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Comparison of dynamic behavior and maturation of neural multipotent cells derived from different spinal cord developmental stages: an *in vitro* study

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Neural progenitor cells (NPCs) are characterized as undifferentiated cells with the ability of self-renewal and multipotency to give rise to other cells of the nervous system. In our *in vitro* study we demonstrate the proliferative and differentiative potential of NPCs isolated from the spinal cord at different developmental stages (embryonal, early postnatal, adult), maintained and expanded within neurospheres (NSs). Using the NSs culture system, we examined the size, number of NSs and their fate when exposed to differentiation conditions. Based on immunocytochemical analyses for cell markers (MAP 2, GFAP, RIP) we evaluated the occurrence of various cell types: neurons, astrocytes and oligodendrocytes. The results show that NSs increased in size during cultivation time *via* NPC proliferation, but proliferation potential decreased during maturation stages. In addition, NPCs derived from spinal cord developmentally different stages gave rise to a consistent ratio of glial and neuronal progeny (3:1), and adult tissues represent a comparable source of NPCs compared to embryonal and early postnatal tissues. These data provide useful information for large-scale *in vitro* expansion of NPCs required for potential cell therapy after spinal cord injury.

Key words: neural progenitor cells, neurospheres, *in vitro* cultivation

The terms neural stem cells and progenitors NSCs/NPCs (herein collectively termed neural progenitor cells – NPCs) refer to the multipotent cells that give rise to other cells of the nervous system. NPCs characterized by multilineage potency and self-renewal capacity are present during embryonic development, as well as in certain regions of the adult central nervous system (CNS), brain and spinal cord (SC) (Reynolds et al. 1992, Palmer et al. 1997, Chiasson et al. 1999, Gage 2000, Alvarez-Buylla et al. 2001, Okano and Sawamoto 2008, Skup et al. 2014). NPCs have been identified in the adult mammalian CNS along with the entire neuroaxis from the forebrain to the spinal cord. There are two constitutive neurogenic sites in the adult CNS, the subventricular zone and subgranular zone (Weiss et al. 1996, Shihabuddin et al. 1997, Temple and Alvarez-Buylla 1999). The adult SC has also been shown to contain progenitors for neurons and glia with proven localization of NPCs in: the white matter parenchyma (Horner et al. 2000, Yamamoto et al. 2001), the region around the central canal (Kulbatski et al. 2007, Mothe et al. 2011), either in the ependyma (Meletis et al. 2008), or subependymally (Martens et al. 2002). The central canal extends into the terminal part of the SC, the filum terminale (FT) (Varghese et al. 2010). The filum terminale is the rudimentary, most caudal part of the spinal cord and it develops as a consequence of the unequal growth rate of the vertebral canal vs. spinal cord. For a long time it was considered that the FT consisted of a fibrous strand bonding the caudal end of the spinal cord to the vertebral canal and played no functional role in the postnatal nervous system. Traditionally, the FT has been regarded as a fibrovascular tag of clinical significance only if it resulted

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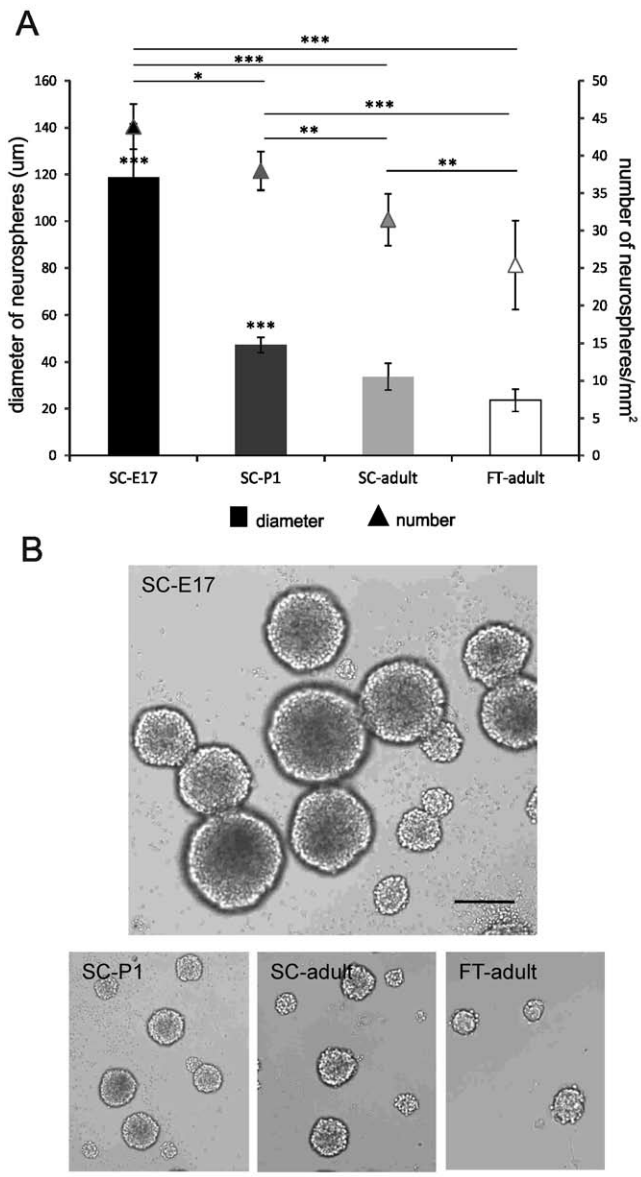

