human cells where induction and maturation can take months. Despite efforts to generate specific spinal cell types, the co-dependence in signaling involved in neuronal diversification necessarily leads to heterogeneity in cultures that use common induction factors. Overexpressing transcription factors directly responsible for neuronal subtype specification is a way to obtain more homogeneous cultures while minimizing induction time. Overexpression of just Ngn2 or NeuroD1 in PSCs results in efficient conversion into functionally active neurons (Zhang et al. 2013). Precise manipulation of transcription factor activation is important for cell type specificity; spinal motor neurons can be generated using a combination of Ngn2, Isl1, and Lhx3 (LIN factors), but replacement of Lhx3 with Phox2a yields cranial motor neurons (Hester et al. 2011, Mazzoni et al. 2013).

Direct reprogramming from one somatic cell type to another has become possible, bypassing an intermediate pluripotent state. Avoiding iPSC generation can potentially reduce the time to generate a specific cell type and minimizes the risk of teratoma formation upon transplantation. Several combinations of transcription factors convert fibroblasts to directly reprogrammed NSCs (iNSCs), though it is possible to generate tripotent iNSCs that can become neurons, astrocytes and oligodendrocytes by expressing just Sox2 or Oct4 (Kim et al. 2011, Han et al. 2012, Lujan et al. 2012, Ring et al. 2012, Mitchell et al. 2014). Vierbuchen *et al*. demonstrated that expression of Brn2 (Pou3f2), Ascl1, and Myt1l (BAM factors) were sufficient to convert mouse fibroblasts to functionally mature, excitatory neurons (iNs) (Vierbuchen et al. 2010). A variety of distinct neuronal and glial populations have since been generated from both mouse and human somatic cells, including dopaminergic neurons, sensory neurons, motor neurons, oligodendrocyte precursor cells (OPCs), and astrocytes. (Caiazzo et al. 2011, Son et al. 2011, Najm et al. 2013, Yang et al. 2013, Marro and Yang 2014, Blanchard et al. 2015, Caiazzo et al. 2015). Of interest for SCI, astrocytes have been directly converted to iNSCs and functional neurons both *in vitro* and *in vivo* (Corti et al. 2012, Guo et al. 2014).

Very low neuronal conversion efficiencies—hovering near 1% using certain protocols—remain a hurdle facing direct reprogramming as a clinically relevant method for cell replacement. Depending on the protocol efficiency, mitotic cells, such as fibroblasts, must be significantly expanded in order to obtain a sufficient quantity of desirable cells, and then purified prior to transplantation, which can be expensive and time consuming. The sub-optimal conversion rates may not induce therapeutic outcomes that overcome the risks associated with reprogramming. Several ways to enhance the reprogramming process are in

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development. For example, microRNAs can behave synergistically with the BAM factors to more efficiently convert iNs (Ambasudhan et al. 2011, Yoo et al. 2011). iPSC reprogramming can be optimized by blocking DNA and histone methylation or modulating chromatin structure to open transcriptional binding sites (Huangfu et al. 2008, Lin et al. 2009, Luna-Zurita and Bruneau 2013, Tso and McKinnon 2015). Direct reprogramming has recently been achieved by delivering small molecules (Hu et al. 2015, Li et al. 2015); this is clinically relevant, as it avoids potentially hazardous viral transductions and presents the opportunity to bioengineer delivery systems to convert endogenous cells into cell types of interest. This approach may be more viable to replenish neurons by transcriptional reprogramming, since transplanted iNSCs suffer from poor engraftment (Hong et al. 2014).

