Progress in the use of induced pluripotent stem cells for cervical and thoracic traumatic spinal cord injuries: Meta-analysis and Review

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Abstract*:*

Induced pluripotent stem cells (iPSCs) are cells that are genetically reprogrammed from human tissues such as blood or skin cells. These cells can be differentiated in vitro into specialized cells such as neurons that can replace damaged neurons in the spinal cord. A handful of studies have analyzed the functional use of iPSC-derived cells in vivo for treatment of cervical/ thoracic spinal cord injuries caused by physical trauma. The hypothesis is that cells derived *from iPSCs are an effective treatment for cervical/ thoracic spinal cord injuries (SCI)*. This meta-analysis determined if significant motor improvement was restored after treatment with iPSC- derived cells compared to control treatments. Overall based on locomotion scales in rodents and monkeys, this approach indicates a therapeutic benefit for SCIs using cells derived from either iPSCs or embryonic stem cells (ESCs). The confirmation that treatment with iPSC-derived cells is as effective as cells derived from ESCs is important due to the controversies existing with current work using ESCs. By piecing together evidence of the successes and limitations of iPSCs in the recovery of motor skills, this intends to elucidate the progress achieved with transforming iPSCs into cells needed for spinal cord repair. Results from our analysis can be used to address questions that are still unanswered in this field and to determine the direction that future research needs to take.

Background:

Introduction to Spinal Cord Injuries

Traumatic SCIs are mostly prevalent in the cervical and thoracic regions of the spinal cord, and these are generally caused by a car accident or sports related injury. Likewise, geriatric patients with osteoporosis or degenerative spondylolisthesis are at an increased risk. These injuries result in contusion or compression of the spinal cord that can lead to impairment of muscle movement depending on the severity of the injury. In worst cases, permanent dysfunction such as paralysis can lead to a person becoming paraplegic or quadriplegic depending on the site of injury.

According to the US National Spinal Cord Statistical Center (NSCSC), the incidence of traumatic SCIs is 17,500 new cases each year and globally between 250, 000-500,000. The average age of injury is 42 years old; however, traumatic SCIs mostly occur in people younger than 30 years old. Males account for 81% of SCIs and the ratio of men to women is 3:1 (NSCSC, 2017). Vehicular accidents are the leading causes, followed by falls, violence, and sports such as skiing, rugby, and horseback riding which pose the greatest risk. Most SCIs at the cervical level are from hockey, skiing, diving, and American football, whereas over half of SCIs from horseback riding and snowboarding occur at the thoracic or lumbosacral region (Weidner, 2017). A SCI is a very expensive traumatic condition which a patient in the first 2 years in the US pays around $200,000 for home care, medical complications, and other services (Johnson, 1996). Therefore, there is a need to find an optimal treatment for a SCI.

Pathophysiology behind a SCI

In a SCI, myelopathy occurs, causing damage to white matter that contains the axons and tracts to and from the brain, and gray matter that results in a loss of motor neurons. This will manifest in deficiencies in motor and sensory skills. In the primary injury phase, the SCI can result from contusion of shattered cervical/ thoracic vertebral bones or compression which results in an increase of pressure due to blood or bone on the spinal cord. Then at the secondary injury phase, cell apoptosis and necrosis, oxidative damage, glutamate excitotoxicity, and axon tracks are destroyed due to immune responses (Ronaghi, 2009). Normally, the spinal cord is "immune privileged" secreting immunosuppressive cytokines and immunotolerant because it is isolated from the rest of the body through the blood brain barrier (BBB) (Itakura, 2015). In addition, lymphatic vessels that carry white blood cells are absent in the central nervous system (CNS), which contains low levels of major histocompatibility complex (MHC) molecules, indicating that only certain immune responses occur in the spinal cord (Itakura, 2015). Once the BBB is broken in the spinal cord, this increases permeability for cells and molecules carried out in the blood to invade the injured tissue, causing inflammation. This leads to platelet and fibrin clots to reduce bleeding. Moreover, astrocytes during injuries become eosinophilic and get involved in the immune response. Their migration increases permeability to leukocytes and causes inflammation (Treuting, 2017). After a SCI, astrocytes proliferate and express glial fibrillary acid protein (GFAP) and congregate to form glial scars during the chronic stage. The neural scar tissue expresses Semaphorin 3A, an inhibitor of axonal regeneration (Nagoshi, 2017). This inhibits recovery of the CNS by creating a physical barrier using scar tissue from gliosis and collagen fibers. These scars also secrete chondroitin sulfate proteoglycans that inhibit axonal growth; thus, using a chondroitinase or semaphorin 3A inhibitor are good regimens to encourage plasticity

(Nagoshi, 2017). Also at this stage, oligodendrocytes and neurons at the SCI site die due to disruption of cell membrane and from hemorrhages. Once oligodendrocytes die, this triggers axon de-myelination, affecting signal transduction and reducing the possibility of generating an action potential.

Considering the complexity of the trauma sustained to the spinal cord and the prevalence of SCIs in society, this medical condition has prompted research into different modes of treatment. Currently, treatment options are limited; however, cell therapy can potentially help improve the quality of life for a SCI patient. Thus, research has already investigated the use of cells derived from ESCs, but due to ethical concerns and immunocompatibility, the use of these cells continues to be under debate. In 1998, the first human (h) ESC line was created and since January 2009, hESC derived tissues have been used in clinical trials in SCI patients (Lukovic, 2012; Abdelalim, 2016). In 2006, an alternative to ESCs was introduced: iPSCs resolve these ethical concerns and immunocompatibility associated with ESCs.

What are iPSCs?

A pluripotent stem cell (PSC) is a general term that describes iPSCs and ESCs. Pluripotency refers to the ability of these cells to differentiate into any of the 3 germ cell lines: endoderm, ectoderm, and mesoderm, hence into all cell types of the body. These cells can be differentiated in vitro into specialized cells such as neurons which can replace damaged neurons. iPSCs are remarkably similar to ESCs but have different origins. IPSCs are cells that have acquired the characteristic of PSCs by genetic reprogramming obtained after the overexpression of key transcription factors: Oct4, Sox2, Kl4, and c-Myc (Takahashi and Yamanaka, 2006). Other combinations of transcription factors, reprogramming molecules, and regulatory gene

networks that promote the expression of the core transcription factors related to pluripotency such as Oct4, Sox2, and Nanog can be used to induce the reprogramming of somatic cells into PSCs. iPSCs can be derived from any somatic cell; however, the most common cells used are blood cells or fibroblasts. iPSCs are suitable for tissue regeneration since they undergo unlimited self-renewal and multi-lineage differentiation, including into neurons by modifying specific molecular signaling pathways (Lukovic, 2012). Moreover, iPSCs downregulate astroglial activity at the injury site which is beneficial because this prevents glial scar formation, generating a microenvironment that is more suitable for recovery (Bahmad, 2017). Therefore, iPSCs have great potential in medicine because these cells have been used to treat neurological, hematological, metabolic, cardiovascular, and immunodeficiency diseases. iPSCs can be differentiated into neuron stem cells (NSC), which are identified through the expression of markers like GFAP (+), Lex(+), CD49f(+), and CD29(+) (Shen et. al 2008). After this stage, the NSCs can be further differentiated into neural cells specific to the spinal cord (i.e. oligodendrocytes and neurons) (Figure 1).

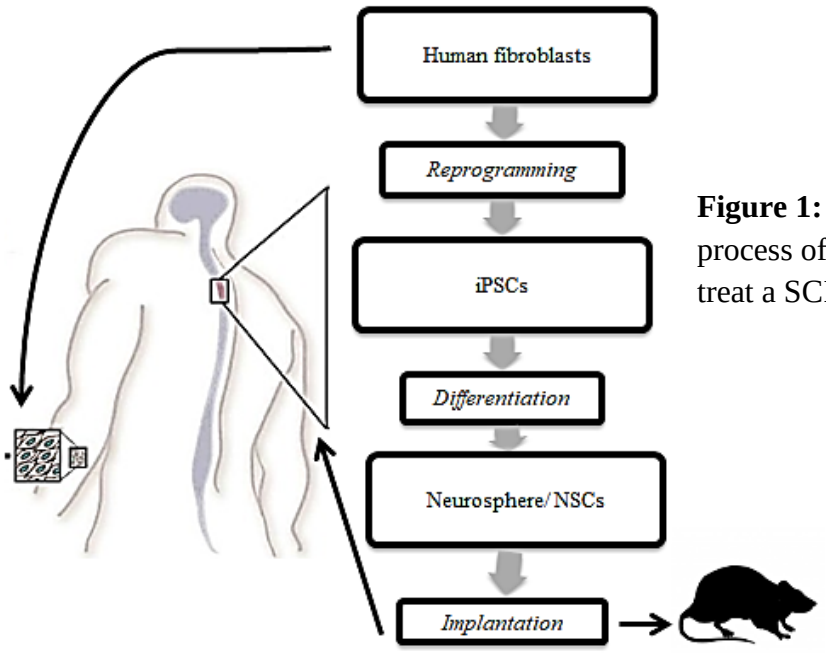


Figure 1: General schematic of process of how iPSCs are used to treat a SCI in rodents and humans

