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Advances in Human Stem Cell Therapies: Pre-clinical Studies and the Outlook for Central Nervous System Regeneration

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Corresponding Author:	Melissa Renee Andrews, PhD University of Southampton Centre for Biological Sciences Southampton, UNITED KINGDOM
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Southampton Centre for Biological Sciences
Corresponding Author's Secondary Institution:	University of Southampton
First Author:	Lindsey H Forbes, PhD
First Author Secondary Information:	
Order of Authors:	Lindsey H Forbes, PhD
	Melissa Renee Andrews, PhD
Order of Authors Secondary Information:	

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Title

Advances in Human Stem Cell Therapies: Pre-clinical Studies and the Outlook for Central Nervous System Regeneration

Key words: *cell transplantation, CNS regeneration, embryonic stem cells, induced pluripotent stem cells, spinal cord injury*

Abstract: Cell transplantation has come to the forefront of regenerative medicine alongside the discovery and application of stem cells in both research and clinical settings. There are several types of stem cells currently being used for pre-clinical regenerative therapies, each with unique characteristics, benefits and limitations. This brief review will focus on recent basic science advancements made with embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Both ESCs and iPSCs provide platforms for new neurons to replace dead and/or dying cells following injury. Due to their capacity for reprogramming and differentiation into any neuronal type, research in preclinical rodent models have shown that ESCs and iPSCs can integrate, survive and form connections in the nervous system similar to *de novo* cells. Going forward however, there are some limitations to consider with the use of either stem cell type. Ethically ESCs are not an ideal source of cells, genetically iPSCs are not ideal in terms of personalised treatment for those with certain genetic diseases the latter of which may guide regenerative medicine away from personalized stem cell based therapies and into optimized stem cell banks. Nonetheless, the potential of these stem cells in CNS regenerative therapy is only beginning to be appreciated. For example, through genetic modification, stem cells serve as ideal platforms to reintroduce missing or downregulated molecules into the nervous system to further induce regenerative growth. In this article, we highlight the limitations of stem cell based therapies whilst discussing some of the means of overcoming these limitations.

Stem cells in nervous system repair

Regeneration of the injured central nervous system (CNS), for example following spinal cord injury (SCI), is hindered by two main factors: an inhibitory environment surrounding the lesion site and the failure of mature neurons to regenerate. Current therapies for CNS injury produce only modest levels of physiological and functional neuronal repair highlighting a critical requirement for new treatments. One research area receiving significant attention involves replacing damaged cells and tissues with cell transplants. A range of cell types have recently been investigated for their potential to promote CNS repair including but not limited to oligodendrocyte precursor cells, olfactory ensheathing cells, bone-marrow derived stromal cells (BMSCs), neural progenitor cells (NPCs) (Reviewed in Tetzlaff et al., 2011), embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Although each of the above cell types have demonstrated some level of regenerative growth in rodent models of CNS injury either modestly or more substantially, it is stem cell transplants that hold the greatest therapeutic potential due to their capacity to produce any cell type. Several different types of stem cells induce regrowth and/or new growth after injury including ESCs, endogenous neuronal stem/precursor cells (NPCs/NSCs), BMSCs and iPSCs. This article, however, will focus on human ESCs and iPSCs and summarize the current approaches used to combat their limitations in promoting integration, survival and regeneration following CNS transplantation in animal models.

Human stem cells

Human embryonic stem cells, or ESCs, develop from mammalian blastocysts and can differentiate into all three germ layers and thus any cell type (Evans and Kaufman, 1981). There are ethical concerns surrounding the acquisition and subsequent use of human ESCs that has limited their use both experimentally and therapeutically. Alternatively, induced pluripotent stem cells, or iPSCs, can be produced by inducing expression of defined transcription factors in somatic cells resulting in their dedifferentiation back to a pluripotent state (Takahashi et al., 2007) which can then be differentiated to any target cell type. Recent advances using iPSCs have demonstrated successful modelling of neurodegenerative and genetic diseases which surpass current methods as most knockout mice or toxin-induced models cannot fully reproduce disease pathology (Reviewed in Wu et al., 2019). Nevertheless, the discovery of iPSCs brought forward the idea of personalized medicine, where one's own cells could be used

as a treatment for diseases or injures, yet there are clear challenges involved in creating tailored cell therapies. Alongside extensive cost and time required to produce the cells, evidence suggests that reprogramming iPSCs has limitations including the epigenetic state of somatic cells as well as potential cellular senescence among others (Reviewed by Haridhasapavalan et al., 2019). Equally, in treatment of genetic diseases such as Huntington's disease, personalized iPSCs are likely to have little benefit to patients without genetic correction prior to therapeutic use (Reviewed by Golas and Sander, 2016). Perhaps a more practical option is the creation of stem cell banks which contain human donor stem cells screened for specific leukocyte antigens which can then be matched to individual patients (Reviewed in Solomon et al., 2015). Despite these limitations, however, iPSCs have the potential to be a significant resource for disease modelling and have thus far been used in rodent CNS transplantation studies with encouraging results.