4. Transplantation Outcomes of Stem Cell-Derived Spinal Types

4.1 Considerations for Transplantation

The optimal window for cell therapy is generally thought to be within two to four weeks of the initial trauma, when the acute phase of the secondary injury has abated and while severed axons and surviving interneuron populations are still capable of responding to the host of axon guidance molecules and neurotrophins upregulated after SCI (Schwab and Bartholdi 1996, Hayashi et al. 2000, Coumans et al. 2001, Widenfalk et al. 2001, Bareyre et al. 2004, Conta and Stelzner 2004, Courtine et al. 2008, Lang et al. 2012, Hollis 2015). Multiple groups have observed that delayed transplantation into a more chronic injury is beneficial compared to acute treatment (Coumans et al. 2001, Karimi-Abdolrezaee et al. 2006), possibly because of alterations in the inflammatory response that allow for better cell survival in both endogenous and transplanted populations (Donnelly and Popovich 2008, Moreno-Manzano et al. 2009, Rolls et al. 2009, David et al. 2012). Beyond that timeframe, the glial scar stabilizes and the upregulation of pro-regenerative factors attenuates, thus reducing the potential for regeneration (Satake et al. 2000, Widenfalk et al. 2001, Silver and Miller 2004, Cregg et al. 2014). The intended mechanism by which the transplant stimulates recovery is an important consideration when choosing both the time of intervention and the specific cell population for transplantation (Figure 3) (Bradbury and McMahon 2006). For example, the replenishment of neuronal populations is critical to take advantage of functional relay mechanisms (Bareyre et al. 2004, Courtine et al. 2008), but post-mitotic neurons suffer upon transplantation, so using plastic progenitor populations may be a more reasonable approach. At the same time, inhibitory proteoglycans and trophic factors secreted by reactive astrocytes have differential effects on stem cell differentiation and survival, which may significantly alter the composition of the transplant. (Silver and Miller 2004, Rolls et al. 2009). Transplanting glia, which have roles in trophic support or immunomodulation, may mitigate some inhibition from the lesion (Zeis et al. 2015, Liddelow and Hoyer 2016). Anecdotal evidence from Asterias' clinical trial suggests that, even after cessation of immunosuppression, human ESC-derived OPCs that successfully integrated into host networks can survive years after transplantation (Asterias 2016).

4.2 PSC-derived NSCs

PSC-derived NSC transplants have numerous neuroprotective and regenerative benefits beyond cell replacement including immunomodulation, the secretion of neurotrophic factors

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to enhance endogenous cell survival, axon regeneration, and remyelination initiated by NSC-derived oligodendrocytes. Most studies demonstrate that transplants are capable of differentiating into neurons, astrocytes, and oligodendrocytes *in vivo*, though the ratios may differ depending on the method of NSC differentiation, the SCI model, and time point of investigation (Nori et al. 2011, Kobayashi et al. 2012). The extent of functional recovery also varies between studies. Significant recovery has been observed after human iPSC-derived NSC transplantation with no tumorgenicity and integration into host neural circuits, prompting consideration for clinical trials (Nori et al. 2011, Kobayashi et al. 2012). However, others have shown only marginal behavioral improvements despite good cell survival, differentiation, migration, and integration (Nutt et al. 2013, Khazaei et al. 2014, Lu et al. 2014, Pomeshchik et al. 2015, Romanyuk et al. 2015).

4.3 PSC-Derived Oligodendrocyte Precursor Cells (OPCs)

Oligodendrocyte death and chronic demyelination are features of the secondary injury cascade that follows the primary trauma after SCI (Kakulas 1999, Totoiu and Keirstead 2005). Adult OPCs have a limited capacity to regenerate or migrate, but a variety of stem cells, including endogenous adult NSCs, preferentially differentiate into oligodendrocytes that are capable of providing trophic support, remyelination and functional repair (Gensert and Goldman 1997, Faulkner and Keirstead 2005, Meletis et al. 2008). Functional recovery after NSC transplantation has been attributed in large part to OPC differentiation. ESC-derived OPCs have been transplanted after SCI with success, resulting in remyelination of host axons, improved electrophysiological and motor functions, and greater white and gray matter sparing (Brustle et al. 1999, Keirstead et al. 2005, Nistor et al. 2005, Sharp et al. 2010). Phase I/2 clinical trials for the transplantation of human ESC-derived OPCs into SCI patients with thoracic and high cervical injuries are currently underway, with demonstrable neurological improvements observed in patients from the initial safety cohort (Khazaei et al. 2014).

4.4 PSC-Derived Astrocytes

Astrocyte diversity and the role of astrocyte phenotype on neural circuit formation and regeneration remains poorly understood (Zhang and Barres 2010, Clarke and Barres 2013). While reactive astrocytes actively inhibit regeneration through the glial scar, other endogenous astrocytes have pro-regenerative roles, including the formation of astrocyte bridges that precede axons extending through the inhibitory environment (Kawaja and Gage 1991, Taylor et al. 2006, Nicaise et al. 2015). It is thus important to consider how the preparation of stem cell-derived astrocytes affects phenotype prior to transplantation; glial-restricted precursors that are pre-differentiated into astrocytes using different growth factor cocktails have opposite effects on recovery after transplantation (Davies et al. 2008). Astrocytes that differentiate from PSC-derived NSCs *in vivo* may contribute to or deter from the regenerative effects seen after transplantation depending on the method of NSC derivation, but the outcome is rarely linked to a specific astrocyte subtype. Protocols to obtain pro-regenerative astrocyte subtypes are still being developed, so studies that investigate the specific effect of PSC-derived astrocytes on spinal cord regeneration have not yet been published (Benveniste et al. 2005, Emdad et al. 2012, Roybon et al. 2013, Falnikar et al. 2015).