How similar are iPSCs to ESCs?

iPSCs and ESCS are functionally and molecular equivalent. iPSCs have identical patterns in gene expression, chromatin methylation, and pluripotency (Ronaghi, 2009). Fully reprogrammed iPSCs express genes including OCT4, SOX2, NANOG which are found in similar levels to ESCs and they reactivate telomerase gene expression, down regulate THY1, and upregulate SSEA1 (Robinton, 2012). iPSCs also have a similar differentiation potential to ESCs in terms of differentiating into the 3 neural lineages (Tsuji, 2010). After differentiation of ESCs to neural progenitors in the neurosphere, stem cell markers Oct4 and Nanog decrease and Nestin increases. (Li H, 2009). Recent studies also show that genetically matched, male hESC and hiPSC lines are transcriptionally and epigenetically highly similar to one another, implying that the variability of the genetic background and sex may account for differences in gene expression and methylation found in between non-matching cell lines (Choi, 2015. Both human iPSCs and ESCs tend to have additions to chromosomes 12 and 17; however, iPSCs have additional gains to chromosome 1 and 9, while ESCs have gains in chromosome 3 and 20 (Robinton, 2012). Whether these gains are advantageous or deleterious is unknown, or if these additions have any important effect on predicting outcome of functional recovery. However, one great advantage of using iPSCs over ESCs is the process of obtaining the parental cells for reprogramming into iPSCs is non-invasive and not detrimental to a patient with a SCI. Since it is easier to obtain parental cells, it is more feasible and an affordable regimen for clinical use, costing $120-200 per sample (Liu, 2017). Later, these cells are reprogrammed into autologous iPSCs and can be differentiated into NSCs and then to terminal neurons to be used to treat lesions site of the donor/patient, becoming a personalized specific therapy (Lee-Kubil, 2015).

Existing concerns over iPSCs

One potential issue with the use of iPSCs and progenitors derived from them is the formation of teratomas if the transplanted cells from iPSCs were not fully differentiated. Specifically is of a concern, if undifferentiated NSC Nestin+ cells with active Oct4 transgene, which is expressed in human gliomas, were to be used (Nagoshi, 2017). The source of the parental somatic cells for reprogramming into iPSCs is predictive of the percentage of undifferentiated Nanog positive cells in the neurospheres and to the likelihood of a teratoma forming (Tsuji, 2011). One study showed that iPSCs derived from mouse embryo fibroblasts (MEFs) resulted in hardly any undifferentiated cells in the neurospheres. Furthermore, teratoma formation in mice transplanted with MEF-iPSC clone derived neurospheres was as infrequent as in ESC derived neurosphere (Tsuji, 2011). In contrast, iPSCs derived from tail tip fibroblasts (TTFs) showed resistance to differentiation and contained undifferentiated cells in the neurosphere after the induction of differentiation and produced a significantly larger teratoma (Tsuji, 2011). Neurospheres differentiated from iPSCs with adult liver cells as parental cells are intermediate between the MEF-iPSC clones and TTF-iPSC clones for neural differentiation and tumorigenesis (Tsuji, 2011). Further risks for developing a tumor occurs after implantation of the neurosphere, due to its remaining potential to differentiate into astrocytes, oligodendrocytes, and neurons in the SCI area (Germano, 2010). A solution for this would be instead of implanting the neurospheres, it may be safer to implant the specific cell type required, i.e. oligodendrocytes which help with re-myelination, rather than an astrocyte which promotes glial scar formation.

Another concern is the use of existing iPSC lines that are not directly derived from the patient. By implanting these cell lines, patients may experience immune rejection, hence will be required to be under immunosuppressant treatment. It is known that immune rejection occurs

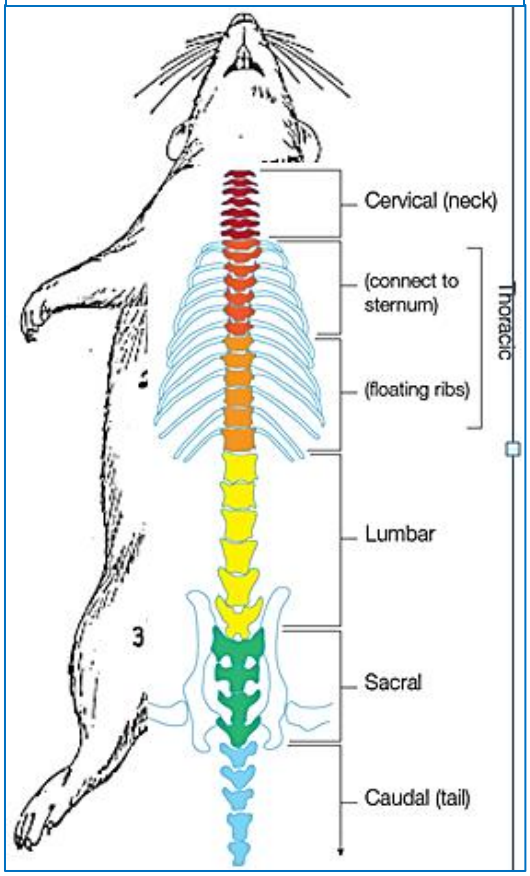
within 3 wks. post-initiation of treatment. In experimental conditions in xenografts in immunocompetent BALB/cA mice mice, graft survival rate was 0% without immunosuppressant and increased up to 100% in the group with immunosuppressants (Itakura, 2015). Immune rejection may be a result of mismatch between the donor and recipient in a locus or in the minor histocompatibility antigen. Yet evidence in mice suggests that after a period of 100 days, it is important to discontinue the use of immunosuppressants, because prolong immune suppression can induce the formation of tumors due to inhibition of normal immune responses. To overcome tumorigenicity, tumors can be ablated after transplantation, immunosuppressants can be discontinued in order to activate immunoreactivity, or Notch signaling can be targeted using a gamma secreting inhibitor (GSI), which has a role in the status of undifferentiated neural progenitor cells (NPCs) and induces their maturation while limiting proliferation (Nagoshi, 2017). The optimal approach is to use patient specific therapy in which the iPSCs are derived from that particular patient.

Another concern regarding the use of iPSCs is the method by which they are derived to avoid permanent insertion of transgenes. Some reprogramming cocktails include oncogenes such as c-Myc and Klf4 which ideally should be avoided. Non-transgene methods such as piggyback transposon system, episomal vector, Sendai virus, or plasmids are common methods to derive transgene iPSCs and have been proven to be relatively safe unlike retrovirus and lentiviral vectors that can result in undesired permanent gene insertion and promote tumorigenicity (Seaung-Ik Oh, 2012).

Humans vs. Rodents

Before making the leap from experimental treatment of SCIs with cells derived from iPSCs in rodents to primates and then to humans, it is important to understand the differences between the pathology and physiology of humans and rodents. First of all, the actual spinal cord terminates at the first lumbar vertebrate (L1); thus, it covers the cervical and thoracic regions, while the lumbar and sacral regions are a part of the cauda equina (figure 2). White matter in the spinal cord contains axons, tracts of nerve fibers, and oligodendrocytes. In contrast, grey matter contains neuronal cell bodies. Unlike rats, humans possess a greater quantity, in general twice the amount, of white matter due to the elevated encephalization quotient of brain size to body weight, due to greater detail and capacity of sensations and motor skills in human limbs (Treuting, 2017). A further complication in using rodents as experimental models reside in that several studies that implanted iPSCs in mice use human iPSCs; thus, the NSCs retain the intrinsic human rate of maturation in the rodent's spinal cord but normal maturation of NSCs in rats occur faster than in humans. Rat biology works at a faster rate than humans, since the gestational period for human is 280 days and for rats is 21 days, so this will reflect in how fast recovery will be evident in rodent versus human populations (Lu, 2017). Hence studies done in rats, which monitor rats post a SCI for several months, may not reflect the length of time it takes to detect functional recovery in a

Figure 2: Vertebrae of the spinal column in a mouse



human. In humans, neurological recovery occurs mostly during the first year with the first 3 months having the steepest curve of recovery of locomotion and later between 1-5 years post the SCI (Weidner, 2017)

Ultimately, given that iPSCs are compatible to ESCs and ESCs have been useful in restoring motor functionality in rodent and human populations, the postulation is that iPSCs should also elicit similar positive outcomes on locomotion. A review and meta- analysis was conducted in order to ascertain whether there is significant evidence that motor function can be rehabilitated using iPSCs after sustaining a debilitating injury to the spinal cord. By reviewing studies that used iPSCs to treat SCIs in animal models, this will shed light onto the consensus over the usefulness of these stem cells in medicine.

