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Chapter 14

Primary Neural Stem Cell Cultures from Adult Pig Brain and Their Nerve-Regenerating Properties: Novel Strategies for Cell Therapy

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Additional information is available at the end of the chapter

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1. Introduction

Neurons are one among the very few differentiated cell types which are strictly post-mitotic and therefore non-renewable by sister cell divisions. The production of new neurons, or neurogenesis, does though occur in limited areas of adult mammalian nervous system, due to the existence of intrinsic stem cells which has been formally demonstrated by a primary culture approach on adult brain tissues from mouse [1], man [2,3] and other species [4]. These "neural stem cells" satisfy the general stemness criteria, i.e.: illimited proliferative activity, self-renewal capacity, multipotency (i.e. capacity to generate all the differentiated cell types of their host tissue, which for neural stem cells encompasses neurons, astrocytes, oligodendrocytes), proliferative reactivity to tissue lesions and physiological signals [5]. This discovery has raised great promise for clinical repair in neurology and led to coin a novel principle in cell therapy, which consists in replacing lost neurons by grafting *in vitro* purified neural stem cells [6,7,8]. This theoretical principle has been challenged by the putative tumour-promoting potential of grafted proliferative cells. Such risk can reasonably be ruled out by the recent demonstration that adult brain-derived neural stem cells are highly resilient to tumorigenesis and senescence, much more than embryonic neuroblasts, embryonic stem cells, induced pluripotent stem cells or any other stem cell type from adult mammalian tissues [9]. However, the use of homotypic neural stem cell transplantation in human clinics seems ethically difficult, since it requires initial sampling of alive brain tissue. In order to overcome this limitation, we have developed primary neural stem cell culture from adult porcine brain. The pig is indeed the non-primate species that is immunologically closest to human [10], which already allowed successful protocols of xenotransplantation in human clinics using *in vitro*-expanded primary cell preparations from several adult pig tissues – but not from the nervous system.

2. General properties of neural stem cells in adult mammals

Neural stem cells are identified and isolated in primary cultures of adult nervous tissues in the presence of epithelial growth factor (EGF) and basic fibroblast growth factor (bFGF), by the growth of cellular masses called "neurospheres" which include the three neural lineages: neurons, astrocytes, oligodendrocytes [1]. This primary culture is termed "neurosphere assay" (NSA) and allows to generate and expand great numbers of proliferative neurogenic cells through successive passages [11]. Recent methodological refinements allowed to isolate and distinguish neural stem cells *per se* from downstream progenitors, i.e. proliferative cells being committed to a single lineage although yet undifferentiated [12].

Beside the two initially recognized and most active neurogenic structures of adult mammalian brain (the subventricular zone of telencephalon and the subgranular layer of hippocampal dentate gyrus), several topographically restricted regions of the central nervous system were shown to yield neurospheres in EGF-bFGF-treated primary culture and therefore to contain neural stem cells [13,14]. Spatial frequency among tissue and *in vivo* yield of neuronal versus glial progenies are highly variable, ranging among: 95% in hippocampus and hypothalamus, 60-70% in SVZ-olfactory bulb system, 40% in dorsal vagal complex, 0% in spinal cord despite as high a proliferation rate as in SVZ [15]. However, *in vitro*-expanded neurospheres display strikingly reproducible properties, regarding both growth dynamics (10-12 days for the primary generation, 6-7 days for the subsequent ones) and lineage proportions after differentiation commitment by growth factor removal: 25% neurons, 70% astrocytes, 5% oligodendrocytes [11].

Neural stem cells generate differentiated progenies, like other types of adult stem cells, through several intermediate stages which can now be distinguished by their respective marker combinations – or "molecular profiles" or "transcriptomic signatures" (Figure 1). The biological properties of neural stem cells from adult nervous tissues are reminiscent of those characterizing neural progenitors from embryos, especially *in vitro*. However, accumulated data in the past decade have revealed important differences between these two categories of neural precursor cells, which led to consider them as distinct cell types. The differences include indeed intrinsic molecular mechanisms of proliferation and differentiation, as well as receptivity to extracellular signals [16,17,18]. Most interestingly, neural stem cells from adults are much less teratogenic than embryonic stem cells and foetal neural progenitors [9,19, 20,21,22]. Primary cultures of neural stem cells from the archetypical subventricular zone of rodent, which is the most highly proliferative of endogenous neurogenic niches in mammalian brain, could indeed be maintained over more than 70 successive *in vitro* generations (or "passages" in the neurosphere assay) without any alteration of growth kinetics, self-renewal capacity and lineage potential [9]. *In vivo* ectopic grafts of *in vitro*-expanded neurospheres never triggered tumors in experimental rodents [9], by contrast to primary cultures of other stem cell categories from adult mammals [19,20,21]. In the recent years, some typical stem cells have been formally characterized in a variety of mammalian tumors, including neural ones; however, these "cancer stem cells" are distinct lineages from intrinsic stem cells of healthy tissues and seem to arise *de novo* as a consequence of DNA damage independently of tissue stem cells [23]. In

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the case of neural tissues, it must be recalled that beyond early post-natal ages (4-years in man) brain cancers are exclusively gliomas [24,25]. This fact renders most unlikely that adult neural cancers might derive from neural stem cells. Indeed, in a recent extensive survey of above 2000 brain metastases from 500 patients, neural stem cells were never found affected [22]. Regarding neurological repair aims for human clinics, the specially high resilience of adult neural stem cells against tumorigenesis is a determinant advantage in view of cell therapy applications.

