Pig Models of Neurodegenerative Disorders: Utilization in Cell Replacement-Based Preclinical Safety and Efficacy Studies

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ABSTRACT

An important component for successful translation of cell replacement-based therapies into clinical practice is the utilization of large animal models to conduct efficacy and/or safety cell dosing studies. Over the past few decades, several large animal models (dog, cat, nonhuman primate) were developed and employed in cell replacement studies; however, none of these models appears to provide a readily available platform to conduct effective and large-scale preclinical studies. In recent years, numerous pig models of neurodegenerative disorders were developed using both a transgenic approach as well as invasive surgical techniques. The pig model (naïve noninjured animals) was recently used successfully to define the safety and optimal dosing of human spinal stem cells after grafting into the central nervous system (CNS) in immunosuppressed animals. The data from these studies were used in the design of a human clinical protocol used in amyotrophic lateral sclerosis (ALS) patients in a Phase I clinical trial. In addition, a highly inbred (complete major histocompatibility complex [MHC] match) strain of miniature pigs is available which permits the design of comparable MHC combinations between the donor cells and the graft recipient as used in human patients. Jointly, these studies show that the pig model can represent an effective large animal model to be used in preclinical cell replacement modeling. This review summarizes the available pig models of neurodegenerative disorders and the use of some of these models in cell replacement studies. The challenges and potential future directions in more effective use of the pig neurodegenerative models are also discussed. J. Comp. Neurol. 522:2784–2801, 2014.

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The utilization of animal models plays a central role in biomedical research. Experimental animals not only provide insight into the pathogenesis of human diseases, but more important, they prove to be fundamental for development and testing of new pharmacological, surgical, or cell replacement-based therapies. Small animal models (such as rodents) have been proven to be invaluable in basic biology research because of their accessibility and relatively low cost. Their ability to adequately model a wide spectrum of human diseases or syndromes, however, is limited. Therefore, the development and use of large animal models which closely recapitulate the pathophysiology of human-related diseases is crucial for the successful transition of prospective therapies into human clinical trials.

The pig represents one of the large animal models currently used in human disease-related translational research. The pig body size, organ(s) physiology, and anatomical dimensions are similar to humans, thus providing a readily accessible large animal system for preclinical translational modeling. Moreover, in comparison to nonhuman primates, the use of pigs offers several specific advantages including: 1) short gestation interval (120 days), 2) generation of multiple piglets from a single sow (up to 6–12 piglets are typically born), and 3) cost-effectiveness. Importantly, the experimental use of pigs, like every other animal used in research, is subject to regulations and proper research conduct, but does not raise the same ethical concerns with the public as the use of nonhuman primates, making it a readily acceptable animal platform for effective preclinical experimental use. Pigs have become important in modeling a number of human diseases, such as diabetes, cardiovascular disease (myocardial ischemia), and multiple neurodegenerative disorders such as stroke, spinal ischemic, and traumatic injury. In addition, the use of pig-harvested solid organs is extensively being studied for its potential use in human organ xenotransplantation (reviewed in Ekser et al., 2012).

With respect to cell replacement therapies and preclinical safety studies, the use of pigs appears to be clearly superior over other existing large animal models. First, a highly inbred miniature pig strain with partial or near full major histocompatibility complex (MHC) match has been generated, permitting effective syngeneic and/or allogeneic solid organ or cell grafting modeling in which most of the transplantation MHC combinations relevant to human transplantation can be mimicked (Utsugi et al., 2001; Mezrich et al., 2003). Second, well-defined short and medium-term (1–2 months) intravenous immunosuppression drug delivery protocols that lead to consistent xenograft survival (solid organs or specific organ-targeted injection of human cell lines) have been developed (Usvald et al., 2010). Third, in recent years the successful generation of porcine embryonic, induced pluripotent or fetal tissue-derived stem cells has been reported and used for in vivo grafting using several porcine disease models (Vodicka et al., 2008; West et al., 2010; Cheng et al., 2012; Haraguchi et al., 2012; Fujishiro et al., 2013). Fourth, because of similarities in organ dimension (such as brain or spinal cord), the pig is being effectively used in preclinical safety studies to define the optimal dosing of different drugs, neuromodulatory compounds, and transplantable human cell lines to be used in prospective human clinical trials (Nakajima et al., 1992; Svendsen, 2006; Bode et al., 2010; Templin et al., 2012). Finally, sequencing of the porcine genome has been recently completed, thus providing a systematic genetic database to expand the basic research as well as production of custom-made transgenic pigs (reviewed in Bendixen et al., 2010; Groenen et al., 2012). No other large animal model, such as sheep, dog, and primate has these characteristics.

This review provides a comprehensive summary of recent advances in the field of pig neurodegenerative disease modeling, current utilization of such models in cell replacement-based therapies, and preclinical safety cell grafting dosing studies. In addition, the current bottlenecks in the more effective preclinical use of miniature pigs is summarized and discussed, including: 1) the need for syngeneic transplant studies using inbred MHC-compatible pig-derived cell lines, 2) limitations of long-term immunosuppression, and 3) limited resource of well-defined embryonic stem cell lines.

MINIATURE PIG AS A DISEASE PRECLINICAL MODEL

In recent years substantial progress has been made in using pigs as a preclinical model of numerous human diseases (reviewed in Whyte and Prater, 2011). Based on how the disease state is induced, the pig disease models can be classified into two principal categories: 1) transgenic disease models which employ knockout, knockin, or random transgene-integration technology, and 2) central nervous system (CNS) lesion models which use regional delivery of a variety of neurotoxins

to induce local neuronal/glial degeneration, or which directly implement surgically induced lesions in a specified CNS structure. In the following section, all reported porcine neurodegenerative models to date are described and discussed in the context of their validity in mimicking the related human disease condition.

Transgenic neurodegenerative models

The possibility of genetically modifying pigs provides scientists with an extremely powerful tool to model virtually any human genetic mutation-based disease in the background of a large animal model. Numerous such transgenic pigs have been generated using various techniques, comprehensively reviewed by Whyte and Prather (2011). These techniques include microinjection of DNA into pronuclei of zygotes collected from superovulated females; sperm-mediated gene transfer (SMGT) (Webster et al., 2005; Lavitrano et al., 2006); and lentivirus and retrovirus-mediated gene transfer into the porcine oocytes and nuclear transfer and cloning (Cabot et al., 2001; Hofmann et al., 2003; Matsunari et al., 2008). More recently, targeted transgene insertion by recombinase-mediated cassette exchange (RMCE) and somatic cell nuclear transfer (SCNT) enabled integration of an intact gene of interest into loci preselected for transcriptional activity (Jakobsen et al., 2013). Using these techniques, several porcine neurodegenerative models have been developed, including models of retinitis pigmentosa, Alzheimer's disease, Huntington's disease, and spinal muscular atrophy. It is, however, important to note that most of these genetically modified pigs have not displayed typical histopathological and phenotypical characteristics of neurodegenerative diseases that they were developed to model. This is strikingly different to what has been observed in genetically modified rodent (mouse, rat) models. While the primary reason for such differences between small and large animal species is not known, a relatively long lifespan in pigs (15–20 years) and complex brain circuitry may be associated with a much slower clinically defined disease manifestation. It is well known that rodent models have numerous limitations and caveats associated with attempting to model complex and still relatively poorly understood human disorders. These caveats are the reason why, to date, transgenic animal models have not been the panacea for drug discovery that many had hoped for (McGonigle, 2014). As the modeling of neurodegenerative diseases in pigs is currently in its beginnings, in-depth characterization and long-term follow-up of current models is extremely important, as the information acquired from these models will be invaluable when designing new, genetically more precise pig models of these devastating human diseases.