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4.5 PSC-Derived Motor Neurons

Motor neurons have some potential for cell replacement because their loss significantly impairs behavioral recovery in several traumatic and neurodegenerative conditions. Many protocols have been developed to obtain enriched, subtype-specific motor neuron pools that closely resemble endogenous neurons and retain the ability to engraft in the developing spinal cord (Davis-Dusenbery et al. 2014). Efforts to transplant enriched motor neurons after SCI have been confined to embryonic or PSC-derived pMNs, since maturation comes with an intrinsic loss in plasticity needed to survive the hostile SCI environment. PSC-derived pMNs are able to survive, migrate, and differentiate after SCI transplantation *in vivo*, but the gliogenic nature of the injury site limits neuronal differentiation despite the ability of these pMNs to robustly differentiate into motor neurons *in vitro* (Erceg et al. 2010, Rossi et al. 2010). PSC-derived pMN transplantation also confers neuroprotective and neuroregenerative benefits akin to OPC transplantation; pMNs secrete neurotrophic factors that enhance endogenous axon sprouting and can result in functional improvements (Erceg et al. 2010, Rossi et al. 2010). Methods to direct maturation of progenitor populations or augment survival of mature neuronal transplants *in vivo* need improvement to take advantage of new PSC derivation methods.

4.6 PSC-Derived Interneurons

Local interneurons have been implicated in the formation of detour circuits that significantly contribute to functional recovery after partial SCI (Courtine et al. 2008, Flynn et al. 2011). Transplanted PSC-derived NSCs differentiate into a variety of interneurons that are capable of reconstructing functional neural relays, but, like astrocytes, the specific identity of those interneurons is rarely investigated beyond neurotransmitter expression profiles. Identifying the contributions of unique subtypes towards regeneration is important, since they respond differentially after SCI (Flynn et al. 2011, Husch et al. 2012). The recent availability of protocols and transgenic ESC lines that enrich for specific interneuron populations should improve access to these subtypes for modeling and transplantation (Murashov et al. 2005, Kim et al. 2009, Brown et al. 2014, Xu et al. 2015, Xu and Sakiyama-Elbert 2015, Iyer et al. 2016).

5. Transplantation Strategies

5.1 Biomaterials Scaffolds

Despite the many benefits of cell transplantation, the hostile, toxic environment of the injury site is a significant detriment to cell survival (Figure 3). A wide variety of biomaterial scaffolds have been investigated as transplantation vehicles and are typically chosen to accomplish one or more goals such as protecting transplanted populations, providing a structural bridge between injured areas, promoting axonal extension and cell migration, and mediating sustained delivery of pharmacological therapies (Raspa et al. 2016).

Protein-based biomaterials tend to have superior biocompatibility and an abundance of cell adhesion sites, which are critically important because they assist in cell survival, migration, proliferation, and differentiation. Collagen not only displays a number of cell adhesion sites, but also has mechanical properties similar to that of most soft tissues (Yoshii et al. 2004).

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When combined with growth factors, ESC-derived NSCs, or Schwann cells, collagen scaffolds promote CST and axon regeneration, remyelination and functional recovery (Hatami et al. 2009, Han et al. 2010, Patel et al. 2010). Fibrin based scaffolds have a particularly high therapeutic potential; they are biodegradable, can be injectable or formed *in situ*, have a multitude of cell adhesion sites, and can easily be modified for growth factor delivery (Itosaka et al. 2009, Kim et al. 2014). Our lab and others have done significant work demonstrating the effectiveness of using fibrin scaffolds to deliver growth factors, anti-inhibitory molecules, and ESC-derived NSCs or pMNs (Johnson et al. 2009, Johnson et al. 2010a, Johnson et al. 2010b, Johnson et al. 2010c, McCreedy et al. 2014b, Wilems et al. 2015, Wilems and Sakiyama-Elbert 2015).

Other natural materials that have been used for SCI can be heavily glycosylated, and are comparatively less adhesive than collagen or fibrin. Agarose has been shown to guide neurite outgrowth *in vitro* (Luo and Shoichet 2004) and to increase axonal regeneration following SCI (Stokols and Tuszynski 2006). Templated agarose scaffolds containing autologous MSCs that ubiquitously express brain-derived neurotrophic factor (BDNF) significantly increase long-range axonal growth after complete transection of the thoracic column (Gao et al. 2013). Alginate is a highly porous material widely used for both cell transplantation and drug delivery, but while alginate scaffolds have shown promise after SCI when seeded with BDNF-expressing MSCs, they may have cytocompatibilty issues and a high degradation rate (Zimmermann et al. 2001, Tobias et al. 2005, Ashton et al. 2007, Gunther et al. 2015). Hyaluronic acid (HA) is a major component of the extracellular matrix within the spinal cord and is commonly used as a foundation for hybrid biomaterial blends (Gupta et al. 2006, Horn et al. 2007, Park et al. 2010, Khaing et al. 2011), HA is often modified to incorporate additional cell adhesion sites or to alter its mechanical properties. For example, chemical modification of HA to allow for covalent crosslinking reduces the degradation rate compared to unmodified HA gels, thus providing utility over a longer period (Fuhrmann et al. 2015). OPCs transplanted in HA scaffolds crosslinked with thiol-functionalized gelatin and poly(ethylene glycol) diacrylate remyelinate endogenous axons in an ethidium bromide-induced demyelination lesion model (Li et al. 2013b).