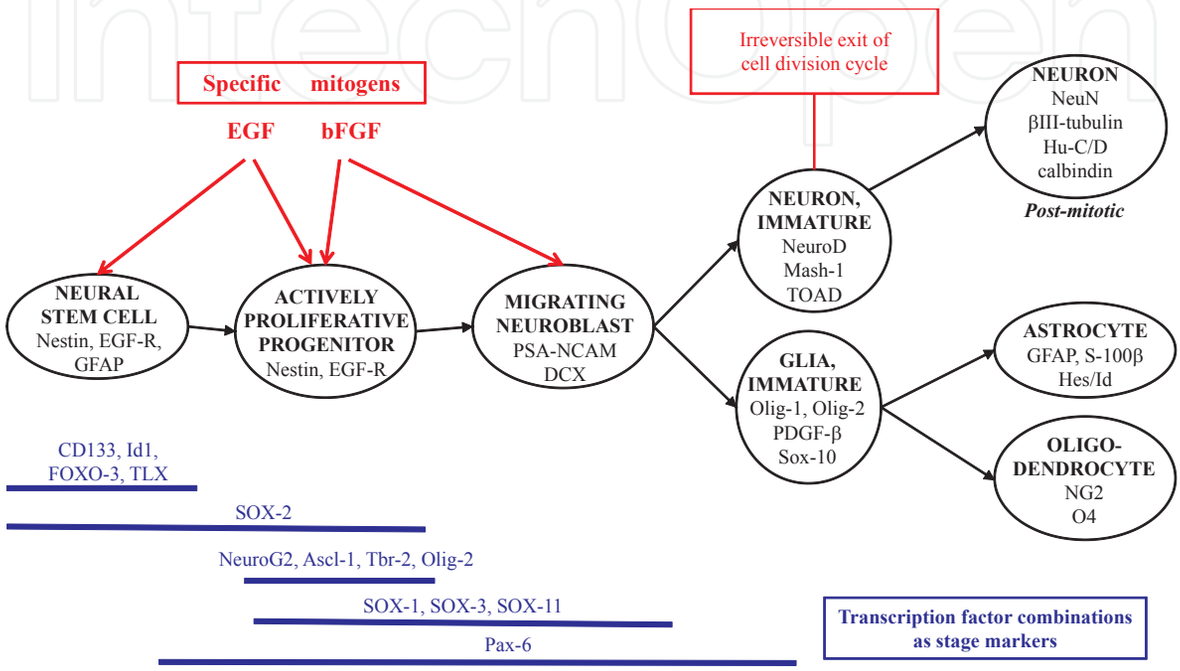

Figure 1. The "neuropoietic system" of adult neurogenesis. Modelized from references 15,38.

3. Neurosphere isolation from adult pig brain subventricular zone

Neural progenitors have been isolated from foetal porcine brain by several laboratories in the last years, by using the neurosphere assay. We decided to develop isolation of adult brain-derived neural stem cells, because of their higher safety than other available proliferative neurogenic cell types in terms of oncogenic potential (cf above). Neural stem cells are found only in rare and discrete structures, the best characterized and most productive of which is the subventricular zone (SVZ) of the forebrain in rodents. We have isolated and characterized for the first time the neural stem cells of adult pig SVZ, by transposing the "neurosphere assay" from routine protocols of rodent studies [26].

3.1. The key to success for the "neurosphere assay": Adequate tissue sampling

This particular primary culture is known to be hampered by two pitfalls: i) delay between *in vivo* blood supply arrest (upon either sacrifice of experimental animals or tissue explantation)

and tissue immersion into appropriate fresh survival medium like the Hank's one or artificial cerebrospinal fluid (see below); ii) excess of non-competent tissue around the NSC-containing area within the microdissected sample.

In order to minimize the duration of brain tissue sampling, which is lengthened in adult pig by the requirement of dorsal skull sawing, we performed the surgical approach of telencephalic subventricular region in deeply anesthetized pig instead of immediately after sacrifice. A rough 10mm-thick, transverse section of midbrain was explanted and immediately transferred into a large Petri dish filled with fresh Hank's medium, and the subventricular zone was then microdissected out of this thick slice on the basis of macroscopical neuroanatomical landmarks by reference to a detailed pig brain atlas [27]. Despite this precaution, our first neurosphere assay attempts on adult pig SVZ failed, which was subsequently attributed to improper tissue sampling. Indeed, the telencephalic subventricular zone is proportionally much larger in adult pig than in adult rodents, and proliferative activity is already known as highly heterogeneous among the whole anatomical extent of SVZ in rodent brain [28]. We performed therefore the subregional mapping of proliferative activity within adult pig SVZ, by Ki-67 immunohistochemistry using a routine protocol [29,30]. The Ki-67 protein is indeed a phylogenetically conserved marker which is expressed exclusively during the cell division cycle of eukaryotic cells, but throughout all its successive phases (G1-S-G2-M); it is therefore widely used in clinical anatomo-pathology and oncology. Proliferative cells, appearing *in situ* as Ki-67 immunoreactive nuclei, were almost exclusively grouped in a narrow ventral subfield of the Nissl-stained subventricular zone, lining the ventral border of lateral ventricle of adult pig brain (Figure 2). The major lateral territory of adult pig SVZ was almost devoid of Ki-67 labelling (Figure 2). We have confirmed the anatomical restriction of the "niche" to such a small subfield of neuroanatomically defined SVZ, by specific labelling of the radial glia which has been extensively characterized in rodent brain SVZ, using double vimentin-glial fibrillary acidic protein (GFAP) immunohistochemistry [26]. *In vitro* culture of neural stem cells from adult pig brain became successful when the SVZ tissue explants were microdissected according to the anatomical lineaments of specific Ki-67 immunoreactive labelling.

3.2. Primary neurospheres from adult pig brain as a vehicle of neural stem cells

For primary cell culture ("neurosphere assay"), a piece of SVZ tissue was microdissected from the pig forebrain slice in low-calcium artificial cerebrospinal fluid (aCSF: 124 mM NaCl, 5 mM KCl, 3.2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 26 mM $NaHCO_3$, 100 mM glucose, pH 7.38, according to Weiss et al., 1996). As previously described [30], the tissue sample was rinsed twice with aCSF, and digested first in 40 U cystein-EDTA-βmercaptoethanol-preactivated papain (Sigma, L'Isle D'Abeau, France) for 10 min at 37°C, then in 250 µL undiluted TrypLE™ Express solution (heat-resistant, microbially-produced, purified trypsin-like enzyme, Gibco cat 12604-013, Invitrogen, Cergy-Pontoise, France) for 10 min at 37°C. After the addition of 750 µL of fresh aCSF and centrifugation for 8 min at 400g at room temperature, the cell pellet was resuspended in 1mL culture medium (Dulbecco's modified Eagle medium [DMEM, Sigma], B27 supplement [Gibco Invitrogen], 200 U/mL penicillin and 200 µg/mL streptomycin [Gibco Invitrogen]) containing 20ng/mL Epidermal Growth Factor (EGF, Gibco Invitrogen) and 20 ng/mL basic