Retinitis pigmentosa model

The first transgenic pig model of human disease was a domestic pig retinitis pigmentosa (RP) model (Petters et al., 1997). A domestic transgenic pig expressing a mutated rhodopsin gene (Pro347Leu) was created using a pronuclear microinjection technique. Similar to human patients with RP, progressive photoreceptor degeneration (of both rods and cones) in the outer nuclear layer of retina as soon as by 3 weeks of age and loss of function by 20 months of age was demonstrated. Recently, Ross et al. (2012) described the successful development of a new model of RP in an inbred miniature pig via SCNT with the most common rhodopsin mutation (Pro23His) found in human autosomal dominant RP. The use of the highly inbred NIH minipig ($SLA^{C/C}$ haplotype) to generate this P23H RP model provided it with several advantages over the P347L model: 1) enables transplantation studies of progenitor or retinal stem cells using syngenic donors due to high histocompatibility, 2) these pigs are about 1/2 to 1/3 of the size of age-matched domestic pigs, and 3) clinical phenotype is expected to be more consistent. Accordingly, this RP model will likely represent a more useful model, particularly once employed in long-term cell replacement-based therapies, mutated gene-corrections and pharmacological studies.

Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disorder, typically manifested by progressive memory loss and corresponding neuronal degeneration in several brain regions, such as in lamina II of the entorhinal cortex and in the CA1 region of the hippocampus (reviewed in Huang and Mucke, 2012). The genetic basis for familial, autosomal dominant AD is linked to mutations in PSEN1, PSEN2, and the amyloid precursor protein gene (APP). These mutations are associated with increased production of the proteolytic fragment of amyloid β (Aβ), which aggregates into fibrils and toxic oligomeric forms, eventually leading to neurodegeneration and corresponding loss of synaptic connectivity (Walsh et al., 2005).

A transgenic porcine model for AD was developed by Kragh et al. (2009) in the Göttingen breed of miniature pigs. After stable insertion of human APP into fibroblasts, one transgenic cell clone was used for SCNT to produce seven healthy transgenic cloned pigs with normal weight gain. The transgenic piglets harbored a single full-length copy of the neuron-specific splice variant of human APP transgene in their genome and showed specific expression of the transgenic protein in brain tissues. This APP splice-variant carried an AD-causing

dominant mutation known as the Swedish mutation. Accumulation of the pathogenic protein and subsequent appearance of a clinically defined functional deficit were predicted to develop with increasing age in 1–2 years (Kragh et al., 2009). However, this prediction was not confirmed, as no significant difference between 1–2-year-old cloned, transgenic AD minipigs and age-matched controls were found using the spontaneous object recognition test (SORT), which is based on behavioral discrimination of familiar and novel objects as a measure of memory and, further, no significant effect of age and IPI (inter-phase intervals) was found (Sondergaard et al., 2012).

In 2013, the same group (Jakobsen et al., 2013) generated AD minipigs with targeted transgene insertion by recombinase-mediated cassette exchange (RMCE) and SCNT. They first produced Göttingen minipigs with four RMCE acceptor loci. By using the Cre-loxP system in combination with minicircles in fibroblasts with all four acceptor loci and followed by SCNT, they then produced piglets with a single cDNA copy of the AD-causing gene *PSEN1M146I* driven by an enhanced human UbiC promoter incorporated into one of the transcriptionally active acceptor loci (Jakobsen et al., 2013). To date, no data are available which would demonstrate an AD behavioral phenotype in this model.

Huntington's disease

Huntington's disease (HD) is a fatal, autosomal dominant, hereditary, neurodegenerative disorder clinically characterized by progressive motor dysfunction, cognitive decline, and psychiatric disturbance (Ross and Tabrizi, 2011). Causative mutation of HD is an expansion of the polyglutamine (CAG) repeat sequence in the coding region of exon 1 of the huntingtin gene localized on chromosome 4 (HTT; IT-15), leading to expression of mutant huntingtin protein with expanded poly-glutamine (polyQ) tract (1993). This expansion induces progressive and devastating neurodegenerative changes in the entire brain with striatum, cerebral cortex (Vonsattel and DiFiglia, 1998), and white matter (Tabrizi et al., 2011; Dumas et al., 2012) being the most affected regions. Wildtype (WT) huntingtin has numerous functions that are important for normal embryonic development and neurogenesis (Cattaneo et al., 2005; Lo Sardo et al., 2012). HD onset and severity is polyQ-length-dependent and is characterized histopathologically by the presence of mutant huntingtin protein aggregates and inclusion bodies (IBs) (Mangiarini et al., 1996; DiFiglia et al., 1997). Intensive research revealed that many factors influence the incidence of aggregated mutant huntingtin, including levels of mutant protein expression, polyglutamine length, the length of the mutant huntingtin fragment, and age of the animal (Hackam et al., 1998; Li and Li, 1998; Chen et al., 2002). Since the identification of the HD gene mutation in 1993 (1993), a wide array of genetic HD models (transgenic, knockin, knockout, humanized) have been produced in mouse, rat, nonhuman primate, and sheep (Mangiarini et al., 1996; von Horsten et al., 2003; Yang et al., 2008; Jacobsen et al., 2010; Yu-Taeger et al., 2012).

Cloned transgenic HD minipigs bearing N-terminal mutant HTT (208 amino acids and 105 Q) were generated via somatic cell (transduced primary porcine fetal fibroblasts) nuclear transfer technology (Yang et al., 2010), but the extremely high expression levels of the transgene led to premature (3 days old) death of three of the five piglets. The fourth lived for only 25 days and the fifth founder was still viable at the beginning of 2013 (Morton and Howland, 2013). Cloned transgenic HD minipigs displayed typical apoptotic neurons with activated Caspase-3 and DNA fragmentation in analyzed brain tissue. The authors suggest that mutant HTT is more toxic to larger animals in comparison to the widely used mouse HD model (Yang et al., 2010). The very short fragment of mutant huntingtin which is expressed in this pig model brings its usefulness into question, although no other data have been published since the initial report. Our group recently reported the first successful generation of a transgenic HD pig model (Baxa et al., 2013). To generate this model, the lentiviral vector encoding human N-terminal-truncated (548 aa) HTT gene with mixed 145 CAG/CAA repeats under the control of human HD promoter was injected into a one-cell stage embryo (microinjection into the perivitelline space) (Baxa et al., 2013). Twenty-nine injected zygotes were transferred to recipient sows via laparoscopy. After standard duration of gravidity, the first HIV1-HD-548aaHTT-145Q manipulated piglets were born. One gilt (F807) in a litter of six live newborns was transgenic (Baxa et al., 2013). Stable transgenic mutant HTT protein levels (548aa-124Q) comparable to endogenous pig HTT levels were confirmed in the whole CNS and other organs and successful germ line transmission occurred through consecutive generations (F0, F1, F2, and F3). No developmental or motor deficits were observed up to 40 months of age and no aggregate formation in brain up to 16 months was detected. Analysis of 16-month-old sibling pairs showed reduced intensity of DARPP32 immunoreactivity in neostriatal transgenic HD neurons compared to WT. Transgenic HD boars also showed reduced fertility and fewer spermatozoa per ejaculate and in vitro analysis demonstrated decreased ability of transgenic HD spermatozoa to penetrate WT miniature pig oocytes (Baxa et al., 2013).

Spinal muscular atrophy

Spinal muscular atrophy (SMA) is an autosomal-recessive neurodegenerative disorder characterized by the degeneration of the motor neurons of the spinal cord leading to skeletal muscle wasting (reviewed in Lorson et al., 2010). SMA is a result of loss-of-function (deletion or mutation) of the survival motor neuron-1 (SMN1) gene. A highly conserved copy, SMN2, is present exclusively in humans and serves as a disease modifier, as increasing SMN2 copy number decreases the severity of the disease. SMN2 alone cannot prevent the disease and mutations in SMN2 have no clinical consequence if SMN1 is fully functional.