Compared to natural materials, synthetic scaffolds offer more customizable attributes and greater control over mechanical properties, but their use can be limited by enhanced inflammatory and immune responses, few cell adhesion sites, and poor engraftment into the host tissue (Straley et al. 2010, Shrestha et al. 2014). For instance, poly(lactic-co-glycolic acid) (PLGA) scaffolds embedded with NSCs and Schwann cells demonstrate robust axonal extension following a complete thoracic transection one month post-transplantation (Olson et al. 2009). However, acidic PLGA degradation byproducts reduce the pH of the local environment, which can lead to reactive gliosis and/or a heightened inflammatory response (Oh et al. 1995, Sung et al. 2004, Kim et al. 2007). Poly(hydroxyethyl methacrylate) (pHEMA) scaffolds can mimic the mechanical properties of the spinal cord and improve axon regeneration, but syringomyelia development and limited integration at the host-transplant interface have been observed (Bakshi et al. 2004, Nomura et al. 2006, Li et al. 2013a).

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Balancing the positive and negative properties of a biomaterial is important when making scaffold design decisions. Scaffolds that combine several materials may emphasize positive attributes while masking negative ones, and therefore optimize the efficacy of a treatment. For example, a study using pHEMA-co-methyl methacrylate channels filled with different materials and growth factors showed that fibrin filled channels led to the greatest regeneration of reticular neuron axons and improved functional recovery compared to unfilled pHEMA controls (Tsai et al. 2006). The incorporation of bioactive peptide sequences into hydrogels is a promising approach that promotes specific cell-material or material-growth factor interactions to prompt regeneration (Woerly et al. 2001, Taylor et al. 2006, Taylor and Sakiyama-Elbert 2006, Park et al. 2010, Kubinova et al. 2015). In one combinatorial study, NSCs were seeded into conduits containing growth factors that were immobilized onto polycaprolactone (PCL) scaffolds using silk fibroin coatings; improved functional outcomes and axonal regeneration were observed 8 weeks after transplantation into a rat thoracic SCI model (Tang et al. 2014). These findings and others indicate that hybrid matrices combined with controlled drug delivery vehicles and cell transplantation can be manipulated to enhance regeneration following injury.

5.2 Controlled Drug Delivery

Systemic delivery, scaffold-based delivery, and micro- and nano-particle based delivery systems are the three main strategies to deliver pharmacological factors after SCI (Willerth and Sakiyama-Elbert 2007, Lee et al. 2010, Wang et al. 2011, Tyler et al. 2013, Zhao et al. 2013)(Figure 3). Neurotrophins and growth factors are often investigated, as well as anti-inhibitory molecules, anti-convulsants, immunotherapeutic molecules, hormones, and antibody treatments, many of which are in current use or in clinical trials (Mohtaram et al. 2013, Silva et al. 2014, Kabu et al. 2015). Commonly used methods such as direct injection, intrathecal infusion, or simple diffusion-based scaffold systems can be invasive, costly, and incapable of delivering bioactive molecules over the time-scale necessary for effectiveness (Jones and Tuszynski 2001). However, because more controllable drug delivery systems are not necessarily compatible with cells—harsh synthesis methods can result cell toxicity or a heightened immune response— combinatorial systems that are able to co-deliver drugs and stem cell-derived populations are still in the early stages of development. We and others have demonstrated that simultaneous, controlled delivery of anti-inhibitory molecules, growth factors, and stem cell-derived neurons is feasible, but still requires refinement to improve overall efficacy (Karimi-Abdolrezaee et al. 2010, Wilems et al. 2015).

5.3 Transgenic and Gene Delivery Approaches

While biomaterials and viral vectors have been designed to exogenously deliver therapeutic factors (Tuinstra et al. 2012, Tuinstra et al. 2014, Thomas et al. 2015) (Figure 3), cells can also be engineered as self-renewing drug delivery vehicles if appropriately modified. This presents an opportunity for sustained local delivery of growth factors or anti-inhibitory molecules at the site of the injury without the need for synthetic particles (Blesch et al. 2002, Thuret et al. 2006, Walthers and Seidlits 2015). The inclusion of tetracycline-inducible systems can help control long-term gene expression and thereby mitigate issues associated with gene overexpression, including the “candy store effect” which limits axon extension out of the graft (Blesch et al. 2002). Transducing stem cells *in vitro* prevents complications from

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direct *in vivo* gene manipulations, but can result in limited cell survival post-transplantation (Walthers and Seidlits 2015).