Materials and Method:

Recollection of Literature

The studies used in the meta-analysis and review were collected from online scholarly, peer reviewed journals or books through databases including PubMed and Web of Science. Searches conducted through these databases used key words such as *behavior assessment, ESCs, iPSCs, motor recovery/ functionality, SCIs, stem cell therapy, and physiology of the spinal cord.* The search included studies published in English from 2000-2018 to narrow down the selection. The next step was to eliminate studies that included use of iPSCs or ESCs in treating neurological trauma sustained to the brain rather than the spinal cord or neurodegenerative diseases because this was not the focus of the investigation. In terms of the site of the injury, studies were screened for cervical and thoracic models only. Furthermore, the final studies had to encompass some scale of behavioral testing to assess the use of iPSC-derived cells before and

after implantation. This strategy identified 17 studies that used iPSCs in treating traumatic SCIs although out of these, 7 were excluded because insufficient information was reported to calculate neither a t-statistic nor effect size. Instead those studies were included for qualitative synthesis and 6 studies comprised the meta-analysis.

Inclusion and Exclusion Criteria for Meta-analysis

A general screening determined which studies were eligible to be used in the meta-analysis was based on the following criteria: all studies should be comparable because of use of the same animal population (rats/ mice), same motor assessment scale (BMS/ BBB), similar location of SCI (thoracic only), blinded observers were used to assign the level of motor recovery, and a mean, SD, and n value was provided. Studies were excluded if they did not report any data assessing motor functionality, if a different animal model was used instead of rodents due to differences in the scales, especially given that greater detailed motor skills can be evaluated in monkeys, and studies using cervical SCI models were not included because the Basso mouse scale (BMS) is not the most appropriate method to detect motor improvement or deficit for this region. Lastly, the overall procedure of inducing a SCI and implantation of iPS-derived cells should be similar with minor variations as reported in Table 1.

Comparative and statistical analysis within studies

Before performing the meta-analysis, a t statistic was used to determine which studies showed significant results. Means were estimated from the data (e.g. graphs) provided for all studies that used the BMS; however, if neither a SD nor SEM was given, the t statistic could not be performed. In those cases, only could it be stated that the study itself reported significance at a certain p value (e.g. 0.05, 0.01); however, significance could not be verified in this analysis. A

one tailed t test was performed at an α level of 0.05 corresponding to the null hypothesis $X_{iPSCs} = X_{control.}$ The alternative hypothesis assumes that the mean BMS score post transplantation in the iPSCs group should be higher than the control group, thus $X_{iPSCs} > X_{control.}$ Given that each scientist recorded repeated measures of BMS scores for different durations e.g. (8 wks. versus 90 days), for consistency means were derived for each study at the 42 day mark for the t- statistic since that was the lowest duration observed. The one tail t statistics were verified for significant difference based on a T distribution critical values table:*easycalculation.com/statistics/t-distribution-critical-value-table.php.*

Comparative and statistical analysis between studies

After performing the t test and determining significance, a weighted mean took into account 6 studies. Their weights were allocated in order to detect the overall effect of the usage of iPSCs as a regimen for a SCI, which can be then generalized to a larger population of studies focusing on this topic. The weighted mean and standard deviation were calculated using the software TI-84 Plus Silver Edition. Finally, the meta-analysis was performed using the BioStat Comprehensive Meta-analysis (CMA) 2.0 Software. The book, Introduction to meta-Analysis by Michael Borenstein was used in conjunction as reference and guidance for the general procedure of a meta-analysis. Like with the t-statistic, a one tailed hypothesis test was conducted under an α level of 0.05 corresponding to the null hypothesis $X_{iPSCs} = X_{control}$ and the alternative hypothesis $X_{iPSCs} > X_{control}$, which assumes that the mean BMS score post transplantation in the iPSCs group should be higher than the control group.

Objectives of the current Meta-Analysis

The current meta-analysis synthesizes research findings in order to address several fundamental questions about recovery in motor functionality: (a) whether there is a difference in the BMS score between rodent populations who were treated with iPSCs compared to control groups, (b) whether the iPSC treated group showed advantageous results compared to the control groups, and (c) to what degree can motor functionality be restored in comparison to a non-injured rat or mouse.

Methods to evaluate locomotor behavior

To assess the locomotor behavior recovery after a SCI, several methods have been developed, and below are some of the most commonly used and their differences. Current research in animal models such as mice uses the BMS or BBB scale. The BBB test is based on a 21 point system that evaluates improvements post-treatment with cells derived from iPSCs: a score of zero is when there is no movement and a score of 21 indicates complete normal limb movement (Basso et al. 2006). The BMS score assesses motor recovery also, but this scale is more commonly used. It is based on a 9 point scale: complete paralysis corresponds to a score of 0. A BMS score of 2 indicates that the mouse could move hind limbs but cannot support weight. A BMS score of 3-4 indicate that the mice can put their paws on the ground and support weight. At a score of 5, coordination skills are considered and are a key marker of significant locomotor recovery in the injured animals, whereas a score of 9 represents animals that have full, normal motor movement (Salewski, 2015).

Figure 3: US Gait Scoring System in humans		
0	***No obvious signs of problems***	Balance
1	***Obvious signs***	Clear limp, awkward but can walk 5ft
2	***Severe signs***	Will not walk 5ft

In humans, the Gait scale goes from 0-2 and is determined after observing video footage of a patient's movement and determining the likelihood to loose balance and fall (Figure 3). Likewise, the American Spinal Injury Association (ASIA) score is used as a complete neurological assessment, motor and sensation, of a patient with a SCI (Furlan, 2008). In contrast, when testing locomotion recovery in monkeys, the open fields rating scale, bar grip test, or the Tarlov criteria are used (Figure 4) (Tang, 2013).

Score	Neurological outcome
0	Spastic paraplegia and no movement of the lower limbs
1	Spastic paraplegia and slight movement of the lower limbs
2	Good movement of the lower limbs but unable to stand
3	Able to stand but unable to walk normally
4	Complete recovery and normal gait/hopping

Figure 4: Tarlov criteria used to assessed locomotion recovery in the treated and control groups in monkeys

Table 1: General information about studies using iPSCs in rodent and monkey populations with SCIs

Study	**Injury site**	**Type of Injury**	**Cell implanted & time post SCI**	**Cell line**	**Animal used**	**Immuno-suppressant**	**Method to Differentiate**
Amemori (2015)	T8	Contusion	NSC 1 wk.	hiPSC-NS/PCs (IMR90)	Adult male wistar rats	Cyclosporine A, azathioprine sodium, methylprednisone	Lentivirus
Hayashi (2011)	T9-T10	Contusion	Astrocytes 3 or 7 days	Mouse iPSCs	Female Sprague dawley rats	Cyclosporine A	NSS method
Kawabata (2016)	T10	Contusion	Oligodendrocyte precur (hiPSCs-OPC) 9 days	201Bh7 iPSCs/ murine	Female NOD-SCID mice	None given	Lentivirus
Kobayashi (2012)	C5	Contusion	NSC/ PCs 9 days	Murine/ hiPSCs	Female marmosets	Cyclosporine A	Lentivirus
Liu (2017)	T9	Contusion	NPCs 9 days	hiPSCs (derived USCs)	Adult SCID mice	None given	Sendai viral vector
Lu (2014)	C5	Lateral	NSCs	hiPSCs	Adult	None given	Retroviral

		Hemi-section	2 wks.		female athymic nude rate & SCID mice		vector
Nori (2011)	T10	Contusion	201B7 neuro-sphere 9 days	hiPSC	Female NOD SCID mice	None given	Lentivirus
Nori (2015)	T10	Contusion	253G1-NS/ neuro-sphere 9 days	hiPSC dermal cells	Female NOD-SCID mice	None given	Lentivirus
Nutt (2013)	C4	Contusion	IMR90 hiPSC-NSCs 4 wks.	hiPSCs from lung fibroblast	Female Long Evans rats	Cyclosporine A	Not specified
Oh (2015)	T11	Compressed	diPSC-NPCs 9 days	hiPSC from interverte bral disc	Adult male ICR mice	Cyclosporine A	Retrovirus
Okubo (2016)	T10	Contusion	NS/PCs, neuro-sphere 9 days	hiPSCs 253G1, clone 836B3, 201B7	Female NOD-SCID Mice	None given	Lentivirus
Pomeshchik (2015)	T10	Contusion	(UEFhfiPS 1.4-NPCs 7 days	hiPSCs from skin fibroblast	female C57BL/6 J mice	Tacrolimus	Lentivirus
Ruzicka (2017)	T8	Compressed	1 wk.	BM-MSCs SPC01 hiPSCs	Male Wistar rat	Cyclosporine A, azathioprine sodium	Lentivirus for hiPSCs line only
Salewski (2015)	T6	Compressed	NSC 6 days	hiPSCs	wild-type (C57BL/ 6J), *Shiverer* mice (C3Fe.SWV-*Mbpshi*/J	Cyclosporine A	PiggyBac transposon
Suzuki (2017)	C6/7	Contusion	NSC 7 wks.	Murine iPSCs from MEF from other rodents	Female C57BL/6 mice	Cyclosporine A 2 days prior transplant-end	PiggyBac transposon
Tang (2013)	T9	Contusion	NSCS 1 wk.	hiPSCs	Rhesus monkeys	Cyclosporine A	Retrovirus
Tsuji (2010)	T10	Contusion	Neuro-sphere 9 days	Mouse ES (EB3 ES SNS)	Female C57BL/ 6J mice	Not given	Lentivirus