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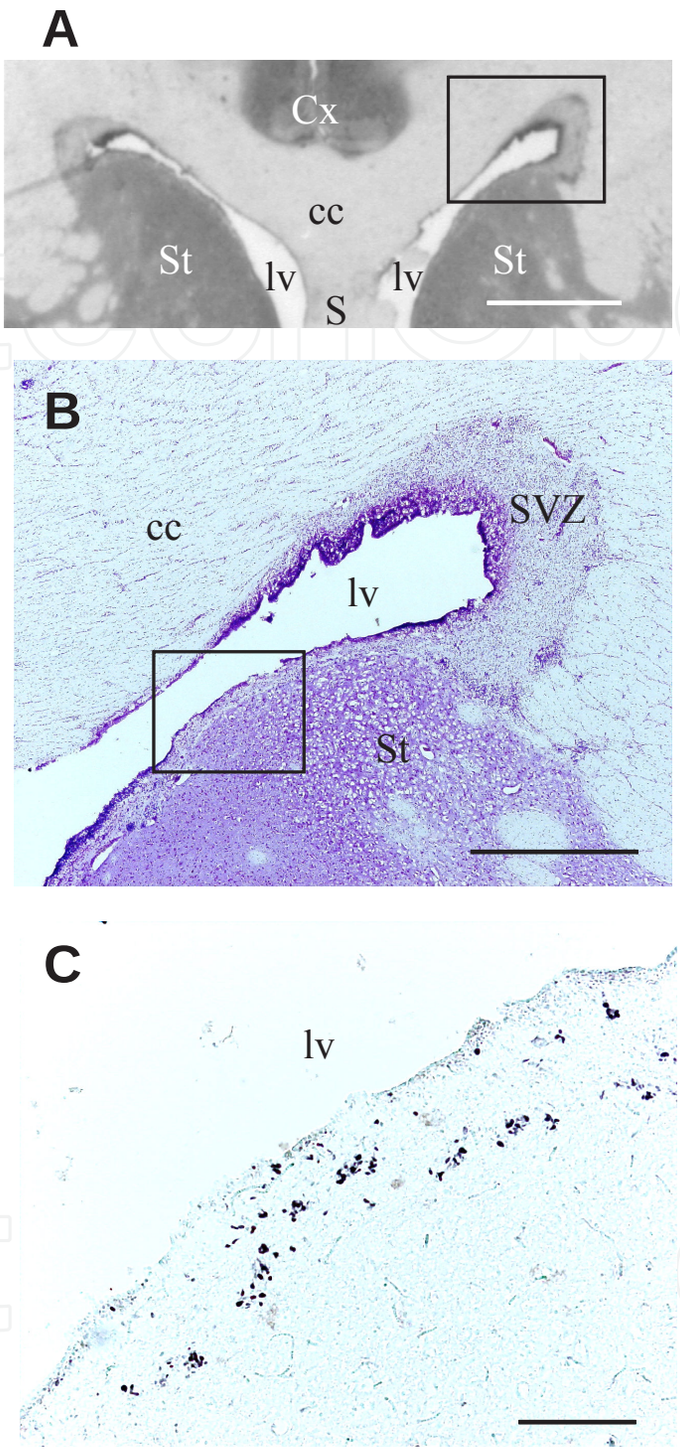

Figure 2. *In situ* localization of the proliferative cells of the adult pig SVZ (modified from original data of reference 26). (A) Macroscopic aspect of Nissl-stained coronal section of adult pig forebrain. (B) Light microscopic aspect of the SVZ region in a Nissl-stained pig brain section. (C) Ki-67 immunohistochemistry on adjacent section of the previous one, with high magnification on the boxed area of B. Note the high density of Ki-67-positive nuclei in this area, as reavealed by brown precipitate, which contrasts with the lateral field of SVZ devoid of Ki-67 immunoreactivity. Legend: *cc: corpus callosum, Cx: cerebral cortex, lv: lateral telencephalic ventricle, S: septum pellucidum, St: striatum, SVZ: subventricular zone.*

Fibroblast Growth Factor (bFGF, Gibco Invitrogen). The cells were dissociated gently with a 26G steel needle mounted on a disposable 1mL syringe, counted on a Malassez slide, and seeded at various densities according to purported utilization. Optimal results were obtained with either Corning 24-well plates or with Falcon 6-well ones. Seedings for basic cell biology kinetics were performed at 3,000 cells per mL of culture medium (subclonal concentration). Maximal neurosphere expansion for transplantation or molecular assays was obtained with seeding at 30,000 cells per mL. Cultures must be monitored daily to follow the morphological growth of the neurospheres; when their diameter reaches 150μm indeed, growth slows down while sphere surface apparently thickens and hardens, and culture proves unable to be expanded any more. Passage (i.e. sphere dissociation, cell resuspension and re-seeding as for primary culture) was performed therefore when the majority of spheres was 100-120μm in diameter.

For passage, the primary spheres were collected in sterile tubes, incubated for 45-60 min at 37°C in 250 μL undiluted TrypLE™ Express solution (Gibco Invitrogen) per 6 mL culture-derived pellet, and dissociated gently with a 26G steel syringe; dispersed cells were centrifuged, resuspended in fresh proliferative medium, counted ant their dilution adjusted to be seeded as above.

Both primary and passaged spheres were prone to two kinds of utilization:

- for neural stem cell expansion, subsequent passages were run in proliferative conditions, i.e. in the presence of 20ng/mL EGF and 20ng/mL bFGF;
- for cell differentiation, spheres were picked with a Pasteur pipette, seeded in wells containing poly-D-lysine (Sigma) precoated sterile coverslips and cultured in the above culture medium (DMEM, B27, penicillin-streptomycin) in the absence of EGF and bFGF for 7-10 days.

