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Single-injection *ex ovo* transplantation method for broad spinal cord engraftment of human pluripotent stem cell-derived motor neurons

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HIGHLIGHTS

- Single injection transplants cells throughout the chick embryo's spinal cord lumen.
- HPSC-derived, HB9$^+$/ChAT$^-$, motor neuron precursors are optimal for chick transplant.
- Transplanted motor neurons display broad engraftment throughout the spinal cord.

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ABSTRACT

Background: Transplantation of human pluripotent stem cell (hPSC)-derived neurons into chick embryos is an established preliminary assay to evaluate engraftment potential. Yet, with recent advances in deriving diverse human neuronal subtypes, optimizing and standardizing such transplantation methodology for specific subtypes at their correlated anatomical sites is still required.
New method: We determined the optimal stage of hPSC-derived motor neuron (hMN) differentiation for *ex ovo* transplantation, and developed a single injection protocol that implants hMNs throughout the spinal cord enabling broad regional engraftment possibilities.
Results: A single injection into the neural tube lumen yielded a 100% chick embryo survival and successful transplantation rate with MN engraftment observed from the rostral cervical through caudal lumbar spinal cord. Transplantation of HB9$^+$/ChAT$^-$ hMN precursors yielded the greatest amount of engraftment compared to Pax6$^+$/Nkx6.1$^+$/Olig2$^+$ progenitors or mature HB9$^+$/ChAT$^+$ hMNs.
Comparison with existing method(s): Our single injection hMN transplant method is the first to standardize the optimal hMN phenotype for chick embryo transplantation, provide a rubric for engraftment quantification, and enable broad engraftment throughout the spinal cord with a single surgical intervention.
Conclusion: Transplantation of HB9$^+$/ChAT$^-$ hMN precursors into chick embryos of Hamburger Hamilton (HH) stages 15–18 using a single luminal injection confers a high probability of embryo survival and cell engraftment in diverse regions throughout the spinal cord.

1. Introduction

The number of neuronal subtypes that can be generated from human pluripotent stem cells (hPSCs) has expanded substantially since their first derivation in 2001 (Zhang et al., 2001). Today's protocols are focused not only on improving the derivation efficiency of novel neuronal subtypes but also refining their phenotypical patterning to distinct anatomical regions of the central nervous system (CNS) (Lemke et al., 2017). Such regional specification has been shown to enhance neuronal engraftment efficiency and regenerative efficacy upon transplantation (Kriks et al., 2011; Ma et al., 2012; Cunningham et al., 2014). However, prior to testing in rodent and large animal studies, transplantation into chick embryos remains a favored preliminary assay to evaluate the hPSC-derived neurons' engraftment potential (Lee et al., 2007b; Son et al., 2011; Amoroso et al., 2013; Du et al., 2015; Fattahi et al., 2016). Chick embryo transplantation is ideally suited for this type of analysis because it is cost effective, requires minimal regulatory oversight, provides an *in vivo* microenvironment with developmental cues yet void of a mature

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immune system, and permits rapid assessment of the transplants' migratory and engraftment capabilities (Lee et al., 2007a; Wichterle et al., 2009; Boulland et al., 2010; Fattahi et al., 2016). However, customizing and standardizing the methodology to enable optimal engraftment profiles for diverse hPSC-derived neuronal subtypes remains a challenge.

Motor neurons (MNs) reside in discrete columns and pools throughout the hindbrain and spinal cord and project peripherally to provide the sole efferent pathway connecting the CNS to rest of the body. Their diverse regional phenotypes are imparted by colinear and combinatorial expression of 39 *HOX* genes that regulate the MNs' columnar and pool identity, positioning within the spinal cord's ventral horn, and trajectory/connectivity of axonal projections (Philippidou and Dasen 2013). In the first reported derivation from mouse embryonic stem cells (mESCs), *in ovo* transplantation of spinal MNs was used to demonstrate their functional phenotype (Wichterle et al., 2002). Post-mitotic cervical MNs were derived using 5 days of embryoid body (EB) culture with Retinoic Acid (RA) and Sonic Hedgehog (SHH) morphogenetic patterning for the last 3 days. Then, MN-containing EBs were transplanted into void spaces within the developing posterior neural tube of Hamburger Hamilton (HH) stage 15–18 chick embryos. The void spaces were created by mechanical lesions that physically removed segments of somite and spinal tissues. Over 2–7 days, the transplanted cells engrafted into that focal region and projected axons out of the ventral roots to connect with skeletal muscles. This powerful demonstration of xenotransplantation and engraftment codified the protocol's methodology (Wichterle et al., 2009), and it has subsequently been used by numerous labs for phenotypical validation of mESC-derived MNs (Soundararajan et al., 2006; Peljto et al., 2010; Toma et al., 2015).