				& mouse iPS (38C2 SNS or PNS); 335D1, 256H13, 256H18			

***Results**:*

Out of a total of 17 studies that assessed iPSCs in treating a SCI, the final studies incorporated into the meta-analysis were Nori (2015), Okubo (2016), Amemori (2015), Salewski (2015), Tsuji (2010), and Pomeshchik (2015). Pomeshchik (2015) followed all the inclusion criteria but the t score didn't support the null nor alternative hypothesis and instead the control group had higher BMS scores than the treated group. Other studies such as Fujimoto (2012), Nori (2011), Hayashi (2011), Kawabata (2010), and Ruzicka (2017), and Oh (2015) followed most of the screening criteria except could not be verified for statistical significance because no SD, SEM, or exact p value was given. Liu (2017) was excluded because this study did not assess motor recovery, but is reviewed because of its innovative source of parental cells. Lu (2014) and Nutt (2013) were excluded because they used a distinctly different scale to assess motor recovery and focused on a cervical SCI. Suzuki (2017) did use the BMS, but instead used a cervical SCI model and did not provide adequate data for their BMS results. Others studies like Kobayashi (2012) and Tang (2013) were conducted in primates using iPSCs and insufficient data were provided to perform a separate t statistic. For comparison, one study in marmosets using ESCs was reviewed (Iwai, 2015). Because of the lack of replication of multiple preclinical trials in primates which are evolutionary and anatomically closer to humans, it is hard to draw a

conclusion about the usefulness of iPSCs in humans with SCIs from rodent populations but these results highlight the potential.

Part 1: t-statistic:

The following studies only include the ones that used the BMS/ BBB scale for behavioral evaluation post transplantation which were incorporated into the t-statistic and are rearranged in alphabetical order, not due to level of significance. If the t-statistic could not be calculated, the p value at which the study expressed significance at was acknowledged. For the few instances in which the BBB scale was used, BBB scores of the control and iPSC groups were converted into compatible BMS scores. This was necessarily because if the BBB scores were averaged with the BMS score, the weighted mean in the second part would be skewed. The BBB scale is from 0-21, while the BMS is from 0-9.

t-statistic: $\frac{\bar{x}_{iPSCs}-\bar{x}_{control}}{\sqrt{\frac{s^2_{iPSCs}}{n_{iPSCs}}+\frac{s^2_{control}}{n_{control}}}}$ $\boldsymbol{df = (n_{iPSCs} - 1) + (n_{control} - 1)}$

<u>Amemori (2015)*</u>

$T_{is}= \frac{11.4-6.8}{\sqrt{\frac{0.6^2}{11}+\frac{0.9^2}{9}}}$ =13.13 d.f. = (11-1) + (9-1) = 18 p<0.0005* significant

$T_{it}= \frac{10.1-8.4}{\sqrt{\frac{0.4^2}{9}+\frac{0.8^2}{9}}}$ =5.70 d.f. = (9-1) + (9-1) = 16 p<0.0005* significant

~Slightly different scale. To be compatible to BMS scores, convert means of the control and iPSCs groups from BBB score to BMS score.

e.g. $\frac{11.4}{X_{iPSCs}} = \frac{21}{9}$ X_{iPSCs}= 4.89 $\frac{6.8}{X_{control}} = \frac{21}{9}$ X $_{control}$ = 2.91

Fujimoto (2012)

$T=\frac{3.1-2.3}{\sqrt{\frac{S^2}{9}+\frac{S^2}{8}}}$ = ? d.f. = (9-1) + (8-1) = 15 Study stated significance at p<0.05

Hayashi (2011)

~Slightly different scale. To be compatible to BMS scores, convert means of the control and iPSCs groups from BBB score to BMS score.

$\frac{9.1}{X_{iPSCs}}=\frac{21}{9}$ X_{iPSCs}= 3.90 $\frac{9.2}{X_{control}}=\frac{21}{9}$ X control = 3.94

$T_{3day}=\frac{3.90-3.94}{\sqrt{\frac{S^2}{20}+\frac{S^2}{10}}}$ = ? d.f. = (20-1) + (10-1) = 28 Study stated no statistical significance, p>0.05

$T_{7day}=\frac{3.56-4.07}{\sqrt{\frac{S^2}{9}+\frac{S^2}{7}}}$ =? d.f. = (9-1) + (7-1) = 14 Study stated no statistical significance, p>0.05

Kawabata (2016)

$T=\frac{4.4-3.3}{\sqrt{\frac{S\,iPSCs^2}{10}+\frac{S\,control^2}{10}}}$ = ? d.f. = (10-1) + (10-1) = 18 Study stated significance at p<0.05

Nori (2011)

$T=\frac{4.4-3.1}{\sqrt{\frac{SD^2}{18}+\frac{SD^2}{16}}}$ = ? d.f. = (18-1) + (16-1) = 32 Study stated significance at p<0.01

Nori (2015)*

$T=\frac{4.7-3.3}{\sqrt{\frac{0.2^2}{16}+\frac{0.2^2}{16}}}$ =19.80 d.f. = (16-1) + (16-1) = 30 p<0.0005* significant

Oh (2015)

$T= \frac{4.2-2.7}{\sqrt{\frac{SD^2}{20}+\frac{SD^2}{14}}}$ =? d.f. = (20-1) + (14-1) = 32 Study stated significance at p<0.05

Okubo (2016)*

(201B7) line $T= \frac{4.2-2.8}{\sqrt{\frac{0.3^2}{10}+\frac{0.5^2}{10}}} = 7.59$ d.f. = (10-1) + (10-1) = 18 p<0.0005* significant

(253G1) line $T= \frac{3.5-2.8}{\sqrt{\frac{0.4^2}{10}+\frac{0.1^2}{10}}} = 5.39$ d.f. = (10-1) + (10-1) = 18 p<0.0005* significant

Pomeshchik (2015)*

$T= \frac{4.18-5.09}{\sqrt{\frac{0.84^2}{11}+\frac{1.11^2}{11}}}$ =-2.17 d.f. = (11-1) + (11-1) = 20 p<0.025 statistical difference but in opposite direction of alternative hypothesis proposed, thus doesn't show treatment having higher scores than the control

Ruzicka (2017)

$T= \frac{4.5-2.4}{\sqrt{\frac{SDiPS^2}{24}+\frac{SD\ control^2}{16}}}$ =? d.f. = (24-1) + (16-1) = 38 Study stated significance at p<0.05

Salewski (2015)*

$T_{wild}= \frac{4.8-2.7}{\sqrt{\frac{0.7^2}{7}+\frac{0.7^2}{5}}}$ =5.39 d.f. = (8-1) + (6-1) = 12 p<0.0005* significant

Suzuki (2017)

$T= \frac{5.6-5.5}{\sqrt{\frac{SD^2}{15}+\frac{SD^2}{15}}}$ =? d.f. = (15-1) + (15-1) = 28 Study stated no significance at p>0.05

Tsuji (2010)*

$T = \frac{4.3 - 2.8}{\sqrt{\frac{0.28^2}{19} + \frac{0.20^2}{12}}} = 17.37$ d.f. = (19-1) + (12-1) = 29 $p < 0.0005$* significant

*Note: * indicates studies that are included in the meta-analysis*

For 6 studies sufficient data was provided to perform a t-statistic and (5/6) refuted the null hypothesis which showed significant evidence of a difference in the BMS scores and that the treated had a better outcome than the control group. Pomeshchik (2015) was the only study that indicated the opposite that the control group did better than the treatment group. On the other hand, others studies that indicated significance but standard deviations were not provided were not incorporated into the meta-analysis; thus, Fujimoto, Hayashi, Kawabata, Oh, Nori (2011), and Ruzicka were excluded in part 3.

