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# Selective neuronal differentiation of neural stem cells induced by nanosecond microplasma agitation☆

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**Abstract** An essential step for therapeutic and research applications of stem cells is their ability to differentiate into specific cell types. Neuronal cells are of great interest for medical treatment of neurodegenerative diseases and traumatic injuries of central nervous system (CNS), but efforts to produce these cells have been met with only modest success. In an attempt of finding new approaches, atmospheric-pressure room-temperature microplasma jets (MPJs) are shown to effectively direct *in vitro* differentiation of neural stem cells (NSCs) predominantly into neuronal lineage. Murine neural stem cells (C17.2-NSCs) treated with MPJs exhibit rapid proliferation and differentiation with longer neurites and cell bodies eventually forming neuronal networks. MPJs regulate ~75% of NSCs to differentiate into neurons, which is a higher efficiency compared to common protein- and growth factors-based differentiation. NSCs exposure to quantized and transient (~150 ns) micro-plasma bullets

*Abbreviations:* CNS, central nervous system; MPJs, microplasma jets; NSCs, neural stem cells; NO, nitric oxide; qRT-PCR, quantitative real-time PCR; NDs, neurological diseases; ROS, reactive oxygen species; RNS, reactive nitrogen species; UV, ultraviolet; GFAP, glial fibrillary acidic protein; APC, adenomatous polyposis coli; OEC, olfactory ensheathing cell; FBS, fetal bovine serum; HS, horse serum; DMEM, Dulbecco's modified Eagle medium; SDS-PAGE, sodium dodecyl sulfate-polyacryl amide gel electrophoresis; TH, Tyrosine Hydroxylase.

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up-regulates expression of different cell lineage markers as β-Tubulin III (for neurons) and O4 (for oligodendrocytes), while the expression of GFAP (for astrocytes) remains unchanged, as evidenced by quantitative PCR, immunofluorescence microscopy and Western Blot assay. It is shown that the plasma-increased nitric oxide (NO) production is a factor in the fate choice and differentiation of NSCs followed by axonal growth. The differentiated NSC cells matured and produced mostly cholinergic and motor neuronal progeny. It is also demonstrated that exposure of primary rat NSCs to the microplasma leads to quite similar differentiation effects. This suggests that the observed effect may potentially be generic and applicable to other types of neural progenitor cells. The application of this new *in vitro* strategy to selectively differentiate NSCs into neurons represents a step towards reproducible and efficient production of the desired NSC derivatives.
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## Introduction

Neural stem cells (NSCs), with their ability to differentiate into mature cell types provide an unlimited source of 'raw material' for regenerative medicine therapies of many incurable diseases (Bergmann and Frisén, 2013). However, applications of NSCs in regenerative medicine are hampered by the lack of insufficient methods for directed differentiation. Cell lineages derived from NSCs including neurons, oligodendrocytes and astrocytes are of significant interest for many applications in regenerative medicine and medical treatments of traumatic injuries of neural system (Hernández-Benítez et al., 2012; Rossi and Cattaneo, 2002).

Neurological diseases (NDs) generally affect the central, peripheral, and vegetative nervous systems. They mainly effect by functional disruption of feelings, motions, and consciousness. NDs include brain trauma, spinal cord injury, crania-cerebral, Alzheimer's and Parkinson's diseases (Bjorklund and Lindvall, 2000; Breunig et al., 2011; Grois et al., 1998; McKay, 1997; Rodriguez et al., 2009; Sleeper et al., 2002). Presently available treatments of nervous system diseases include: 1) traditional drug therapy in which drugs are used as a source of nutrition for the recovery of damaged nerve cells; 2) gene therapy; and 3) transplantation of central nervous tissues (Sunde and Zimmer, 1981). However, the progress in approaches based on nervous tissue transplantation is quite limited, in part due to ethics regulations related to embryonic brain. Recently, a new method based on neural stem cells isolation and cloning has been employed as a successful *in vitro* strategy for the treatment of nervous system diseases (Reynolds and Weiss, 1992; Temple, 2001). NSCs exhibit the potency to differentiate into the main phenotypes in the nervous system (*e.g.*, cortical neurons) and as such show great potential to resolve neurological and neurotraumatic disorders without raising ethics issues (Breunig et al., 2011).

The key purpose of cultivating neural stem cells *in vitro* is to control and enhance the cell fate commitment, differentiation into well-resolved cell types, proliferation, and migration. Therapeutic use of NSC differentiation into neurons for tissue transplantation to treat nervous system disorders is an important hallmark of stem cell research (Temple, 2001). This method is considered one of the most promising therapies for neurodegenerative and neurotraumatic diseases. Previous reports have shown that cytokines, chemicals, Chinese medicines, selected proteins, hormones, electrical stimulation, and local environmental factors could affect the differentiation of NSCs (Boyer et al., 2005; Chambers et al., 2003; Flanagan et al., 2006; Hwang et al., 2004; Okada et al., 2004; J.-H. Wang et al., 2006; J. Wang et al., 2006). For example, excessive nephroblastoma gene expression may enhance NSCs proliferation and differentiation (Shi et al., 2006). Carbon nanotube ropes with electrical stimulation may also stimulate the differentiation of NSCs into the neuronal lineage (Huang et al., 2012). Olfactory ensheathing cell (OEC) conditioned medium was found to promote axonal regeneration and functional recovery after transplantation (Barnett, 2004; Pellitteri et al., 2009). However, these biochemical (*e.g.*, based on the presentation of differentiation factors in the medium) and more recent, nanotechnology-based methods of NSCs differentiation have quite significant drawbacks including chemical toxicity, insufficient selectivity of cell-type-specific differentiation, glial scar formation after transplantation, and several others (Rossi and Cattaneo, 2002). Therefore, there is a strong demand for new techniques for cell-type-specific, efficient, and safe NSC differentiation.

Non-thermal (non-equilibrium) atmospheric-pressure plasmas (Ostrikov et al., 2013), including atmospheric-pressure room-temperature microplasma jets (MPJs) of interest here have recently been very effective in cancer therapy, blood coagulation, root canal treatment, wound healing, antimicrobial and other applications (Keidar et al., 2011; Kim et al., 2010; Kolb et al., 2008; Laroussi, 1996; Lloyd et al., 2010). There are two most common types of the atmospheric-pressure plasmas that are used in biomedical treatments, namely "direct" and "indirect" plasma devices. In the first type of devices, a living tissue acts as an electrode, while the second type of devices, which include the MPJs, produces the plasma plumes that emerge outside the area where the electrodes are located. However, the reactive species and the biological effects produced by the both plasma device types are quite similar. The factors produced by the plasmas include reactive oxygen species (ROS), reactive nitrogen species (RNS), ultraviolet (UV), as well as charged ions and electrons (Kong et al., 2009).

ROS such as OH and O could disrupt cells from the cell membrane to the inner nucleus through oxidation, which results in the effective inactivation of bacteria, fungi and viruses, or even programmed death of cancer cells (Dobrynin et al., 2009; Imlay et al., 1988; Lu et al., 2008b; Xiong et al., 2011). Non-thermal atmospheric-pressure plasmas can also enhance endothelial cell proliferation (Kalghatgi et al., 2010). These effects can be achieved by optimizing the treatment conditions such as the plasma parameters, working gases, and treatment time, which affect the dose of energy exposure, which in turn determine cell fate and behavior

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