All culture media were renewed by the replacement of 500 μL of medium per well every 2-3 days.

Stemness criteria fulfilment by adult pig SVZ-derived spheres in the presence of EGF and bFGF.

We have observed in our primary neurospheres from adult pig SVZ the properties which have been established as the seminal criteria of neural stem cells since their pioneering discovery in adult murine brain [1,11].

The existence of EGF/bFGF-dependent proliferative cells in the ventro-medial SVZ from adult pig brain was demonstrated by the growth of spherical masses from dissociated pieces of this tissue [26] which displayed similar morphology and size distribution to neurospheres obtained from the rat SVZ [30]. Each pair of SVZ samples from 1 pig brain yielded routinely 1 million dispersed cells for seeding, i.e. the total number of cells collected per pig SVZ was about 10 times that of mouse SVZ [11,31]. The pig SVZ spheres appeared though after a longer delay in culture (9-12 days in vitro [DIV]) than primary neurospheres from adult rat SVZ (3-4 DIV: 30). Each pair of SVZ from one pig generated 3000 primary spheres after 2 weeks in the present culture conditions and at initial seeding densities of 30,000 cells per mL. These primary cells

could be expanded linearly on this base through several passages (up to 6 with no change of growth slope) [26].

The self-renewal capacity of primary sphere-generating cells from adult pig SVZ was established by the obtention of novel spheres from dissociation of mature primary Spheres. Re-seeding of dispersed neurosphere cells in the same proliferative medium after 12-14 DIV led to novel "secondary" spheres that were morphologically similar to the primary spheres and grew slightly faster than primary spheres. A second passage at 8-10 DIV led to tertiary spheres that displayed similar morphology and growth rate to secondary spheres. In two independent cultures from 2 pigs each, 5-6 subsequent passages could be performed with similar aspects and cell number amplifications of respective generations. The second passage regularly triggered a 3-time-magnification of total cell number, while other passage increments (I, III and next ones) averaged 20-30% [26].

The multipotentiality and neural character of EGF/bFGF-dependent proliferative cells of adult pig SVZ were demonstrated on mature spheres which were deprived of the 2 mitogens after having reached proliferative maturity, by immunocytochemical detection of neuronal and neuroglial markers in three distinct subpopulations of each sphere – according to the classical lineage test of neurosphere assay [1,11,30]. Quantitative analysis with confocal microscopy indicated that all neurospheres from adult pig SVZ yielded similar proportions of the three neural lineages: 25% neurons, 70% astrocytes, 5% oligodendrocytes. A discrete distribution of the three cell lineages was also reproducibly observed inside the neurospheres, with oligodendrocytes being buried in the core of the spheres while both astrocytes and neurons were concentrated at the periphery [26].

3.3. Transcriptomic signature of neural stem cells in adult pig SVZ neurospheres

We have assayed the expression of phenotypic markers by RT-PCR on total RNA from proliferative, undifferentiated SVZ neurospheres of adult pig SVZ, which were collected at the second or third generation as described above. Pig SVZ neurospheres displayed positive expression of RNAs encoding for:

- nestin, an intermediate filament which is systematically expressed by neural stem cells and embryonic neural progenitors;
- the proliferation marker cyclin D1;
- the neural stem cell proteins Sox-2, Hes-1, $p75^{NTR}$ (the low-affinity receptor of neurotrophins), Lhx2;
- the neuronal proteins βIII-tubulin, doublecortin (DCX), neuron-specific enolase (NSE), Internexin α, Pbx1, Mash1, NeuroD1;
- the oligodendrocyte markers CNPase, Olig-2, PDGF-Rα (Figure 3).

The astrocyte marker glial fibrillary acidic protein (GFAP) has been detected on differentiated neurospheres from adult pig SVZ by immunocytochemistry using the antibody against cow

