EFFECTS OF HUMAN INDUCED PLURIPOTENT STEM CELL-DERIVED NEURAL PROGENITOR CELLS ON FUNCTIONAL OUTCOME IN A PORCINE ISCHEMIC STROKE MODEL

by

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(Under the Direction of SIMON PLATT)

ABSTRACT

Stroke is the leading cause of disability in North America. Current therapies are extremely limited and carry no potential for tissue regeneration. Human induced pluripotent stem cell derived neural progenitor cells (iNPCs) have shown therapeutic promise in several rodent models of stroke. Previous translational failures between rodent models and human clinical trials, however, have highlighted the need for transitional large animal models of stroke where potential therapies are investigated for effects, not only on structural, but also on functional outcomes. In this study, a functional outcome scale is developed for a porcine ischemic stroke model. This scale was used to assess the effects of iNPCs on functional outcome in pigs following permanent middle cerebral artery occlusion. iNPC treatment hastened recovery across multiple functional parameters in pigs following ischemic stroke. Results were repeatable between different observers supporting the use of the developed scale in future investigations of regenerative therapies in pigs following neurologic injury.

INDEX WORDS: Porcine ischemic stroke model, Post-stroke assessment scale, Induced pluripotent stem cell therapy, Neural progenitor cell therapy

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CHAPTER 1

INTRODUCTION

Stroke is defined as focal injury in the central nervous system secondary to a disruption of blood flow[1]. It is the leading cause of disability and the fifth leading cause of death in the United States [2]. Nearly 800,000 people in the United States experience a stroke each year [2]. Despite significant scientific effort, there remains a shocking dearth of therapeutic options for such a prevalent and devastating condition. Over 1000 preclinical studies and 100 clinical trials have been completed, but for nearly 20 years, tissue plasminogen activator remained the only FDA-approved therapy for stroke [3, 4]. It is estimated, however, that less than 10% of stroke patients are eligible to receive tPA due to the risk of intracerebral hemorrhage and a narrow therapeutic window (within 4.5hours of stroke) [5]. In 2012, two clot-retrieving devices, Trevo (Stryker) and Solitaire (Medtronic), also became FDA-approved but while effective, their use is also limited by eligibility and a narrow therapeutic window (within 6 hours post-stroke) [3].

The overwhelming translational failure between the laboratory and the clinical setting prompted a meeting of the Stroke Therapy Academic Industry Roundtable (STAIR) in 1999 to make several recommendations regarding the effective development of stroke recovery drugs[6]. Central to the recommendations was a call for rigorous preclinical testing using sound, unbiased study designs in more than one animal model [6]. Most preclinical studies are completed in rodents, which possess a lissencephalic brain[6]. Further updates to the STAIR recommendations elaborated that transitional animal models, ideally those possessing a gyrencephalic brain with a

similar white matter content to the human brain, should be a necessary part of preclinical testing [7].

Another core concept in the STAIR recommendations is the need for therapies with multiple mechanisms of action [8]. Ideal therapies would offer not only neuroprotection, but also functional recovery. Stem cell therapy is unique in that it offers the potential for functional recovery, in addition to neuroprotecion through the release of beneficial factors [9, 10]. With the advent of induced pluripotent stem cell capability, freedom from the ethical dilemmas associated with stem cell use has allowed the field of regenerative medicine to excel [10]. Several groups have already demonstrated the beneficial effects of human-induced pluripotent stem cell-derived neural progenitor cells in rodent models of ischemic stroke [11-18]. In keeping with the STAIR recommendations, prior to proceeding with human clinical trials, cell therapy should be tested in a transitional gyrencephalic animal model.

The pig has garnered considerable interest as a laboratory model for neural injury due to similarities in the anatomy, growth, and development between pig and human brains[19]. In following with STAIR recommendations, the pig is a species that possesses a gyrencephalic brain with a grey to white matter ratio nearly identical to that of humans making it an ideal transitional animal model for stroke [19]. A robust and repeatable model of ischemic stroke has recently been developed in the pig [20]. The purpose of this study is to investigate the effects of human induced pluripotent stem cell-derived neural progenitor cell (iNPC) therapy in a pig ischemic stroke model. Specifically, this research was aimed at elucidating the effect of iNPCs on functional recovery following permanent middle cerebral artery occlusion in pigs.

With stroke being the leading cause of long-term adult disability worldwide, functional recovery is of particular importance as an outcome measure in any preclinical study [2]. In

humans, functional recovery following a stroke is typically assessed using a variety of scales and indices including the modified Rankin Score, the Barthel index, the NIH Stroke Scale, the Glasgow Outcome Scale and the Canadian Neurologic Scale[21]. The goal of these evaluations is to determine the degree of neurologic recovery and function through quantification of patient neurologic deficits, quality of life, and functional independence in activities of daily living[21].

Many of the measured outcomes and scoring parameters are unique to humans including speech, visual, and verbal comprehension, which do not translate well into animal models of stroke. As such, several animal-specific scales and tests have been designed to assess similar representative outcomes in laboratory species[22, 23].

The first specific aim of this study was to create a pig post-stroke functional recovery scale that is easy to use, repeatable, pertinent, and reliable. The expected result is that pigs that have undergone a middle cerebral artery occlusion will reliably demonstrate detectable neurologic deficits, not present in non-stroked animals, as determined by the pig post-stroke scale.

The second specific aim of this study is to apply the pig post-stroke scale to determine the effect of iNPCs on the long-term functional recovery of pigs following an ischemic stroke. In accordance with the use of iNPCs in rodent models [11, 12, 14, 16], it is expected that pigs receiving iNPC therapy will demonstrate less severe neurologic deficits in addition to faster recovery following an ischemic stroke.

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CHAPTER 2

LITERATURE REVIEW: FUNCTIONAL OUTCOME SCALES IN STROKE

Stroke is the leading cause of long-term disability worldwide with estimates of one in six people experiencing a stroke in their lifetime [1]. An important aspect of both preclinical and clinical trials for stroke therapy is functional outcome measures [1-3]. Current scales used to assess humans following stroke include the American Heart Association Stroke Outcome Classification Score (AHA.SOC), Barthel index (BI), the Canadian Neurologic Scale (CNS), the Glasgow Outcome Scale (GOS), the modified Rankin Score (mRS), and the NIH Stroke Scale (NIHSS) [4]. The application of these scales provides an attempt to quantify the degree of neurologic recovery and function through evaluation of neurologic deficits, quality of life, and functional independence in activities of daily living[4]. Quantification of these parameters on an ordinal or interval scale provides a means to evaluate effect of novel therapies in clinical trials. Currently, the modified Rankin Score and/or Barthel index are used as standards in human clinical trials to differentiate good from poor outcomes[5].

The failure of many stroke therapy clinical trials can partially be blamed on a lack of long-term functional outcome evaluation in the preclinical animal model [2, 3, 6, 7]. Following stroke, human functional outcome scales are designed to assess changes categorized under cognition, language, emotion, motor, and sensory abilities [8]. Unfortunately, the anthropomorphic nature of these scales renders them unsuitable for use in animal stroke models. As such, functional outcome in rodent models of stroke are evaluated through either a modified composite scoring system, such as the Bederson Scale or modified Neurologic Severity Score

(mNSS), or through the amalgamation of separate tests such as the Morris water maze, Staircase test, Rotarod, and Sticky tape test, to evaluate specific cognitive or motor skills [2, 6, 9, 10]. These tests have been designed to assess learning, memory, motor skills, and asymmetrical neurologic deficits [11].

Specifically for stroke, where the ultimate goal is translation from animal models to human clinical trials, it is important to assess functional outcomes in animals that are representative of all the neurologic domains and relevant to long-term outcome in humans. The vast majority of ischemic strokes in humans involve the middle cerebral artery and several well-established rodent models of middle cerebral artery occlusion exist [9, 12, 13]. In middle cerebral artery occlusion, affected areas of the brain include large areas of the sensorimotor cortex, basal ganglia, and internal capsule[9, 11, 14]. Symptoms following middle cerebral artery occlusion in people include hemiparesis, dysphagia, hemineglect, aphasia, impaired cognition, and urinary/fecal incontinence [15].

A variety of functional outcome tests have been designed for rodent models of stroke, but there is no consensus on a gold standard [2, 3, 6]. Most studies apply the use of a composite scoring system, such as the Bederson Scale or mNSS, in addition to or in lieu of individually scored tests such as the Rotarod test, Apomorphine induced rotation test, Morris Water Maze, and Sticky tape test [2, 3, 10, 16]. The Bederson scale represents one of the oldest composite grading systems and provides the basis of many newer grading systems in rodents [3, 9]. In this scale, limb placement and circling are scored as measurements of sensorimotor deficits[9, 10, 13, 17]. The mNSS is another popular composite scoring system comprised of cumulative scores assigned to evaluations of sensorimotor function, reflex, and balance [10]. Separately developed tests for sensorimotor and balance function in rodents include the Grid Walking, Accelerated

Rotarod, Ledged Tapered Beam, and Ladder Rung Walking tests[2, 10]. The Adhesive Removal ("Sticky Label"), Pasta, Staircase, and Reaching Chamber/Pellet Retrieval tests are designed to assess dexterity and fine motor skills following neurologic injury [10]. To evaluate cognitive function, several tests have been developed to assess working and reference memory[2]. These tests include Open Field tests, the Morris Water Maze, Radial Arm Maze, and Stepdown Avoidance tests [2, 18]. The specifics of these tests for sensorimotor, balance, and cognitive function in rodents have been previously reviewed by Corbett and Nurse [2], Hunter et al.[3], and Schaar et al. [10].

In designing an assessment scale, it is important to demonstrate the test-retest reliability, inter-rater reliability, clinical sensitivity, and validity of the assessment[15]. Test-retest reliability refers to a demonstrable consistency in results between trials using the same raters, study population, and assessment scale. Inter-rater reliability refers to a consistency in results between different raters when applying the same assessment scale to the same study population[15]. This is of particular importance in a clinical trial setting where different raters would be relied upon to provide consistent measurements across a large study population. In a previous study that applied the modified Rankin Scale to stroke patients, it was demonstrated that training the raters and providing them with a structured interview for the modified Rankin Scale significantly improved inter-rater reliability[19].

The clinical sensitivity of an assessment scale is the ability of the scale to perceive a clinically significant change[15]. In the context of functional outcome following ischemic stroke injury, clinical sensitivity of an assessment scale would allow discernment between healthy patients and patients that had experienced a stroke. Especially in clinical trials, clinical

sensitivity would also need to discern changes in functional outcome both between patients and within the same individual during the course of their recovery[20].

The validity of an assessment scale is defined as the ability of a scale to assess the outcome it was intended to measure[15, 21]. In the context of medical assessment scales, three main types of validity are applied: convergent (criterion), construct, and content validity[15, 21]. When a gold standard test exists, convergent or criterion validity can be demonstrated if results of the two tests are in agreement[21]. In the case of functional neurologic outcome following ischemic stroke, a gold standard of measurement does not exist- even for humans[15, 21]. The challenge is that successful functional outcome is a subjective assessment and objective quantifiable measures of stroke, such as size and location, are not always predictive of clinical outcomes[6, 21, 22].

If a gold standard does not exist, construct validity is applied which evaluates agreement of the scale in question with other testing methods used to assess the same "construct" or outcome[21]. For human stroke patients, new stroke assessment scales are compared against established scales such as the modified Rankin Scale and Barthel index[4, 23]. These scales, in turn, are measured against parameters such as stroke imaging measurements and acute injury stroke assessment scales such as the National Institute of Health Stroke Scale (NIHSS) [4, 15, 24-27].

When neither a gold standard nor other existing assessments for an outcome or construct exist, content validation is necessary. Content validation involves the integration of expert opinion and a review of current literature to create a viable assessment scale[21]. Without other tests or standards for comparison, this method of validation is intuitively prone to bias and error;

however, when no other alternatives exist, its use must be considered. Several human stroke scales, including the Canadian Neurologic Scale, were developed using content validation[21].

As there is no gold standard, current functional outcome assessment scales in animal models of stroke rely on a mixture of content validation and construct validation. This is especially true in large animal models where few post-stroke functional assessment tools exist [28-31]. The first specific aim of this study was to create a functional recovery assessment scale in a pig ischemic stroke model. Applying the constraints described above, the scale would be validated based on expert opinion and a review of current literature on stroke outcome scales in humans, rodents, and existing large animal outcome scales. The assessment scale should also demonstrate test-retest reliability with the same observer being able to repeat assessments on the same animals and providing scores that are statistically consistent between observations. Inter-observer reliability would also be demonstrated with no statistical significance between scores assigned by two different observers on the same study animals. Finally, the pig post-stroke assessment scale should be clinically sensitive with the expected result being a reliable and statistical distinction in the scores between non-stroked vs stroked animals and animals with acutely injured vs quiescent/chronic stroke injuries.

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CHAPTER 3

INDUCED PLURIPOTENT STEM CELL-DERIVED NEURAL CELL TYPES IN TREATMENT OF STROKELITERATURE REVIEW: CELL THERAPY FOR ISCHEMIC STROKE[1]

[1] Lau, V.W., S.R. Platt, S.L. Stice, and F.D. West. (2015). Induced pluripotent stem-cell-derived neural cell types in treatment of stroke. In D. C. Hess (Ed.), *Cell therapy for Brain Injury* (pp. 147-172). Springer.

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3.1 Introduction

Every year, approximately 800,000 individuals in the USA alone suffer a stroke, making stroke the leading cause of long-term disability and the fourth leading cause of death, and adding to the millions of stroke victims and families that care for them [1–4]. Despite considerable efforts to develop pharmacological treatments and devices, developed approaches are grossly inadequate. These treatments are predicated on limiting damage that occurs during an ischemic event, yet none of them enable large-scale tissue regeneration. The promise of stem cell therapies is the potential to replace ablated cells and damaged tissue, to form new functional neural networks that make appropriate connections and lead to the restoration of sensory, motor, and cognitive function in patients. The regeneration and replacement of lost tissue and improvements in functional deficits will enable the many stroke victims to return to a more productive lifestyle and relieve the family burden of long-term care. Adult and embryonic stem cells (ESCs) and neural stem cells derived from ESCs have all been of keen interest to the stroke field. However, adult stem cells pose inherent difficulties, including isolation and expansion for some therapies, while ESCs have been mired in controversy since they were first isolated [5]. Some patients and practitioners may object to the use of ESCs and seek alternatives, despite publications demonstrating that viable embryos do not need to be used or destroyed in order to isolate ESCs [6].