Recently, the first step in development of a porcine animal model for SMA has been undertaken and heterozygous SMN1 knockout pigs were generated (Lorson et al., 2011). The authors pointed to the importance of conservation of alternative splicing events of both human SMN1 and SMN2 in the pig in order to produce a successful transgenic model of SMA. The porcine model of human SMA is being produced in several stages: First, a knockout (KO) of the SMN1 allele produced SMN1+/− pigs. Nuclear transfer of SMN1 using fetal fibroblasts produced healthy SMN1+/− piglets that, like their human SMN1+/− counterparts, are phenotypically normal. The second stage of generation of the porcine model of SMA is currently in progress and includes the addition of human SMN2 transgene to the pig genome. After this task is accomplished, SMN1−/−; SMN2 pigs will be generated through breeding and using a second round of nuclear transfer (Lorson et al., 2011). At present, efforts are underway to introduce the human SMN2 transgene into pig fetal fibroblasts, leading to completion of the pig model of SMA. Importantly, effective SMA therapeutics do not currently exist, highlighting the value of a genetically modified pig model for this disease (reviewed in Lorson and Lorson, 2012).

Surgically induced neurodegenerative models

Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disease, characterized by the loss of more than ~50–70% of the dopaminergic neurons in the substantia nigra pars compacta (SNc), a profound loss of dopamine (DA) in the striatum, and the presence of intracytoplasmic inclusions called Lewy bodies (LB), which are composed mainly of α-synuclein and ubiquitin. Although mutations in the α-synuclein gene have thus far been associated only with rare familial cases of PD, α-synuclein is found in all LBs (Spillantini et al., 1997). The clinical manifestation of PD includes tremor, rigidity, bradykinesia, and postural instability, and can be accompanied by nonmotor symptoms such as olfactory deficits, sleep impairments, and neuropsychiatric disorders. Importantly, L-DOPA was found to reverse many of the symptoms and is currently used as a drug therapy in early Parkinson's disease, temporarily improving patients' motor symptoms (reviewed in Cools, 2006).

To date, PD falls into the category of idiopathic diseases, although some atypical cases have a genetic origin. Several different genetic mutations (α-synuclein, parkin, LRKK2, PINK1, DJ-1) have been identified, and this has led to the development of genetic models of PD, including mice, *Drosophila melanogaster*, and *Caenorhabditis elegans* (Dawson et al., 2010). To our knowledge, however, a transgene pig model of PD has not yet been fully established, although direct stereotaxic intracerebral transfection of nigra cells with viral vectors encoding for α-synuclein has been accomplished in the Gottingen minipig (Glud et al., 2011). Only ~10% of PD cases are due to genetic mutations, however, while the vast majority of PD cases arise without apparent genetic linkage and are referred to as "sporadic" (Dauer and Przedborski, 2003). Due to the well-defined appearance of regional neurodegeneration in dopaminergic neuronal populations in atypical PD, several "neurotoxic" models which target these neuronal populations in vivo have been developed (in addition to transgenic models). In these models, neurotoxin is delivered systemically or directly injected into targeted brain regions (Fig. 1) to induce local neuronal degeneration (reviewed in Blesa et al., 2012). Among the systemic neurotoxins, the 1-methyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) are the most commonly used. In 1999, Mikkelsen et al. first reported the MPTP-induced Parkinsonism in minipigs. The authors reported that nine pigs were administered 1 mg/kg of MTPT subcutaneously for 6 consecutive days. All MPTP-treated animals developed Parkinsonian symptoms, i.e., muscle rigidity, hypokinesia, and impaired coordination within 5 days; animals with the lowest striatal DA concentrations showed the most severe signs of Parkinsonism (Mikkelsen et al., 1999). Bjarkam et al. (2008b) developed a stereotaxic technique (Fig. 1) allowing precise implantation/injections of stem cells (Danielsen et al., 2000; Cumming et al., 2001; Dall et al., 2002; Bjarkam et al., 2008b, 2010), viral vectors (Glud et al., 2011), encapsulated genetically modified cells (Fjord-Larsen et al., 2010), and deep brain stimulation electrodes (Bjarkam et al., 2008a) into specific brain nuclei using the same surgical set-up and computerized targeting devices as used for human stereotaxic procedures (Bjarkam et al.,

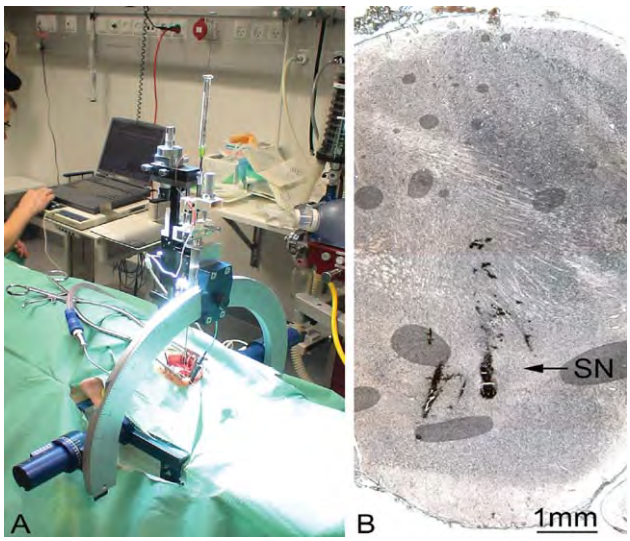

Figure 1. Stereotaxic targeting of the minipig substantia nigra (SN). **(A)** The stereotaxic procedure is similar to the procedure in humans based on a stereotaxic MRI and subsequent computerized image analysis providing the coordinates for the SN, whereafter the targeting is performed isocentric with a mounted modified Leksell frame. **(B)** Postmortem histological image of three lesion tracts targeting the SN (black arrow).

2004, 2009). We have likewise developed a model of progressive PD based on continuous intoxication with MPTP delivered by a subcutaneously implanted pump, and evaluated the degree of parkinsonism using both the previously developed and validated clinical scoring system and infrared computerized walking analysis (Mikkelsen et al., 1999; Nielsen et al., 2009). The work on a symptomatic pig model of PD based on unilateral lesioning of the nigrostriatal pathway with stereotaxic injection of 6OH-dopamine–displaying rotational behavior after amphetamine injections proportional to the degree of nigrostriatal damage and thus unilateral parkinsonism–is ongoing.

Spinal trauma models

Recently, several acute or chronic spinal trauma injury models have been developed in pigs. In these models, a segmental spinal cord injury is induced after surgical exposure of trauma-targeted spinal segment(s) using weight drop, computer-controlled contusion/compression devices, or calibrated vascular clips (Jones et al., 2012; Navarro et al., 2012; Zurita et al., 2012; Lee et al., 2013). Depending on the severity of local segmental injury, the degree of neurological dysfunction is typically manifested by partial or complete loss of ambulatory and sensory function. Using a computer-controlled contusion system, we have recently developed a chronic spinal trauma model in adult Gottingen-Minnesota (G-M) miniature pigs (Navarro et al., 2012). In this model, we have shown that, after 2.5 kg compression force delivered at a velocity of 3 cm/sec on the dorsal aspect of the exposed Th12 segment, there is consistent development of paraplegia with loss of body weight-bearing capacity and that such a deficit persists for up to 9 months after injury. Importantly, the histopathological analysis of spinal cords at and around the injury epicenter showed progressive development of syringomyelia (Fig. 2), loss of myelinated axons and neurodegenerative/inflammatory changes which are similar to human patients with chronic spinal trauma. Because of the consistency of neurological dysfunction and spinal histopathological changes similar to those found in human patients with chronic spinal traumatic injury, the pig spinal trauma models in general are becoming important models in testing new therapies (pharmacological or cell replacement-based) for treatment of both acute and chronic spinal trauma.