Gene delivery can also be used to direct differentiation of transplanted stem cells or endogenous adult NSCs and reactive astrocytes into more pro-regenerative glia or neurons (Tang and Low 2007, Corti et al. 2012, Guo et al. 2014). Transgenic modifications can help to enrich specific PSC-derived neuronal populations of interest and/or eliminate undifferentiated PSCs that could generate teratomas. We have developed several transgenic ESC lines to obtain specific ventral spinal populations that are difficult to isolate from primary tissues, but may also suffer from low differentiation efficiencies (McCreedy et al. 2012, McCreedy et al. 2014a, Xu et al. 2015, Iyer et al. 2016).

6. Conclusions, Challenges, and Future Outlook

The potential for stem cell therapies has never been greater. Ethical impediments caused by stem cell research have largely been mitigated by advancements in iPSC and direct reprogramming technologies. Human stem cells can be sourced from autologous patient tissue, permitting personalized strategies that reduce the risk of immune rejection as well as enabling disease modeling. *In vitro* methods to obtain specific spinal cell types have been made possible by the significant strides in research parsing spinal cord development and the genetic events that contribute to cell fate determination. After transplantation, stem cell-derived populations remyelinate host axons, administer trophic support, and contribute to relay circuits that promote functional recovery. Combinatorial transplantation strategies are increasingly comprehensive as biomedical engineers design scaffolding systems capable of supporting stem cell-derived populations while simultaneously mediating drug delivery and providing mechanical and chemical cues to promote regeneration.

Challenges persist. Reprogramming somatic cells into iPSCs has historically required viral overexpression of oncogenic transcription factors that compound the issue of stem cell tumorgenicity. Non-viral methods that minimize the tumorgenic potential have been developed, but reprogramming efficiencies are insufficient for clinical use, and the time needed to isolate and expand these populations remains unfeasibly high. There is also a risk that reprogrammed cells may revert to a pluripotent state. A reliance on animal products and feeder cells in either stem cell culture media or biomaterials matrices also reduces the potential clinical utility.

Differentiation methods face similar issues; neuronal induction efficiencies are lower than desirable, human cells can require weeks to months to mature in culture, and the yields can be variable, especially between *in vitro* and *in vivo* studies. Such inconsistencies can result in large histological and behavioral differences in SCI models, which ultimately harm the advancement of stem cell therapies. Our ability to refine induction methods is limited in part because the impact of various experimental conditions on differentiation and subtype specification is poorly understood. These may include the cell media composition, the types of morphogens and growth factors being applied, the material substrate and format, the environmental and atmospheric conditions, the cellular heterogeneity and density, etc.—slight changes may dramatically alter induction efficiencies in ways that are currently

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unknown. Furthermore, the signaling cascades responsible for spinal diversification and maturation are still being determined. More markers need to be available to identify unique neuronal and glial subtypes, as well as research suggesting how these populations may contribute to motor function and regeneration.

Active engagement between translational researchers and basic scientists will be important for the field to move forward. High throughput gene expression methods, single-cell sequencing, proteomics, and similar techniques will allow for a more comprehensive investigation and comparison of cells generated during development and in the dish. The biology ought to inform both differentiation and therapeutic strategies. The complex interplay between transplanted cells, materials, pharmacological agents, and the *in vivo* environment will require combinatorial systems where the components complement, rather than detract from one another, as can so often be the case. Creative design is necessary to get these novel strategies from the bench top and into the clinic.