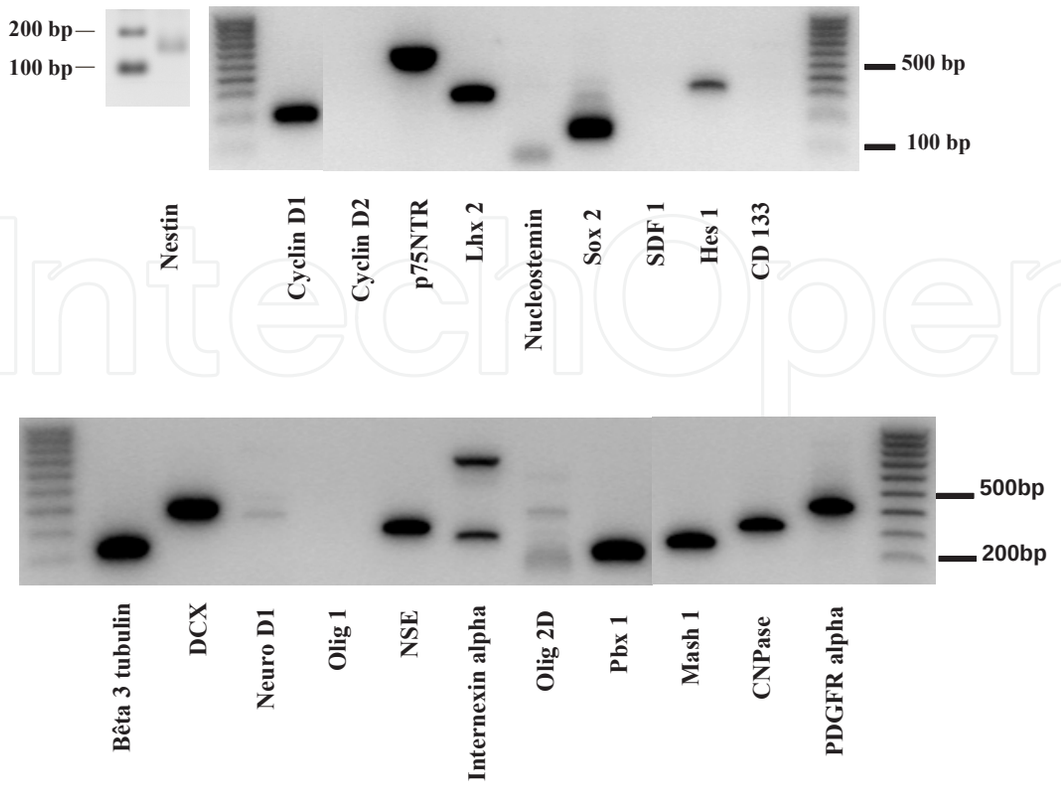

Figure 3. Transcriptomic profile of proliferative neurospheres from adult pig SVZ (modified from original data of reference 26). Agarose gel electrophoretic analysis of amplicons from RT-PCR on proliferative neurosphere RNA with primers designed on the basis of human and/or pig genomic sequences. Stem cell markers: Nestin, Hes-1, Lhx-2, p75NTR, Sox-2, Cyclin D1, Cyclin D2. Committed progenitor markers: Pbx1, βIII-tubulin, internexin-α, NeuroD, NSE, DCX, Mash-1, CNPase, PDGFR-α. PCR products are shown beside co-electrophoresed commercial DNA ladders.

GFAP (cf above) but its transcript could not be detected in the proliferative neurosphere RNAs (probably due to unknown specificity of porcine gene sequence and primers therefore).

The lack of Olig-1 transcripts is expected at this pro-differentiating stage since this gene is expressed in the late phase of oligodendroglial lineage maturation [32,33]; it is also in keeping with Hes-1 detection in the present proliferative spheres, since Hes-1 overexpression was shown to correlate with Olig-1 down-regulation during differentiation [34,35].

In the persistent absence of any specific marker of either neural stem cell or neural committed progenitors, this series of transcripts fits best with the molecular profile of neural stem/precursor cells which has been characterized in rodents and human [36,37,38]. On these bases, we may assume that the major cell type in our adult pig SVZ-derived neurospheres correspond to neural multipotent progenitors, i.e. a stage of the neural lineage that is slightly more mature than the primitive neural stem cells but equally multipotent [26]. This interpretation is supported by additional RT-PCR data in our neurospheres: the absence of the stemness gene transcripts CD133 and SDF-1 [39], the weakness of nucleostemin expression [40], and the lack of detectable cyclin D2, which was demonstrated to specifically drive neural stem cells into division onset by assaying *in vivo* adult neurogenesis in knockout mice for each of the three

cyclins D [41]. Cyclin D1, which was readily expressed in the present material, conversely operates in actively dividing neural progenitors like in most of proliferating cell types in adult organisms.

The present data can be compared with published molecular profiling of neural progenitor cultures from the pig foetal brain [42,43] or adult retina [44,45]. Among the 13 markers that have been addressed by RT-PCR on mRNA extracts of proliferating neurospheres, nine yielded similar results between foetal and adult SVZ, i.e. either negativity (CD133, SDF-1) or positivity (nestin, Sox-2, Hes-1, Pbx-1, β-III-tubulin, doublecortin, Nogo-A). Two differences were encountered between expanded neural cells from adult and foetal brains. The first of these was the detectable expression of cyclin D2 in foetal, but not adult, pig neurospheres. This indicates that neural stem cell expansion is higher in foetal than in adult neurosphere cultures [42]. Furthermore, the lack of Ki-67 detection in pre-differentiation neurospheres from foetal [42], but not adult (present data) supports the same interpretation, since primitive neural stem cells are much less actively dividing than neural precursors at the immediately subsequent maturation stage [46]. Interestingly, the published molecular profile of *in vitro* expanded human embryonic neural stem cells (hNSC) is very close, but not identical, to those of pig neural precursor cells. The major difference was the expression of the stemness gene CD133 in hNSCs that otherwise expressed both Ki-67 and GFAP like adult and foetal pig cells, respectively [42].

In the future, production of neural stem/progenitor cells from the adult pig by the present procedure can promote cell therapy improvements, first in animal models, but also in clinical attempts to repair neural lesions in the use of adult human. By analogy with other tissues [47, 48], adult pig neural precursor cells should be feasible for transplantation into diseased humans without immune rejection [49,50]. The present study thus provides the substratum for an alternative source to human allografts for transplantation in neurodegenerative disorders or injuries, which is now open to experimentation.

3.4. *In vitro* lineage labelling

In order to track *in vivo* the fate of transplanted neural stem cells and their progenies, *in vitro*-expanded neurospheres must be labeled prior to transplantation with a tag which will be retained permanently and transmitted through division without affecting survival and differentiation of labeled cells.

The easiest and long-established tagging method for living cell tracking after transplantation, is incubation of dividing cells with a labeled analog of DNA biosynthesis precursors: nucleotides. Two tools are available: tritiated thymidine (^{3}H-Thy) and bromo-deoxy-uridine (BrdU), and can be detected *in situ* by autoradiography and immunohistochemistry respectively. These tags are easy to apply and detect, but they have the major inconvenient to fade across subsequent rows of cell division because of the semi-replicative mechanism of DNA replication. Practically, this nuclear labeling becomes undetectable after 2 successive divisions of initially labeled cells.

Another, more recent procedure allows true lineage-labeling without fading across successive rows of division: it consists in applying a viral vector of the green fluorescent protein (GFP) gene, either by *in vivo* stereotaxic surgery into the stem cell-containing niche [51] or *in vitro* on expanded primary neurospheres [52]. In collaboration with a retrovirology laboratory (U421 INSERM, Ecole Normale Supérieure, Lyon, France), we have chosen the latter approach [53] and used a lentiviral vector of green fluorescent protein gene (LV-GFP) which had already been validated for human dendritic cells [54]. LV-GFP was freshly synthetized, titrated and tested as previously described [55] stored at -80°C and unfrozen just before use. Adult pig SVZ-derived neurospheres were initially expanded through at least 2 passages. Then, 4-5 days after the last passage, the growing neurospheres were preincubated 30 minutes in the presence of 1mM polybrene (Sigma) and void lentiviral particles (VLP for optimal integration yield; 55) at 0.1 x MOI (multiplicity of infection), and further incubated 2h with fresh culture medium containing 1mM polybrene and LV-GFP at 0.3x MOI; incubation medium was then replaced by fresh standard culture medium. LV-GFP-infected neurospheres were allowed 3 days culture in standard conditions for optimal lineage labeling (Figure 4).

