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# Transgenic enrichment of mouse embryonic stem cell-derived progenitor motor neurons

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**Abstract** Embryonic stem cells (ESCs) hold great potential for replacing neurons following injury or disease. The therapeutic and diagnostic potential of ESCs may be hindered by heterogeneity in ESC-derived populations. Drug selection has been used to purify ESC-derived cardiomyocytes and endothelial cells but has not been applied to specific neural lineages. In this study we investigated positive selection of progenitor motor neurons (pMNs) through transgenic expression of the puromycin resistance enzyme, puromycin N-acetyl-transferase (PAC), under the Olig2 promoter. The protein-coding region in one allele of Olig2 was replaced with PAC to generate the P-Olig2 cell line. This cell line provided specific puromycin resistance in cells that express Olig2, while Olig2$^-$ cells were killed by puromycin. Positive selection significantly enriched populations of Olig2$^+$ pMNs. Committed motoneurons (MNs) expressing Hb9, a common progeny of pMNs, were also enriched by the end of the selection period. Selected cells remained viable and differentiated into mature cholinergic MNs and oligodendrocyte precursor cells. Drug resistance may provide a scalable and inexpensive method for enriching desired neural cell types for use in research applications.


## Introduction

In most neurological disorders, neurogenesis is insufficient to replenish lost neuronal populations. Endogenous stem cell populations are hindered by limited numbers, variable proliferation in response to disease, and in some cases, differentiation into glia rather than neurons (Barnabe-Heider et al., 2010; Meletis et al., 2008; Yang et al., 2006; Baker et al., 2004). Embryonic stem cells (ESCs) can be differentiated into specific neuronal subtypes and may be useful for cell replacement strategies in the central nervous system (Sonntag et al., 2007). Transplantation of ESC-derived dopaminergic neurons and cholinergic motoneurons (MNs) has been shown to promote partial recovery from Parkinson's-like symptoms and spinal cord injury, in rodent models (Roy et al., 2006; Erceg et al., 2010). Heterogeneous populations arising from differentiation of ESCs, however, currently limit the efficacy of such treatments (Gogel et al., 2011). Strategies for controlled differentiation of ESCs and the subsequent

*Abbreviations:* pMNs, progenitor motor neurons; PAC, puromycin N-acetyl transferase (PAC); NF, neurofilament; ChAT, choline acetyl transferase, MN, motoneuron; RA, retinoic acid; Shh, sonic hedgehog; Isl1, Islet1; GRE, gene regulatory elements; O4, oligodendrocyte marker 4; EB, embryoid body.

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enrichment ESC-derived cells types are therefore critical to the development of ESC-based therapies and diagnostic screening tools.

Directed differentiation of ESCs into spinal MNs can be achieved following exposure to retinoic acid (RA) and sonic hedgehog (Shh) (Wichterle and Peljto, 2008; Wichterle et al., 2002). During this process, ESCs first differentiate into progenitor motor neurons (pMNs) expressing the basic helix–loop–helix transcription factor Olig2 (Mizuguchi et al., 2001; Novitch et al., 2001). These cells can commit to the MN fate by downregulating Olig2 and expressing the homeodomain (HD) transcription factors Islet 1 (Isl1) and Hb9, also known as Mnx1 (Mizuguchi et al., 2001; Novitch et al., 2001; Arber et al., 1999; Pfaff et al., 1996). Despite optimization, differentiation protocols for pMNs result in a heterogeneous population of cells including other ventral spinal progenitor cells (Wichterle et al., 2002). $Hb9^+$-committed MNs compose only 15–50% of the total culture after differentiation of ESCs (Wichterle and Peljto, 2008; Deshpande et al., 2006). Low-purity cultures give rise to multiple types of spinal interneurons, therefore subsequent enrichment may be necessary (Deshpande et al., 2006).

Greater pMN purity can be obtained by fluorescence-activated cell sorting (FACS) of a transgenic ESC line that expresses GFP under the Olig2 gene regulatory elements (GRE) (Xian et al., 2005; Xian and Gottlieb, 2004; Xian and McNichols, 2003). This method, however, requires expensive equipment and must be performed at a centralized facility, risking contamination. Gradient centrifugation can enrich spinal MNs from the mouse embryonic lumbar spinal cord and human ESCs, but has not been optimized for mouse ESC-derived MNs (Wada et al., 2009; Wiese et al., 2010). Transgenic selection may provide a low-cost alternative and can be performed directly in the culture dish. Puromycin resistance through expression of the enzyme puromycin N-acetyl-transferase (PAC) has been shown to allow enrichment of ESC-derived cardiomyocytes and endothelial cells in transgenic lines (Marchetti et al., 2002; Kim and von Recum, 2009; Kolossov et al., 2006; Anderson et al., 2007), but has not been used to enrich specific neural populations.