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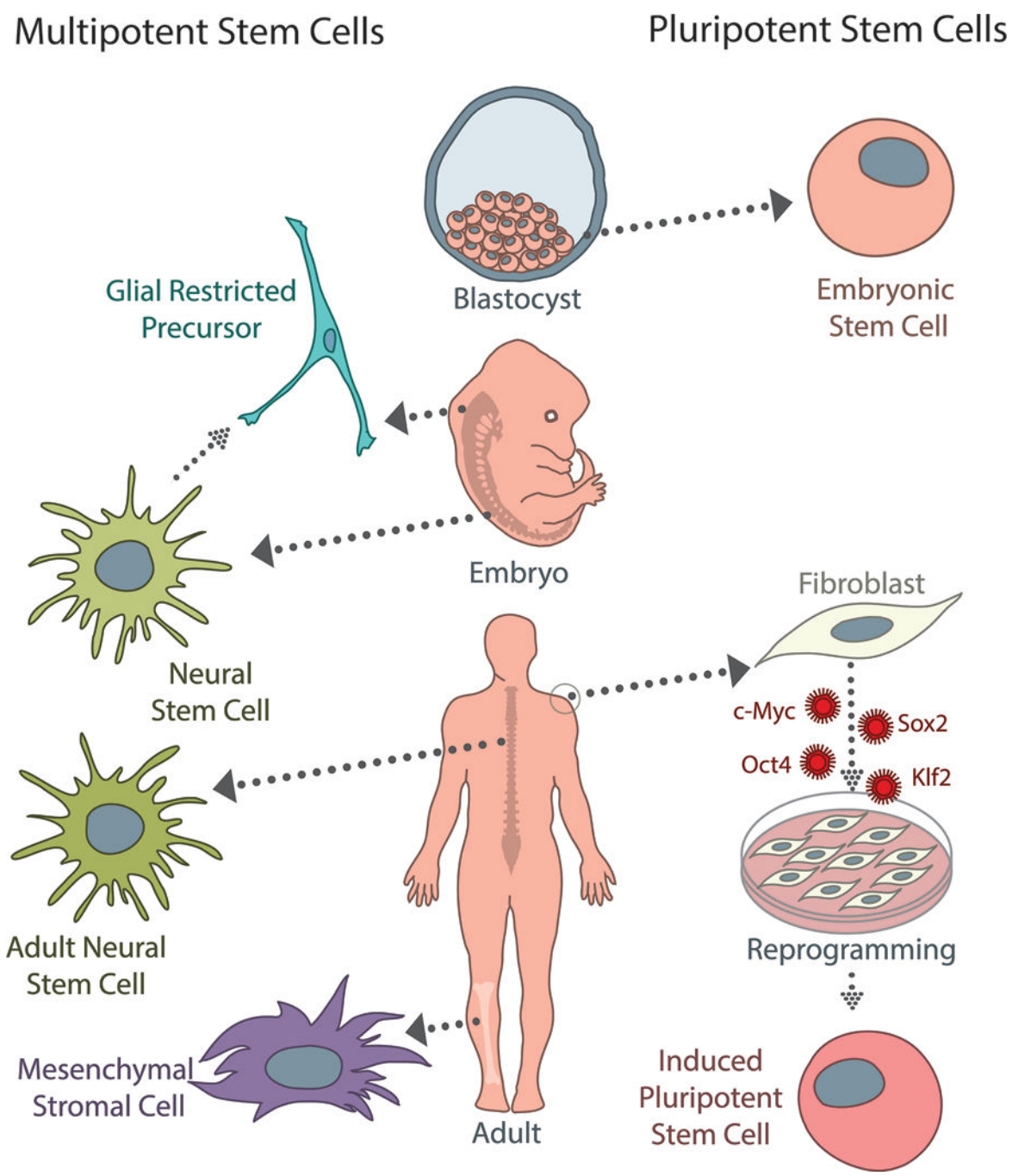

Figure 1.

There are several sources of multipotent (left) and pluripotent (right) stem cells currently used for spinal cord injury. Neural stem cells (NSCs) can be derived from fetal or adult tissue, and are capable of differentiating into neurons, oligodendrocytes, and astrocytes. While not typically considered stem cells, glial-restricted precursors (GRPs) are a commonly studied, tri-potent population that can be isolated from neural stem cells or fetal tissue directly. GRPs differentiate into oligodendrocyte progenitor cells and two types of astrocytes. Mesenchymal stromal cells (MSCs) are an appealing population clinically

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because they can be isolated from adult bone marrow or peripheral blood; however, while they are capable of differentiating into a wide variety of cells types, the efficacy of neuronal differentiation is a specific concern for SCI treatment. Embryonic stem cells (ESCs) are a pluripotent population, which can give rise to cell types from all three germ layers; however, because they are derived from the inner cell mass of early blastocysts, ethical considerations limit their clinical potential. Induced pluripotent stem cells (iPSCs) can be generated from adult somatic cells (fibroblasts, melanocytes, cord or peripheral blood cells, adipose stem cells, etc.) by several different reprogramming methods using the Yamanaka factors (c-Myc, Sox2, Oct4, Klf2). While induction and reprogramming efficiencies remain a concern, iPSCs represent an autologous, patient-specific population that has significant clinical potential as the field progresses.