In parallel, a new type of pluripotent stem cell has been generated—induced pluripotent stem cells (iPSCs). Although relatively new, iPSCs are believed to harbor all the same beneficial properties as ESCs, with both being pluripotent stem cells capable of forming any cell type in the body. It is a common belief that iPSCs will eventually be derived from the patient's own somatic cells so that immunological rejection associated with transplantation of any foreign cells or

tissues may be averted. iPSCs are highly plastic and can be easily differentiated into neural stem cells (NSCs) that can be expanded to someday provide the volume of cells needed for therapeutic applications. Data in rodent stroke models have been very positive with induced pluripotent stem-cell-derived neural stem cell (iNSC) transplantations leading to functional recovery and decreased infarct sizes [7–10]. However, many challenges and questions remain before iNSC cell therapies can be deemed a safe and effective treatment in human patients.

iNSCs have the potential to transform the way researchers and physicians approach stroke treatments; transitioning from a paradigm of merely limiting further ischemic injury to one where lost tissue can be regenerated. For well over 50 years, tremendous effort has been committed to producing stroke therapies that limit the extent of injury through pharmaceutical and mechanical means with limited success.

These approaches lead to recanalization of occluded vessels to restore blood flow to ischemic tissues or function as neuroprotectants that reduce cytotoxicity from inflammatory responses, damaging free radicals, or similar elements [11–14]. These efforts have had limited success with tissue plasminogen activator (tPA) being the only Food and Drug Administration (FDA)-approved pharmacological treatment in addition to a handful of FDA-approved clot-retrieval devices [14, 15]. These approaches are effective yet suffer from significant shortcomings. Only about 5 % of ischemic stroke patients receive tPA due to its restrictive 4.5 h window of use. The mechanical embolus removal in cerebral ischemia (MERCI) system (an FDA-approved clot-retrieval device) can be used in patients up to 8 h post stroke, but often fails to restore blood flow in ~50% of occluded vessels [14, 15]. Neither of these clot-removal approaches can be utilized to treat patients that have suffered a hemorrhagic stroke, thereby excluding approximately 15 % of the stroke patient population [16]. A host of other

neuroprotective treatments reducing secondary injury caused by inflammatory and immune responses have been developed yet have never made it beyond clinical trials (reviewed in [17, 18]). Even assuming that thrombolytic, neuroprotective, or similar approaches were 100 % effective, these treatments only prevent further damage, but have little regenerative capabilities. Therefore, the tissue damage caused by the initial ischemic event remains unchanged beyond normal healing.

An assessment of the litany of failed treatments by the Stem Cell Emerging Paradigm in Stroke Consortium meetings (STEPS I, II, and III), modeled on the stroke therapy academic industry roundtable (STAIR) model where leaders from academia, industry, and the FDA and National Institute of Neurological Disorders and Stroke (NINDS) participate, resulted in publications identifying several major factors needed to improve the development of stroke treatments. One of the major conclusions was the need for a regenerative cell therapy that will not only protect cells from ischemic injury but also replace lost and damaged tissues [19, 20]. This has resulted in a growing interest in potentially restorative treatments centered on stem cell therapies.

Recent studies have demonstrated that iNSCs may serve as an excellent regenerative therapy with a dual function: (1) acting as a cell-replacement therapy and as (2) a producer of regenerative paracrine factors (e.g., vascular endothelial growth factor, VEGF) that enhance endogenous tissue regeneration in rodent stroke models [7–10, 21–23] (Fig. 10.1). Transplanted cells migrate to the site of injury, differentiate, and functionally integrate forming new electrically active neural networks leading to improvement in neurological scores and motor function. These exciting and encouraging results have spurned considerable interest in the stroke community as a step forward in personalized regenerative

medicine. In this chapter, we examine the development of iPSCs and derived NSCs, the current state of the art and areas of emphasis for improved translation to human medicine.

3.2 Development of Induced Pluripotent Stem Cell Technology

iPSCs are a recent discovery where mature somatic cells can be reprogrammed into pluripotent stem cells capable of differentiating into any cell type in the body through the over-expression of defined genes [24, 25]. The development of iPSC reprogramming technology resides at the convergence point of the fields of cellular reprogramming and ESCs where the conceptual framework to understand the genetic, epigenetic, and functional pluripotency networks were pioneered [26–32]. Based on prior knowledge, Yamanaka's research team hypothesized that "the factors that play important roles in the maintenance of ES cell identity also play pivotal roles in the induction of pluripotency in somatic cells" [25]. In the mouse, embryonic fibroblasts were retrovirally transduced with 24 pluripotency-associated genes resulting in the formation of nine colonies exhibiting ESC character with cells growing in colonies and displaying a rounded morphology, large nucleoli, and high nucleus-to cytoplasm ratio. They went on to demonstrate that only four critical factors (Pou5f1 (also known as Oct3/4), Sox2, c-Myc and Klf4) were necessary to achieve complete reprogramming of embryonic and adult fibroblast cells. iPSCs demonstrated morphology, immunoreactivity, global gene expression, and epigenetic status indicative of a pluripotent state similar to ESCs. Functional tests of plasticity demonstrated that iPSCs were capable of forming embryoid bodies (EBs; Fig. 10.2a) in vitro and teratomas in vivo consisting of all three germ layers, ectoderm, endoderm, and mesoderm.

iPSCs ultimately passed the most stringent of tests and were found capable of incorporating into all tissues of chimeric mice including the germline (Fig. 10.2b) and were

successful in the tetraploid complementation pluripotency assay—a test where all cells of the embryo proper are derived solely from transplanted iPSCs [25, 33–35]. The significant value of this iPSC technology for basic mouse genetics was soon recognized. However, of perhaps even greater interest was their obvious potential in human medicine for cell-replacement therapy. In 2007, the Yamanaka lab was successful in deriving the first human iPSCs using the same reprogramming genes that were successful in the generation of mouse iPSCs, thus opening the door a bit wider for personalized medicine [24].

Intuitively, patients treated with their own iPSCs would be less immunogenic than those treated with allogeneic iPSCs (iPSCs derived from other patients) or ESCs; therefore, autologous iPSCs are thought to be similar to autologous human adult stem cell therapies used today in the clinic. However, there is still debate as to their immunogenicity. An early publication demonstrated that mouse iPSCs transplanted into a syngenic recipient animal, an animal that is genetically identical and transplant compatible to the mouse from which the iPSCs were derived, resulted in a T-cell-dependent immune response [36]. The researchers attributed this response to aberrant gene expression resulting from the reprogramming process. In contrast, recent studies showed little or no evidence of increased T cell proliferation or integration, antigen-specific secondary immune activity, or graft rejection in response to undifferentiated or differentiated mouse iPSCs transplanted into syngenic animals [37, 38]. These studies support the premise that autologous iPSCs may be safely transplanted into human patients without rejection; however, additional studies are needed to confirm these findings.

iPSC technology has made considerable advancements with alternative reprogramming strategies aimed at improving safety and efficiency. The initial Yamanaka lab reprogramming approach utilized spontaneous retrovirus integration of known oncogenes, including c-Myc. This

random integration approach raised major concerns that insertion of genes could lead to insertional mutagenesis in addition to spontaneous reactivation of the c-Myc oncogene, which could potentially lead to tumor formation in human patients. However, recent advancements have led to novel nonintegrating approaches including minicircle DNA, modified mRNAs, and protein strategies to generate iPSCs without the need for permanent incorporation of reprogramming genes or the use of viral techniques [39–41] (Table 10.1). Efforts have also led to combinations of reprogramming factors that do not require the use of c-Myc [42]. These advances significantly improved many of the initial safety concerns that limited the potential of iPSC technology.

3.3 Differentiation of Induced Pluripotent Stem Cells into Therapeutic Cells for Stroke Treatment

Stroke results in the active recruitment of endogenous NSCs in the brain leading to proliferation and migration of NSCs from the subventricular zone to the ischemic region [43–46]. This natural regenerative cell response is insufficient, however, to restore most stroke patients to their normal pre-stroke function [45, 46]. iNSCs can act as a supplemental cell source to increase the number of NSCs and the regenerative capabilities of the stroked brain. It is preferential to differentiate iPSCs into iNSCs as the direct transplantation of undifferentiated cells is likely to lead to tumor formation. Previous studies by Kawai et al. and Chen et al. showed that transplantation of undifferentiated stem cells into middle cerebral artery occlusion (MCAO) stroke models led to the development of large tumors containing cells of the ectoderm, endoderm, and mesoderm lineages [47, 48]. The differentiation of iPSCs into iNSCs has been successfully achieved using a number of different protocols originally developed for hESCs [7–10]. Oki et al. utilized a previously developed ESC approach where iPSCs were detached and

grown in suspension as EBs to enhance spontaneous differentiation [9, 49]. To better direct these cells down the neural lineage, EBs were then cultured in chemically defined neural medium composed of DMEM/F12, supplemented with insulin, transferrin, progesterone, putrescine, sodium selenite, and heparin in the presence of FGF-2. Plated EBs flattened and formed small, elongated cells that generated rosette structures resembling the early neural tube. In addition to the typical neural stem cell markers SOX2 and Nestin, neural rosette cells expressed the rosette-associated transcription factors DACH1 and PLZF with apical expression of ZO-1. Neural rosettes were isolated and ultimately lost the rosette morphology and further developed into NSCs. However, these iNSCs are capable of long-term expansion, while maintaining SOX2 and Nestin expression [9]. Yuan et al. used a similar EB approach, where EBs were formed and plated but were also exposed to retinoic acid (RA) leading to the formation of rosettes [10]. Upon removal of RA, neural rosette cells detached, continued to grow in suspension and formed neural spheres. These spheres were then plated on poly-ornithine and laminin-coated dishes in serum-free media with derived cells being a homogeneous population of NSCs. Other groups have used similar systems with variations including the addition of unique growth factors, inhibitors, supporting stromal cells (e.g., PA6) and changes in timing of differentiation steps [7, 8]. Despite the variability in protocols, iNSCs are SOX1 and Nestin positive and should be capable of differentiating into multiple lineages of neurons and glia.

Intuitively it may seem that iNSCs would be the best cell type for transplantation to regenerate lost and damaged tissue. However, the plasticity of iNSCs is such that they may differentiate into any neural cell type and may differentiate into cells that are regionally incorrect. Therefore, it is of potential value to generate iPSC-derived progenitors that are regionalized. A recent report described the derivation of telencephalic progenitors, which may be

valuable for treating stroke regionalized to the forebrain [21]. iPSCs were differentiated using a serum-free EB approach. Telencephalic progenitors expressed the pallial telencephalic marker PAX6 and the telencephalic marker BF1 in addition to the neural stem cell markers SOX1 and Nestin. Tornero et al. recently generated cortical neuron progenitors for the treatment of stroke noting that "Clinical and imaging data showing the distribution of ischemic cell loss underlying the most severe symptoms in stroke patients indicate that cell replacement approaches should focus on the reconstruction of damaged cortex" [23]. To produce cortically fated cells, Tornero et al. differentiated iPSCs in the presence of Wnt3A, BMP4, and cyclopamine. These cortical progenitors expressed the cortex-specific neuronal marker TBR1 and cortex markers CTIP2 and CDP (markers associated with the deeper and superficial cortex layers respectively). hESCs and iPSCs have been found to be capable of differentiating into numerous specialized neural cell types making the potential cell type options and combinations for therapeutic use numerous. The ability to transplant multiple combinations of various neural cell types to match regional-specific areas of the brain is intriguing yet adds an additional layer of complexity that will take significant consideration.

3.4 Direct Reprogramming of Fibroblasts into NSCs

Two major limitations of transplanting iNSCs into stroke patients are (1) the potential of transplanting a contaminating iPSC subpopulation that spontaneously develops into a tumor and (2) the somewhat lengthy time period it takes to generate iNSCs. Typically, it can take months to generate and sufficiently characterize iNSCs with the need to first isolate and expand the somatic cells, then reprogram the cells into iPSCs, differentiate these cells into iNSCs and then perform the necessary quality control tests on these cells prior to transplantation (e.g., cellular phenotyping, functionality assessments, karyotype analysis). A recent breakthrough in

reprogramming has led to the development of technologies where somatic cells can be directly reprogrammed into neurons and NSCs without a pluripotent stem cell intermediate [50–53]. Direct neural stem cell reprogramming has been accomplished with various combinations of reprogramming factors (Table 10.2). Ring et al. was the first to show that both mouse and human fibroblasts could be reprogrammed into iNSCs with simple culture manipulations and the overexpression of the single reprogramming gene SOX2 [53]. Human cells formed clusters of SOX2 and Nestin positive cells 5 days after SOX2 retroviral transduction. These cells then underwent multiple rounds of neurosphere culture and could be maintained under standard NSC conditions. Human iNSCs were capable of differentiation into TUJ1/MAP2 + neurons, glial fibrillary acidic protein (GFAP) + astrocytes and O4/OLIG2 + oligodendrocytes.

Mouse cells showed similar developmental plasticity and upon further differentiation were proven to be functionally active. Neurons derived from mouse iNSCs formed synapses marked by synapsin with patch-clamp recordings showing functional membrane properties and activity. Neither human nor mouse cells formed tumors upon transplantation into noninjured animals. Direct iNSC reprogramming provides a rapid and safe reprogramming approach with the only major limitation being the need for viral delivery and integration of reprogramming factors. Yet, building upon nonviral and nongenomic DNA-integrating approaches created for generating iPSCs, it is very likely that similar approaches can be developed for direct reprogramming of somatic cells into iNSC.