Fig. 1. Number and size of neurospheres (NSs) derived from individual spinal cord tissues of embryonal/SC- E17, postnatal day 1/SC-P1, adult/SC-adult and filum terminale/ FT-adult during cultivation in proliferation media on the tenth day *in vitro*. The number of NSs was counted in 10 different fields/each group and calculated *per* mm^2. The number of NSs ranged from 28 to 46 spheres/mm^2 with the required statistical significance (*P<0.05, **P<0.01, ***P<0.001). The measured diameter of formed NSs in ten random fields/each group was in the interval of 29–104 μm, with the vast majority of the largest spheres occurring in culture derived from SC-E17 (***P<0.001) (A). Brightfield images of neurospheres obtained from individual spinal cord tissues illustrating the size and amount of cultivated NSs. Scale bar is 100 μm (B).

in the overstretching or compression of the SC, giving rise to the tethered cord syndrome (Yamada et al. 2004). Treatment often includes surgical removal of the FT with beneficial outcomes for patients (Garces-Ambrossi et al. 2009). Subsequent findings produced evidence controverting this opinion, and recent histological studies have shown that the FT contains an ependymal-lined canal, glial and neuronal cell bodies, as well as stem/progenitor cells in rats (Rethelyi et al. 2004, 2008, Boros et al. 2008), and also in humans (Varghese et al. 2009, Arvidsson et al. 2011).

NPCs can be isolated and expanded *in vitro* to study their characteristics. The possibility to cultivate NPCs through the assay of neurospheres (NSs) was first described by Reynolds and coworkers (1992) and is now widely used for determining the presence of NPCs *in vitro*. NPCs can be expanded in serum-free medium supplemented with epidermal growth factor and fibroblast growth factor, which act as mitogens for these cells both *in vitro* (Reynolds et al. 1992, Gritti et al. 1996, 1999) and *in vivo* (Craig et al. 1996). Generally, NSs are derived from a single-cell suspension of neural stem and progenitor cells isolated from the adult or fetal CNS, but NSs culture can also be established from embryonic stem cells (ESCs) (Tropepe et al. 2001, Jensen and Parmar 2006). NSs are spheroid, free-floating cultures, 3D structures that consist of cells with a rich extra-cellular matrix, composed of a heterogenous mix of neural stem cells, neural progenitor cells and more differentiated cells at different stages of mitotic division (Alam et al. 2004) with varying capability to form NSs. Although both neural stem cells and neural progenitor cells are able to give rise to NSs, only neural stem cells can retain the ability to form NSs over long periods of the time in culture (Reynolds and Rietze 2005). Until recently, no specific markers were available to explicitly identify NPCs, because of their paucity and primitive nature (Kim and Morshead 2003), so these cells were identified by their self-renewal capacity. The ability to form NSs is still a good indicator of the presence of NPCs (Louis et al. 2008). When floating NSs are plated on adhesive substrate and the mitogens are removed, progenitor cells migrate out of the spheres and differentiate into neurons and glia (Gage et al. 1995)

In our *in vitro* study using rats, we demonstrate and compare the proliferative and differentiating potential of NPCs isolated from embryonic day 17 (SC-E17), postnatal day one (SC-P1) and adult spinal cord (SC-adult) and filum terminale (FT-adult).

All experiments conformed to the Slovak Law for Animal Protection No. 23/2009, which is transposed from Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes, and were approved by the Institutional Ethical Committee for animal research.

