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Research Report

Fetal mouse mesencephalic NPCs generate dopaminergic neurons from post-mitotic precursors and maintain long-term neural but not dopaminergic potential *in vitro*

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ABSTRACT

Stem cells have one major advantage over primary cells for regenerative therapies in neurodegenerative diseases. They are able to self-renew making sufficient quantities of cells available for transplantation. Embryonic stem cells and fetal neural progenitor cells (NPCs) have been transplanted into models for PD with functional recovery of motor deficits. However, their precise characteristics are still unknown and ideal conditions for their long-term expansion and differentiation into dopamine neurons remain to be explored. Mouse fetal NPCs are commonly grown as characteristic neurospheres, but they also proliferate under monolayer culture conditions. We investigated the proliferative behavior and dopaminergic differentiation capacity of fetal mouse midbrain NPCs derived from E10 to E14 embryos expanded either as neurosphere or monolayer culture. We found similar proliferation capacities in NPCs of all embryonic stages. Neuronal differentiation capacity is higher in neurosphere cultures compared to monolayer NPCs and persists in long-term cultures. We did not find dopaminergic differentiation in long-term expanded mouse NPC types, which is in contrast to rat and human fetal midbrain NPCs. Mouse NPCs generate dopaminergic neurons until up to three weeks *in vitro* but they do not incorporate BrdU. Quantitative analysis showed that they were not just primary neurons from the isolation process but formed to a great extent *in vitro* during differentiation suggesting that they are formed by promotion of post-mitotic neuroblasts. A detailed transcription profile reveals de-specification processes during *in vitro* cultivation, which matches their NPC behavior. We provide the constitutive work for studies using fetal midbrain NPCs of mouse including transplantation studies and transgenic models.

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1. Introduction

Neural stem cells (NSCs) in the developing and adult brain generate neural tissue and are able to regenerate the brain making them a very attractive tool towards treatment of neurodegenerative diseases or ischemic injuries (Arenas, 2010; Lindvall and Kokaia, 2006; Meyer et al., 2010). Pluripotent stem cell-derived neural progenitor cells (NPCs) can be expanded to obtain higher numbers and generate more dopamine neurons than tissue-derived NPCs (Bjorklund et al., 2002; Kim et al., 2002). Tissue-derived NPCs lack tumor formation after grafting (Arnhold et al., 2004; Carvey et al., 2001; Chung et al., 2006a). NPC cultures *in vitro* have been successfully established from a number of organisms and regions of the brain by addition of epidermal growth factor (EGF) and/or basic fibroblast growth factor (FGF-2). They have been characterized by neurosphere forming assays (Reynolds and Weiss, 1992), clonal growth and prolonged self-renewal (Reynolds and Weiss, 1996; Storch et al., 2001; Uchida et al., 2000) as well as their ability to form neurons and glia *in vitro* (Cai et al., 2002; Reynolds and Weiss, 1992; Storch et al., 2001, 2003). In general, fetal NPCs show an early rostro-caudal specification of their differentiation capacity and only NPCs from midbrain tissue are able to differentiate into functional dopamine neurons (Potter et al., 1999; Storch et al., 2001). Indeed, functional dopamine neurons have been generated from expanded fetal human NPCs (Milosevic et al., 2006; Storch et al., 2001; Wang et al., 2004). Likewise, rat midbrain NPC cultures were successfully established, expanded and differentiated into dopaminergic neurons (Carvey et al., 2001; Ling et al., 1998). Fetal NPCs from mouse brain seem to have different characteristics: it was reported that establishing mouse ventral mesencephalic NPC cultures was infrequent (Smith et al., 2003) and Chung et al., (2006b) were not able to generate dopamine neurons from fetal mouse mesencephalic NPCs after four weeks in culture.