3.5 iNSC Transplantation into Stroke Models Leads To Promising Yet Mixed Success

iNSCs have been transplanted into mouse and rat MCAO reperfusion models with cells showing promising results [7–10, 21, 22]. However, it is difficult to compare outcomes and efficacy across studies as transplantation parameters were variable with transplant cell numbers

ranging from 100,000 to 1,000,000, and timing of transplantation ranging from immediately post-reperfusion to 7 days later. The site of injection was also variable with cells being injected proximal to the lesion or in the contralateral hemisphere to the site of injury. These differences may also account for the significant amount of variability with respect to results. In general, 200,000–250,000 cells were injected 7 days later, avoiding the extreme levels of cytotoxicity immediately after stroke, into the ipsilateral lobe of stroked animals. Transplants showed survival in most studies, but the exact cell number is questionable with one study estimating 10 % cell survival [9]. Transplanted cells regularly showed differentiation into neurons, astrocytes, and oligodendrocytes with quantitative data showing higher levels of neuron differentiation than glia [9, 22]. iNSC-derived neurons showed specialization with cells differentiating into dopaminergic and gabaminergic neurons [7–9]. Functionally, whole-cell patch-clamp recordings of brain slices from iNSCs-treated mice at 5 months showed that the majority of iNSC-derived neurons tested were able to produce action potentials in response to depolarizing current and were sensitive to the voltage-gated Na+ channel blocker tetrodotoxin (TTX) and the voltage-gated K+ channel blocker tetraethylammonium (TEA) [9]. iNSC-derived neurons were also sensitive to type-A γ-aminobutyric acid (GABAA) receptor and glutamate receptor antagonists. These results and additional findings showed that iNSC-derived neurons were capable of receiving synaptic input from host neurons and functionally integrating into the neural circuitry [9].

The effect of iNSCs on endogenous tissue was inconsistent between studies. iNSCs had a protective and regenerative effect on host tissues, likely caused by paracrine signaling with the release of factors such as VEGF, as demonstrated in the Cheng et al. study [7]. They found that cell transplantation resulted in a 36 and 11 % reduction in Iba-1 + and ED1 + immune cells

respectively—cells that are often associated with increased cytotoxicity. At week 8, they demonstrated a 55 % reduction in gliosis and a 17 % reduction in apoptosis. However, Oki et al. found no significant effect on immune cells (Iba-1 or ED1) or gliosis [9]. Similar studies showed no significant difference in stroke volume suggesting a minimal neuroprotective effect [9, 22].

Functional assessments again showed mixed results across studies, yet were promising. Studies showed improvements in modified neurological scores, rotarod, stepping, and staircase assessments in animals treated with iNSCs relative to nontreated controls [7–9, 21]. Animals showed mixed results with the tape removal test and failed to show significant improvement over control in the corridor, elevated body swing, and cylinder tests [9, 22]. Interestingly, the study that demonstrated the most significant functional improvement also showed the largest decrease in immune cell number, apoptosis, and gliosis [9]. This suggests a strong correlation between tissue-level improvements and positive functional outcomes.

3.6 iNSCs in Stroke May Confer Neuroprotection and Enhance Neuroplasticity and Angiogenesis Through Trophic Factor Effects

Transplantation of human iNSCs has been associated with improved functional recovery and a reduction in secondary neural degeneration in various models of ischemic stroke [7–9, 21, 23]. Most of the beneficial effects of iNSCs are observed shortly after transplantation and appear to be independent of iNSC survival suggesting that beneficial effects of iNSCs are not all attributable to cell replacement [8, 9, 21]. While the exact mechanisms through which iNSCs are able to contribute to neural recovery are not well understood, proposed mechanisms include secretion of angiogenic factors such as VEGF, neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and glial cell-line-derived neurotrophic factor (GDNF), and downregulation of inflammatory mediators such as interleukin-6 (IL-6), IL-1β, and tumor

necrosis factor alpha (TNF-α) [8, 9, 54]. These neuroprotective trophic factors may be secreted by the stem cells themselves or act through stimulation of endogenous protective pathways decreasing inflammation, promoting neural regeneration, angiogenesis, plasticity, and recruitment of axons from ipsilateral and contralateral hemispheres [8, 9, 55].

3.7 Routes of Cell Delivery

A variety of approaches to delivering therapeutic cells to sites of neural injury have been described [56–58]. These include intraparenchymal, intravascular, intracisternal, and intracerebroventricular injections (Fig. 10.3). To date, delivery of iNSCs for the treatment of ischemic stroke in animal models has been limited to intraparenchymal injection through transcranial approaches. Intraparenchymal approaches have also been used in several human clinical trials involving the administration of fetal porcine cells and cultured human neuronal cells to patients suffering from chronic stroke injuries [59, 60]. It is important to note, however, that intraparenchymal injections are by no means the only method of delivering cell therapy to sites of neural injury. To appreciate alternative delivery options, it is necessary to explore methods utilized with other stem cell therapies (e.g., mesenchymal stem cells, embryonic stem cell-derived NPCs, and umbilical cord-derived cells). There are pros and cons to each delivery method, which will be described in more detail in the sections below.

3.7.1 Intraparenchymal

Intraparenchymal injections are the most commonly reported approach. This may be due to the advantages conferred by this method such as site specificity, guaranteed cell delivery to the site of injury, and direct penetration through the blood–brain barrier [58]. Unfortunately, intraparenchymal injections typically require more invasive approaches to the site of injury through burr hole craniectomies. Intraparenchymal injections may also result in more clustered,

uneven distributions of cells within injured tissue relative to other cell delivery techniques such as intra-arterial (IA) injections that can accomplish a diffuse, extensive spread of cells throughout an injured region [57]. The location of the injection is dependent on the specific injury with a large proportion of MCAO models targeting injections at the site most consistently associated with infarction—the striatum [8, 9, 21]. With advanced imaging techniques such as magnetic resonance imaging (MRI), it is also possible to target injections into the peri-infarct tissue rather than into the infarct core, which may allow for improved cell survival and engraftment [61]. Implantation into either the ipsilateral or contralateral hemisphere to the injury has resulted in beneficial effects, with evidence that cells are able to migrate across midline from the contralateral hemisphere towards the site of injury [7, 62]. Injected neural progenitor cells display a predilection for injured tissues—a trait described as pathotropism [63, 64].Ischemic injured brain tissue can secrete a variety of signals such as stromal derived factor-1 (SDF-1) and monocyte chemotactic factor 1 (MCP-1), which attract cells, including iNSCs, carrying the receptors CXCR4, CXCR7, and CCR2 [65–67]. This pathotropism will likely enhance the ability of cells to treat ischemic tissue through trophic factor signaling and improve engraftment of cells.

3.7.2 Intravenous

Intravenous (IV) injections are another popular route as they are generally less invasive and pose less of a technical challenge. The number of cells that need to be administered is normally greatly increased from what is permissible via intraparenchymal delivery [57]. In general, IV cell delivery results in the reduced cell engraftment and is associated with cell-uptake by systemic, non-target organs with many of these cells being trapped in the lungs and liver [68]. The common occurrence of cells being trapped in the small vasculature of lungs is commonly

referred to as the pulmonary first-pass effect [69]. Nonetheless, studies with NPCs (not of human induced pluripotent stem cell origin) have shown that IV administration of cells in ischemic neural injury models can result in a reduction of ischemia-associated learning dysfunction, even when administration was delayed beyond the typical acute therapeutic window [70]. It is believed that these benefits arise from the production of anti-inflammatory and regenerative factors that have a systemic effect including the injured brain. In rare instances, IV delivery of cells has resulted in detectable cell engraftment within the brain [56, 71]. The ability of cells to travel from the vasculature into the brain may reflect the permeability of a compromised blood–brain barrier at the site of injury.

3.7.3 Intra-arterial

A means of avoiding the pulmonary first-pass effect is IA delivery of cells. This method is generally more invasive with higher patient risk for morbidity (due to hemorrhage and thrombosis) and mortality than intravenous approaches [57]. While riskier, cells administered IA have demonstrated increased migration, dissemination, and transplantation success than cells administered IV or intrathecally (IT) [57]. In a study where human mesenchymal stem cells (MSCs) were delivered IA in an MCAO rodent model, it was demonstrated that the location of transplanted cells was dependent on the timing of cell delivery [72]. Cells delivered 1day post injury were distributed to the peri-infarct region and core of the stroke. Cells delivered on day 4 post injury demonstrated only a peri-infarct distribution. No functional improvements and only very few cells were successfully delivered when injections were administered 7 days post stroke. This would imply a limitation in the timeframe in which IA treatments are effective, although this remains to be shown with iNSCs. Timing of delivery IA may also have an impact on the phenotypic fate of transplanted NSCs with cells transplanted in the first 24 h expressing

significantly more GFAP and cells transplanted at 7 and 14 days expressing more βIII-tubulin, indicating astrocyte and neuron differentiation respectively [73].

3.7.4 Intracerebroventricular and Intracisternal

Intracerebroventricular or intrathecal injections have also been reported as a means of delivering cells to the ischemic brain [56, 57]. These are generally associated with less patient risk than IA and intraparenchymal approaches and permit the injection of higher cell numbers. Following intra-ventricular injections, the cells are able to adhere to the walls of the ventricles and migrate through the ependymal lining into the damaged tissues, especially through the lateral versus medial walls of the ventricles [56, 57]. The exact mechanism through which the cells traverse through the ependymal lining is unknown but theories include transport through macrophage-associated regional specializations termed “fractones” [56, 74].

With intrathecal (IT) injections, cells are delivered into the cisterna magna. Again, larger cell numbers can be delivered than with intraparenchymal approaches. Unfortunately, given the flow of cerebrospinal fluid, cells can be lost to other parts of the central nervous system (CNS). In comparisons between IA, IV, and IT in rodent models, IT injections were more effective at delivering cells than IV injections, but IA was considered the superior delivery method in terms of total number of NPCs successfully delivered to the targeted tissue and the achievement of a diffuse, widespread distribution of cells within the injury [57, 75].

3.7.5 Delivery with an Extracellular Matrix

Survival rates of engrafted cells, regardless of the method of cell delivery, are typically low with less than half of injected cells surviving for any period of time ([76]; reviewed in [58]). One method of increasing cell survivability in the cytotoxic acute ischemic injury environment is through implantation of iNSCs with supportive extracellular matrices (ECMs). ECMs can be

derived from natural materials like collagen, polyglycosaminoglycans, and ornithine/laminin or from synthetic polymers and hydrogels including polyglycolic acid (PGA), polyethylene glycol (PEG), and poly-L-lactic acid (PLLA) [77, 78]. These materials can be transformed into scaffolds with a variety of shapes and sizes with various porosities and stiffnesses to promote engraftment and recovery. In areas of severe or cystic tissue loss, as is seen in ischemic stroke, biomaterial scaffolds can act as bridging substrates to allow cell attachment and engraftment [79]. There is also suggestion that biomaterial scaffolds can play a part in inhibiting glial scar formation in some neural injury models [80].

Encapsulating scaffolds can act as barriers for grafted cells protecting from host immune rejection, while permitting signaling factors to diffuse between the graft and the injured environment [81]. As neural injuries often possess irregular boundaries, malleable and liquid substrates such as injectable hydrogels and microspheres have been particular targets of investigation [79, 82, 83]. In addition to acting as structural support, scaffolds can also be engineered to contain various growth factors, peptides, and chemical signals such as heparin and hyaluronan to promote microenvironments conducive to graft survival [78, 83–86]. Multiple studies demonstrated that ECM or NSC alone did not improve sensory motor function recovery nor decreased infarct size after focal cerebral ischemia in rodents. However, when ECM and NSCs were combined, there was a significant improvement in both functional and anatomical outcomes [82].

Despite their anticipated benefits, biomaterials can also present unique challenges including inhibition of neurite outgrowth by the scaffold [79, 87–89] and variable matrix degradation times [90]. In some cases, the scaffold may interfere with graft cell differentiation and integration [91]. For synthetic polymers, there is particular concern about harmful

degradation by-products that can increase local acidity, inflammation, and tissue damage [92]. Immunoreactivity and tumorgenicity of biomaterials are also of concern, especially with undefined natural materials harvested from plant and animal sources [77]. In addition to the materials in its composition, the macro-architecture of the implants appears to play a role in the host-immune response [93]. With some scaffolds, fibrous tissue buildup and foreign-body reactions around the implant can also cause interference with tissue integration, angiogenesis, and trophic factor diffusion to and from the grafted cells ([93]; reviewed by [92]). Nonetheless, ECMs offer an exciting and viable option for improving the success of iNSC transplantation in the ischemic-stroke environment.

3.8 Cell Dosage

Currently, there are no clear guidelines on how to determine the therapeutic number of cells to be transplanted for any cell therapy to achieve optimum treatment of stroke. Albeit there are some key factors that are likely to be critical in the development of guidelines for therapeutic dose. These potential factors include:

1. Severity, localization and type of stroke injury
2. Whether the therapeutic is acting through paracrine signaling as a producer of neuroprotectants, regenerative factors or as a replacement therapy
3. Comorbidities such as hypertension and diabetes
4. Patient age, sex, and size
5. Delivery mechanism

When delivering cells through the vasculature, cistern, or ventricle, it is possible to administer higher cell numbers with some rodent IV dosages approximating 5×10^6 cells [56, 94, 95]. The beneficial effects may also be dose-dependent as shown in a rat ischemic stroke study

involving IV administration of bone marrow stromal cells (BMSCs); rats receiving higher cell numbers displayed better outcomes [96]. In rodent models of ischemic stroke, cell numbers for intraparenchymal implantationhave ranged anywhere from 5000 to 1.5 million [97]. When translating this to human patients, consideration should be given to the significant size disparity between rodents and humans. A cells-per-body-mass dosage can be extrapolated from rodent studies but may not be the best method for determining an optimal dose in human patients similar to pharmacokinetic studies where differences in species metabolism and physiology contribute significantly to appropriate dose scaling [98, 99]. Some clinical trials have adopted this approach by calculating the effective IV dose of BMSCs in rodents and determining an equivalent dosage in humans as about 1x 10^8 cells/patient [100]. In one study looking at intraparenchymal cell delivery in a rodent model, the injection of higher numbers of cells resulted in higher total number of cells surviving [97]. Cell survivability on a percentage basis, however, was actually higher when lower numbers of cells were injected, suggesting an optimal threshold for cell numbers to be engrafted. It is thought that beyond this threshold, cell survivability decreases due to limited supply of local nutrients.