Spinal ischemia models

To model spinal ischemia-induced neurodegenerative changes which develop during transient aortic cross-clamping (i.e., surgical procedure to replace aortic aneurysm), several pig transient spinal ischemia models have been developed. To induce spinal ischemia, the descending thoracic aorta is occluded using 1) aortic clamps in a thoracotomized animal (Colon et al., 1987; Dapunt et al., 1994; Meylaerts et al., 2000), or 2) a

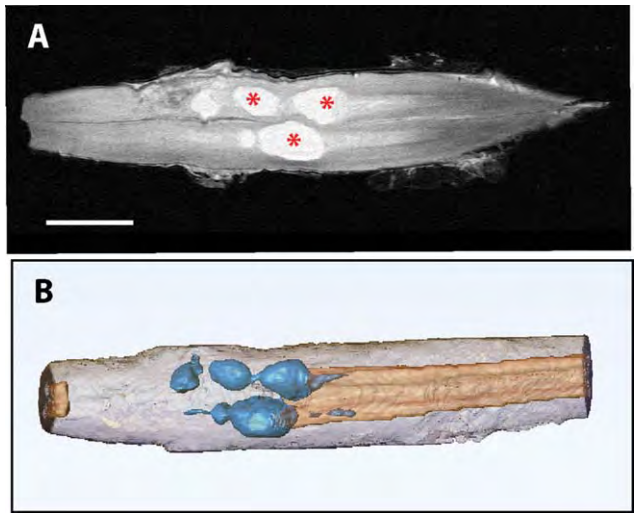

Figure 2. Development of spinal cavitation (syringomyelia) in an adult pig at 4 months after Th12 paraplegia-inducing contusion injury. **(A)** 2D FSPGR image from 7-T MRI demonstrating the presence of bilateral septic cavitation (red asterisks) and areas of high density structures around the injury epicenter. **(B)** 3D rendered image of the same spinal cord shown in (A). Scale bar = 8 mm.

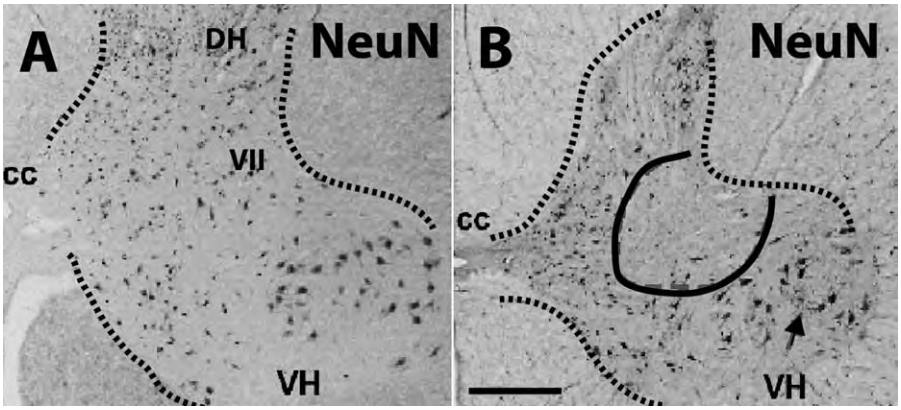

Figure 3. Selective loss of small neurons in lumbar spinal cord after transient spinal cord ischemia in an adult pig. Animal developed spastic type of paraplegia at 3 weeks after 40 minutes of transient aortic occlusion. **(A)** Transverse spinal cord section taken from L3 spinal cord segment of a control naïve animal and stained with neuron-specific antibody NeuN. Normal distribution of small interneurons in dorsal horn (DH), in the intermediate zone (lamina VII) as well as large α-motoneurons in ventral horn (VH) can be seen. **(B)** Transverse spinal cord section taken from L3 spinal cord segment of an animal with developed ischemic spastic paraplegia and stained with neuron-specific antibody NeuN. A selective loss of small interneurons in dorsal horn and in the intermediate zone (lamina VII; black semicircle) can be seen. In contrast, α-motoneurons in the ventral horn (VH) show continuing survival. Scale bar = 1 mm; cc, central-canal.

balloon catheter previously placed via the femoral artery is inflated with saline, inducing intraluminal aortic occlusion (Papakostas et al., 2006). On average, 30–45 minutes of aortic occlusion is required to induce irreversible spinal neuronal degeneration in previously ischemia-exposed spinal segments. Depending on the duration of ischemic episode, the neurological dysfunction is typically characterized by paresis or fully developed spastic or flaccid paraplegia. Histopathological changes in previously ischemia-exposed spinal cord segments are characterized by a selective loss of small inhibitory interneurons (in the case of spastic paraplegia) (Fig. 3) or pan-necrotic neuronal degeneration (in the case of flaccid paraplegia), affecting populations of both small interneurons and large, ventrally located α-motoneurons. These behavioral/histopathological data in pig spinal ischemia models are similar to previously reported spinal ischemia models in rodents (mice, rats, rabbits), (Zivin and DeGirolami, 1980; Taira and Marsala, 1996; Kakinohana et al., 2011), dogs, baboons (Gelfan and Tarlov, 1955; Svensson et al., 1986), and in human patients with previous spinal ischemic injury (Tarlov, 1967). As in the spinal trauma model, the pig spinal ischemic injury model may represent a large preclinical model of choice to evaluate the efficacy of cell replacement therapies in modulating motor dysfunction after transient spinal cord ischemia.

Air decompression-induced spinal degeneration model

Decompression illness (DCI), commonly known as diver's disease or "the bends," is a systemic disease resulting from formation of bubbles in the tissues or circulation because of inadequate elimination of inert gas, mostly nitrogen, after diving. Clinically, patients typically present with two common types of symptoms. Type I: joint pain only (shoulder, elbow, hip, and/or knee), cutaneous (cutis marmorata), lymphatic (pitting edema), or Type II: cardiopulmonary (chokes) or neurologic (paralysis) (Barratt et al., 2002; Barratt and Van Meter, 2004). The most commonly affected areas of the central nervous system are the lower thoracic spinal cord (Tournebise et al., 1995; Barratt et al., 2002) and/or the brain territory supplied by the middle cerebral artery or vertebral-basilar arterial systems (Barratt et al., 2002).

The most widely used model of DCI is the pig model. In this model, using a compression chamber, a simulated dive to 200 feet of seawater (612.6 kPa) with a decompression rate of 60 fsw.min^{-1} is typically used to induce DCI. Animals show signs of motor dysfunction and dysesthesia during the first 2–7 minutes after decompression (Broome and Dick, 1996). Histopathological analyses in pigs with DCI show the presence of spinal parenchymal hemorrhages and spongiosis at 24 hours after induction of DCI (Broome and Dick, 1996) and with fully developed necrotic foci several days after injury (Barratt et al., 2002).

Focal brain ischemia models

Several focal brain ischemia (FBI) models have been developed in the pig. First, FBI is induced by transorbital frontotemporal permanent middle cerebral artery occlusion. In this model, consistent and predictable

neurodegenerative changes have been identified in affected brain regions using MRI and histopathological analysis (Imai et al., 2006; Zhang et al., 2007). Second, FBI was produced in male infant piglets (2–4 weeks old) by photothrombotic occlusion of the middle cerebral artery. In this model, both a significant decrease in regional blood flow and the appearance of apoptotic positive cells were seen at 4 hours after injury (Kuluz et al., 2007).