Human-derived ESCs and iPSCs in rodent models of CNS repair

The generation of efficient human ESC-directed differentiation into specific neuronal subtypes *in vitro* well over a decade ago has opened new avenues for CNS disease modelling and cell replacement therapies. Transplantation of human ESC-derived cells into rodent models has had great success (Denham et al., 2012; Espuny-Camacho et al., 2013; Espuny-Camacho et al., 2018). For example, Denham and colleagues transplanted pre-differentiated neurons derived from human ESCs into the uninjured neonatal rat striatum at postnatal day 2. ESC-derived axons were detected along host white matter tracts, with *ex vivo* patch clamping of grafted neurons displaying action potentials, and immunohistological analysis revealing expression of synaptic proteins suggesting functional integration (Denham et al., 2012). Further advancements with human ESC-derived neurons indicate that matching the phenotype of transplanted stem-cell derived neurons to the cortical areal identity significantly improves graft viability and integration (Espuny-Camacho et al., 2018). For example, transplanted human ESC-derived visual cortical cells functionally integrated into the lesioned mouse visual cortex forming functional synapses with host circuitry compared to visual cortical cells transplanted into the mouse motor cortex which had limited integration with host circuitry (Espuny-Camacho et al., 2018).

In vitro studies of human iPSCs have further paved the way for the advancement of stem cell transplantation into the CNS. For example, cerebral cortical neurons can be generated from human iPSCs *in vitro* (Shi et al., 2012). These iPSC-induced pyramidal cells not only form

neurons from several cortical layers, they produce a glutamatergic phenotype, form excitatory synapses and possess electrical activity *in vitro* (Shi et al., 2012). Similarly, human iPSCs and their derivatives have shown great promise following CNS transplantation (Tornero et al., 2013; Forbes and Andrews, 2019). In the context of injury, transplants of human cortically-fated iPSC-derived NPCs into the rat somatosensory cortex following stroke-induced injury resulted in functional recovery with evidence of integration into the immunocompromised host brain (Tornero et al., 2013). Interestingly, transplanting cortically-fated iPSC-derived cells compared to un-fated iPSC-derived neural progenitors resulted in fewer proliferating cells (detected with Ki67 immunohistochemistry) likely reducing the risk of tumour formation although both graft types resulted in comparable functional recovery (Tornero et al., 2013). On the other hand, only the cortically-fated iPSC-derived cells extended high density projections following grafting suggesting that cellular identity increases integration and function as described for human ESCs. This was shown in a recent study where human iPSC-derived NSCs were injected alongside an artificial extracellular matrix into the sensorimotor cortex of perinatal rats following induced focal ischemia (Basoudan et al., 2018). A month after grafting, instead of dispersing and projecting into the host environment as documented *in vitro*, transplanted cells formed cerebral organoids characterized by neural stem and progenitor cells generating rosettes, failing to extend long distance projections. As these cells were un-fated neural stem cells, they likely were unable to differentiate further and instead remained in a stem cell niche highlighting the requirement for areal specific fated cells for CNS transplantation. Furthermore, both inhibitory and excitatory cortical neurons can be generated from human iPSCs *in vitro* and, following transplantation into the uninjured adult rat forebrain, can exhibit both inhibitory and excitatory post-synaptic currents (Yin et al., 2019). This suggests human iPSC-derived neurons could re-establish damaged signaling pathways following injury.

Combating the limitations of stem cell grafts

There are limitations surrounding stem cell transplantation research which in turn can substantially limit their use long-term. Two of the major limitations are graft survival and integration into the host environment. Much can be learned from mouse stem cell transplants into mouse hosts, but with the current availability of human iPSCs it is crucial to explore the survival and regenerative capacity of human cells in a rodent host. To fully understand the capabilities of human iPSCs to promote CNS repair, pre-clinical xenogenic transplant models are frequently chosen but often require modification of the host immune system. Currently this

modification involves the use of immunodeficient hosts such as NOD/SCID transgenic mice, or the use of immunosuppressant drugs such as cyclosporine. However, transplantation of human stem cells into naïve neonatal rodents (postnatal day 0 – postnatal day 3) increases graft survival due to the immature state of the immune system and allows fundamental characterization of grafted cells *in vivo*. For example, we have observed that human iPSC-derived NPCs could survive up to 8 weeks post-transplantation into the uninjured neonatal (P0-P2) cerebral cortex (Forbes and Andrews, 2019). Others have reported similar survival rates with human ESC-derived neurons, with survival up to 10 weeks post-transplantation into the neonatal uninjured rat striatum (Denham et al., 2012).