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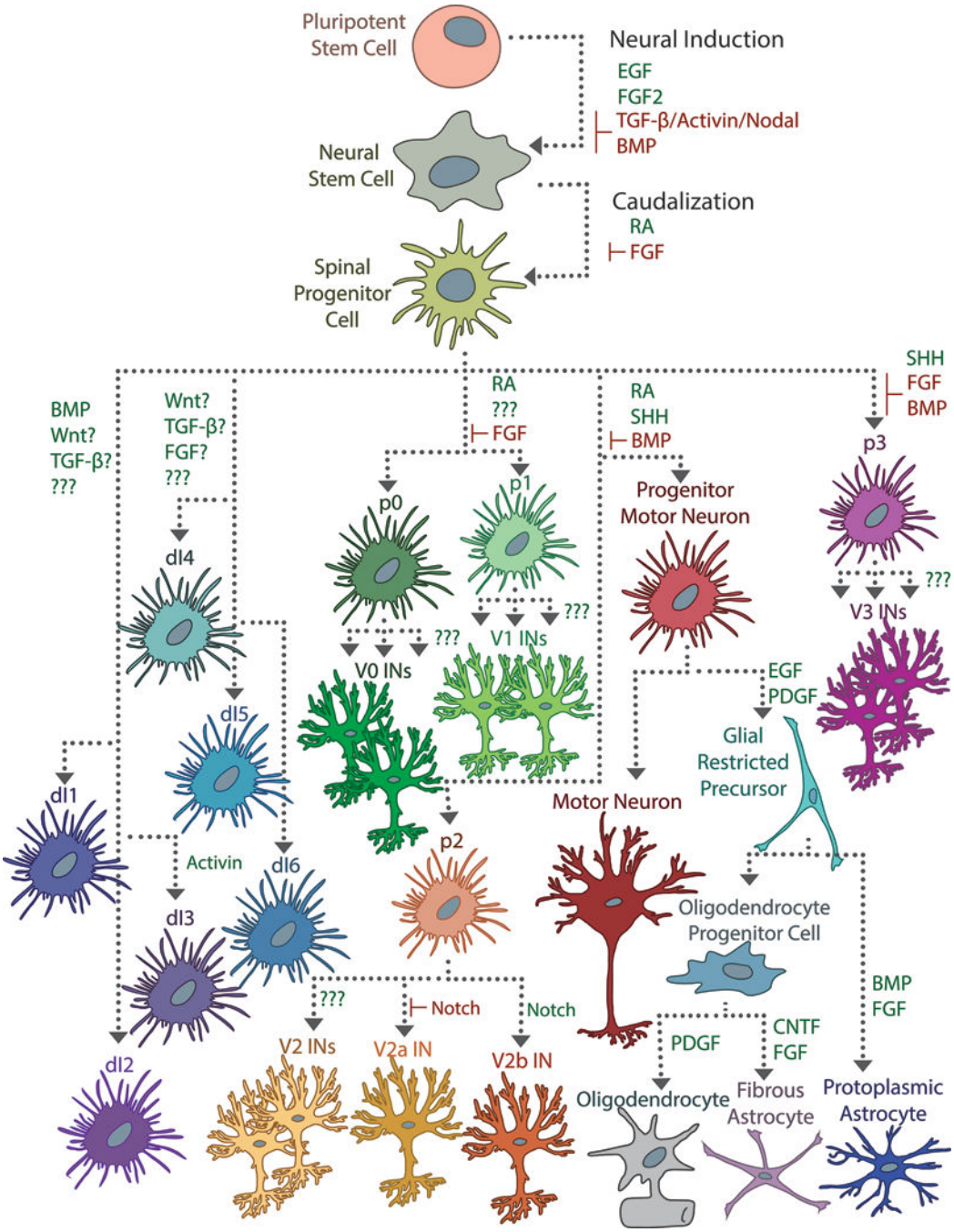

Figure 2.

Directing differentiation of pluripotent stem cells is traditionally achieved by triggering the signaling cascades responsible for cell fate determination. Neural induction from pluripotent cells typically begins with conversion into a neural stem cell (NSC), which may involve stimulating EGF and FGF2 signaling and inhibiting BMP and TGF-β/Activin/Nodal signaling. Subsequent exposure with RA caudalizes the NSCs and ensures a spinal identity. A complex interplay of signaling events is responsible for the dorsoventral patterning of the spinal cord, made more opaque by the importance of exposure duration and cell-cell

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signaling events. In the developing spinal cord, Shh from the notochord and floor plate interact with RA to induce differentiation of ventral progenitor cells (p0–p3, pMN), which mature into motor neurons, oligodendrocytes, astrocytes, and a variety of ventral interneuron populations. A combination of BMP, Wnt, FGF, and TGF-β signaling induces differentiation of the dorsal progenitor populations (dI1–dI6), but the precise mechanisms are poorly understood. Some specific factors that influence progenitor subspecialization have been determined experimentally *in vitro*, especially with regards to glial-restricted progenitor cells (GRPs), but for most interneuron populations, these are unknown.