Global brain ischemia models

Global brain ischemia (GBI) occurs when cerebral blood flow is reduced in most or all areas of the brain and is commonly caused by cardiovascular failure (Hossmann, 1991). GBI causes neuronal injury to selectively vulnerable brain areas (Kurth et al., 1999; Traystman, 2003). The clinically most relevant model of GBI is cardiac arrest (Hossmann, 1991) caused by ventricular fibrillation (Traystman, 2003). Several pig models of GBI induced by cardiac arrest were developed using young piglets (LeBlanc et al., 1993; Thoresen et al., 2001; Wiklund et al., 2005) or adult animals (Mayr et al., 2001; Yannopoulos et al., 2005). Cardiac arrest in pigs is commonly induced by ventricular fibrillation produced by AC current (Tadler et al., 1998; Liu et al., 2003; Yannopoulos et al., 2005). In addition, asphyxial cardiac arrest (Mayr et al., 2001; Agnew et al., 2003), hypoxic global insult (Thoresen et al., 2001), bupivacaine-induced cardiac arrest (Mayr et al., 2001), or hypothermic cardiac arrest (Ye et al., 1996; Kurth et al., 1999; Rimpilainen et al., 2000; Kornberger et al., 2001) are also reported methods to induce cardiac arrest in pigs. As such, pigs with previously induced cardiac arrest are often used as a model for cardiopulmonary resuscitation (CPR) and therefore are commonly referred to as cardiac arrest/CPR models (Traystman, 2003). Brain histopathological analysis shows that the severity of neuronal degeneration directly correlates with the length of the experimental GBI. Cardiac arrest and the resulting GBI in piglets cause blood–brain barrier (BBB) breakdown, and subsequent albumin leakage contributes to brain edema, which ultimately leads to selective rapid and progressive neuronal damage (Sharma et al., 2011). Affected areas also exhibit the myelin loss and appearance of activated glial fibrillary acid protein (GFAP)-positive astrocytes typically seen several days to weeks after ischemic insult (Sharma et al., 2011). The most vulnerable brain regions in animals with GBI induced by ventricular fibrillation are (severity in descending order) thalamus, cerebral cortex, hippocampus, hypothalamus, and brain stem (Sharma et al., 2011). In GBI induced by (deep) hypothermic circulatory arrest it is hippocampus, cerebral cortex, striatum (caudate nucleus, putamen), cerebellum (Ye et al., 1996; Kurth et al., 1999), thalamus, pons, and mesencephalic gray matter (Ye et al., 1996; Kurth et al., 1999). Ischemia-damaged neurons displayed apoptotic cell death in the cerebral cortex (with some rare clusters of infarction with necrotic cells), and a mixture of necrotic and apoptotic neurons was found in the hippocampus in brains of DHCA piglets (Kurth et al., 1999). Brain damage in DHCA piglets resulted in neurological deficit which included (severity in descending order) disturbed gait, feeding difficulty, abnormal tone, and impaired consciousness. Neurological deficit after DHCA progressively improves even during the period of ongoing neuronal cell death (Kurth et al., 1999). Pig models of cardiac arrest/CPR were used to study the effect of various drugs such as epinephrine (Schleien et al., 1986; Lindner et al., 1991; Kornberger et al., 2001), methoxamine (Brown et al., 1987), vasopressin (Mayr et al., 2001), or xenon (Schmidt et al., 2005) or the effect of hypothermia after cardiac arrest on cerebral and/or myocardial perfusion and neuroprotection (Thoresen et al., 2001; Agnew et al., 2003).

Recently, a new isolated GBI model was developed by occlusion of the innominate, proximal left subclavian, and both the internal mammary and distal subclavian arteries (Allen et al., 2012). In this model 30 minutes of ischemia led to the development of multiple postreperfusion seizures, raised NDS, and postmortem macroscopic swelling was observed, accompanied by edema in the cortex, cerebellum, and hippocampus (Allen et al., 2012).

Traumatic brain injury models

Numerous experimental studies were reported which employ a fluid-percussion-induced brain injury in pigs. Using this model system in juvenile or adult pigs, the extent of local cortical injury can be well calibrated and lead to predictable pathological changes ranging from focal neurodegenerative changes to more diffuse injury, including loss of axonal integrity (Brodhun et al., 2001; Fritz et al., 2005). More recently, a controlled cortical impact model was developed in adult Yorkshire pigs in which the velocity of cortical impact and the dwell time can be preprogrammed (Manley et al., 2006). Using this model, it was demonstrated that following injury there was a progressive increase in intracranial pressure and heart rate and these changes were more pronounced in animals with the 12 mm depth of depression. Animals exposed to 11 mm depth of depression displayed the presence of edema, inflammatory cells, and pericapillary and petechial hemorrhages similar to those seen in human patients. Similarly as seen in human patients, the histopathological analysis

showed the presence of degenerating neurons and axonal injury in and around the injury epicenter (Manley et al., 2006).

Use of pig models in cell replacement modeling

Immunosuppression protocols and immunodeficient pigs

A critical requirement for the successful utilization of experimental cell replacement modeling is the development of effective immunosuppression protocols which would lead to consistent grafted cell (xenografts or allografts) survival. Several routes of immunosuppressive drug delivery were reported to be successfully used in pigs, each having its pros and cons. First, immunosuppressive drug can be delivered orally, typically mixed with liquid food (Jones et al., 1999; Sano et al., 2002; Jensen-Waern et al., 2012). While the noninvasive nature of orally delivered drugs is preferable, such an approach can be hampered by inconsistencies in the drug absorptions, particularly if severely injured animals with variable food appetite are to be employed. Second, repetitive bolus delivery or continuous intravenous infusion of immunosuppressive drugs using a chronically placed intravenous catheter was successfully used (Griesemer et al., 2008; Usvald et al., 2010; Kakinohana et al., 2012). While being more labor-intensive, this approach permits the achievement of a well-controllable blood drug concentration and can effectively be used for several months after initiation. Third, we have recently reported the successful use of long-term (up to 3 months) tacrolimus (Tac)-releasable subcutaneous pellet-induced immunosuppression in rat spinal xenograft studies (Sevc et al., 2013). The initial use of the Tac pellet formulation in adult (25–30 kg) or young (5–8 kg) piglets shows comparable long-lasting immunosuppression after a single subcutaneous Tac pellet implantation (unpubl. obs.).

It is of note that utilization of severe combined immunodeficient (SCID) pigs would not only greatly facilitate experimental cell replacement modeling but also allow the production of humanized tissues and organs. Interestingly, the first successful generation of immunodeficient pigs was only recently reported by Suzuki et al. (2012). SCID pigs were generated by nuclear transfer using X-linked interleukin-2 receptor gamma chain gene (Il2rg)-targeted embryonic fibroblasts as donor cells. Germline transmission of the Il2rg deletion produced athymic hemizygous Il2rg (-/Y) males with impaired immunoglobulin production and the absence of T and NK cells (Suzuki et al., 2012). However, immunodeficient pigs were short lived (<2 months) even under controlled laboratory conditions and thus not suitable for long-term experimental studies (Suzuki et al., 2012). Watanabe et al. (2013) also recently generated Il2rg KO pigs which, similar to above-mentioned study and human X-linked SCID patients, lacked thymus and were deficient in T and NK cells. Importantly, they demonstrated that the combination of Zinc Finger Nuclease-encoding mRNAs and SCNT can provide easier and robust method for producing KO pigs without genomic integration and thus greatly facilitate the development of porcine SCID model in the future (Watanabe et al., 2013).

Use of miniature pig in spinal cell dosing and safety studies

The nondiseased naïve miniature pigs are frequently used in preclinical safety and toxicity cell replacement-based or pharmacological studies (Nakajima et al., 1992; Svendsen et al., 2006; Bogde et al., 2010; Templin et al., 2012). The primary goal in those studies is the definition of a safe dosing regimen to be used in prospective human clinical trials. Because of the similarities in spinal cord (and to some extent brain) dimensions to humans, pigs represent an effective model to perform detailed cell grafting dosing studies.