In order to prolong the survival of human stem cell grafts *in vivo* we can gain insight from mouse stem cell transplant studies. A recent study has utilized a strategy for inducing immunological tolerance of transplanted mouse-derived stem cells to increase graft survival. Specifically in this study, Li and colleagues examined allogeneic stem cell transplantation together with modulation of T-cell activation in the host, results of which demonstrated a significant increase in grafted cell survival (Li et al., 2019). In other studies, Ballout and colleagues have shown that a mouse stem cell transplant can modify the local environment, whereby transplanted cells increased recruitment of astrocytes (Ballout et al., 2019), which are known to be associated with axon growth and guidance, suggesting cell grafts stimulate a pro-regenerative environment. They also demonstrated that delaying the timing of transplantation after injury in an adult mouse host improves graft survival as this avoids the immediate immune response occurring in the injury. For example, one week following lesioning of adult mouse motor cortex, embryonic-derived motor neurons were transplanted into the lesion site. At one week post-lesion, there was an increased presence of pro-regenerative cells, such as Arg1-immunoreactive microglia, and a decrease in pro-inflammatory cytokines, such as interleukin-1β, likely resulting in increased chances of graft integration and viability. At 21 days post-injury however, pro-regenerative microglia switch to a pro-inflammatory phenotype narrowing the window for therapeutic intervention (Ballout et al., 2019). These results highlight the pro-regenerative aspects of neuroimmune cells, such as microglia and astrocytes, but recognize the limitations of the host immune system. Together these results suggest that repair of CNS injuries using stem cell grafts may be possible, yet highlights the host immune system may dictate therapeutic timing.

In animal models of CNS injury, although graft survival can often be prolonged with immunosuppression or neonatal immune-deficient windows, survival is also influenced by the ability of grafts to integrate within host tissue. In some CNS injuries, for example SCI, the environment of the injury site can prevent cell integration and thus survival due to an increase in inhibitory proteins within the lesion including those associated with the glial scar, containing reactive astrocytes that secrete the proteoglycan tenascin-C and chondroitin sulphate proteoglycans (CSPGs). Modifying or pre-conditioning stem cells prior to transplantation may better equip cells to adapt within an inhibitory environment and result in better repair. For example, overexpression of growth-promoting proteins such as integrins to promote axonal growth (Forbes and Andrews et al., 2019), specifically the α9 integrin subunit in human iPSC-derived NPCs, resulted in increased neurite outgrowth on a tenascin-C substrate *in vitro*. In other studies, co-delivery of Chondroitinase ABC, an enzyme which degrades CSPGs, has been shown to increase in transplant survival when delivered to a spinal cord lesion together with human induced pluripotent stem cell-derived neuroepithelial cells (Führmann et al., 2018). In addition to increased stem cell transplant survival, ChABC delivery also led to the development of functional synapses and behavioural recovery after SCI when delivered with mouse iPSC-NPCs (Suzuki et al., 2017). This highlights the potential for combining treatments that can combat the inhibitory environment created after CNS injury with a human stem cell replacement therapy to promote repair when used in an injury model.

To further enhance human graft viability, cell replacement therapies however could evolve from single stem cell transplants and instead focus on tissue-based transplantation consisting of more developed stem cell structures, such as pre-developed axon tracts (Chen et al., 2019) or organoids (Daviaud et al., 2018). Human ESCs organoids have been established from human ESCs *in vitro* and following transplantation into the lesioned mouse cortex demonstrate enhancement of graft viability compared to single cell transplants (Daviaud et al., 2018). Further benefits of this organoid tissue-based transplantation include a reduction in microglia/macrophage activity and increased vascularization to the grafted organoids compared to human NPC grafts.

New methods are further being developed to promote axon growth prior to transplantation. This includes axon stretch growth which elongates stem cell-derived axons prior to transplantation (Chen et al., 2019). By growing human stem cell-derived NPCs on a moving membrane and exposing them to mechanical tension, axon tracts can be grown within the

laboratory extending up to 1 cm in a month. *In vitro* these tracts demonstrate functional activity with spontaneous calcium waves (Chen et al., 2019). This offers new *in vitro* methods for modeling CNS injury and a further possibility for transplantation of axon tracts providing new hope for navigating the intrinsic inabilities of axons to promote growth after injury. Pre-formed axon tract replacement however, does not address how transplanted tracts would merge and synapse with existing, potentially multiple, damaged tracts *in vivo* and would likely also require modification of the local injury environment.