3.9 Timing

A potential benefit of cell therapy is that it offers a broader therapeutic time window than current FDA-approved therapies like tPA, which require administration in the hyperacute phase (< 6 h) from the time of injury [101]. The precise optimal therapeutic time window for stem cell treatment of stroke is still unclear and likely varies between stroke conditions. One factor that should be considered is the route of cell delivery. For routes of administration like intravascular injection that rely on a compromised blood–brain barrier and inflammatory signaling for cells to home to the site of injury, therapy within the acute period post stroke may be more relevant as

reviewed in Bliss et al. 2010 [101]. For IA routes, the timing of the injection can affect the distribution, survival, and the phenotypic fates of the injected cells [73]. There are also concerns for cell survivability with transplantation during the acute stroke phase due to the cytotoxic environment, which suggests that the subacute or chronic injury periods may be more optimum transplant points [101]. This is highly dependent, however, on the anticipated primary effect of the transplanted cells. In some cases where the primary effect is through neuroprotection via trophic effects rather than cell differentiation and replacement, transplantation during the acute post-stroke period may be optimal. Whereas if a cell is predicted to have an anti-inflammatory or neuroplasticity effect, it is perhaps more relevant to transplant cells during the subacute stroke phase [101]. Earlier intraparenchymal injection times are also supported by reports where the beneficial effects of intraparenchymal injections were independent of cell survival [8, 9, 56]. One thought is that earlier intracerebral injection times may improve cell survivability as the microglial response has not yet had a chance to establish itself [97]. Rosenblum et al. [73] compared injection of neural progenitor cells (NPCs) at various time points in a hypoxia–ischemia rodent model and demonstrated that intra-striatal injections 3 days post injury yielded the highest cell engraftment as compared to injections administered at 6 and 24 h and 7 and 14 days. Current clinical trials have surveyed the effects of BMSCs and Human NT2N neurons (derived from the NTera2 teratocarcinoma cell line) on stroke injuries in the late subacute period (4–5 weeks post stroke) and chronic periods, respectively [100, 102]. While some patients appeared to benefit from the treatment, the benefits were not considered to be statistically significant [102]. The exact mechanisms of action of cell therapy at these later treatment points have not been specified. To date, studies investigating the optimal therapeutic time frame for administration of iNSCs have not been investigated.

3.10 Future Directions

While there have been successful transplantations of iNSCs into rodent models of ischemic stroke, there are still many unanswered questions regarding the specifics of cell dosage, use of ECM, transplant location, optimal timing for transplantation, or the best vehicle and approach for cell delivery. The lack of consensus in a relatively homogeneous model species like the rat or mouse suggests that optimal cell transplantation conditions are likely varied and case dependent.

Long-term studies on the safety and efficacy of iNSCs have yet to be completed in non-rodent models. As outlined by the STAIR [103] and STEPS II [104] meetings, it is vital that successful rodent therapies be confirmed in other animal models of stroke prior to advancing to human clinical trials. Several large-animal models of ischemic stroke have been developed and iNSC transplantation studies in these species are eagerly awaited [105–107]. Due to similarities between humans and primates, the primate model would seem to be a natural fit for studies of iNSC treatments. However, the cost, specialized facilities, regulatory burden, and ethical issues associated with primate models make alternative large-animal models such as sheep and pigs more attractive in some respects. The pig stroke model offers a significant advantage over rodent models as pigs have much larger gyrencephalic brains with gray–white matter composition more similar to humans [108, 109]. Utilization of animal models with similar white matter composition is of significant importance as white matter injuries uniquely contribute to clinical deficits in stroke patients and it will be important to determine if iNSC treatment will be able to appropriately differentiate and integrate in both gray- and white-matter compartments [110, 111]. Both the human and pig brain is composed of > 65 % white matter, while white matter in the rodent brain is < 10 %, making the pig a potentially excellent surrogate [107, 110, 112–115]).

Moreover, human and pig brains are both gyrencephalic, while the rodent brain is lissencephalic, a key architectural difference that has a direct correlation with brain connectivity and complexity [107, 112, 113].

Brain size is also another major variable when considering a cell therapy. The human brain is approximately 650 times the size of the average rodent brain, while only being 7.5 times the size of the pig brain—a size comparable to typical nonhuman primate models [116]. Size affects the number of cells to be transplanted, the sites of injection, the ability of the graft to be vascularized, and the distances axons must travel to form connections. To achieve maximum clinical translatability, using animal models as similar to humans as possible will be of critical importance in testing additional factors affecting iNSC therapy efficacy and safety. iNSC treatment of stroke in rodent models have led to justified enthusiasm with cells showing long-term integration and functionality with treated animals showing improvement in functional deficiencies [7–10]. In the light of these initial successes, and with an eye towards clinical applications, additional studies are now needed to assess basic questions such as cell dosage, treatment window, and route of delivery in suitable large animal models. These studies should be performed as randomized double-blinded trials to prevent any unintended bias from researchers, under the most stringent testing conditions possible. Utilizing strict testing protocols, regenerative iNSC therapy will hopefully move from promise and potential to a realized clinical therapy that will help millions of stroke victims lead more normal and productive lives.

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Table 3.1: **Methods for Reprogramming Somatic Cells to iPSCs**

Vector type	Cell type	Advantage	Disadvantage	References
Integrating	Retroviral	Efficient, highly successful with numerous cells types	Genomic integration, incomplete proviral silencing and slow kinetics, formation of large numbers of partially reprogrammed colonies	[24, 25, 117, 118]
	Lentiviral	Efficient and transduces dividing and non-dividing cells, highly successful with numerous cells types	Genomic integration and incomplete proviral silencing, formation of large numbers of partially reprogrammed colonies	[119-122]
Excisable	Transposon	Efficient and integrated regions can be removed	Screening of excised lines is labor intensive	[123]
	LoxP-flanked lentiviral	Efficient and integrated regions can be removed	Exogenous genes are removed, but loxP sites are retained in the genome	[124]
Non-integrating	Adenoviral	No genomic integration	Low efficiency	[125]
	Plasmid	Occasional genomic integration	Low efficiency and occasional vector genomic integration	[126, 127]
DNA Free	Protein	No genomic integration, direct delivery of transcription factors and no DNA-related complications	Low efficiency, short half-life, and requirement for large quantities of pure proteins	[128, 129]
	Modified mRNA	No genomic integration, faster reprogramming kinetics, controllable and high efficiency	Labor Intensive	[130]

Table 3.2. Direct Reprogramming into Neural Progenitors

	Factors	Mode of Reprogramming	Lineages	Species	Reference
1	ASCL1 & BRN2 & MYT1L or ZIC1	Lentivirus	Neuron	Mouse	[50]
2	ASCL1 & BRN2 & MYT1L & NEUROD1	Lentivirus	Neuron	Human	[51]
3	miR-124 and BRN2 and MYT1L	Lentivirus	Neuron	Human	[131]
4	SOX2 & FOXG1 & BRN2	Lentivirus	Neurons, Astrocytes and Oligodendrocytes	Mouse	[52]
5	SOX2 & BRN4, KLF4, C-MYC (4 Factor) or with E47 (5 Factor)	Retrovirus	Neurons, Astrocytes and Oligodendrocytes (low)	Mouse	[132]
6	OCT4 & SOX2 & KLF4 & c-MYC +Significant media manipulations	Lentivirus (pre-transduced dox controlled TEFS)	Neurons and Astrocytes	Mouse	[133]
7	ASCL1 & BRN2 & MYT1L	Lentivirus	Neurons	Mouse	[134]
8	ASCL1 & NGN2 & HES1 & ID1& PAX6 & BRN2 & SOX2 & C-MYC & KLF4	Retrovirus	Neurons, Astrocytes and Oligodendrocytes	Mouse	[135]
9	SOX2 & KLF4 & C-MYC & highly regulated OCT4	retrovirus	Neurons, Astrocytes and Oligodendrocytes	Mouse	[136]
10	SOX2	retrovirus	Neurons, Astrocytes and Oligodendrocytes	Mouse and Human	[53]

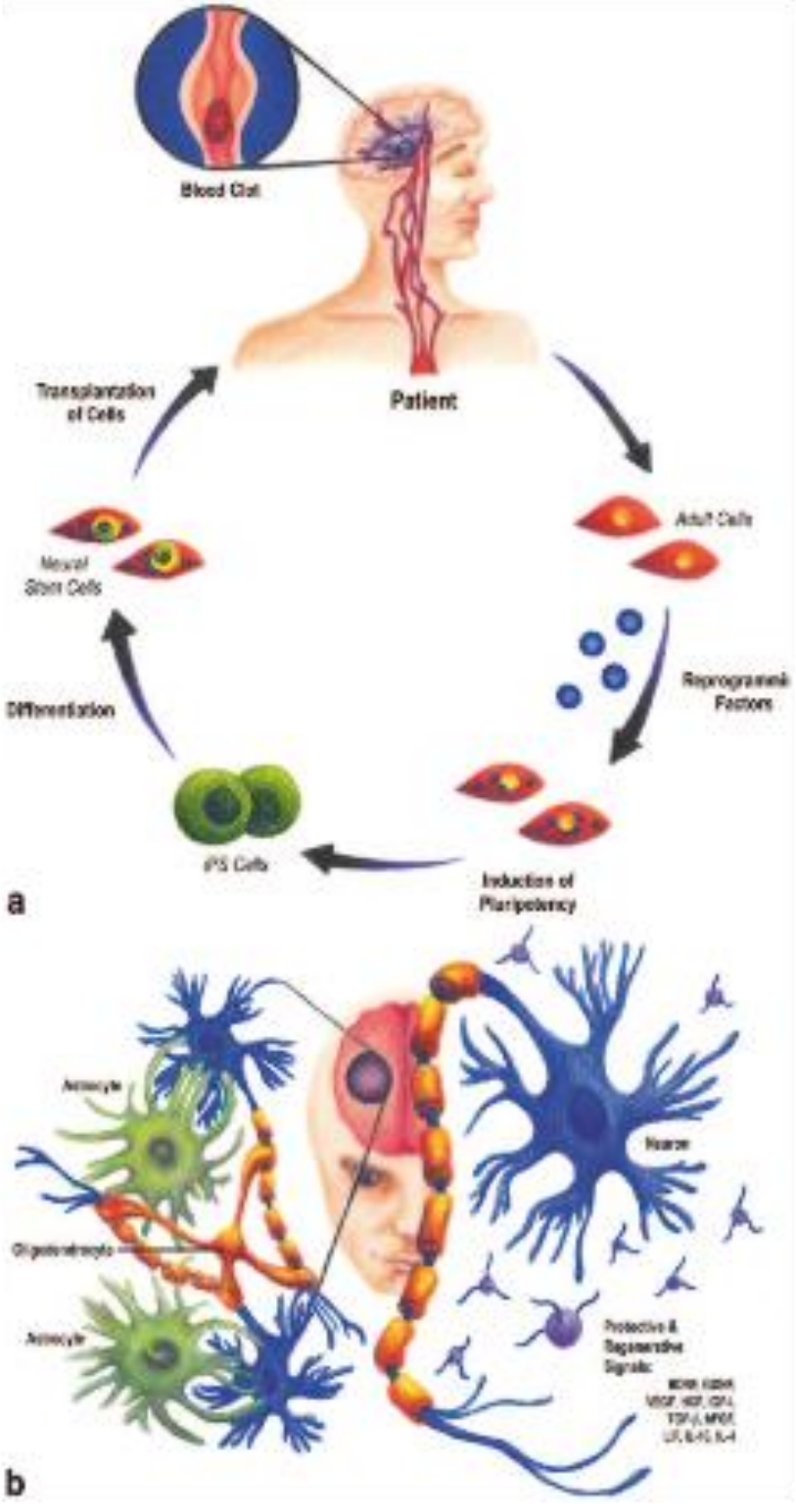
Blood Clot
Patient
Transplantation of Cells
Adult Cells
Neural Stem Cells
Reprogramming Factors
Differentiation
iPS Cells
Induction of Pluripotency
a
Astrocyte
Neuron
Oligodendrocyte
Astrocyte
Protective & Regenerative Signals:
b

Fig. 3.1 iNSC functioning as a cell-replacement therapy and as a producer of regenerative therapeutics for stroke patients. **a** A patient who has an ischemic or hemorrhagic stroke (ischemic stroke shown) experiences significant brain tissue damage and loss. iNSCs could be generated from the patient's own body by collecting adult somatic cells and reprogramming these cells using pluripotency transcription factors into induced pluripotent stem cells (*iPSCs*). iPSCs could then be differentiated into iNSCs and transplanted back into the patient where they would differentiate into neurons and glia that functionally integrate into the site of injury. **b** Transplanted iNSCs and dif- ferentiated cells have been shown to produce and may generate other regenerative and protective signaling factors including vascular endothelial growth factor (*VEGF*) and interleukin-10 (*IL-10*). (Illustration by Leah K. Schultz)