Neural progenitor cells were harvested from embryonic day 17 (E17) (n=12), postnatal day one (P1) (n=12) spinal cord, adult spinal cord (SC) (n=6) and filum terminale (FT) (n=6) of male Wistar rats. The overlying meninges of spinal cords were removed, the dissected individual tissues were cut into small pieces and transferred into a solution (Worthington Biochemicals, New Jersey) containing 0.01% papain and 0.01% DNase for 1.5 hour at 37°C and then mechanically dissociated into a cell suspension which was centrifuged using a discontinuous density gradient to remove cell membrane fragments. Harvested single cells were cultivated in Nunc T25 culture flasks (5 000 cells/cm^2), grown in proliferation culture medium composed of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 (1/1 v/v) (Biowest, Nuaillé,France) supplemented with 5 mg/ml streptomycin, 5 IU/ml penicillin (Biochrom AG, Berlin, DE), B27, N2 (Gibco; Invitrogen, Carlsbad, CA) and growth factors: basic fibroblast growth factor-2 (bFGF-2) and epidermal growth factor (EGF) (both 20 ng/ml) (AppliChem GmbH, Darmstadt, Germany) to allow the formation of neurospheres (37°C, 5% CO_2). Using the NSs culture, we investigated the qualities of NSs: the number of NSs/mm^2 and the size of NSs (μm) on the tenth day of cultivation (10DIV-day *in vitro*). We counted the number of neurospheres in 10 different fields/each group and calculated the number of NSs *per* mm^2. Considering NSs size, we measured the diameter of formed NSs in ten random fields/each group. After analysis, these neurospheres were dissociated by means of mechanical trituration and cultured in growth factors free and 3% foetal calf serum (FCS) (Biowest, Nuaillé, France) enriched differentiation medium on laminin-coated (Gibco; Invitrogen, Carlsbad, CA) plates for an additional 14 days to induce final differentiation. Using immunocytochemistry and applying specific antibodies, the populations of neurons (MAP 2 1:200), astrocytes (GFAP 1:500) and oligodendrocytes (RIP 1:1 000) were analyzed. The number of cells positive for each specific marker was counted as a percentage of total DAPI + nuclei in 10 random visual fields of cells (400–600 cells)/tissue/per each marker. In subsequent NSs passages the self-renewal was determined as the number of secondary neurospheres/number of seeded cells ×100, i.e how many precursors capable of generating a "daughter" neurosphere were contained in each "mother" neurosphere (Lu et al. 2010). Data are presented as mean ± SEM. Statistical differences between groups were evaluated with one-way analysis of variance (ANOVA). Values of *P<0.05, **P<0.01, ***P<0.001 were considered to be statistically significant.

After 2–3 DIV of cultivation in proliferation media, the first NSs were observed, mainly in cultures derived from SC- E17, SC-P1 and SC-adult tissues. In FT-adult derived culture, the NSs developed after 3–5 DIV. The NSs initially appeared as smaller clusters of 2–4 cells and later grew into larger NSs. The NSs were free-floating and were identified by their spherical structure and bright phase appearance. During the cultivation we observed time-dependent changes in the neurospheres. During cultivation time NSs increased in size *via* NPCs proliferation, but proliferation potential decreased with maturation and no obvious differences were observed in the morphologies of types of NSs, e.g. shape, density. The number of NSs *per* primary culture varied depending on the tissues they were derived from. The highest number of NSs was found in culture derived from embryonic tissue (45 NSs/ mm^2 on average), and during maturation the proliferation activity of NPCs expressed by NS formation ability

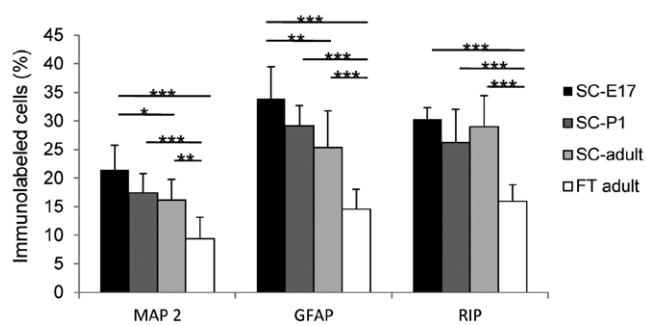

Fig. 2. Phenotype of neural progenitor cells (NPCs) obtained by mechanical dissociation of neurospheres and cultivated in differentiation medium for 14 days. Based on immunocytochemical analyses, the occurrence of neurons (MAP 2), astrocytes (GFAP) and oligodendrocytes (RIP) was counted as a percentage of total DAPI+ nuclei in 10 random visual fields of cells (400–600 cells)/tissue *per* each marker. Percentages of phenotypes changed during the developmental stages, with preservation of glia:neuron ratio 3:1 (*P<0.05, **P<0.01, ***P<0.001).

went into decline (see Fig. 1). The same pattern was observed in NSs size (see Fig. 1). Moreover the self-renewal capacity of NPCs decreased during the developmental stages (SC-E17=3.4; SC-P1=2.5; SC-adult=1.7; SC-FT=1.3). So the most and the largest NSs were formed in culture derived from SC-E17, which means that NPCs derived from embryonic tissue are the most potent. Interesting findings were that the FT exhibited similar characteristics in NSs formation and proliferation, while the number of neurospheres from the FT is almost half the number of SC-E17, and the NSs of the FT are six times smaller than the embryonic NSs.