Our group has developed and used a spinal cell injection technique in chronically immunosuppressed naïve pigs to define the optimal cell dosing regimen of human fetal tissue-derived neural precursor cells (NPCs) grafted into the lumbar spinal cord (gray matter) as analyzed by changes in neurological function and local spinal histopathological changes (Fig. 4) (Usvald et al., 2010). This model was then used further in other laboratories to perform additional detailed toxicity studies after lumbar and cervical grafting of human NPCs and to test a spinal cell injection device subsequently used in a human clinical trial. The data from both studies were used for designing the clinical cell dosing protocol and then used in a Phase I clinical trial in human amyotrophic lateral sclerosis (ALS) patients receiving spinal grafts of human spinal stem cells. Phase I of this trial was successfully completed (Boulis et al., 2011; Donnelly et al., 2012; Glass et al., 2012; Riley et al., 2012).

To our knowledge there are no experimental data available at present which would employ a pig model(s) of neurodegenerative disorders in cell replacement-based therapies. We have recently employed a previously described Th12 spinal contusion model in pig and have tested the survival and differentiation of human spinal stem cells once grafted into and around the injury epicenter at 24 or 72 hours after injury. Consistent cell survival was seen at 4 weeks after cell grafting

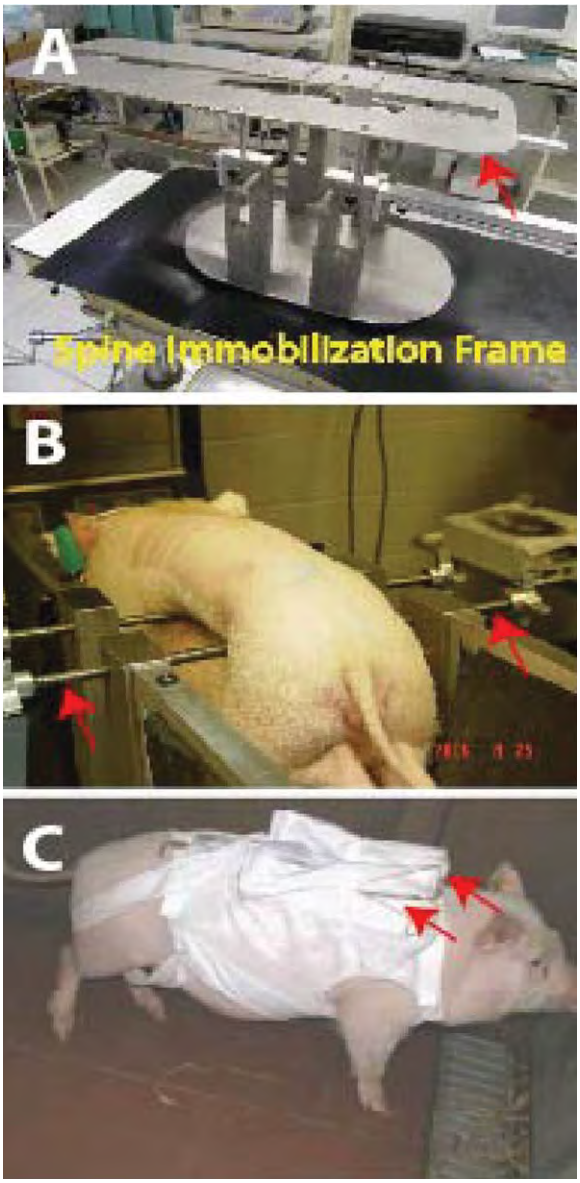

Figure 4. Instrumentation and technique used to induce/perform spinal trauma, spinal cell injections and chronic intravenous immunosuppression in adult pig. **(A)** To immobilize the lumbar spine, a custom-made spinal immobilization frame which can accommodate pigs between 10–40 kg of body weight is used. The frame is equipped with a removable horizontally oriented steel platform (red arrow) which serves to attach a spinal compression apparatus or spinal injection devices. **(B)** To immobilize the lumbar spine, the anesthetized animal is lifted from the operating table and four horizontal steel bars (two on each side; red arrows) are slid against the lumbar paravertebral muscles bilaterally with the spine resting on the top of the bars. **(C)** For chronic intravenous immunosuppressive drug delivery, a chronic vein catheter is implanted into the jugular or femoral vein and drug is delivered continuously using 5- or 7-day lasting external infusion pumps. One pump or two infusion pumps (if continuous delivery of two different drugs is required) are placed and secured in pockets (red arrow) of custom-made pig jacket.

in continuously immunosuppressed animals (Weingarten, 2013).

Current limitations on effective use of pig model in autologous, syngeneic, and allogeneic cell grafting modeling

Currently used protocols in human solid organ transplantation or bone marrow grafting use a well-developed crossmatch and HLA genotype-screening platform between donor and graft recipient (Sheldon and Poulton, 2006). These protocols have allowed for optimal HLA matching between donor and recipient tissues, resulting in significantly improved long-term survival of allogeneic grafts (Dyer et al., 1989; Opelz et al., 1999). Despite these advances, it is not known whether allogeneic stem-cell-derived grafts would induce similar immune responses, since these cell-based grafts do not carry passenger leukocytes and express low levels of HLA, thereby reducing the likelihood of inducing direct allorecognition (Boyd et al., 2012). To advance our understanding and to optimize immunosuppression protocols to be used in prospective clinical trials, the availability of similarly MHC-characterized large animal models (fully or partially MHC-matched) which would mimic the MHC transplantation combinations relevant to the human population is required. An inbred fully or partially MHC SLA-matched miniature pig strain was recently developed (Pennington et al., 1981; Mezrich et al., 2003) and successfully used in solid organ (kidney) transplantation modeling as well as in development of immune tolerance (allogeneic kidney transplant) protocols using short course immunosuppression across a two-haplotype, fully MHC-mismatched barrier (Utsugi et al., 2001).

However, in contrast to solid organ transplantation, there are no experimental data available at present which would use the porcine embryonic stem cells (ESCs)-induced pluripotent stem cells (iPSCs)- or fetal tissue (FT)-derived NPCs obtained from WT or fully MHC-matched or partially MHC-matched pigs and used in cell replacement modeling in pig neurodegenerative models. The availability of such cell lines and MHC-matched adult pig recipients is critical for several reasons: 1) A primary planned utilization of iPS-derived lines is their use in autologous transplantation in the absence of immunosuppression. However, at present, no optimal reprogramming protocol is defined (i.e., integrative vs. nonintegrative approach) and typically several clones of such iPSCs are prepared in vitro from a single donor for potential in vivo grafting. The availability of multiple genetically identical (MHC genes in particular) recipients is therefore critical in the effective screening of individual clones and to study the diversity