4. Improvement of post-lesional neural outcome with primary pig neurosphere transplantation inside a venous autograft

Cell therapy for the nervous system has encountered a major empirical obstacle: in adult mammalian brain, the neurogenic potential of neural stem cells is inhibited by intrinsic tissue microenvironment except for the very few naturally neurogenic areas, which has been formally demonstrated by heterotopic transplantation studies [56]. To overcome this problem, we developed a novel paradigm in order to improve post-lesion regeneration in peripheral nerves: transplantation of *in vitro*-expanded, proliferating neurospheres from adult pig subventricular zone inside a homotypic venous graft which is sutured at both ends onto the lesioned nerve ends.

Bridging a long nerve gap with a homotypic venous graft is already used in human neurology in order to ameliorate post-lesional recovery of peripheral nerves [57,58,59,60], but it was barely explored for neuronal cell therapy applications. The disabilities resulting from peripheral nerve lesions in adults typically display no neuron loss but pathway disruption, upon disappearance of distal axonal segments, which was extensively documented to lead to restricted or aberrant synaptic reconnexion of surviving neurons [61]. We postulated that neural stem cell grafting on the lesion site might overcome this limitation, by generating chains of interconnected neurons that would functionally replace the lost nerve substance more efficiently than existing severed neuronal axons.

To test this hypothesis, we attempted transplantation of the proliferating neurospheres from adult pig subventricular zone (SVZ) which we had characterized above, inside an autologous venous graft, following surgical transsection of nervis cruralis with 30mm-long gap in adult pig [53]. The following section summarizes the hallmarks of this study, methods and results [53].

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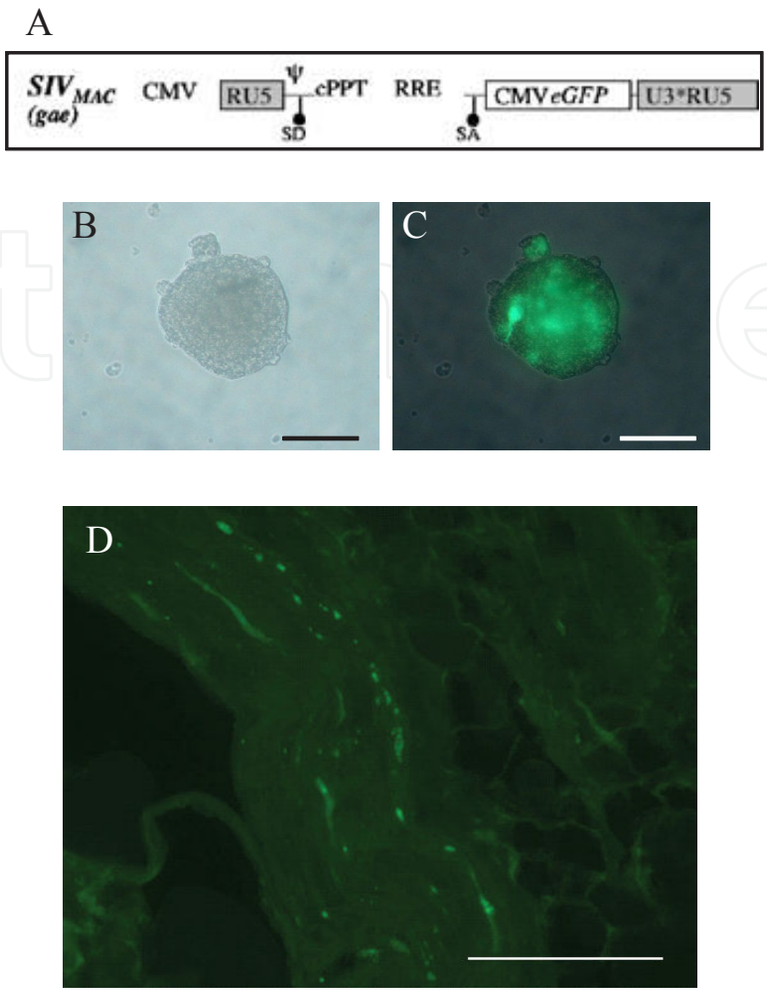

Figure 4. Lineage labelling of neurospheres from adult pig SVZ by *in vitro* viral transfer of GFP gene (modelized from references 54, 55]. The functional structure of the lentiviral GFP vector is schematized in (A) (see 54 for more details). (B,C) Labelled neurospheres 3 days after *in vitro* infection with the lentiviral vector of GFP, as observed under a photonic microscope with natural light (B) or green fluorescence (C). (D) *In situ* detection of neurosphere-derived GFP in a transplanted venous bridge at 8 months after lesion, by fluorescent light microscopy. Legend: *CMV: cytomegalovirus; gag: infection competency coding sequence; GFP: green fluorescent protein; PPT: purine-rich element (to increase exon recognition); RRE: RNA export responsive element; SIV: Simian immunodeficiency virus*. Scale bars: 50 µm (B,C), 0.5mm (D).

4.1. The experimental cell therapy paradigm and its functional outcome

Our lesion model was unilateral transsection of nervis cruralis with 30 mm-long gap in adult pig, under anesthesia. This lesion induces a major motor defect, consisting in the loss of the right leg extension over the thigh, which can be quantified by electromyography of the crural-innervated muscle quadriceps. Our experimental cell therapy paradigm consisted in bridging the nerve gap immediately after damage with an autologous venous segment (sampled from vena mammalian externalis) which was sutured at the proximal end over the perinevre of the severed nerve, filled through the opposite end of the venous graft with freshly prepared suspension of *in vitro*-expanded neurospheres from adult pig SVZ (300 µL, 1000 neurospheres) which we had previously characterized (see section II) and sutured the venous neuroguide

over the distal end of the severed nerve (Figure 5). Control animals did not receive neurospheres in the venous shaft before saturation. The transplanted neurospheres had been expanded *in vitro* from adult pig SVZ through 2-3 passages and have been labeled *in vitro* with either BrdU (for short-term post-lesional survival times) or lentivirally-transferred fluorescent protein gene (see above, section II.4) prior to transplantation (Liard et al 2012).