Together these studies stress the potential that human stem cells have for overcoming both graft survival and integration into the injured CNS. Yet there are still a number of stark challenges surrounding cell replacement therapy namely ethical use of human stem cells and ensuring proper quality control. Similarly, in this article we have focused on pre-clinical human stem cells *in vitro* and following transplantation into CNS injury animal models yet the physiological processes underlying human CNS injury are vastly more complex highlighting the requirement for more reliable and robust pre-clinical and clinical models to ascertain the extent to which human stem cells can promote CNS repair.

Conclusions

Human stem cell grafts hold great promise for CNS treatments with potential for patient-specific therapies in CNS diseases and injuries. Due to the complexity of many CNS conditions, such as SCI, it is likely research will focus on structured tissue-based transplantation approaches due to promising integration and survival. Furthermore, careful consideration of the immune response must be tailored to provide a pro-regenerative window for therapeutic intervention. This timed treatment would likely be in combination with pre-conditioning e.g. cell adaptations *in vitro* prior to transplant, or pharmacological interventions to modify the inhibitory injured environment.

It is expected that the different diseases will have different requirements for stem cell therapy. For example, where neuronal cell death is a crucial disease characteristic, such as Alzheimer's and Parkinson's disease, the focus for therapy would likely be replacing injured or damaged neurons, whereas in other conditions the therapeutic focus may center on promoting endogenous repair or via secretion of pro-regenerative factors. In some injuries, such as SCI, a combination of these strategies may be required to promote functional repair.

Figure legend: Recent stem cell transplantation studies. Embryonic stem cells are derived from the inner cells of mammalian blastocysts. This is in comparison to induced pluripotent stem cells which are derived from somatic cells, such as fibroblasts, that are dedifferentiated back to a pluripotent state *in vitro*. Both ESCs and iPSCs have the potential to be differentiated into any cell type with iPSCs holding a great advantage for prospective tailored cell therapy.

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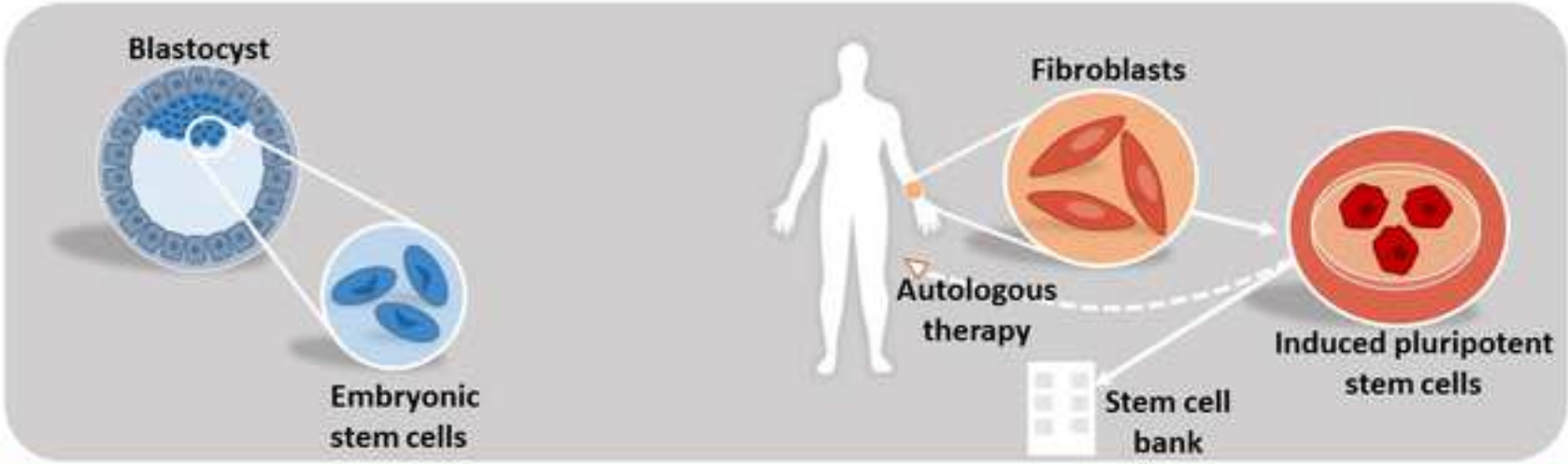
Figure



Species Origin	Cell type	Transplant model	Reference
Human	ESC-derived neurons	Neonatal mouse striatum	Denham et al., 2012
	ESC-derived progenitors & neurons	Neonatal mouse frontal cortex	Espuny-Camacho et al., 2013
	ESC-derived visual cortical neurons	Adult mouse visual cortex	Espuny-Camacho et al., 2018
	iPSC-derived NPCs	Adult rat cerebral cortex	Tornero et al., 2013
		Neonatal rat cerebral cortex	Forbes and Andrews et al., 2019; Yin et al., 2019
	iPSC-derived NSCs	Neonatal rat cerebral cortex	Basoudan et al., 2018