Part 2: Weighted mean:

Next, a weighted mean and standard deviation was computed for both the control and treated (iPSC) group's BMS scores in order to determine how the weight of each study reflects its relative importance on assessing the overall effect of motor recovery. Two weighted means were calculated. The first one considers all the studies that used the BMS/ BBB scale regardless if the studies included sufficient data to calculate a t statistic or effect size. The purpose of this mean was to consider and predict what the weighted means of the treament and control group would have been if all studies assessing motor recovery in rodent populations could have been incorporated. This way averages a larger number of studies (n value), which may be more representative of studies using iPSCS for SCIs in rodents.

weighted mean: $x = \frac{\sum_{i=1}^{n} (x_i * w_i)}{\sum_{i=1}^{n} w_i}$ → $\bar{x} = \frac{w_1 x_1 + w_2 x_2 + \ldots + w_n x_n}{w_1 + w_2 + \ldots + w_n}$

weighted standard deviation is:

$$sd_w = \sqrt{\frac{\sum_{i=1}^{N} w_i (x_i - \bar{x}_w)^2}{\frac{(N' - 1) \sum_{i=1}^{N} w_i}{N'}}}$$

Figure 5. Weighted mean and standard deviation for all studies that used the BMS/BBB scale **only**

A. The weighted average and SD for BMS scores for iPSC group (above) and for control group (below)
B. Mean BMS score from each study and the n value for iPSCs group (above) and control group (below)

A

L1	L2
3.1	8
3.9	20
3.56	9
4.4	10
4.8	10
4.4	18
4.7	16

L2(1)=9

B

1-Var Stats
x̄=4.365065502
Σx=999.6
Σx²=4429.2888
Sx=.537902538
σx=.5367267934
↓n=229

$\overline{x_{iPSCs}} = 4.37 \pm 0.54$

L1	L2
2.3	8
2.91	9
3.6	9
2.79	9
3.1	16
3.3	16
5.09	11

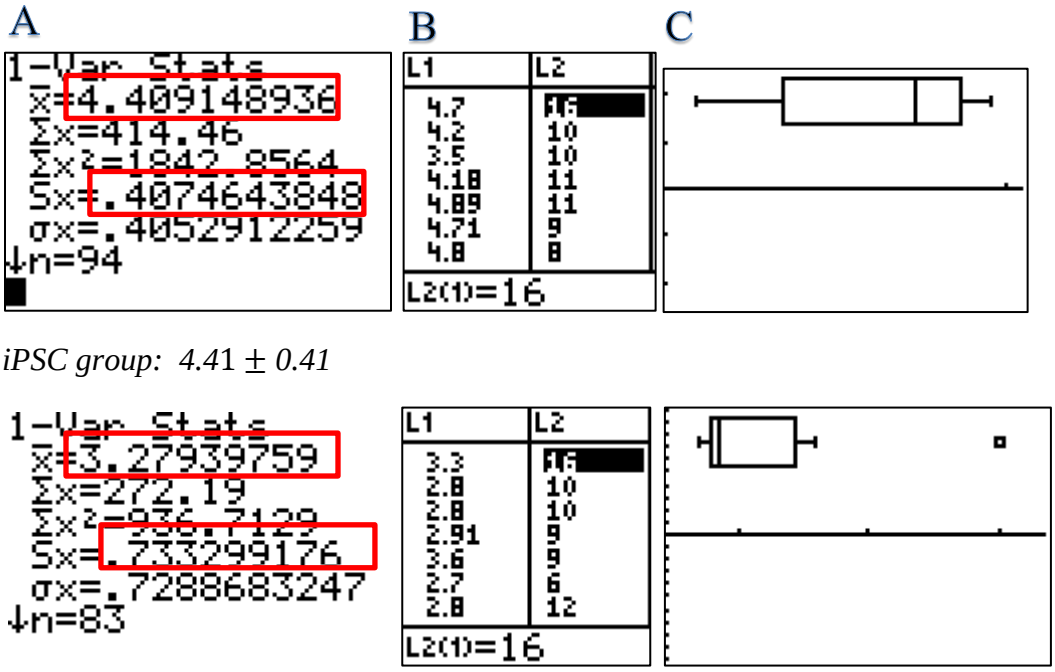
L2(1)=8

1-Var Stats
x̄=3.341016043
Σx=624.77
Σx²=2242.8423
Sx=.9142706916
σx=.9118228407
↓n=187

$\overline{x_{control}} = 3.34 \pm 0.91$

In contrast, the second weighted mean and standard deviation only consists of studies that are analyzed in the meta-analysis.

Figure 6: Weighted Mean and standard deviation of studies **exclusively** included in Meta-analysis



iPSC group: 4.41 ± 0.41

control: 3.28± 0.73

A. The weighted average and SD for BMS scores for iPSC group (above) and for control group (below)
B. Mean BMS score from each study and the n value for iPSCs group (above) and control group (below)
C. Modifed box whisker plot of mean BMS score for iPSC groups is negatively skewed (above) and control group is positvely skewed (below). One outlier present in the control group.

In both scenarios for weighted means, the treatment group has a higher mean than the control group. For the second scenario which pertains to studies used in the meta-analysis, only the treatment group means had one outlier. Pomeshchik (2015) was the only study which had a higher BMS score for the control as compared to other studies. The error bars for the iPSC group and control didn't overlap, signfiying that there is indeed a difference between their BMS average scores. Even though the weighted means for the iPSCs group is 4.41 and control group is 3.28, it is important to note that these scores were taken at the 42 day mark and some studies do indicate mild further improvement after this time point.

Part 3: Peforming the meta-analysis using the fixed effect size model

Given that the scale used to assess locomotor functional recovery was the same across studies and the overall procedure was homogenous, a fixed random effect model was used but other components including the use of immunosuppressed mice versus the administration of an immunosuppressant has random effects. There are 6 studies incorporated into the meta-analysis; however, 8 studies are indicated in figure 9 because of two study subgroups. Okubo (2016) evaluated different iPSC lines while Amemori (2015) examined different methods for transplantation of the cells at the injury site (intraspinal versus intrathecal). In addition for Okubo, two treatment groups used iPSCs, although one included the addition of a GSI, which prevents overgrowth of cells, while the other only included the cells alone. For consistency purposes compared to the other studies, the group that only used iPSCs was used for in the meta-analysis. Otherwise, the iPSC group that is positive for GSI would be a confounding variable. The effect size was based on means and ultimately was calculated using the standard difference of means. On the forest plot, the scale used was from -10 to 10 for a 99% confidence interval.

Figure 7: Calculations for effect size based on standard difference on means and forest plot

Model	Study name	Subgroup within study	Comparison	Statistics for each study							Std diff in means and 99% CI
				Std diff in means	Standard error	Variance	Lower limit	Upper limit	Z-Value	p-Value	-10.00 -5.00 0.00 5.00 10.00
	Nori (2015))	Blank	Blank	7.000	0.944	0.891	4.569	9.431	7.417	0.000	
	Okubo	201B7	vs. PBS	3.395	0.699	0.488	1.596	5.195	4.859	0.000	
	Okubo	253G1	vs. PBS	2.401	0.587	0.344	0.890	3.912	4.093	0.000	
	Amemori	is	Blank	6.106	1.065	1.134	3.363	8.849	5.734	0.000	
	Amemori	it	Blank	4.130	0.834	0.696	1.981	6.278	4.950	0.000	
	Salewski	Blank	wild type	3.000	0.783	0.613	0.983	5.017	3.831	0.000	
	Tsuji (2010)	Blank	Blank	5.937	0.839	0.704	3.775	8.099	7.073	0.000	
	Pomeshchi	Blank	Blank	-0.925	0.449	0.201	-2.080	0.231	-2.061	0.039	
Fixed				2.549	0.247	0.061	1.913	3.184	10.326	0.000	
Random				3.812	1.035	1.071	1.146	6.478	3.683	0.000	

Based on the forest plot, zero was not included in the confidence interval for any of the studies except for one, indicating that the p values are less than 0.05 and in fact the p value is very close to zero and the Z value is 10.326 (figure 7). Additionally, all the intervals except for Pomeshchik (2015) are to the right of zero, indicating that the treatment has a larger mean than the control. More importantly, the overall fixed effects size standard difference of mean is 2.549, which indicates that there is a significant difference in the means between the BMS score of the iPSC group and the control which answers the first objective, so the null hypothesis can be rejected; hence, the alternative hypothesis was failed to be rejected. This indicates that the iPSC treated group performed better on the locomotion scales at the 42 day mark than the control group, which answers the second objective of the meta-analysis. A total of 94 rodents were in the iPSCs group and 83 in the control or an overall total of 177 animal participants (figure 8).