The transition from *in ovo* transplantation of mESC to hPSC-derived MNs (hMNs) has been accompanied by increased variability in methodological details, partly as a result of inherent differences in derivation protocols. For example, MN derivation from hPSCs *versus* mESCs is a protracted process, *e.g.* 2–4 weeks *versus* 5 days respectively. Hence, the stage of differentiation at which hMNs have been transplanted into the chick embryo's spinal cord ranges from pluripotency (Goldstein et al., 2002) to $HB9^+$ post-mitotic precursors (Amoroso et al., 2013) to mature, $ChAT^+$ cells (Lee et al., 2007b; Du et al., 2015). Also, both EB/neurosphere (Amoroso et al., 2013; Du et al., 2015) and monolayer (Lee et al., 2007b; Lippmann et al., 2014) hMN derivation protocols are widely used. Thus, hMNs have been transplanted as both spheroids (Amoroso et al., 2013; Du et al., 2015) and cell suspensions (Lee et al., 2007b). Additionally, while transplantation has consistently been performed in HH stage 15–18 embryos, the MNs' regional phenotype as defined by *HOX* gene expression, the rostrocaudal transplant location, and the use of a somitic *versus* midline neural tube lesion has varied amongst different studies (Lee et al., 2007b; Son et al., 2011; Amoroso et al., 2013; Du et al., 2015). Variations in any of these methodological details could significantly affect the efficiency of hMN transplant engraftment. However, evaluating the effects of such protocol alterations is not possible because engraftment results are typically presented as proof-of-principle images instead of being quantitatively assessed. Moreover, these studies demonstrate successful transplantation within only a few focal rostrocaudal spinal regions, while MNs reside at every vertebral segment and derivation of these diverse regional phenotypes from hPSCs is now possible (Gouti et al., 2014; Lippmann et al., 2015). Therefore, there is a need to develop a standardized chick embryo transplantation method that is quantitatively benchmarked and capable of efficiently testing hMN engraftment throughout the entire spinal cord.

To address this need, we have developed a single-injection *ex ovo* transplantation methodology for hMNs that yielded 100% embryo survival and engraftment at diverse regions throughout the spinal cord. The *ex ovo* culture system was used to facilitate embryo access and visualization (Yalcin et al., 2010). Also, a rubric was established for quantitative assessment of engraftment levels. $HB9^+/ChAT^-$ post-mitotic precursors were identified as the optimal differentiation stage for hMN transplantation. Using our deterministic *HOX* patterning protocol, we specifically transplanted *HOXC6* expressing cervical cell populations, and interestingly, observed the greatest levels of engraftment in the embryo's cervical spinal cord proximal to the wing buds. Collectively, these results provide a standardized and quantitatively benchmarked methodology for effective transplantation of hMNs to achieve broad engraftment throughout the chick embryo spinal cord, thereby obviating the need for multiple focal engraftments as with prior methods. Also, it facilitates hMN engraftment studies investigating whether region-specific (*i.e.* defined *HOX* profile) hMN populations display optimal engraftment behaviors at correlated rostrocaudal spinal positions, as suggested by our results.

2. Materials and methods

2.1. Animal subjects

Fertilized chick embryos were obtained from Sunnyside Hatchery (Beaver Dam, WI). Upon receipt, the chick embryos were placed with their apex vertical and placed inside a humidified egg incubator set to 37 °C. The eggs were allowed to develop for up to 72 h or until HH stage 15–18.

2.2. hPSC culture and motor neuron differentiation

The HUES3 HB9:GFP cell line (Harvard Stem Cell Institute) was used for all experiments and maintained in E8/feeder free conditions as described elsewhere (Lippmann et al., 2014). The cells were cultured on Matrigel™ coated plates and routinely passaged with Versene (ThermoFisher). Motor neuron (MN) differentiation was also conducted as previously described (Fig. 1**A**) (Lippmann et al., 2015). In brief, hPSCs were seeded onto Matrigel-coated 6-well plates at a density of 1.5×10^5 cells/cm^2 in E8 media (ThermoFisher) with 10 μM Y-27632 (ThermoFisher). The culture media was changed to E6 media (ThermoFisher) on Day 0, and then changed to E6 media containing 200 ng/mL FGF8b (PeproTech) the following day. On Day 2, the cells were gently subcultured and re-seeded at a 2:3 ratio in E6 media containing 10 μM Y-27632, 200 ng/mL FGF8b and 3 μM CHIR99021 (CHIR, R&D Systems). On Day 4, the media was changed to E6 media containing 1 μM Retinoic acid (RA, Sigma Aldrich), 2 μM Purmorphamine (PM, R&D Systems), and 2 μM SAG (R&D Systems) for 48 h to pattern a cervical, $Pax6^+/Nkx6.1^+/Olig2^+$ MN progenitor phenotype by Day 6. For continued differentiation, the cells were subcultured and re-seeded at a 1:12 ratio in a 12-well plate with E6 media containing 1 μM RA and 10 μM Y-27632. On Day 7–9, the media was changed daily and supplemented with 100 nM PM and 100 nM SAG. On Days 10–15, the media was further supplemented with 5 μM DAPT (R&D Systems) to generate post-mitotic, $HB9^+/ChAT^-$ MN precursors by Day 16. For maturation, the cultures were maintained for two additional weeks in E6 media supplemented with neurotrophic factors (NTFs) including 10 ng/mL BDNF (PeproTech), 10 ng/mL GDNF (PeproTech), N-2 and B-27 supplement (ThermoFisher), and cAMP (Sigma) as well as Glutamax and PenStrep (ThermoFisher). The media was changed every third day and yielded $HB9^+/ChAT^+$ MNs by Day 30. Each culture's regional patterning along the spinal cord's rostrocaudal axis was evaluated by RT-PCR using *HOXA1*, *HOXB4*, *HOXC6*, *HOXC9*, and *HOXD10* Taqman gene expression assays (Table S1).