After ten days of NPC cultivation and NSs formation, these NSs were dissociated and the final differentiation was induced. After replacing growth factors with FCS in the cultivation media, the dissociated cells started to attach to the laminin-coated surface and underwent differentiation into neurons, astrocytes and oligodendrocytes at different rates, because not only proliferation, but also differentiation of NPCs is mediated by the effects of external stimuli coming from medium components (Cameron et al. 1998, Hung and Young 2006). NPCs derived from all tissue began to differentiate in the same manner, but occurrence of different neural cell types changed during the developmental stages (see Fig. 2). No morphological differences in neurons, astrocytes and oligodendrocytes were seen between any experimental groups (see Fig. 3) . In our FT cultures, and in fact in all cultures (E17, P1, SC-adult), the glia:neuron ratio 3:1 was preserved. Although in younger stages (E17, P1) in the glia population the astrocytes predominated over the oligoden-

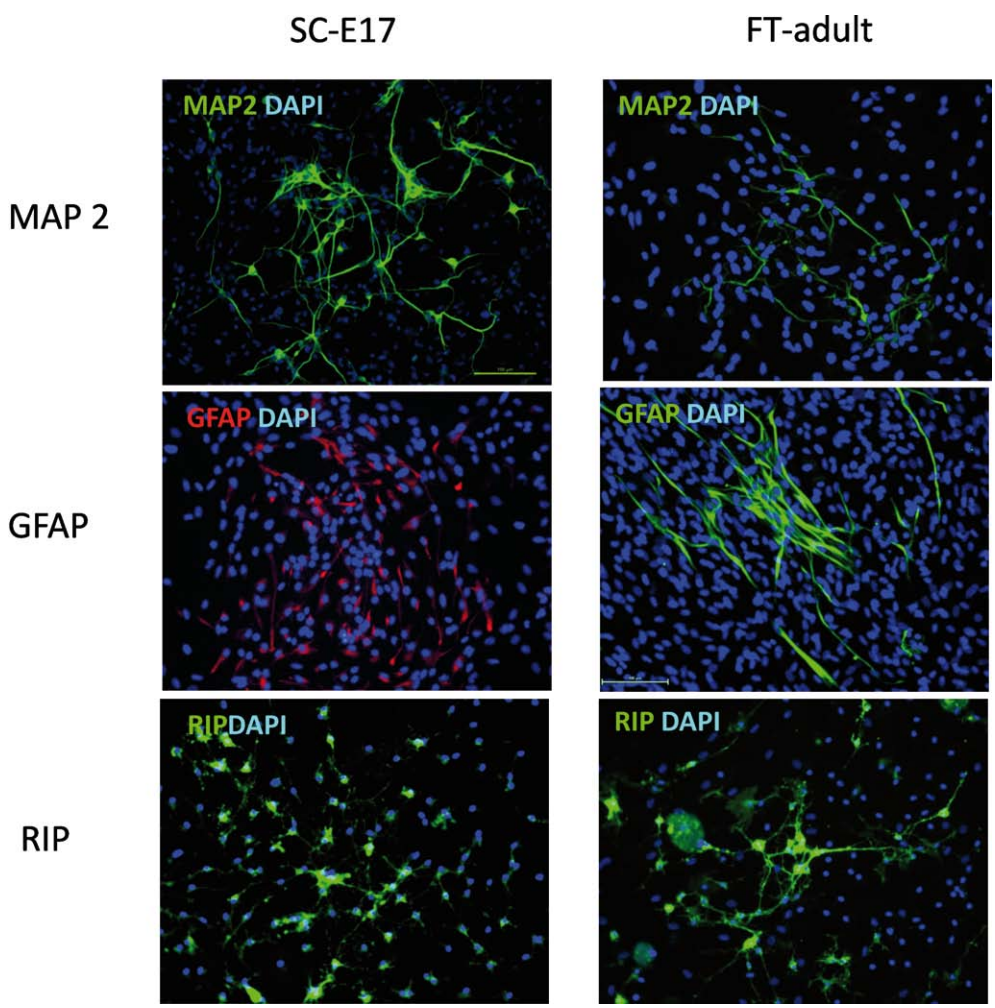

Fig. 3. Immunocytochemical identification of the phenotype of neural progenitor cells (NPCs) obtained from embryonal (SC-E17) and adult (FT-adult) filum terminale that were cultivated in differentiation medium for 14 days. Expression of neuronal (MAP2), astroglial (GFAP) and oligodendroglial (RIP) markers. Scale bar equals to 100 μm.