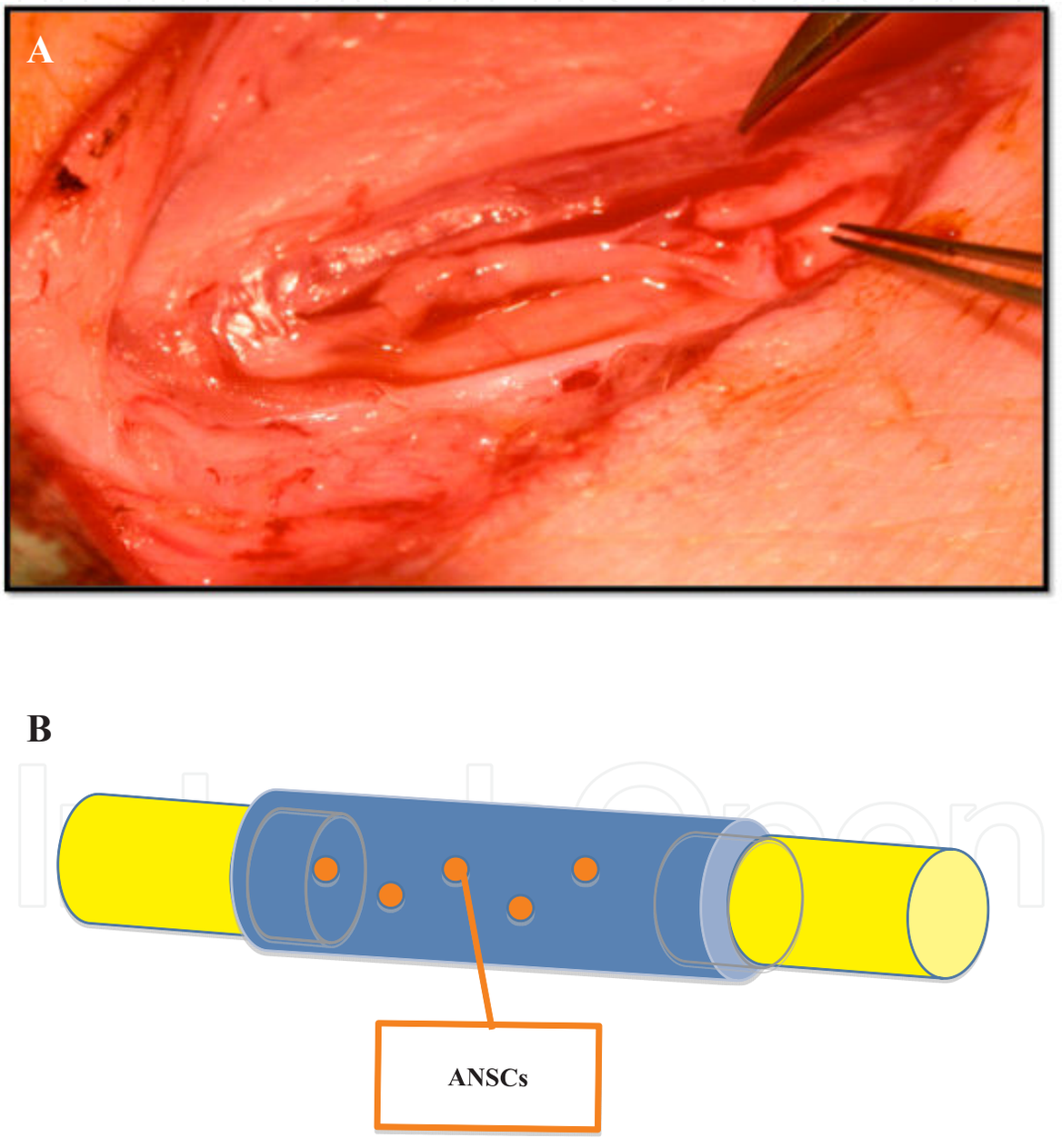

Figure 5. Surgical paradigm for adult pig neural stem cell (ANSCs) transplantation inside a venous graft (modelized from reference 53]. (A) Surgical bridging of lesioned nerve by a venous graft (below the forceps tips; the arrow points to the distal, unsutured end of the graft). (B) Procedure schematization.

Lesion-induced loss of leg extension on the thigh became definitive in controls (up to 8 months after lesion) but was reversed by 90-180 days after neurosphere-filled vein grafting. Prior to surgery, electromyograms of muscle quadriceps vast internal displayed amplitudes of 4.9 to 6.1 mV and post-stimulus latencies of 0.89 to 3.7 ms. Immediately after surgical realization of nervis cruralis substance loss, electromyograms of muscle quadriceps were negative for all experimental animals. Electromyography showed stimulo-detection recovery in neurosphere-transplanted pigs which was partial at 180 days after lesion and almost complete by 240 days, while electromyograms of controls were still negative (Liard et al 2012). Interestingly, in neurosphere-transplanted pigs, post-stimulus latencies returned earlier to normal (i.e., at 180 days post-lesion) than electromyogram amplitude (which was fairly detectable although much lower than on intact leg).

Therefore, transplanting *in vitro* purified-expanded adult neural stem cells inside a venous neuroguide on the site of a peripheral nerve lesion promoted efficient functional recovery in adult pig [26]. We further addressed the underlying mechanisms by post-mortem immunohistochemical analysis of the bridged nerve (see below).

4.2. Exclusively neuronal fate of grafted neurosphere cells

In order to assess the fate of transplanted neurosphere cells [53], we euthanized the experimental animals at various post-lesion intervals, sampled the experimental vein-bridged nerve segment and processed it for immunohistochemistry (fixation by 24-hour-immersion at 4°C into a buffered 4% paraformaldehyde solution, cryoprotection in 30% sucrose-containing phosphate buffer). After snap-freezing in isopentane at -45°C, serial sagittal sections were collected from each frozen vein-bridged nerve segment in a cryostat and processed for phenotypic marker immunohistofluorescence. Fluorescent dyes were chosen so as to be compared with either intrinsic green fluorescent protein (GFP) or with BrdU immunohistofluorescence, depending on the method for neurosphere cell labeling prior to transplantation [53].