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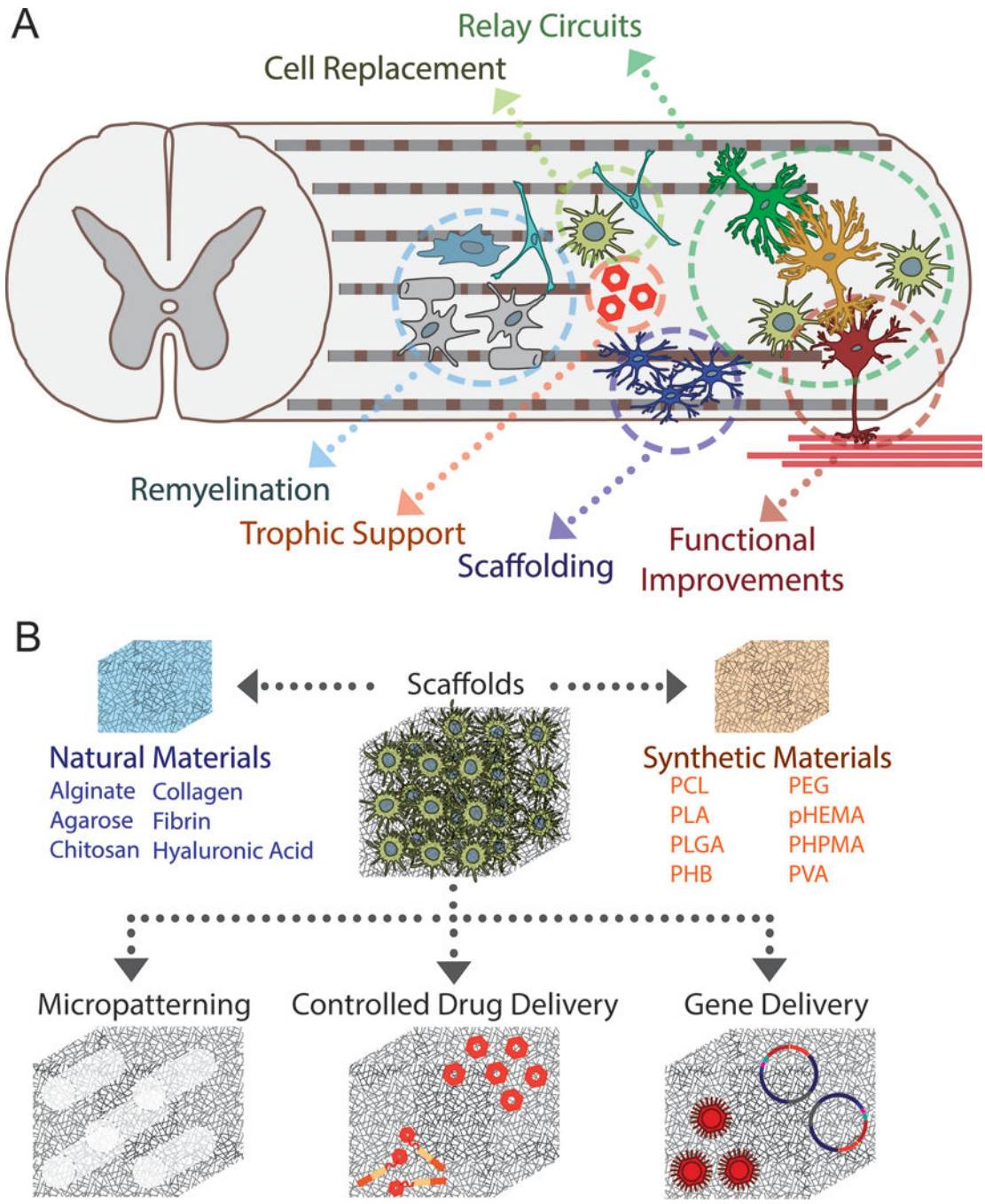

Figure 3.

(A) Transplantation of stem cell-derived spinal populations into the SCI lesion has multiple benefits. NSCs can differentiate into mature neuronal and glial populations to replace lost or damaged cells. These populations can serve as relay circuits between intact tissue across the lesion, and thus promote functional improvements as determined by electrophysiological and behavioral means. Stem cell-derived oligodendrocytes and astrocytes can remyelinate damaged axons and provide scaffolding for regenerating axons, as well provide trophic support through the secretion of growth factors. (B) Compared to direct injection of cells

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into the lesion or the intrathecal space or the delivery of micro-encapsulated cells, biomaterial scaffolds represent a better opportunity for a combinatorial therapy. Modifications including micropatterning, drug delivery, and gene delivery can be achieved to support and guide regeneration at the site of injury.