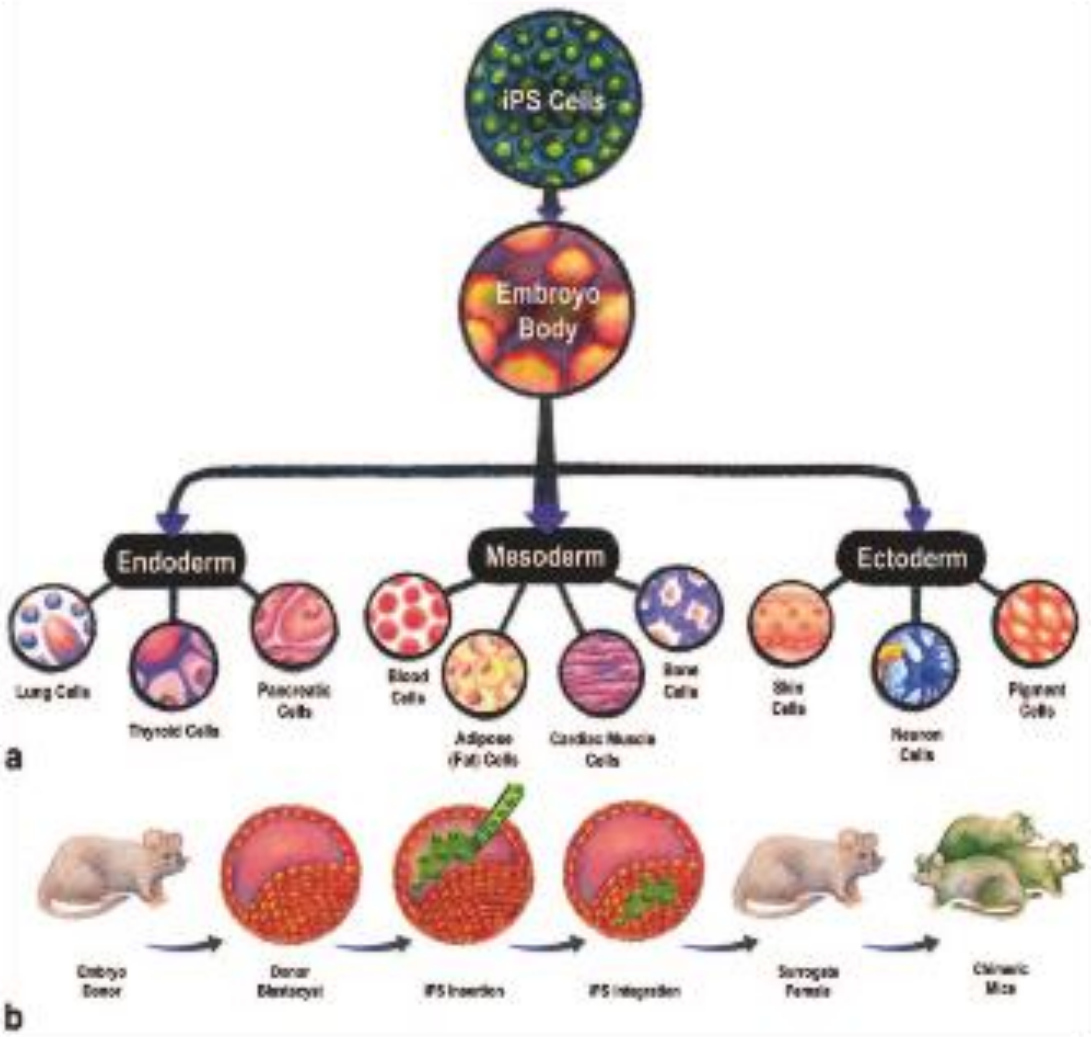
iPS Cells
Embryo Body
Endoderm
Mesoderm
Ectoderm
Lung Cells
Thyroid Cells
Pancreatic Cells
Blood Cells
Adipose (Fat) Cells
Cardiac Muscle Cells
Bone Cells
Skin Cells
Neuron Cells
Pigment Cells
a
Embryo Donor
Donor Blastocyst
IPS Insertion
IPS Integration
Surrogate Female
Chimeric Mice
b

Fig. 3.2 iPSCs are Capable of Differentiating into Any Cell type in the Body In Vitro and In Vivo. (A) To test iPSCs for their ability to form cell types of all three germ layers, a defining characteristic of iPSCs, EB differentiation is commonly used. iPSCs are induced to form large masses of cells reminiscent of developing embryos that induces spontaneous cell signaling that leads to the formation of endoderm, mesoderm and ectoderm. Cell types representative of these lineages are commonly confirmed by immunocytochemistry of specific cell type marker expression. For example, cells can be immunostained for the neuron marker MAP2 to identify cells of the ectoderm lineage. **(B)** To more stringently test the functional capacity of iPSCs, chimera formation is commonly performed. Embryos are collected from donor animals and are injected with iPSCs. iPSCs integrate and are transferred to a surrogate female. As the embryos develop, the integrated iPSCs are incorporated into tissues throughout the animal's body. Chimeric offspring are then composed of cells from the donor embryo and the inserted iPSCs.

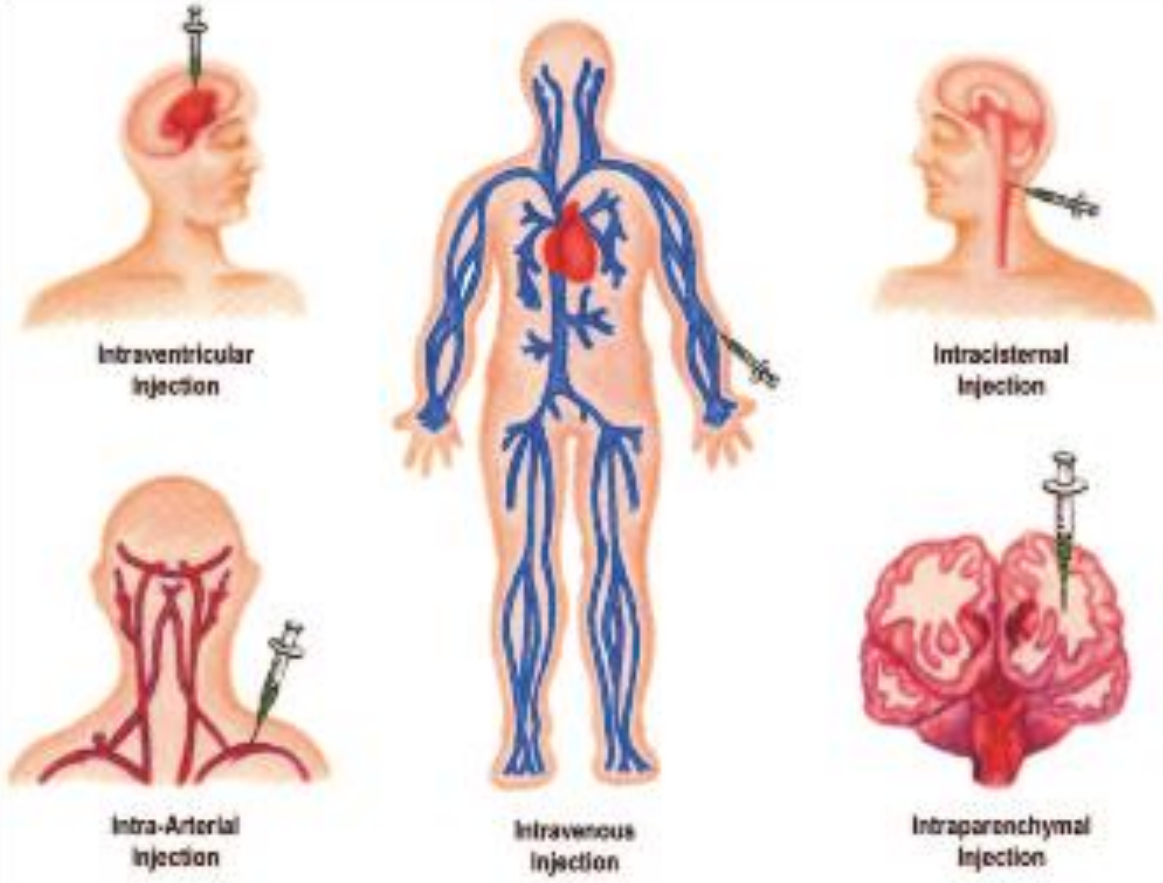
Intraventricular injection
Intracisternal injection
Intra-Arterial injection
Intravenous injection
Intraparenchymal injection

Fig. 3.3 Routes of iNSC transplantation. iNSCs can be transplanted utilizing a number of approaches: intravenous, intra-arterial, intracisternal, intraparenchymal, and intraventricular. Intravenous routes are the least invasive and least technically challenging approach with cells being injected into a peripheral vein of the patient. Successfully transplanted cell numbers are generally low with IV injections as many cells are lost to the pulmonary first-pass effect. Intra-arterial injections of iNSCs provide superior cell delivery but are associated with increased morbidity from thrombosis and hemorrhage. Intracisternal injections are moderately invasive with cells being injected nto one of the subarachnoid cisterns (injection into cisterna magna shown in figure). Intraparenchymal and intraventricular injections allow more direct cell delivery but are relatively more invasive and require a transcranial approach with injection directly into the brain matter or lateral ventricles respectively. (Illustration by Leah K. Schultz)

CHAPTER 4:

HUMAN iNPC THERAPY LEADS TO IMPROVEMENT IN FUNCTIONAL NEUROLOGIC OUTCOMES IN A PIG ISCHEMIC STROKE MODEL[1]

[1] Lau, V.W., S.R. Platt, H.E. Grace, E.W. Baker, and F.D. West. To be submitted to *Cellular Reprogramming*

4.1 ABSTRACT

Stroke is the leading cause of disability in the United States but current therapies are extremely limited and exhibit no regenerative potential. Previous translational failures from bench to beside have highlighted the need for large animal models of ischemic stroke and for improved assessments of functional outcomes. The aims of this study were first, to create a post-stroke functional outcome assessment scale in a porcine model of middle cerebral artery occlusion (MCAO) and second, to use this scale to determine the effect of human induced-pluripotent cell derived neural progenitor cells (iNPCs) on functional outcome in this large animal stroke model. The developed scale was able to consistently determine differences between healthy and stroked pigs at all time points. iNPC-treated pigs showed a significantly faster recovery in their overall scores relative to vehicle-only treated pigs with the parameters of appetite and body posture exhibiting the most improvement in the iNPC-treated group. In this study, we developed a robust and repeatable functional assessment tool that can reliably detect stroke and recovery, while also showing for the first time that human iNPC therapy leads to functional recovery in a translational pig ischemic stroke model. These promising results suggest that iNPCs may one day serve as a first in class cell therapeutic for ischemic stroke.

4.2 INTRODUCTION

Stroke is the leading cause of disability in the United States and the second-leading cause of death in the world [1]. Despite considerable efforts, the vast majority of laboratory-developed stroke therapies have failed to translate into clinically effective treatments [2, 3]. Current Food and Drug Administration (FDA)-approved stroke therapies are limited to tissue plasminogen activator (tPA) and a limited number of stent-retriever devices [4]. These therapies are only

effective in patients during the acute phase of injury and do not carry any regenerative potential for damaged tissues [5].

Induced-pluripotent stem cell derived neural progenitor cells (iNPCs) have recently garnered significant interest as a personalized regenerative cell therapy for stroke [5, 6]. iNPCs can potentially be derived from the patient's own body, thus eliminating the potential of rejection upon transplantation [7, 8]. Several groups have already demonstrated beneficial effects of iNPCs in rodent models of ischemic stroke [9-15]. iNPCs grafts implanted into rodents after middle cerebral artery occlusion (MCAO) were able to survive for up to five months with no evidence of tumorigenesis [10, 12]. In addition, rodents receiving iNPCs following MCAO demonstrated improved functional recovery on various tests including the sticky tape/adhesive removal test, staircase test, rotarod test, and the modified neurologic severity score (mNSS)[10, 12-14, 16]. While iPSC-derived therapies hold great promise in rodent stroke models, previous translational failures between rodents and humans indicate the need for further evaluation of this therapy before proceeding to clinical trials.

The repeated failures in translation between the laboratory and the clinical setting prompted the development of the stroke therapy academic and industry roundtable (STAIR) recommendations [2, 3, 17, 18]. One of the recommendations included pre-clinical testing of developed therapies in multiple animal models, preferably with inclusion of a gyrencephalic species [18]. To address this need, our research group recently developed a gyrencephalic pig model of permanent right middle cerebral artery (MCA) ischemic stroke [19]. Repeatable and reliable structural lesions in the pig model were demonstrated with both magnetic resonance imaging (MRI) and histology; however, functional outcome assessment was only limited to gait analysis [20]. Another STAIR roundtable recommendation was to report the effect of proposed

therapies on the acute and long-term functional outcomes of tested animal models [17]. The failure of many stroke therapy clinical trials can partially be blamed on a lack of long-term functional outcome evaluation in the preclinical animal model [18, 21-23]. In humans, functional recovery can be thought of in terms of various neurologic domains: motor, sensory, vision, affection, cognition, and language [24]. Following a stroke, these domains are assessed using a variety of scales and indices including the modified Rankin Score (mRS), American Heart Association Stroke Outcome Classification Score(AHA.SOC), Barthel index (BI), NIH Stroke Scale (NIHSS), the Glasgow Outcome Scale (GOS) and the Canadian Neurologic Scale (CNS) [25]. The goal of these evaluations is to determine the degree of neurologic recovery and function through quantification of patient neurologic deficits, quality of life, and functional independence in activities of daily living [25]. The importance of these scales is most evident in the context of randomized clinical trials, most of which use the mRS or BI as the standard to dichotomize good from poor outcomes[26].

The vast majority of ischemic strokes in humans involve the MCA, and several well-established rodent models of MCAO exist [27-29]. In MCAO, affected brain regions usually include large areas of the sensorimotor cortex and basal nuclei [28, 30, 31]. Humans affected by MCAO can develop symptoms including, but not limited to, aphasia, hemiparesis, hemineglect, dysphagia, impaired cognition, and urinary/fecal incontinence [32]. Many of the criteria and scoring parameters assess language, emotion, and cognition, however, they do not translate well into non-human species. As such, several animal-specific scales and tests have been designed to assess similar representative outcomes in laboratory species[22, 23, 33]. These tests have been designed to assess learning, memory, motor skills, and asymmetrical neurologic deficits [30].

Functional outcome assessments in rodent models of stroke are extremely variable and a gold standard does not exist [21-23]. Many studies employ a composite scoring system (Bederson Scale or mNSS) and/or perform a multitude of specific tests (e.g. Rotarod, Morris Water Maze) and grade each individually [21, 23, 34, 35]. One of the earliest composite neurologic grading systems is the Bederson Scale [21, 28]. A large number of grading systems are based on this scale, which uses limb placement and circling as measurements of sensorimotor deficits[28, 29, 34, 36]. Another popular composite score scale is the mNSS, which takes into account sensorimotor function, reflex, and balance tests [34]. However, there currently is no commonly performed composite scoring system for the pig stroke model.