drocytes, in adult stages the representation of glia converted in favor of oligodendrocytes with preservation of the total volume of glia vs. neurons. This may reflect some internal programming of progenitors specific for their region of origin. In studying NPCs from the human FT, Varghese and colleagues (2009) came to similar results, obtaining a 2:1 glia:neuron ratio. However, not all NPCs are identical since the proliferative and differentiation capacity of NPCs is dependent upon the location and developmental stage from which these cells are derived. *In vitro* experiments with NPCs originating from different CNS regions have revealed that the original NPCs were indeed regionally specified; for example in cultures obtained from brain, a 2:7 glia: neuron ratio was found (Hitoshi et al. 2002, Moe et al. 2005, Sypecka et al. 2013). Cells derived from NSs are plastic in their differentiation, and by changing the media composition the proportion of the three neural cell types can be altered. But in our experiment, all cells derived from different tissues were cultivated under the same conditions.

The discovery of NPCs, followed by deeper understanding of their characteristics and behaviour, opens up the potential for new treatments of devastating neurodegenerative diseases and damage such as stroke and spinal cord injuries. Currently there are several treatments available for these diseases involving the preferential use of ESCs (Nistor et al. 2005). ESCs probably have greater plastic potential than adult stem cells; however, ethical concerns and their potential for unwanted and possibly dangerous continued growth and tumor formation limit their application (Nussbaum et al. 2007). An alternative for ESCs are stem cells obtained from tissue after birth. NPCs have been harvested from the adult brain (Lois and Alvarez-Buylla 1993, Nunes et al. 2003) and spinal cord (Mayer-Proschel et al. 1997). However, adult stem cells are less plastic than ESCs and divide less frequently in culture (Doetsch et al. 1999). On the other hand, they offer the advantage that they can be transplanted without genetic modifications. Moreover, adult stem cells show a high degree of genomic stability during culture (Foroni et al. 2007). Finally, there is much less moral concern surrounding the use of adult stem cells because they can be harvested from patients, so they can be used in an autologous fashion (Nandoe Tewarie et al. 2009).

In our study, putative NPCs were isolated from FT samples using both mechanical and enzymatical dissociation. The neurosphere culture system remains an extremely useful tool to analyze the behavior of NPCs, self-renewal capacity and multipotency of neural stem and progenitor cells, and represents a potential source for cell replacement therapy (Hung and Young 2006, Jensen and Parmar 2006, Mothe et al. 2008, Parr et al. 2008, Sulla et al. 2010)

Our results support the suggestion that NPCs derived from the FT are easy to obtain, proliferate readily, and are able to differentiate into the cells of gliogenic lineages and neurogenic (motor neurons) *in vitro* (Jha et al. 2013), which represents a promising way for their utilization in cell-based therapies for CNS injuries and diseases. FT-derived NPCs could be utilized for their next purpose immediately after their isolation, or could be stored by freezing, because cryopreservation does not affect the proliferation and multipotency of NPCs (Milosevic et al. 2005). The FT is an excellent candidate as a source of autologous multipotent cells with putative biological and clinical implication. Because the FT is abundant, reproducible and easily accessed without moral concern, FT-derived NPCs offer a more clinically feasible source than embryonal stem cells.

In conclusion, our findings add further evidence for the existence of cells with neural progenitor nature within the filum terminale. In all tissues, SC-E17, SC-P1, SC-adult, FT-adult, the presence of neural progenitor cells was determined *via* neurosphere formation, as a good indicator of NPCs proliferation. Another indicator of NPCs proliferation in culture is the increase in neurosphere size with cultivation time. NPCs from SC-E17 were more potent, producing the largest neurospheres, but the NPCs proliferation potential decreased during subsequent developmental stages. The NPCs' ability of terminal differentiation into neurons, astrocytes and oligodendrocytes decreased during maturation, while the glia:neuron ratio 3:1 was preserved, which may reflect some internal programming specific for cells isolated from the spinal cord. The FT exhibits the same features in neurosphere formation, proliferation and terminal differentiation as other parts of the adult spinal cord, which suggests that the FT is an equivalent part of the spinal cord and not just a problematic remnant; in fact it may be a potential source of NPCs with promising biological and clinical implications.

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