Figure 8: Sample sizes of the treatment and control group for each study and weights for fixed effect sizes model

Model	Study name	Subgroup within study	Comparison	Statistics for each study							Sample size		Weight (Fixed)
				Std diff in means	Standard error	Variance	Lower limit	Upper limit	Z-Value	p-Value	iPSCs	Control	Relative weight
	Nori (2015))	Blank	Blank	7.000	0.944	0.891	4.569	9.431	7.417	0.000	16	16	6.84
	Okubo	201B7	vs. PBS	3.395	0.699	0.488	1.596	5.195	4.859	0.000	10	10	12.48
	Okubo	253G1	vs. PBS	2.401	0.587	0.344	0.890	3.912	4.093	0.000	10	10	17.70
	Amemori	is	Blank	6.106	1.065	1.134	3.363	8.849	5.734	0.000	11	9	5.37
	Amemori	it	Blank	4.130	0.834	0.696	1.981	6.278	4.950	0.000	9	9	8.75
	Salewski	Blank	wild type	3.000	0.783	0.613	0.983	5.017	3.831	0.000	8	6	9.94
	Tsuji (2010)	Blank	Blank	5.937	0.839	0.704	3.775	8.099	7.073	0.000	19	12	8.65
	Pomeshchi	Blank	Blank	-0.925	0.449	0.201	-2.080	0.231	-2.061	0.039	11	11	30.27
Fixed				2.549	0.247	0.061	1.913	3.184	10.326	0.000			

Figure 9: Hypothesis and heterogenity testing for a fixed effect size model

Model		Effect size and 95% confidence interval					Test of null (2-Tail)		Heterogeneity			
Model	Number Studies	Point estimate	Standard error	Variance	Lower limit	Upper limit	Z-value	P-value	Q-value	df (Q)	P-value	I-squared
Fixed	8	2.549	0.247	0.061	1.913	3.184	10.326	0.000	115.100	7	0.000	93.918
Random effects	8	3.812	1.035	1.071	1.146	6.478	3.683	0.000				

In terms of heterogeneity, since the Q score is 115.10 and the I^2 score is 93.92, this suggests that there is some heterogeneity (figure 9). This may be due to within study error because the dispersion was wider than expected given that Q-df> 0. The null hypothesis for heterogeneity assumes that all studies share the same common effect size, while the alternative hypothesis states that studies do not share a common effect size. The p value was close to zero, indicating that there is some evidence to reject the null and accept the alternative, suggesting that either there is some statistical heterogeneity of effect size due to observed dispersion or minor observed dispersion with precise studies. However because of the small number of studies, a conclusion cannot be adequately reached nor are the heterogeneity tests very reliable in this case and may not reflect the true between study variance.

Likewise, according to the funnel plot (figure 10), it seems that there may be some publication bias, although only a small number of studies were included in the meta-analysis. This assertion is not definitive. Just because the heterogeneity is statistically significant does not indicate that the random effects model would be more appropriate in this case.

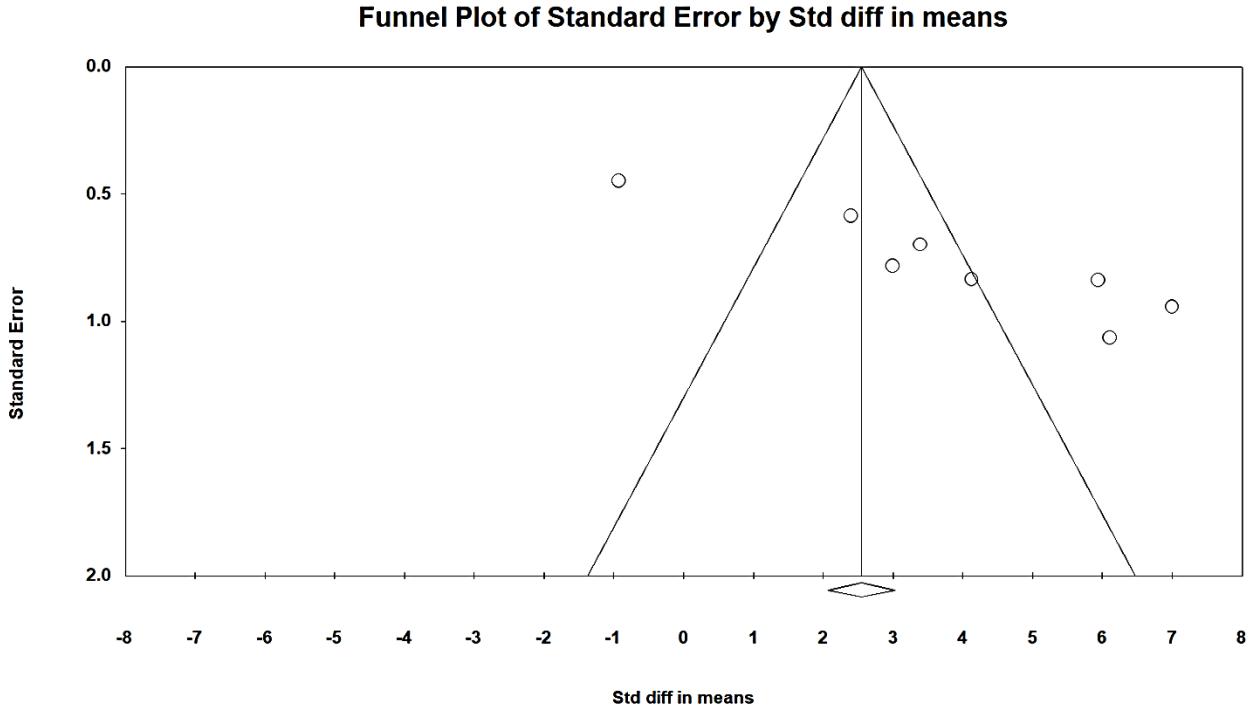


Figure 10: Funnel plot of standard error by effect size for standard difference in means at a 99% CI.

Discussion

Each study assessed contributed a unique element of information over the consensus of iPSCs. The following studies (Tables 3) analyzed the use of iPSCS in rats or mice (Fujimoto, Nutt, Salewski, Amemori, Nori, Pomeshchik, Ruzicka, Tsuji, Okubo, Kawabata, Oh, Suzuki, Liu, Lu, & Hayashi) while the following analyzed them in monkeys (Kobayashi, Tang). Something to note is that most of the control groups did indicate a slight increase in BMS/ BBB scores over time because incomplete SCIs caused from contusion or compression experienced mild neuroplasticity and some regeneration. In addition, the pre-implantation scores were 0-1, indicating paralysis except for Suzuki (2017).

The following studies took into consideration iPSCs and ESCs for sources of cells to transplant. Tsuji and collaborators compared the functional recovery between murine iPSC and ESCs as well as looked at whether it was more beneficial to implant primary neurospheres (PNS) or secondary neurospheres (SNS). Their study concluded that the BMS scores 42 days post SCI between the ESCs and iPSCs were similar. Additionally, there was a significant difference ($p<0.01$) between the iPS-SNS treated group and the control and the BMS scores showed therapeutic benefit in using the SNS over the PNS ($p<0.01$) (Tsuji, 2010). Moreover, the authors analyzed 3 iPSC lines to determine their safety: 335D1, 256H13, and 256H18 clones, all derived from TTF. They found the safe cell line to be 335D1 which showed recovery in motor functions, while 256H18 showed deterioration starting 42 days post injury. Lastly, even though the 256H13 line did not display deterioration within the 42 days, clusters of Nanog+ cells were observed, speculating that a tumor could form if observed for a longer period of time (Tsuji, 2010). Similarly, Fujimoto and collaborators compared the functional recovery using ESCs and iPSCS and found the BMS scores at the endpoint of 56 days to be 3.5-3.6, meaning that they provide comparable outcome results (Fujimoto, 2012). Ruzicka compared three types of stem cells (BM-MSCs, SPC01, iPSCs) to determine their efficacy in treating a SCI and determined that the highest locomotor recovery was in the iPSC-NP grafted group with more white matter ($p<0.05$) and gray matter ($p<0.001$) compared to the control, bone marrow derived mesenchymal stem cells (MSCs), and neural progenitors from spinal fetal cell lines (SPCs) (Ruzicka, 2017). Additionally, 2 implantation techniques were considered, intrathecal and intraspinal, which showed the BBB scores to be identical between the saline controls for these two methods.

Amemori and collaborators also wanted to determine whether the location of the implantation of iPSCs, intrathecal (it) or intraspinal (is), made a difference in locomotion

recovery. Both were effective methods of transplantation as evident by similar end outcomes of BBB scores but only iPS-NPCs injected intraspinally after 2 months were positive for the neuronal marker microtubule-associated protein 2 (MAP2), increased gray and white matter, axonal sprouting, and reduced astrogliosis. Intrathecally injected cell showed improvement in white matter and axonal sprouting, indicating that both methods have therapeutic benefit; however, intraspinal cells promote enhanced long-term spinal cord regeneration (Amemori, 2015). Instead of considering the method of transplantation of derived cells, Salewski and collaborators (2015) examined the role of exogenous myelination in iPSCs on functional recovery by comparing *shiverer* mutant mice (*shi*-iPS-dNSC) to wild type (wt-iPS-dNSC). *Shi*-iPS-dNSC behave like the wild type in vitro and in vivo but are non-myelinating, meaning that oligodendrocytes can be derived but myelin protein is not expressed. This defect in myelination was reflected negatively in the outcome of functional recovery for the *shiverer* group and results were significantly lower than the wild ($p=0.0008$) More importantly, the wild iPSCs treated group achieved significant motor recovery compared to the control ($p<0.00001$). Even though the wild type group iPSC treated group reached a BMS score of 5, a normal mouse has a score of 9, meaning that the mouse with the SCI wasn't able to be restored to the motor capacity of a non-injured rodent. Lastly, Oh and collaborators (2015) reprogrammed iPSCs from human intervertebral disk cells and concluded that the treatment group showed significant motor recovery as compared to the control ($p<0.05$).