The purpose of this study is to create a post-stroke assessment scale for pigs that have undergone stroke surgery. We demonstrate that this scale is able to consistently determine differences between non-stroked and stroked animals with high repeatability between multiple assessors. In addition, we show the effects of human iNPCs on postural reactions, posture, mental status, and appetite in a pig model of permanent right MCAO.

4.3 MATERIAL AND METHODS

All procedures were conducted under guidelines approved by the University of Georgia Institutional Animal Care and Use Committee.

4.3.1 Animals

Eight male castrated six-month old Landrace pigs were used in the study. All pigs were obtained from the University of Georgia Swine unit and weighed between 65-80kg at the start of the study and between 110-130kg at the end of twelve weeks.

4.3.2 Permanent Right Middle Cerebral Artery Occlusion

Pigs were sedated with an intramuscular injection containing xylazine (5mg/kg), ketamine (5mg/kg), midazolam (0.2mg/kg) and butorphanol (0.2mg/kg). All pigs were intubated with a cuffed endotracheal tube and maintained under gas anesthesia with administration of 1 to 2% isoflurane and oxygen. Mechanical ventilation was performed at a rate of 8 to 12 breaths/minute using a tidal volume of 5 to 10mL/kg throughout anesthesia. Intravenous fluids (Lactated Ringers Solution) were administered at a rate of 5 to 10mL/kg throughout anesthesia. Further analgesia was provided with intramuscular injections of flunixin meglumine (Banamine®-S) 2.2mg/kg administered 30 minutes prior to surgery and every 24 hours thereafter for three days post-operatively. Ceftiofur sodium (Naxcel®) 4.4mg/kg was administered intramuscularly at least 30 minutes prior to surgery. Doses were repeated every 24 hours for three days following surgery.

The surgical technique used to crate a permanent right MCAO has been described in detail previously [19]. Briefly, a right frontotemporal craniectomy and orbital rim ostectomy with partial zygomatic arch resection was performed on each animal. Bipolar cautery was used to permanently occlude the right MCA and some of its collateral branches near its origin. A 2cmx2cm piece of porcine urinary bladder mucosa (ACell Vet ^{TM}) was placed over the craniectomy prior to closure.

4.3.3 Magnetic Resonance Imaging (MRI)

Twenty-four hours following induction of right MCAO, each pig underwent MRI evaluation of the brain using a Siemens 16-channel fixed-site 1.5T MRI system. Pigs were sedated with xylazine (5mg/kg), midazolam (0.2mg/kg), and butorphanol (0.2mg/kg) administered intramuscularly. All pigs were intubated with cuffed endotracheal tubes and

maintained on 1-2% isoflurane throughout the MRI procedure. Peripheral intravenous catheters (18 to 22g) were placed in the left or right auricular vein and in the left or right accessory cephalic veins for administration of Lactated Ringers Solution (LRS) at a rate of 5-10mL/hour during anesthesia.

T1-weighted, T2-weighted, T2 fluid attenuated inversion recovery (FLAIR), and diffusion weighted imaging (DWI) were performed in sagittal, transverse, and dorsal planes. Apparent diffusion coefficient (ADC) maps were generated from the DWI sequence.

4.3.4 Human induced pluripotent-cell derived neural progenitor cells (iNPCs)

HIP™ human neural stem cells (GlobalStem®, Rockville, MD; hereafter "iNPC") were expanded on Matrigel ™ diluted 1:100 with Neurobasal Medium (Thermo Fisher Scientific) and kept under a passage number of 20. Daily media changes were performed using Neurobasal media (Thermo Fisher Scientific) supplemented with 2% B-27 Supplement (Thermo Fisher Scientific), 1% non-essential amino acids (Life Technologies), 2mM L-glutamine (Life Technologies), 1% penicillin/streptomycin (Invitrogen), and 20ng/mL basic fibroblast growth factor (bFGF) (R&D systems). Upon confluence, cells were enzymatically suspended for passage using Accutase (Innovative Cell Technologies) and replated at a density of 1:4.

4.3.5 Immunocytochemistry

iNPC were plated onto Matrigel-coated four-chambered glass slides for immunocytochemistry. Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 15 minutes and permeabilized with 0.1% Triton X-100, 1% Polyvinylpyrrolidone (PVP; Sigma-Aldrich) in a 3% serum blocking solution. Cells were incubated with primary antibodies diluted in blocking solution for one hour at room temperature. Primary antibodies used were Nestin (Neuromics, 1:200) and Sox1 (R&D Systems, 1:20). Alexa Fluor (invitrogen,

1:1000) fluorescently-conjugated secondary antibodies were applied for one hour at room temperature to detect primary antibodies. Cells were washed and mounted with Prolong Gold with DAPI (Life Technologies) and imaged using SlideBook software (Intelligent Imaging innovations) on an Olympus IX-81 microscope with Disc-Spinning Unit (Olympus, Inc.).

4.3.6 Cell Transplantation

For all eight animals that underwent MCAO, the surgical site was reopened five days post-stroke for either a PBS-only injection or an iNPC injection. Four pigs were assigned to each treatment group to receive either a PBS-only (non-treated) or an iNSC injection (iNPC-treated). Pigs were anesthetized using the same protocol as described previously and the surgical site reopened via the same incision. Tissues were gently blunt dissected to the level of the prior craniectomy and the previously placed porcine urinary bladder mucosa (ACell Vet ^{TM}) was removed to expose the brain. For cell injections, the iNPCs were suspended in PBS at a concentration of 150,000cells/µL. Two injections of 33.3µL of the cell solution were administered through a glass syringe (Hamilton Co.) and 24g needle using a microinjector set to deliver the volume at a rate of 2µL/minute. Injections were administered at least 5mm apart in the penumbra region of the stroke (as determined through 24-hour post-stroke MRI evaluation for each individual pig) at a depth of 6mm from the surface of the brain at the junction of cortical gray and white matter. The needle was retracted at a rate of 1mm/minute following injection to prevent backflow of cells. For PBS-only treatments, two injections of 33.3µL of sterile PBS were injected in lieu of the cell solution in an identical manner. Following injections, a 2cmx2cm piece of porcine urinary bladder mucosa (ACell Vet ^{TM}) was placed over the craniectomy site prior to closure. All pigs were treated with flunixin meglumine (Banamine®-S) 2.2mg/kg administered 30 minutes prior to injection surgery and every 24 hours thereafter for three days

post-operatively. Ceftiofur sodium (Naxcel®) 4.4mg/kg was administered intramuscularly at least 30 minutes prior to surgery with doses repeated every 24 hours for three days following surgery.

4.3.7 Post-stroke assessment scale

A porcine post-stroke neurologic assessment scale was created to include evaluation of individual parameters such as mentation, posture, gait, postural reactions, cranial nerves, appetite, and circling (Table 1). This scale was based on previously published post-stroke clinical assessment scales in both pigs and dogs [37, 38]. Postural reactions were assessed by shifting the animal's weight over the center of balance for each individual limb through steady pressure applied by the assessor on the contralateral side of the animal. This was meant to mimic hopping tests performed in veterinary neurologic exams in dogs to assess conscious and unconscious proprioception. The maximum score associated with the highest degree of neurologic deficits was set at 30 and a normal neurologic exam score was set as zero.

Evaluations were performed at least one to three days prior to induction of stroke and repeated 1 day, 3 days, and 5 days post-stroke as well as 1 day, 3 days, 1 week, 2 weeks, 4 weeks, 6 weeks, 9 weeks, and 12 weeks post-injection. All examinations were physically performed by one individual (VL) and filmed with a digital camera (Canon Powershot D10). Filmed examinations were later viewed and scored by a non-blinded observer who was aware of the time points at which pigs were evaluated. All filmed examinations were viewed and scored again by the same non-blinded observer at a later time. Filmed exams were then relabeled and placed in random order for evaluation by an observer blinded to the treatment group and time points of each examination and to the first observer's scores. Only results from the scoring of the

blinded observer were used in the final analysis to determine significance between iNPC-treated and non-treated pigs.

4.3.8 Statistics

All statistical analysis was performed using SAS 9.3 (Cary, NC). A two-way ANOVA and a Tukey's post-hoc t-test were used to compare results between treatment groups and between the first and second assessments of the non-blinded observer and the blinded observer. A p-value < 0.05 was used to determine significance between groups.

4.4 RESULTS

4.4.1 MRI 24-hours post-surgery reveals ischemic stroke in the middle cerebral artery territory

iNPCs showed normal neural stem cell morphology (Fig. 1A) and were found to be positive for the neural progenitor markers SOX1 and Nestin (Fig. 1B-D). Greater than 95% of iNPCs were positive for SOX1 and Nestin. Twenty-four hours following bipolar cauterization of the right MCA, all eight animals underwent MRI evaluation of the brain. An area of increased signal intensity was noted in the distribution of the right MCA in each pig on T2-weighted (Fig. 2A), T2-FLAIR (Fig. 2B), and DWI sequences (Fig. 2C). A corresponding region of decreased signal intensity on ADC maps consistent with cytotoxic edema confirming ischemic infarction was identified in each animal (Fig. 2D).

4.4.2 The post-stroke assessment scale reliably detects functional changes in pigs after MCAO stroke and iNPC treatment

Overall scores obtained through the post-stroke assessment scale were able to demonstrate significant ($p<0.05$) differences between pre-stroke and post-stroke animals at every time point for both observer A (non-blinded) and observer B (blinded) at all time points (Fig. 3A). No detectable differences were noted between initial and repeat assessments performed by

the non-blinded observer ($p<0.05$). Likewise, no significant differences were detected between the overall scores assigned by the blinded observer and the non-blinded observer at any time point ($p<0.05$). This indicates that the assessment scale is reliable both between different observers and between assessments performed by one observer. Furthermore, iNPC-treated pigs showed significant improvement in their overall total post-stroke score relative to one day post-stroke by two weeks post-injection (Fig. 3B). Non-treated pigs did not demonstrate significant improvement until nine weeks post-injection.

4.4.3 iNPC treatment hastened recovery of postural reactions, posture, mental status and appetite following MCAO

Overall, iNPC-treated MCAO stroke pigs demonstrated a faster functional recovery relative to non-treated control pigs; however, unique parameters showed varying outcomes (Fig. 4). An improvement in postural reactions was noted in iNPC-treated pigs in the score between one day post-stroke and scores at two and six weeks post-injection while non-treated pigs did not exhibit improvement in their postural reaction scores over the twelve week testing period (Fig. 4A). iNPC-treated pigs also demonstrated a significant improvement in their body posture scores by one week post-injection compared to five days post-stroke, while non-treated pigs did not show any significant improvements in their body posture scores (Fig. 4B). A similar trend was observed in head posture scores; iNPC-treated pigs exhibited improvement in head posture six and nine weeks post-injection compared to one day post-stroke, whereas non-treated pigs did not show an improvement until twelve weeks post-injection (Fig. 4C). iNPC-treated pigs also showed more rapid recovery of their mental status scores with significant improvements by four weeks post-injection compared to one day post-stroke while this improvement was not observed in the non-treated group until nine weeks post-injection (Fig. 4D). There was also a significantly

improved appetite score in the iNPC-treated pigs by four weeks post-injection relative to one-day post-stroke while no significant improvement in appetite was ever noted in control pigs over twelve weeks (Fig. 4E).

4.4.4 iNPC treatment does not improve rate of recovery of circling tendency, cranial nerve function, or gait

For circling and gait, both treatment groups did not exhibit any significant improvement by twelve weeks post-injection compared to their one-day post-stroke scores (Fig. 5A and 5B). However, the post-stroke scores for both parameters were only mildly elevated, and spontaneous recovery to pre-stroke scores for gait and circling was noted in both treatment groups by one to five days post-stroke. No significant difference was noted between treatment groups at any time point for these parameters. Cranial nerve function scores were significantly different from pre-stroke scores at all time points with no evidence of recovery from one day post-stroke scores over twelve weeks in either treatment group (Fig. 5C).

4.5 DISCUSSION

In this study, we have developed a porcine post-stroke functional outcome assessment scale that was sensitive enough to detect changes between normal and stroked animals, and between iNPC-treated and non-treated pigs with high intra- and inter-observer repeatability. Furthermore, we utilize this assessment scale to show that human iNPC therapy leads to significant improvement in functional neurologic outcome across multiple parameters in a pig ischemic stroke model. Scores for the individual parameters showed more variability between observers; however, this did not affect repeatability of the overall scale score. This supports the use of the overall score of this scale in future studies to assess the effect of iNPC therapy or other novel therapies on neurologic function in pigs following stroke.

Animals that received iNPC-injections demonstrated significantly faster recovery of postural reactions, body posture, head posture, mental status, and appetite relative to non-treated animals. Body posture, head posture, and postural reactions are a reflection of sensorimotor status. The faster rates of recovery noted here are similar to findings in rodent studies of neural progenitor cell therapy following stroke [13, 14, 16]. Head and body posture scores were designed to broadly assess the sense of balance as well as unconscious and conscious proprioception similar to the beam walk and rotarod tests used in rodents. The improvements in these parameters are similar to improvements in of the aforementioned tests noted in rodents following iNPC therapy as shown by Eckert et al. (2015) and Chang et al. (2013). Postural reaction assessments in the pigs in this study were scored similarly to the sensory test portion of the rodent mNSS and were designed to test unconscious and conscious proprioception. Rodent mNSS scores were previously demonstrated to improve more rapidly following iNPC injections by Gomi et al. (2012) and Chang et al (2013). In contrast to the rodent studies, however, the rapid recovery in the iNPC-treated pigs did not result in significant differences in neurologic scores between iNPC-treated and non-treated groups by the endpoint of the study (twelve weeks post-stroke) due to spontaneous recovery of the non-treated pigs. This difference may be due to the much shorter follow-up times in rodent studies with endpoints being nine weeks or fewer, thus allowing less time for spontaneous recovery to occur in the chronic stage post-stroke. An exception to this is the study by Polentes et al. in which rodents were followed for four months following stroke and cell transplantation [9]. In their study, animals receiving cell grafts demonstrated sustained improvements over vehicle-injected animals for tape-removal and apomorphin-induced rotation behavioral tests. However, both grafted and non-grafted animals demonstrated spontaneous recovery simultaneously on assessments of the Montaya stair case test

and the mNSS within one month post-stroke [9]. Scores for individual parameters within the stroke scale did not retain significant differences from pre-stroke scores throughout the twelve week test period indicating spontaneous recovery in both treatment groups. This is similar to spontaneous recovery rates in humans which has been reported to occur by about ten weeks post-stroke; albeit the degree of recovery in humans is significantly less [39].