Several factors have been discovered that are important for the ability to self-renew and differentiate *in vitro*. We and other groups reported the importance of reduced oxygen for the long-term survival and retaining the potential to generate neurons (Milosevic et al., 2005; Studer et al., 2000; Zhang et al., 2006). Tgf-β and Wnts play important roles only in short-term expanded rodent NPC cultures (Castelo-Branco et al., 2003, 2006; Farkas et al., 2003; Roussa et al., 2006). We have shown previously that IL-1 is the crucial factor for dopaminergic differentiation in tissue-specific NPCs of rat and human origin (Carvey et al., 2001; Ling et al., 1998; Storch et al., 2001). Moreover, IL-1 was successfully used to generate dopaminergic neurons from embryonic stem (ES) cell-derived NPCs (Kania et al., 2005; Rolletschek et al., 2001) and is one of the factors that mediate astrocyte-driven neuronal differentiation of adult NPCs (Barkho et al., 2006).

The purpose of the present study was to define the characteristics for *in vitro* cultivated tissue-specific NPCs of mouse midbrain origin over time. We determined their potential to expand, producing high numbers of precursors and to subsequently generate a neurogenic culture suitable for transplantation studies.

2. Results

2.1. *Generation of dopaminergic neurons in short-term expanded NPCs in vitro*

Fetal mouse NPC cultures were efficiently initiated with the growth factor combination Egf/Fgf-2, but also with single mitogens Egf or Fgf-2 (Fig. 1). Quantitative neurosphere forming assay for NPCs that were cultivated with Egf, Fgf-2 or the combination of both revealed a significant increase of neurosphere-forming cells in Egf/Fgf-2 cultures compared to both single mitogen cultures (see Fig. 1B for quantitative and statistical results) indicating that the combination of mitogens generated more NPCs from the midbrain tissue source.

When E14 neurosphere cultures were significantly expanded for one or two weeks using Egf, Fgf-2 or both mitogens, subsequently differentiated for one week and analyzed by immunocytochemistry (Fig. 1A), fetal midbrain NPC cultures displayed dopaminergic neurons, which were double positive for tyrosine hydroxylase (Th) and Map2ab; (Fig. 1C). NPCs expanded for one week and differentiated thereafter for another week did not display more dopaminergic neurons than the preceding NPCs. However, NPCs that were expanded with Egf, Fgf-2 or the combination of both mitogens for two weeks and subsequently differentiated with IL-1/Forskolin, yielded more dopaminergic neurons than control (no factor differentiation) samples and preceding NPCs (Fig. 1C). This effect was specific for dopaminergic differentiation, because the general neurogenic potential ($Map2ab^+$ cells) at one or two weeks *in vitro* was not influenced by IL-1/Forskolin (Fig. 1C). To investigate the origin of these newly generated Th^+ dopamine neurons, we performed BrdU labeling of the expanding NPCs for 18 h with subsequent differentiation. This procedure showed dopaminergic neurons that were co-labeled with Nurr1, Lmx1 as well as DAT, demonstrating the midbrain-derived origin and maturity of dopaminergic neurons (Fig. 1D–F). However, those dopaminergic neurons were not co-labeled with BrdU in up to three weeks expanded E14 midbrain cultures (Fig. 1G). Similarly, no single Th/Map2ab/$BrdU^+$ cell was detected in differentiated NPCs from E12 midbrain tissue. In E10 NPC cultures one of 3794 NPCs (12 out of 45,529 counted cells from three experiments) was Th/Map2ab/$BrdU^+$. Together, these data show that the Th^+ dopamine neurons specifically generated from NPC cultures by IL-1/Forskolin treatment evolved from post-mitotic dopamine precursor cells in culture. The significant increase of dopamine cell counts by differentiation conditions rules out cell survival of primary dopamine neurons from the isolated tissue.

On the other hand, oligodendrocytes (GalC), astrocytes (Gfap) and neurons (Map2ab) were evidently formed from previously mitotic NPCs (Fig. 1H–J).

2.2. *Fetal mouse mesencephalic NPCs maintain proliferative potential as neurosphere or monolayer culture for long periods in vitro*

For long-term experiments fetal NPCs were grown as typical neurosphere culture or as a monolayer on poly-L-ornithine/fibronectin-coated dishes for at least 8–12 weeks prior to

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