In this study, we investigated whether transgenic selection could help to enrich low-purity populations that commonly result from pMN differentiation protocols. We generated a new heterozygous "knock in" mouse ESC line (P-Olig2) where the protein-coding region in one allele of Olig2 was replaced with PAC, allowing for positive selection of $Olig2^+$ pMNs during the differentiation. Olig2 expression was analyzed during directed differentiation of ESCs into pMNs using the Shh signaling agonist, purmorphamine (Sinha and Chen, 2006; Wu et al., 2004). Puromycin-treated cells were assessed for expression of pMN-specific markers and differentiation into pMN progeny, including MNs and oligodendrocytes. This study demonstrates the first use of puromycin resistance for positive selection of a specific population of neural progenitor cells.

## Results

### Olig2 expression during differentiation of ESCs

To determine the effect of Shh signaling levels on directed differentiation of ESCs into pMNs, we analyzed mRNA levels in response to increasing concentrations of purmorphamine, a Shh agonist, using quantitative real time (RT)-PCR. ESCs were exposed to 2 μM retinoic acid (RA) and 250 nM, 500 nM, or 1 μM purmorphamine. Relative mRNA levels were analyzed at the end of the $2^-/4^+$ differentiation protocol and were compared to control cells that did not receive RA or purmorphamine ($n=3$ for all conditions). Increasing the purmorphamine concentration from 250 nM to 1 μM led to downregulation of Dbx2 and Irx3, two transcription factors found in p1 and p2 progenitor (more dorsal) domains, respectively (Figs. 1A–B). The mRNA levels for Pax6, which is expressed in the p1, p2, and pMN domains, did not change with concentration. Nkx2.2 mRNA levels were too low for detection even at the highest concentration of purmorphamine (data not shown). Olig2 expression significantly increased with exposure to 1 μM purmorphamine compared to 250 nM and 500 nM purmorphamine (Fig. 1C). HD transcription factors Isl1 and Hb9 expressed during commitment of pMNs to the MN fate were upregulated with 1 μM purmorphamine, similar to Olig2. Finally, 1 μM purmorphamine led to an increase in mRNA for choline acetyltransferase (ChAT), an enzyme found specifically in mature MNs.

### Heterogeneity in differentiated cultures

To characterize the heterogeneity of the cell population resulting from differentiation of ESCs, we utilized a transgenic ESC line expressing GFP under the Olig2 GRE, G-Olig2 (Xian and Gottlieb, 2004). GFP fluorescence can persist for several days after transcription, allowing for identification of pMNs and their recent progeny during differentiation. By visual inspection of GFP fluorescence, pMNs could be easily separated from other spinal progenitor cells that differentiate into spinal interneurons rather than MNs.

G-Olig2 ESCs were differentiated using 1 μM purmorphamine and 2 μM RA and analyzed by flow cytometry. At the end of the $2^-/4^+$ differentiation protocol, 61.6±4.5% of cells expressed GFP ($n=3$). This percentage was approximately 3-fold higher than expression of Olig2 found with traditional antibody staining, suggesting that GFP fluorescence persists in recent pMN progeny as expected. $GFP^-$ cells were consistently found migrating away from EBs and displayed a broad flat morphology typical of astrocytes or astrocyte precursor cells (ASPs) (Figs. 2D–F; white arrows). These cells may originate from the p2 progenitor domain that gives rise to ASPs in vivo (Muroyama et al., 2005). Additional $GFP^-$ cells were present within EBs with a similar morphology to $Olig2^+$ pMNs and may represent progenitor cells from adjacent progenitor domains (white asterisk).

### Generating the P-Olig2 cell line

The P-Olig2 cell line was generated using a targeting vector with a resistance cassette in the open reading frame of the Olig2 gene surrounded by two regions homologous to the Olig2 locus. RW4 ESCs were electroporated with the P-Olig2 targeting vector and homologous recombination occurred as illustrated in (Fig. 3A). To confirm targeted insertion, novel junctions were detected using short arm junction PCR. Successful integration resulted in a 2.1 kb fragment spanning from inside the targeting construct into neighboring genomic