Most of the studies examined used the immunosuppressant, Cyclosporine A or no immunosuppressants were given when the grafted cells were derived from that same animal population. In contrast, Pomeshchik and collaborators (2015) administered a different immunosuppressant drug, Tacrolimus because this drug has less nephrotoxic effects. This study

used the cell line UEFhiPS 1.4-NPCs and showed no significant results indicating improvement in locomotion with transplanted cells ($p>0.05$). However, the study was limited to 42 days, which is a shorter length of time in comparison to the rest of the studies reviewed. In addition, some studies do not see significant results until after the 42-day mark.

The following studies experimented with the same cell lines. Nori (2011) and collaborators originally used the 201B7-hiPS cell line, while in 2015 used the 253G1-NS, both lines come from the same donor but from different parental cells. His first study showed significant improvement in the BMS scores of the treated group ($p<0.01$), while the 253G1 line showed deterioration in motor skills after the 47 day mark and a tumor was formed from undifferentiated NSCs based on detecting an increase in Nestin + cells. Initially, motor improvement was consistent with his previous study in 2011, which noted that Nestin+ cells decreased from 10.7+/-2.2% at 47 days to 7.5+/-1.0 at 103 days, but in the 253GI line Nestin+ cells increased from 19.6+/-0.5% at 47 days to 33.1+/-7.4% at 103 days (Nori, 2011; Nori, 2015). Okubo (2016) like Nori (2011, 2015) used the 253G hiPSC-NS/PC cell line which was deemed as tumorigenic; however, no tumors were formed when GSI was administered in conjunction with the iPSCs because this reduces overgrowth of cells and inhibits tumor formation. Although in the control group that used 253 hiPSCs alone, this showed deterioration in motor function accompanied by a tumor like overgrowth and Nestin+$^{+}$ cells increased to 30.3% ± 1.6%, while in the GSI^{+} group they decreased to 5.3% ± 0.8% (Okubo, 2016). GSI is used to ensure that all NSCs have been fully differentiated into the appropriate subtypes by targeting Notch signaling which promotes neuronal differentiation and maturation (Okubo, 2016). When GSI was given to the 253 hiPSC line, significant motor recovery was detected ($p<0.01$). This indicates that even though this cell line may be tumorigenic, it can still provide therapeutic benefit only if GSI is administered.

Additionally, Okubo used 2 other cells lines: clone 836B3 and 201B7 hiPSC-NS/PC and gave one iPSCs group GSI and the other just contained iPSCs. In this experiment, no tumor was detected regardless if GSI was given and the BMS scores were identical between the GSI+ group (iPSCs & GSI) and the control (iPSCs only) group. Both the GSI+ and control groups had superior BMS scores to the PBS (negative control) group; thus, this indicates that in this cell line a GSI is not necessary. Kawabata and collaborators (2016) like Okubo (2016) and Nori (2011) used the 201B7 hiPSCs cell line but focused on implanting intraspinally hiPSCs-OPC enriched NS/ PCs and indicated significant locomotion recovery in the treated group ($p<0.05$).

Hayashi (2011) used murine iPSCs and was the only study to exclusively implant a differentiated cell (astrocyte) rather than the entire neurosphere or NSCs either at 3 or 7 days post the SCI. The first aim was to determine which timeframe is optimal for implantation and for functional recovery, during the acute (3 days) or sub-acute stage (7 days). The second objective was to determine if astrocytes are useful in repairing spinal neural connections. The results showed no significant improvement in the BBB scores for both the 3 and 7 day groups, indicating that the sub-acute and acute stages elicit similar motor outcome results. This study also suggests that astrocytes are the not the ideal cell type to implant for a SCI because the BMS scores of the groups containing astrocytes are no different from the control groups.

Unlike the previous studies that focused on a thoracic SCI, the following studies concentrated on a cervical model. Suzuki and collaborators (2017) implanted iPSC derived NSCs 7 wks. during the chronic stage, and the results showed some motor improvement on one scale. Motor recovery was evident only for the combinational therapy group (iPSCs with chondrotinase ABC (C-ABC)) on the CatWalk scale, specifically for forelimb stride length but improvement was inconsistent and there was no difference in hind limb stride length or BMS scores. iPSCs alone

were not able to restore motor control during the chronic stage; however, when chondroitin sulfate proteoglycans were degraded using the C-ABC treatment, axonal regeneration was improved (Suzuki, 2017). In this study, the BMS was not as effective in assessing behavior. Prior to sustaining a SCI rodents had a score of 9 and then dropped to 3-4 post the SCI, indicating that unlike injuries at the thoracic region which caused the BMS score to drop to 0-1 post injury, in the cervical region this scale didn't detect as much of a decline in motor abilities. One reason is the BMS is designed to assess behavior deficits at the thoracic level and is not as sensitive to detect changes in the cervical region, suggesting that another motor assessment scale would be more appropriate. Future studies using cervical SCI models should be considered because they account for more than 60% of cases in humans (Bahmad, 2017). This study also elucidates that grafted cells can survive at the chronic stage but at a low rate and even though neurons can be differentiated from NSCs, their capacity to integrate a synapse is hindered. Besides Suzuki, Nutt and collaborators (2013) assessed behavioral recovery for a cervical model but used different scales: the limb-use asymmetry test (LUAT) and forelimb reaching task which assess forelimb preference. The scores between the human iPSC-NPC group was not significantly different from the control groups but comparison of pre-transplant to final LUAT scores indicated significant improvement in the NPC treatment group ($p=0.0032$). Lastly, Lu and collaborators (2014) used the scales vertical exploration and grid walking but found no statistical difference between scores of the control and hiPSCs treated group.

More recently, Liu and collaborators (2017) derived iPSCs instead from urinary stem cells (USCs), which is different from the conventional approach of using fibroblasts. Some benefits of using USCs as parental fibroblasts over fibroblasts is that their isolation is less invasive since 2,000-7,000 cells are removed from the body daily by urine and takes less time to be converted

into iPSCs (Zhou, 2012). Normally, it takes 1-3 months to obtain viable dermal fibroblasts, 2-3 months to reprogram these cells, and 1-2 months to get neural lineage specific cells, so the total process takes 4-8 months. Even though it would be optimal to use a person's own fibroblasts to generate an iPSC line, it might not be practical since there is a short window to maximize functional recovery. However, in contrast to dermal fibroblasts that need at least 21 days to be fully reprogrammed into iPSCs, cell cultured from urine can produce a sufficient number of stem or somatic cells for iPSCs and be reprogrammed in 2-3 wks.

The next step after using rodent populations as experimental animal models is to transition into using non-human primates such as monkeys which few studies have considered the effectiveness of iPSCs for treating a SCI. Unlike rodent populations, the motor assessment scales in monkeys are different and assess finer motor skills. Kobayashi and collaborators (2013) noted that the hiPSC-NS treated group significantly outperformed the control in the open field rating scale, bar grip test, and cage climbing test ($p<0.05$) for a cervical SCI. Tang and collaborators (2013) showed that on day 1 the monkeys had paralysis and no movement at both limbs but on Day 30 post cell-transplantation, the monkeys were able to climb and were almost fully recovered. For comparison, Iwai and collaborators (2015) like Kobayashi used the cervical model in marmosets and ESCs instead of iPSCs. Their research highlighted the compatibility of the behavioral outcome results between iPSCs and ESCs populations, given that at the 42 day mark both studies showed that marmosets achieved a bar grip score of ~45% and the overall trend of improvement was similar .

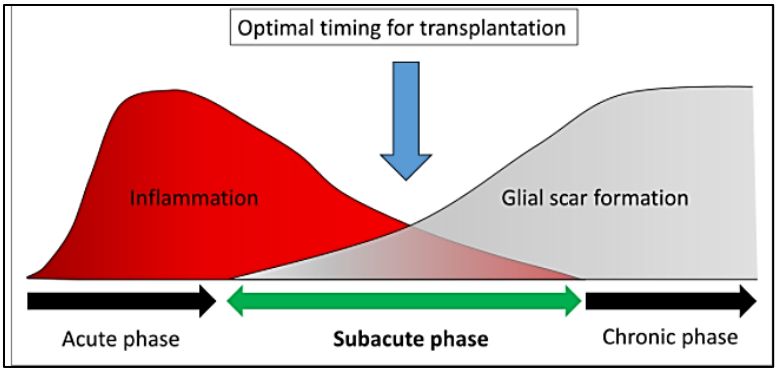


Figure 11: Stages of SCI and critical window for transplantation of iPSCs differentiated cells

After reviewing the variations in the procedure for implantation of iPSCs derived cells and evaluating the level of improvement detected, this investigation highlights the importance of implanting these cells during an optimal time in order for the SCI treatment to be most effective. This period (figure 11) seems to be during the subacute phase where the microenvironment is most conducive for grafted cell survival and for reestablishment of neural connections because inflammation has decreased and glial scars haven't been formed (Nagoshi, 2017). Nishimura (2013) showed motor recovery based on BMS scores was maximized for neural sphere-progenitor cells (NS- PCs) during the sub-acute stage: the control and the chronic TP (iPSC transplanted group) plateaued at a score of 3, while the sub-acute TP group reached a score of 4.8 in 7 wks. ($p<0.01$). In mice, the subacute phase is between 7-14 days, while the chronic phase is around 42 days (Nishimura, 2013). If the cells would be instead transplanted during the acute stage, meaning immediately after sustaining a SCI, improvement in hind limb movement is evident 1 wk. post SCI followed by gradual recovery, but long lasting recovery is more evident during the sub-acute stage (Okada, 2005). The acute stage is not suitable for implantation because of the upregulation of inflammatory cytokines and free radicals. Even though it is evident that the sub-acute stage is the best option to implant the iPSC-NS, it may not be realistic

in patient specific therapy. iPSC generation for clinical use is time consuming and the time frame for the subacute phase in humans is narrow; thus, by the time iPSCs are made, the patient will be in the chronic stage phase. It takes about 6 months to establish hiPSC-NS/PCs from a patient's autologous somatic cells and the therapeutic window for sub-acute SCI in humans is 2-4 months post injury, while in mice it is only a few days (Nishiyama, 2016).

