Progenitor Motor Neurons for Transplantation after Spinal Cord Injury

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Statement of Purpose: Sprouting of cholinergic axons has been associated with functional recovery following spinal cord injury (SCI). Cholinergic motor neurons, therefore, may be beneficial as a cellular bridges and may help rewire local circuitry following transplantation into the injured spinal cord. Obtaining sufficient motor neurons for transplantation studies, however, is not practical through simple dissection procedures. Embryonic stem (ES) cell-derived progenitor motor neurons (pMNs) may provide a source of motor neurons and induction of pMNs from ES cells has been shown using the ventralizing agent sonic hedgehog (Shh). In this study, we evaluated the induction of ES cells into pMNs using a simple suspension culture and a Shh signaling pathway agonist, purmorphamine. The goal of this work was to assess the potential for pMNs to differentiate into motor neurons that could be transplanted in combination with biomaterial scaffolds after SCI.

Methods: Mouse embryonic stem cells were induced to form embryoid bodies (EBs) containing pMNs using a newly developed 2-/4+ purmorphamine differentiation protocol [1,2]. Briefly, ES cells were grown on agar plates for two days in the absence of inducing agents (2^-). EBs were then induced for four days (4^+) using 20 mM retinoic acid (Sigma) and 250 nM, 500 nM, or 1 µM purmorphamine (CalBioChem). EBs not receiving retinoic acid or purmorphamine were used as uninduced controls.

Following induction, EBs containing pMNs were lysed and the RNA was purified with an RNeasy kit (Qiagen). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to determine the relative expression levels of the transcription factors (TFs) Olig2, Hb9, Irx3, and Dbx2. For each concentration of purmorphamine transcript levels were determined as a fold increase in expression over uninduced controls using the delta delta C_t method [3]. Flow cytometry was used to analyze EBs stained with primary antibodies against Olig2 (Millipore) and Hb9 (Abcam) followed by the appropriate AlexaFluor secondary antibodies (Millipore).

Results: Markers found during the development of cholinergic motor neurons were analyzed to determine the potential for purmorphamine-induced ES cells to differentiate into motor neurons for transplantation studies. Induction using purmorphamine led to increased expression of Olig2, a TF specific to the pMN domain in the developing vertebrate cord (Figure 1) [4]. Hb9, a fate specific marker for early motor neurons, was also up-regulated. TFs for dorsal regions (Irx3 and Dbx2) were downregulated with increasing concentrations of purmorphamine, as expected. Flow cytometry confirmed the presence of Olig2 and Hb9 TF proteins in EBs induced with 1µM purmorphamine (Figure 2). Greater expression was observed in induced EBs compared to the uninduced control group.

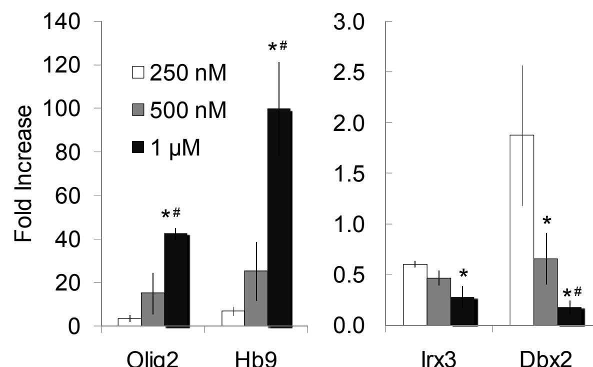

*Figure 1 Relative transcription factor expression levels for EBs induced with 250 nM, 500 nM, and 1 µM purmorphamine. Error bars represent standard deviation. * indicates $p<0.05$ versus 250 nM EBs. # indicates $p<0.05$ versus 500 nM EBs.*

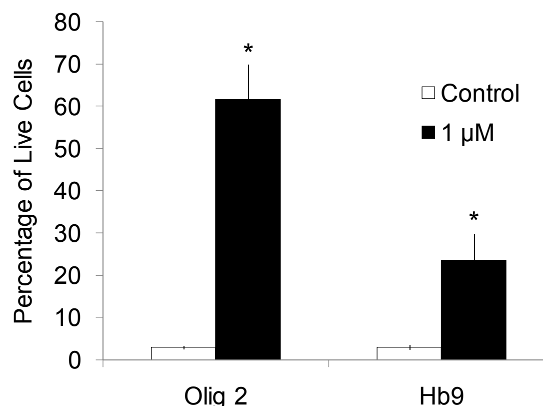

***Figure 2** Percentages of cells within EBs expressing TFs for pMNs (Olig2) and early motor neurons (Hb9). Error bars represent standard deviation. * indicates $p<0.05$ versus uninduced control.*

Conclusions: High concentrations of purmorphamine (1 µM) were found to mimic the ventralizing signal of Shh. These results suggest our new induction protocol with purmorphamine can efficiently generate pMNs that have the potential to differentiate into cholinergic motor neurons. Future work will characterize percentages of cholinergic motor neurons resulting from the induction and will determine the effect of growth factors on pMN differentiation. These cells will then be transplanted with biomaterial scaffolds to evaluate their ability to promote functional recovery after SCI.

References: [1] Li, X et al. Stem Cells. 2008: 26: 886-893. [2] Wichterle, H et al. Cell. 2002: 110: 385-397. [3] Schmittgen, T et al. Nature Protocols. 2008: 3: 1101-1108. [4] Dessaud, E et al. Development. 2008; 135: 2489-2503.