In addition to faster sensorimotor recovery, we show that iNPC-treated pigs also demonstrated faster recovery of appetite over control animals. Human post-stroke outcome assessment scales such as the BI and Functional Independence Measure (FIM) account for activities of daily living such as feeding and urinary/fecal continence [39, 40]. It has been proposed that scales incorporating activities of daily living, such as feeding, are more sensitive to the level of disability and recovery following ischemic stroke than scales like the Modified Rankin Score (MRS)[40]. If tasks like feeding are more sensitive, the rapid improvement of appetite noted in the iNPC-treated pigs and lack of improvement in the control pigs may be the most compelling indicator that iNPC therapy improved recovery in pigs following MCAO.

Cranial nerve function did not exhibit significant improvement in either treatment group over the twelve-week testing period. The most common cranial nerve deficits were contralateral menace response and facial hypalgesia, which are consistent with injury to the sensorimotor cortex. The lack of significant improvement may be due to the grading scheme for cranial nerve deficits where higher scores were more reflective of midbrain and medullary dysfunction, which are areas that are not injured by MCAO. Future cranial nerve scores may be more sensitive if more weight is placed on menace response and facial hypalgesia rather than testing cranial nerves that originate in the midbrain or medulla.

Both treatment groups showed spontaneous improvement in their gait and circling scores within a few days post-stroke before iNPC treatment occurred. The spontaneous recovery of gait scores by three days post-MCAO in this study may be a reflection of the predominant role of extrapyramidal brainstem centers in gait generation in pigs rather than corticospinal tracts [41]. In addition, the method of gait assessment used in this study (gross visual observation) may not have been as sensitive to dysfunction as computerized gait analysis performed with limb stride or step height measurements [20]. More specialized gait analyses, however, require special equipment and setup, which would make the post-stroke assessment scale less user-friendly and globally transferable. Circling was placed in the scale as a crude measurement of cognitive dysfunction and hemi-inattention and was graded on a scale from 0 to 2, making it one of the smallest contributors to the overall score. The narrow grading scale for this parameter may have altered its sensitivity and accuracy. Specifically, this test may not have been sensitive if the pigs were not observed for prolonged periods of walking. An induced-rotation test, such as the apomorphine test used in rodents, may need to be developed in order to detect more subtle differences between treatment groups [34, 42, 43]. The development of such tests for pigs following MCAO warrants further investigation.

The faster recovery noted in the overall score of iNPC-treated pigs could be a reflection of the anti-inflammatory and trophic factors secreted by iNPCs. The rapid onset of improvement in the cell-treated group and lack of significant difference between treatment groups at 12 weeks post-injection would argue against neuronal regeneration being the major mechanism of action. iNPCs have previously been reported to reduce inflammatory cytokines such as TNF-α, IL-6, and IL-1β, in addition to reducing microglial activation and mitigating neuronal loss which is correlated to improved neurological outcome [9, 10, 15, 16].

Given the spontaneous recovery seen in gait and circling, these parameters may be excluded from future post-stroke functional outcome assessment scales. Alternatively, more sensitive testing for these parameters, possibly through development of a porcine apomorphine-induced rotation test and/or computerized and measured gait analyses, may allow for better detection of disability and differences between treatment groups in future studies. The disparity between appetite scores of the iNPC-treated and non-treated pigs may indicate that this is the most sensitive parameter in the functional outcome scale [40]. In addition, appetite may also be the most translatable to human functional outcome scales, and future modifications to the porcine scale should be weighted accordingly.

The post-stroke assessment scale designed in this study offers a robust and repeatable means of evaluating functional outcomes in a large animal model of stroke. This will be of significant value to the field as future studies focus on the pig as a translational animal model for neural disease and injury. In addition, we demonstrated for the first time that iNPC therapy shortened functional recovery time in a large animal model. Shorter recovery times could have significant effects on human quality of life and the cost associated with post-stroke hospitalization and long-term care.

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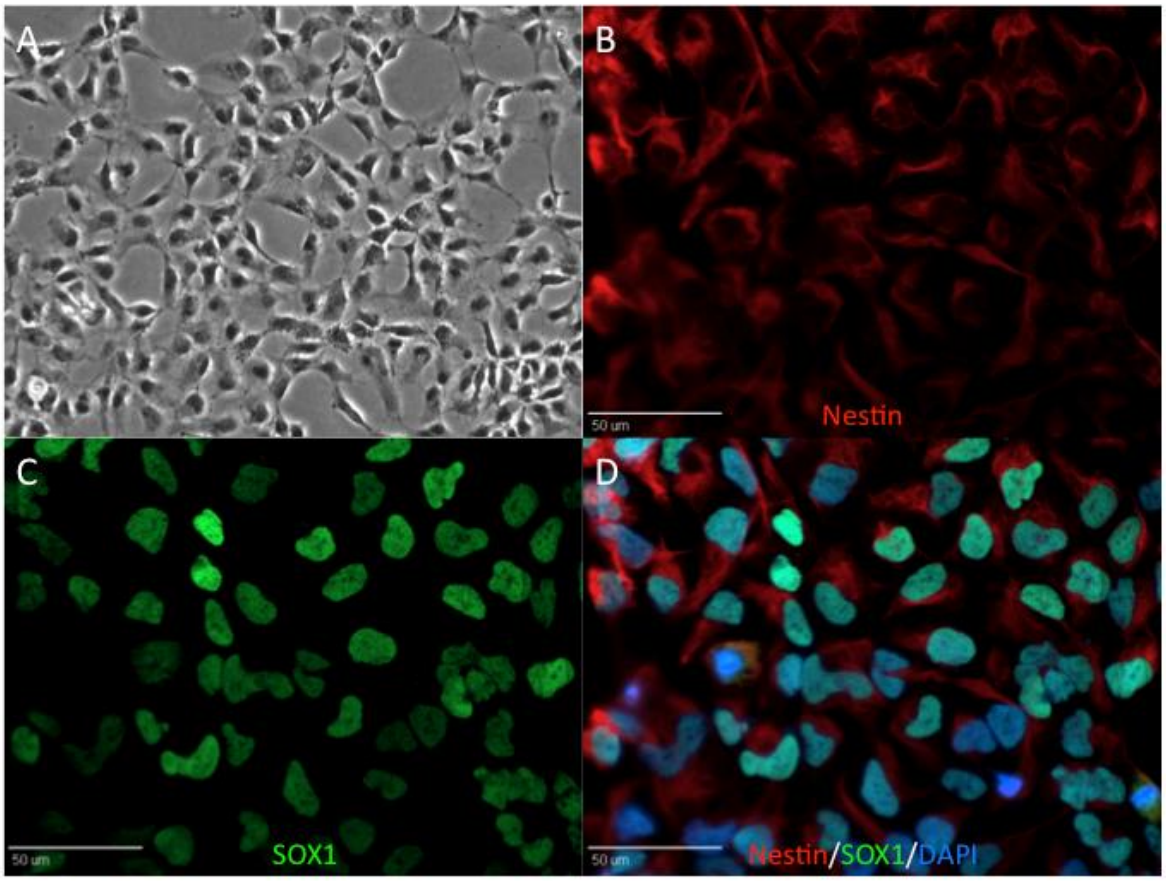
A
B
Nestin
50 um
C
SOX1
50 um
D
Nestin/SOX1/DAPI
50 um

FIG. 4.1. iNPCs demonstrate typical neural stem cell morphology on phase contrast at 20X magnification (A). Immunocytochemistry demonstrates positive expression of neural stem cell markers Nestin (B) and SOX1 (C). Merged image with DAPI (D).

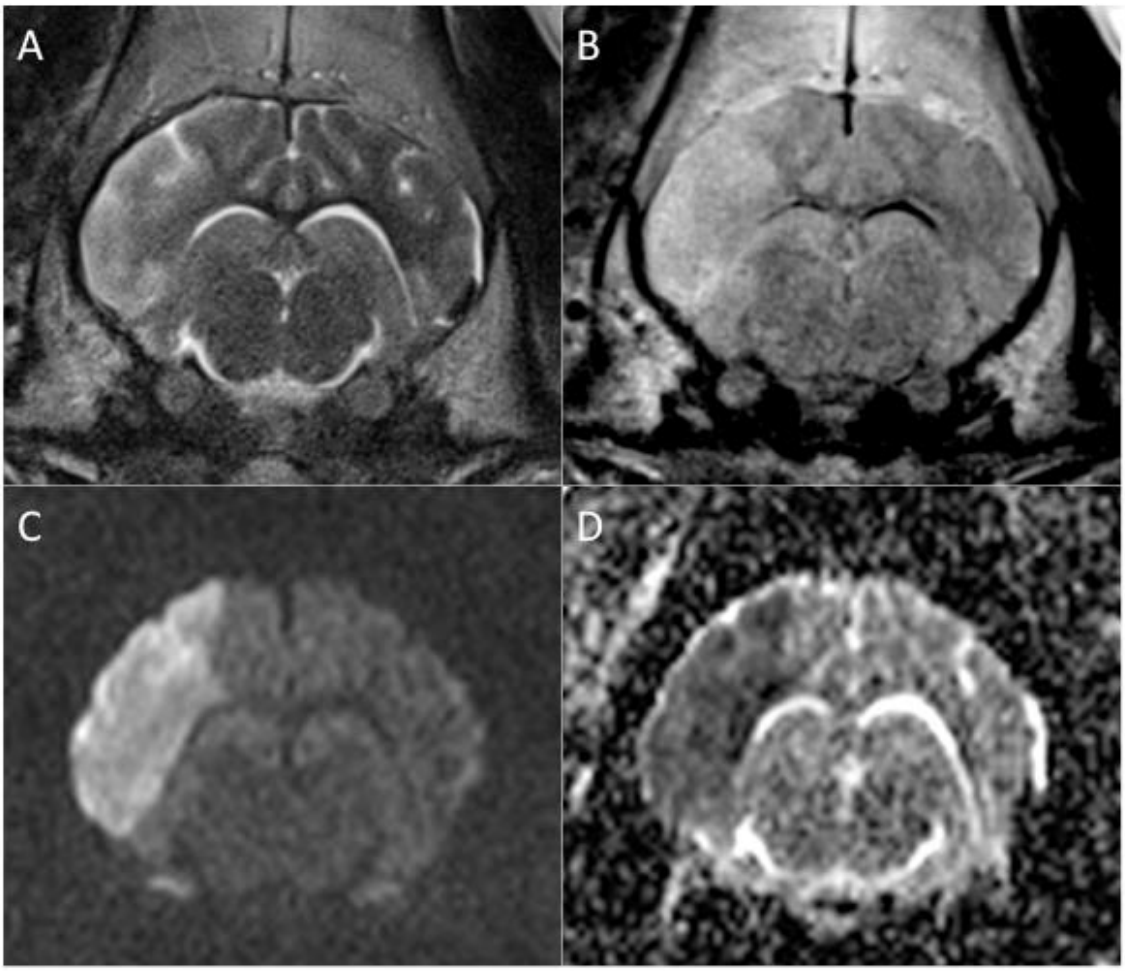
A
B
C
D

FIG. 4.2. MRI performed 24 hours following MCAO demonstrates ischemic stroke in the territory of the middle cerebral artery. The affected area is hyperintense on T2-weighted imaging (A) and T2 FLAIR (B) relative to normal grey matter. The region is hyperintense on DWI (C) with corresponding hypointensity on the ADC map (D) confirming cytotoxic edema.

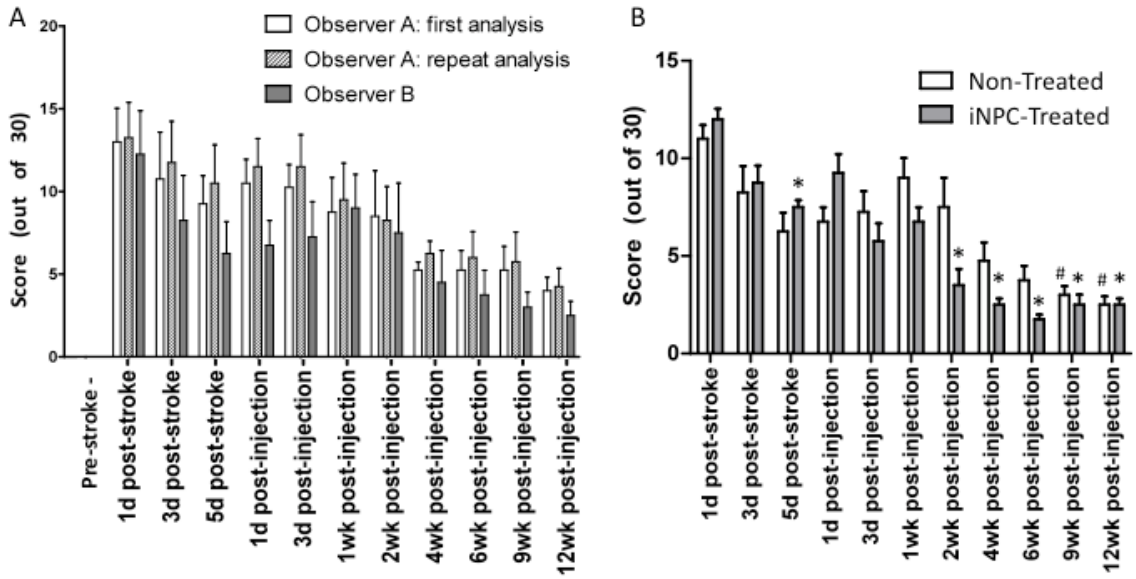
A
Observer A: first analysis
Observer A: repeat analysis
Observer B
Score (out of 30)
20
15
10
5
0
Pre-stroke -
1d post-stroke -
3d post-stroke -
5d post-stroke -
1d post-injection -
3d post-injection -
1wk post-injection -
2wk post-injection -
4wk post-injection -
6wk post-injection -
9wk post-injection -
12wk post-injection -
B
Non-Treated
iNPC-Treated
Score (out of 30)
15
10
5
0
1d post-stroke
3d post-stroke
5d post-stroke
1d post-injection
3d post-injection
1wk post-injection
2wk post-injection
4wk post-injection
6wk post-injection
9wk post-injection
12wk post-injection

FIG. 4.3. All post-stroke time points for both treatment groups were significantly different from pre-stroke scores (A). No significant differences were noted between observers or between different assessments by the same observer at any time point in pigs following MCAO (A). iNPC-treated animals showed significant improvement (*) from one-day post-stroke scores by two weeks following iNPC-injection ($p<0.05$) whereas non-treated animals did not reach this improvement level until 9 weeks post-injection (#) (B).