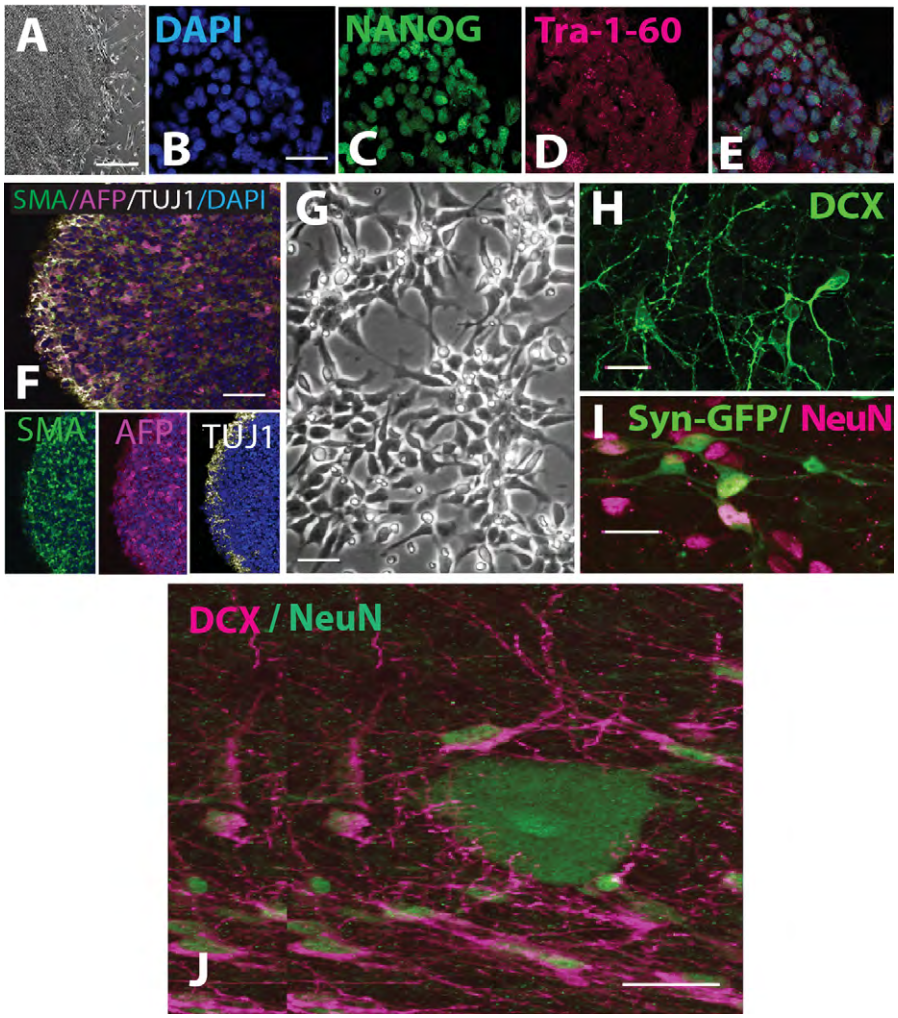

Figure 5. Generation and in vitro and in vivo postgrafting characterization of porcine iPS-derived neural precursors (NPCs). **(A)** Appearance of established iPS colony cultured on MEFs after skin fibroblasts reprogramming with retroviruses encoding Oct4, Sox2, Klf4, and c-myc. **(B–E)** Proliferating iPS colonies expressed pluripotent markers Nanog and Tra 1–60. **(F)** In vitro induced embryoid bodies show expression of markers of all three germ layers including SMA (mesoderm), AFP (endoderm), and TUJ1 (ectoderm). **(G)** Proliferating porcine iPS-NPCs in culture show a typical NPCs morphology. **(H,I)** In vitro induced NPCs show expression of early neuronal marker DCX (H) and mature neuronal marker NeuN (I). **(J)** Survival and differentiation of porcine iPS-NPCs at 4 weeks after spinal grafting in immunosuppressed allogeneic pig. A high density of grafted NeuN/DCX-immunoreactive neurons surrounding NeuN+ host α-motoneuron can be seen. Antibodies: Nanog (rabbit anti Nanog: Cat. No. 21624-100, 1:1000, Abcam, Cambridge, MA), Tra 1–60 (mouse anti Tra 1–60; Cat. No. FCMAB115F, 1:100, Millipore, Bedford, MA) SMA (goat anti alpha smooth muscle actin, Cat. No. 21027, 1:250, Abcam), AFP (rabbit anti alpha-fetoprotein, Cat. No. AM31985PU-S, 1:50, Acris Antibodies, San Diego, CA), TUJ1 (mouse anti-tubulin beta III, Cat. No. MAB1637, 1:250, Millipore), DCX (goat anti-doublecortin, Cat. No. sc-8066, 1:500, Santa Cruz, Santa Cruz, CA), NeuN (mouse anti-neuron-specific nuclear protein, Cat. No-MAB377, 1:250, Millipore). Scale bars = 100 μm in A,F; 30 μm in B; 10 μm; in G–I; 50 μm in J.

in clone engraftment properties if different reprogramming strategies are used. 2) The use of ESC- or FT-derived cell lines (such as neural precursors) in MHC-matched or partially matched recipients can substantially facilitate our understanding of the need for transient or continuous immunosuppression once cells are grafted into the injured-inflamed spinal cord or brain milieu. In the face of imminent human clinical spinal and brain trauma trials with planned use of human fetal tissue or ES-derived NPCs, such data are of critical importance. Recent clinical data show that the use of continuing immunosuppression is a major limiting factor because of its side effects and poor long-term tolerability in ALS patients treated with spinal human stem cells (Glass et al., 2012). Thus, the definition of potential immune tolerance after using only transient immunosuppression protocols in large animal allogeneic grafting design is important for the optimization of human clinical immunosuppressive protocols by mimicking a similar allogeneic-immunosuppression treatment design.