Most research supports that motor improvement is limited or not evident when iPSCs are implanted during the chronic stage. One study used the enzyme C-ABC in conjunction with exercise and detected motor recovery and extension of serotonergic and new neuronal fibers (Shinozaki, 2016). This indicates that the injured spinal cord even in this stage retains capacity to regenerate if axonal growth inhibitors are suppressed, because C-ABC ameliorates the microenvironment of the spinal cord and exercise enhances neuroplasticity (Shinozaki, 2016). Likewise, Nagoshi found that mice that undergone physical therapy such as treadmill training after transplantation experienced greater locomotor recovery (Nagoshi, 2017). Even though combined therapy of treadmill training and NS/PC transplanted during the chronic stage indicates improvement in BMS scores compared to the control ($p=0.035$), BMS scores are still significantly lower (mean= 3.5) than cells implanted during the sub-acute stage (mean score>4) without any rehabilitation therapy (Tashiro, 2016). This indicates that even with the use of physical therapy which works synergistically with NS/PCs to induce neuronal differentiation and maturation, it is still not as optimal as transplantation during the sub-acute stage.

One way to overcome the chronic stage is through excision and immunization. Previous research has found that immunization with neural derived peptides (INDP) and glial scar excision can be beneficial (Rodriguez-Barrera, 2017). By modifying the immune system with INDP and scar removal, a better microenvironment is created for recovery in the spinal cord.

INDP activates T lymphocytes to induce an anti-inflammatory response that reduces the amount of free radicals. The INDP and scar removal group resulted in the greatest increase ($p<0.05$) in motor recovery and 55.5% of the animals achieved a BBB score of 9 or higher (Rodriguez-Barrera, 2017). In contrast, INDP alone or scar removal alone were not as effective in achieving the therapeutic benefit of restoring locomotion (Table 2).

		Mean BBB	***Standard deviation***
pre	Scar removal	6	1.031
	PBS immunization	6.16	0.125
	Scar removal + INDP	6.33	1.47
post	scar removal	6.22	1.85
	Scar removal + INDP	**8.11**	1.69
	PBS immunization	6.38	0.48

Table 2. Locomotion results before and after use of INDP and/ or scar removal during the chronic stage of a SCI

Another option is to prolong the subacute stage by using a glial scar inhibitor such as olomucine or rolipram (Ronaghi, 2009). The aim is to encourage re-myelination with oligodendrocytes, axon elongation, and repair of neuron circuits (Lukovic, 2012). During re-myelination, key neurotrophins such as BDGF, PDGFA, and VEGFA and angiogenesis are up-regulated (Salewski, 2015). During adaptive immune responses, T cells use these neurotrophins to reduce further degeneration of the spinal cord.

Table 3 : Outcomes for studies using iPSCs in rodent and monkey populations with SCIs			
Study	**Length of Evaluation**	**Tumor Formed in iPSCs treated group(s)**	**Motor Recovery, scale(s) used**
Amemori (2015)	9 wks.	Not detected	Yes BBB, Plantar test, Beam walking
Hayashi (2011)	8 wks.	Not detected	No BBB, Inclined plane
Kawabata (2016)	12 wks.	Not detected 22.6% ± 2.5% were $Nestin^{+}/HNA^{+}$ cells	Yes BMS, Rotarod Test
Kobayashi (2012)	12 wks.	Not detected 23.9±2.8% Nestin+	Yes Open field, Bar Grip, Cage Climbing test
Liu (2017)	8 wks.	Not assessed	Not assessed
Lu (2015)	12 wks.	Not assessed	No Vertical Exploration, Grid waling
Nori (2011)	112 days	No, Nestin+ decreased from 10.7+/-2.2% at 47 days to 7.5+/-1.0 at 103 post-transplant	Yes BMS, Rotarod Test, DigiGait system (Treadmill gait)
Nori (2015)	103 days	Yes (253G1) cell line Nestin+ increased from 19.6+/-0.5% at 47 days to 33.1+/-7.4% at 103 days	No, deterioration BMS, Rotarod test, stride length
Nutt (2013)	8 wks.	Not detected	Limited LUAT (limb use asymmetry test)
Oh (2015)	6 wks.	Not detected	Yes BMS, stride length, stance length, sway length
Okubo (2016)	89 days	No tumor for 201B7 cell line Yes for 253G1 cell line control group (iPSCs only), not for (iPSCs + GSI) group, $Nestin^{+}$ cells increased to 30.3% ± 1.6% at 89 days	Yes BMS, Rotarod test, Treadmill Gait
Pomeshchik (2015)	42 days	Not detected	No BMS, CatWalk
Ruzicka (2017)	9 wks.	Not detected	Yes BBB, Flat Beam , Rotarod, Plantar test
Salewski (2015)	8 wks.	Not detected	Yes BMS, CatWalk, Hind limb Intensity, stride length
Suzuki (2017)	16 wks.	Not detected	Limited, (for iPS-NSC+ChABC group) BMS, CatWalk, forelimb grip strength, inclined plane test
Tang (2013)	30 days	Not detected	Yes, Tarlov criteria
Tsuji (2010)	42 days	Not in 38C2 iPSC line or 335DI iPS cell line Not in 256H18 cell line	Yes in 38C2 iPS-SNS Yes in 335D1 iPS SNS Not in other cell lines or in 38C2 iPS-PNS, BMS

***Conclusion**:*

This meta-analysis and review allowed us to recognize commonalities and variations with the approaches in the use of iPSCs derived cells and reinforced the value of these cells in treating SCIs. Based on these independent studies conducted in animals with cervical or thoracic SCIs, there is a significant benefit to their use, as evident of the BMS scores in the iPSC group compared to the control. These pluripotent stem cells play an important role in restoring vital neurological structures such as the spinal cord. A caveat is that to achieve the most therapeutic benefit using these cells, iPSC differentiated cells should be transplanted intraspinally during the sub-acute phase. If transplantation will be necessary during the chronic stage, the microenvironment needs to be altered to optimize the survival rate of these cells. Additionally, physical therapy should be encouraged since data suggests that it can stimulate neuronal regeneration. In terms of teratomas and other tumors formed which has been a concern, only two cases out of 15 studies detected a tumor, suggesting that incidence is low and this is only evident in one cell line. Based on the cell line, some were shown to be safe, while others were deemed as tumorigenic; thus, this stresses the importance of investigating the characteristics and properties of the iPSCs lines that make them safer and promotes better outcomes. For future analysis, studies need to use a standardized scale to measure locomotion in rodents and non-human primates in order to compare effect sizes between studies, scales need to be specific and appropriate for the location of the SCI, thoracic or cervical, and there needs to be a consistent emphasis in reporting sufficient data in order to perform quantitative assessments. The knowledge obtained through these studies in populations will be translated to human studies.

Future perspectives

Before iPSCs can reach clinical trials in humans, more studies need to be performed non-human primates because they are closer in respect to humans in respect of anatomy, size, and physiology. Secondly, these findings alone do not support the conclusion that the use of iPSCs can restore full motor functionality to what it once was prior to sustaining a SCI. There is still a significant difference between a normal non-injured rat which has a score of 9 and a rat with a SCI treated with iPSCs with a weighted mean score between 4-5. Another aspect to be considered is to optimize the use of iPSCs derived from a patient's own fibroblasts or USCs in order to avoid suppressing the immune system. If an immunosuppressant treatment is needed, it should be determined which one is the most efficient and has the least amount of side effects, such as nephrotoxicity. Given that the optimal window for implantation is narrow, the grafted cells will be required to be produced in a faster way and the sub-acute phase will need to be extended to allow for optimal time for transplantation. Lastly, to avoid a tumor from forming, it is important to have proper screening to ensure that all the iPSCs have been fully differentiated.

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