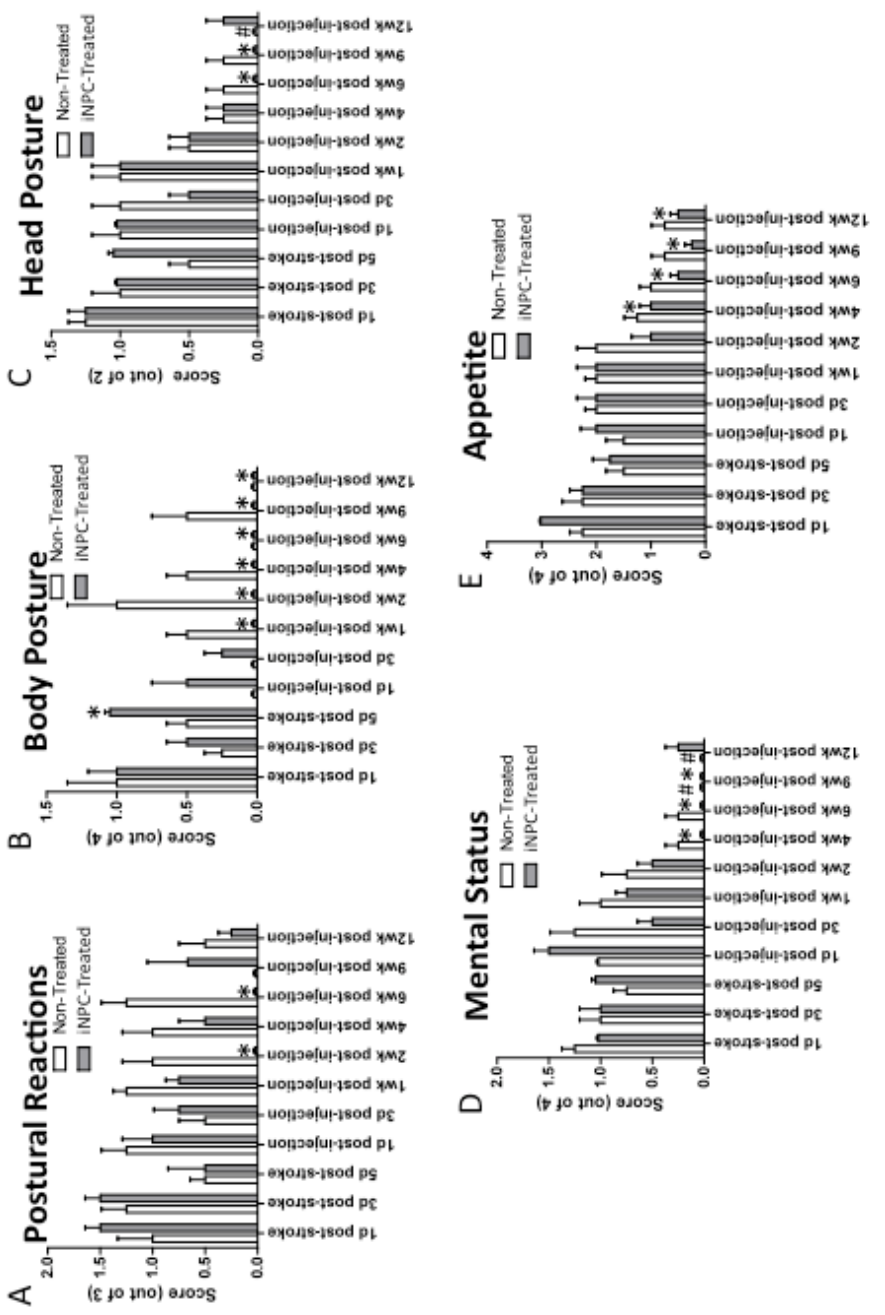
A
Postural Reactions
Non-Treated
iNPC-Treated
Score (out of 3)
B
Body Posture
Score (out of 4)
C
Head Posture
Score (out of 2)
D
Mental Status
Score (out of 4)
E
Appetite
Score (out of 4)
1d post-stroke
3d post-stroke
5d post-stroke
1d post-injection
3d post-injection
1wk post-injection
2wk post-injection
4wk post-injection
6wk post-injection
9wk post-injection
12wk post-injection

FIG. 4.4. Significant improvement in postural reaction scores were noted in the iNPC treated group by two and six weeks post-injection (A) whereas non-treated pigs did not exhibit any improvement over twelve weeks. The body posture scores appeared to improve by one week post-injection in iNPC-treated pigs (B) with no improvement ever noted in the non-treated pigs. Significant improvements in head posture scores were noted by six weeks post-injection in iNPC-treated pigs but not until twelve weeks post-injection in non-treated pigs (C). Improvements in mental status scores were noted by four weeks post-injection in iNPC-treated pigs whereas non-treated pigs did not show improvement until nine weeks post-injection (D). Appetite scores of iNPC-treated pigs improved by four weeks post-injection while non-treated pigs did not show significantly improved appetites throughout the twelve-week period (E). * represent time points in the iNPC-treated group where scores were significantly different from one day post-stroke scores ($p<0.05$). * in the body posture graph (B) are an exception in that these were time points significantly different from five days post-stroke ($p<0.05$). # represent time points where the non-treated scores were significantly improved from one day post-stroke scores.

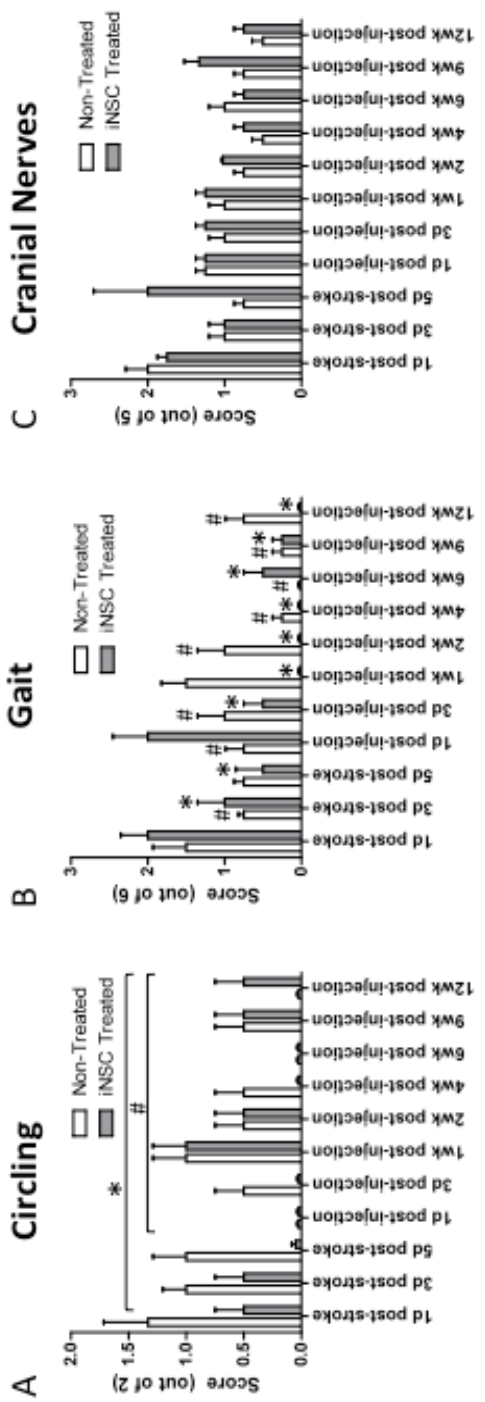
A
Circling
Non-Treated
iNSC Treated
Score (out of 2)
2.0
1.5
1.0
0.5
0.0
1d post-stroke
3d post-stroke
5d post-stroke
1d post-injection
3d post-injection
1wk post-injection
2wk post-injection
4wk post-injection
6wk post-injection
9wk post-injection
12wk post-injection
B
Gait
Non-Treated
iNSC Treated
Score (out of 5)
3
2
1
0
1d post-stroke
3d post-stroke
5d post-stroke
1d post-injection
3d post-injection
1wk post-injection
2wk post-injection
4wk post-injection
6wk post-injection
9wk post-injection
12wk post-injection
C
Cranial Nerves
Non-Treated
iNSC Treated
Score (out of 5)
3
2
1
0
1d post-stroke
3d post-stroke
5d post-stroke
1d post-injection
3d post-injection
1wk post-injection
2wk post-injection
4wk post-injection
6wk post-injection
9wk post-injection
12wk post-injection

FIG. 4.5. Spontaneous recovery was noted in circling and gait scores within a few days post-MCAO (A and B). * represent time points where scores recovered to pre-stroke levels in iNPC-treated pigs. # represent time points where scores recovered to pre-stroke levels in non-treated pigs.

TABLE 4.1. The post-stroke assessment scale. Individual parameters were scored out of a range of 2 to 6. The highest possible score for animals most severely affected is 30 with normal or pre-stroke animals scoring 0. More points were allotted to parameters that would have a more serious consequence for the pig such as appetite, gait, and mental status whereas parameters such as head posture were allotted fewer points. Cranial nerves were given a higher allotment of points to incorporate the significance of brainstem deficits as assessed in coma-scores in the acute stroke patient.

MENTAL STATUS	
Alert	0
Depressed/lethargic	1
Demented	2
Stuporous	3
Comatose	4
APPETITE	
Eating well with no assistance (consumed all feed in 30 minutes)	0
Consumed more than 50% of food with no assistance in 30 minutes	1
Consumed 50% of food in 30 minutes with no assistance	2
Consumed less than 50% of food in 30 minutes with no assistance	3
Anorexic (not eating)	4
HEAD POSTURE	
Erect/normal	0
Head raised on stimulation	1
Unable to raise head	2
BODY POSTURE	
Normal	0
Leaning to one side	1
Falling to one side	2
Extensor rigidity of limbs and alert (decerebellate)	3
Extensor rigidity of limbs and stuporous (decerebrate)	4
CIRCLING	
No circling	0
Intermittent circling (note side)	1
Consistently circling (note side), or non-ambulatory	2
GAIT	
Normal all four limbs	0
Ambulatory with weakness of one limb (note limb)	1
Ambulatory with weakness/ataxia of both limbs on one side (note side)	2
Ambulatory with weakness/ataxia of all four limbs	3
Non ambulatory with intact motor movement of all limbs	4
Non ambulatory with paralysis of any of the limbs and intact nociception (note limbs)	5
Non ambulatory with paralysis of any of the limbs and loss of nociception (note limbs)	6
POSTURAL REACTIONS	
Hopping normal in all four limbs	0
Hopping slow in one limb (note limb)	1
Hopping slow in more than one limb (note limb(s))	2
Hopping absent in one or more limbs (note limb(s)) or non-ambulatory	3
CRANIAL NERVES	
Normal (no deficits)	0
Absent menace response unilaterally (note eye) with normal palpebral reflexes	1
Facial palsy with reduced palpebral reflexes and/or facial hypalgesia (note side)	2
Any of the above deficits with slow pupillary light reflexes and normal to reduced oculocephalic reflexes	3
Pinpoint pupils with reduced to absent oculocephalic reflexes +/- pathologic/spontaneous nystagmus	4
Unilateral or bilateral unresponsive mydriasis with reduced to absent oculocephalic reflexes	5

CHAPTER 5

CONCLUSIONS

Stroke is the leading cause of disability in the United States with one in six people in the world experiencing a stroke in their lifetime[1]. Despite intense research efforts into effective stroke therapies, limited FDA-approved treatment options exist. The Stroke Therapy Academic Industry Roundtable (STAIR) recommendations outline the need for rigorous testing in transitional large animal models of all proposed therapies [2]. These recommendations also highlight the need for therapies that work through multiple mechanisms of action to improve, not only structural, but functional outcome in patients following stroke[2-4]. Thus, the aims of this study were firstly, to develop a post-stroke functional outcome assessment scale that could be used in a porcine animal model of stroke and secondly, to use the developed scale to evaluate the effects of human induced pluripotent stem cell-derived neural progenitor cells (iNPCs) on functional outcome in pigs following middle cerebral artery occlusion.

To meet the first aim of this study, we developed a post-stroke functional assessment scale to be used in a porcine model of ischemic stroke. This scale was shown to have repeatable intra-observer results in addition to inter-observer results. For every time point within the 12 week study period following ischemic stroke, the developed scale was able to show a significant difference between pre-stroke (unaffected) function and post-stroke function (affected) in pigs.

To meet the second aim of this study, the developed scale was used to assess the effect of iNPC treatment on functional outcome in pigs following middle cerebral artery occlusion. Pigs that received iNPCs demonstrated faster recovery of postural reactions, body posture, head

posture, mental status, and appetite relative to control animals. The faster recovery of appetite is especially exciting as this is an important functional outcome parameter in humans following stroke[5].

The developed post-stroke functional outcome assessment scale provides a robust and repeatable means of evaluating functional outcomes in a large animal model of stroke. This will be of significant value to the field as future studies focus on the pig as a translational animal model for neural disease and injury. In addition, we demonstrated for the first time that iNPC therapy shortened functional recovery time in a large animal stroke model. Shorter recovery times have monumental implications for human quality of life and the cost associated with prolonged hospitalization and long-term care.

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