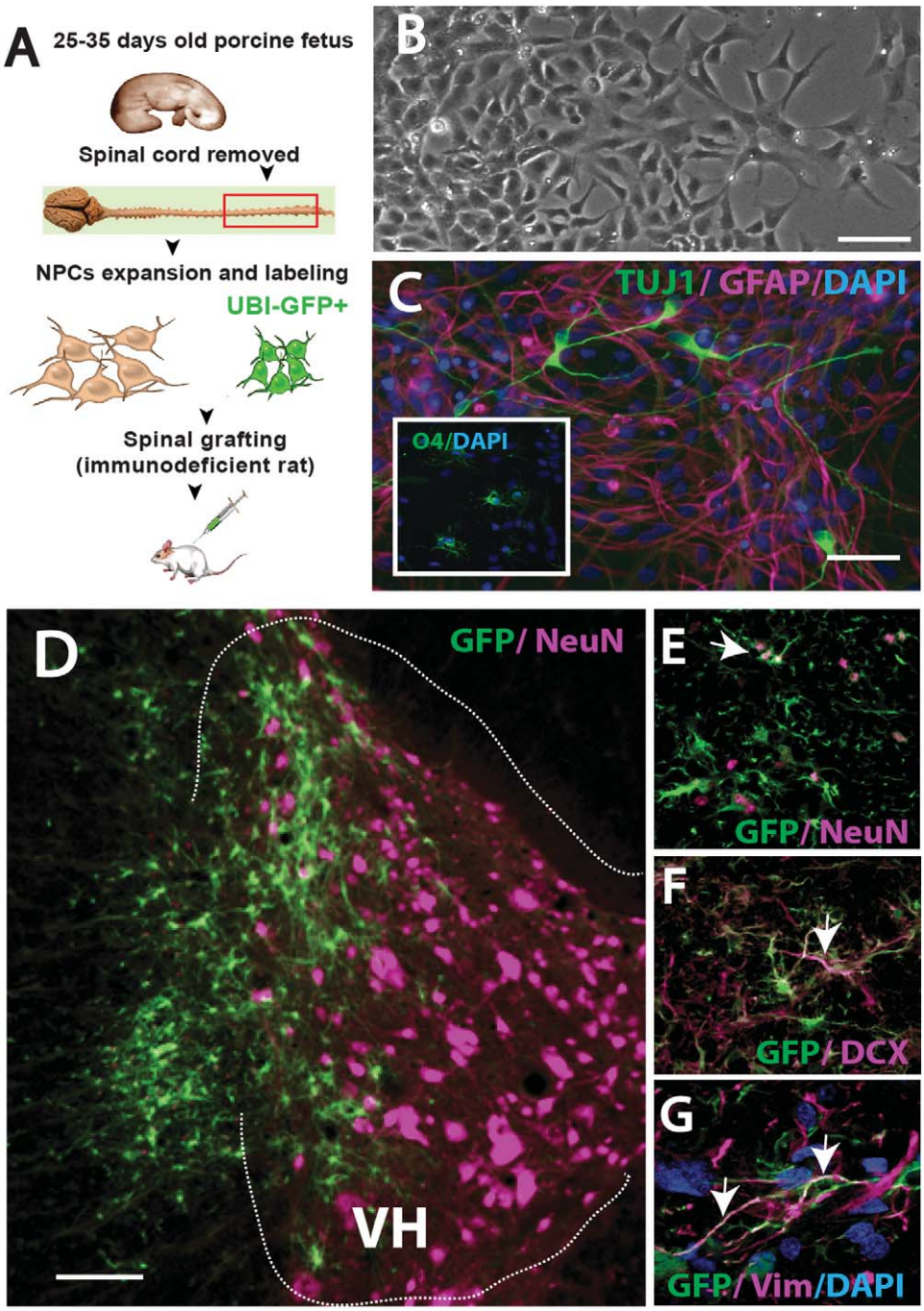

Figure 6. Isolation, expansion, labeling, and in vitro and in vivo post-spinal grafting characterization of porcine fetal spinal cord-derived NPCs. **(A)** NPCs are isolated from the lumbar portion of 25–35-day-old fetuses, expanded, and labeled with GFP-encoding lentivirus under ubiquitin promoter. (**B**) Proliferating NPCs show typical multipolar morphology. **(C)** In vitro induced NPCs show differentiation towards neurons (TUJ1), astrocytes (GFAP), and oligodendrocytes (O4-insert). **(D–G)** At 4 weeks after spinal grafting into lumbar spinal cord of an immunodeficient rat, GFP-labeled grafts and differentiation of grafted cells to neurons (NeuN; E), (DCX; F), and glia (vimentin; Vim; G) can be seen. Antibodies: TUJ1 (mouse anti-tubulin beta III, Cat. No. MAB1637, 1:250, Millipore), GFAP (mouse anti-glial fibrillary acidic protein, Cat. No. C9205, 1:500, Sigma, St. Louis, MO), NeuN (mouse anti-neuron-specific nuclear protein, Cat. No-MAB377, 1:250, Millipore). DCX (goat anti-doublecortin, Cat. No. sc-8066, 1:500, Santa Cruz), VIM (mouse anti-vimentin, Cat. No. 18–0052, 1:1000, Zymed, San Francisco, CA). Scale bars = 30 μm in C; 100 μm in D.

Pig embryonic stem cell-derived, fetal tissue-derived, and iPS-derived neuronal precursors.

Embryonic stem cells

Despite extensive investigation in recent years, it has proven difficult to obtain bona fide blastocyst-derived pluripotent porcine cells in vitro. Putative porcine ESCs have been derived by several groups; however, their "stemness" (i.e., self-renewal and pluripotency) and stable expression of pluripotency markers have not been shown. Moreover, their ability to form teratomas or contribute to formation of pig chimeras remains to be demonstrated (Strojek et al., 1990; Notarianni et al., 1991; Piedrahita et al., 1999; Chen et al., 1999; Li et al., 2003; Vackova et al., 2007; Kim et al., 2007).

Induced pluripotent stem cells

Generation of porcine iPSCs using integrating viral vectors as well as a nonintegrative approach for reprogramming has been recently reported (Wu et al., 2009; Esteban et al., 2009; Ezashi et al., 2009; Telugu et al., 2010; Montserrat et al., 2012; Park et al., 2013). However, newly derived cell lines often displayed variable expression of pluripotency related genes or a tendency for spontaneous differentiation in vitro (Esteban et al., 2009; Ezashi et al., 2009; Wu et al., 2009). Moreover, the ability of porcine iPSCs to form teratomas after in vivo grafting seemed to be limited to cells with active transgenes and, furthermore, similar to ES-derived pluripotent lines, germline chimeras derived from pluripotent iPSC lines still remained to be generated. Importantly, several recent studies showed that porcine iPSCs can indeed be successfully generated (West et al., 2010; Telugu et al., 2011; Cheng et al., 2012; Montserrat et al., 2012; Fujishiro et al., 2013). Our group has also recently produced porcine iPSCs using both integrating and nonintegrating reprogramming vectors (Kakinohana et al., 2010). We have also successfully isolated several clones of expandable iPS-NPCs after in vitro differentiation. These porcine iPSC-derived NPCs showed long-term in vivo survival and expression of neuronal markers after grafting into the spinal cord of immunosuppressed naïve pigs (Kakinohana et al., 2010) (Fig. 5). Despite the latest promising advances, hurdles still remain if these cells are to achieve their expected biotechnological potential.

Fetal tissue-derived NPCs

Several groups have reported that multipotent porcine NPCs can be derived directly from the porcine blastocyst (Puy et al., 2010) and/or fetal brain tissue (Deacon et al., 1997; Schwartz et al., 2005; Harrower et al., 2006). Such derived and in vitro expanded NPCs show long-term (up to 7 months) survival and integration after in vivo intrastriatal grafting in a rat unilateral 6-OHDA lesion model of PD and in PD patients receiving unilateral grafts of NPCs into the caudate-putamen (Deacon et al., 1997; Harrower et al., 2006). Our group has recently derived expandable NPCs from E-25 or E-35 developing porcine cortex and spinal cord. Such derived NPCs can be expanded for at least 16 passages and retain their neurogenic potential upon differentiation in vitro. Similarly, we have seen consistent engraftment and neuronal and glial differentiation of the same porcine GFP-tagged NPCs after spinal grafting in naïve immunodeficient rats at 4 weeks after cell grafting (Fig. 6). Jointly, these data demonstrate that current NPC derivation protocols can effectively be used to generate transplantable populations of porcine NPCs to be used in autologous, syngeneic, or allogeneic cell grafting studies.

SUMMARY

The use of cell replacement-based therapies is heralded as a promising treatment approach to modulate functional deficits associated with a variety of neurological disorders including stroke, spinal trauma, or ALS. While cell replacement therapies are rapidly advancing into the human clinic, several refinements such as defining the optimal cell dose, immunosuppression protocols, and/or the use of new cell delivery devices are needed. Accordingly, there is an imminent need for development and characterization of a large animal model(s) of different neurodegenerative disorders which can effectively be used to define the efficacy of cell replacement therapies and can also serve to develop/validate new cell delivery technologies. As reviewed in this article, significant progress has been made over the past decade and numerous transgenic or surgically induced neurodegenerative models have been developed in pigs. More important, using a pig model, preclinical spinal and brain cell-grafting data and long-term immunosuppression protocols were generated and then successfully used in designing human clinical trials. In addition, the availability of a fully or partially MHC-matched pig strain (in contrast to other large animal species) and the use of these animals in cell replacement modeling will permit the study of comparable MHC combinations as seen in human patients (HLA compatibility). Collectively, these data demonstrate that the use of pig neurodegenerative models can represent an important and highly effective preclinical in vivo platform to validate cell-replacement-based therapies before their transition to human clinical practice.

CONFLICT OF INTEREST

Karl Johe, Michael P. Hefferan, and Tom Hazel are employees of and receive salary from Neuralstem, Inc.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: DD, MM, JDC; Acquisition of data: MHP, CRB, JCHHS, MC, MPH, TH, CC, MC, DW, JS; Analysis and interpretation of data: SJ, JJ, CC; Drafting of the article; DD, MHP, MM, KJ; Critical revision of the article for important intellectual content: CRB, JCHHS, AM; Statistical analysis: SJ, JJ, JS, JB; Obtained funding: MM, JM, JCHHS, CRB, AM; Study supervision: MM, JM, JCHHS, CRB, AM.

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