At 8 days after lesion-transplantation, BrdU-positive cells were localized inside the venous tube as flattened spherical groups and were all immunoreactive for the specific marker of immature migration-prone neurons: doublecortin (DCX) [53]. At longer survival delays assessed (45 to 240 days) all neurosphere-derived cells, whether labeled with BrdU or virally-transferred GFP, expressed the specific marker of mature neurons: neuronal nuclear antigen (NeuN) or neurofilament protein NF-68 (Figure 4; modified from ref 53). By contrast, none of labeled neurosphere-derived cells coexpressed any of glial markers assessed: CNPase (for myelinating glia, i.e. oligodendrocytes or Schwann cells), S-100β and GFAP (for astrocytes), at either of post-lesional delays. Therefore *adult neural stem cell-derived progenies of grafted neurospheres inside the venous bridge survived and differentiated into neurons exclusively* [53]. Moreover, newly-formed neurons distributed inside the venous graft along the longitudinal axis of the severed nerve, which suggests that new neurons would have created gap-filling interconnected chains in-between the proximal and distal ends of the severed nerve. However, such issue remains to be investigated by using a more resolutive approach (electron microscopy).

Our finding contrasts with numerous previous attempts to improve post-lesional repair of peripheral with putatively regenerating cell transplantation. The latter strategy, or cell therapy,

has been attempted with primary cultures of Schwann cells [62,63], olfactory bulb enshealting cells [64], or various types of stem cells [65]. In all these studies, progenies of grafted stem cells mostly proved to be glial cells, which indirectly enhance axonal regeneration [65,66,67]. Since none of these paradigms used vascular bridge for stem cell transplantation, our results suggest that the venous wall *per se* stimulates neuronal differentiation of stem cell progenies. It is in keeping with the recent demonstrations that vascular walls favor neurogenesis from adult neural stem cells [68,69,70], which is mediated in rodent models by the vascular endothelium-derived growth factor (VEGF) [71,72,73,74]. Our results suggest that choosing a venous trunk versus an artificial neuroguide to bridge a nerve gap is an interesting solution because of intrinsic property. Furthermore, all surgeons are able to take a vein trunk on superficial vein network and this is much less expensive than neurotubes in emergency conditions.

4.3. Graft-induced activation of intrinsic Schwann cells in the lesioned nerve

At 180 and 240 days after nerve lesion and GFP-expressing neurosphere transplantation, CNPase immunohistofluorescent labeling was much higher than in vein-bridged lesioned controls which had not received neurosphere transplantation [53]. Both the number of CNPase-immunoreactive Schwann cell processes and their labeling intensities, as quantified by computerized image analysis, were significantly higher in neurosphere-grafted nerves than in controls [26]. Our result is in keeping with a previous report showing that a venous graft favored Schwann cell proliferation after nerve lesion [75]. In our paradigm though, since controls have received a neurosphere-devoid venous graft, our results indicated that neurosphere cells emit diffusible signals that stimulate Schwann cells. Therefore, *adult neural stem cell-derived progenies of grafted neurospheres inside the venous bridge promoted activation of intrinsic myelinating Schwann cells*. This result is in keeping with accumulating reports demonstrating that primary neurospheres from adult brain tissues do secrete *in vitro* some neurotrophic factors [76,77].

Altogether, our novel cell therapy paradigm in adult pig promotes efficient functional recovery of a peripheral nerve after lesion with long substance loss, correlatively with genesis of new neurons in-between the lesional gap and activation of intrinsic myelinating cells.

Similar results have been obtained in adult rat by using a similar strategy (Xu et al 2012) or mesenchymal stem cells combined with acellular conduits (Zhao et al 2012, Jia et al 2012). From both our and others' studies though (as reviewed in ref 66), a pending issue remains unaddressed: which is the long-term fate of ectopic graft-derived new neurons? The putative formation of ectopic multisynaptic neuronal nets bridging the gap between the two sides of the lesional gap, as suggested by the topographical distribution of new neurons (see above), is made plausible by previous experimental demonstrations of synaptic connectivity between ectopic stem cell-derived new neurons and host neural net, both in neurogenic [56] and non-neurogenic (Lu et al 2012) adult structures of mammalian central nervous system. However, this issue requires to be directly addressed by means of electron microscopy.

Another question arising from our results, concerns the potential long term negative effects of grafted stem cell-derived ectopic neurons. Such transplantation might indeed result into neuromas: either neoplastic neuromas deriving from the grafted proliferative stem cells

(Johnson et al 2012), or non-neoplastic neuromas consisting in aberrant axonal swellings (Rajput et al 2012).

5. Future applications of these new paradigms

The results above (sections II & III) bring about a proof-of-concept for future improvements of neurological clinics. However, before it can be tested in human patients, our paradigm deserves some improvements.

5.1. Alternative source of adult neural stem cells

The direct transposition of the present cell therapy strategy to human patients would raise ethical problems, regarding either autologous sampling of patient's subventricular zone as a source of neurogenic neurospheres, or xenotransplantation of neurospheres from adult pig SVZ due to currently discussed virological risks of porcine tissues [10]. A convenient alternative can be provided by the recently discovered neurogenic stem cells of biopsied human olfactory mucosa [84]. This tissue has been demonstrated indeed to harbor a population of mesenchymal stem cells which displays the same neurogenic properties as original neural stem cells after isolation and expansion in primary culture. To this aim, olfactory mucosa biopsies are enzymatically and mechanically separated from the olfactory neuroepithelium, and the cell suspension from the resulting tissue (olfactory chorion) is seeded in a Dulbecco's medium (DMEM) supplemented with F12 supplement and 10% fetal bovine serum. This primary culture yields adherent proliferating cells that are self-renewable under passage into the same medium. Upon seeding into serum-free DMEM supplemented with insulin, transferrin, selenium, EGF and bFGF, olfactory chorion-derived cells grow into typical neurospheres that exhibit the same morphology, growth kinetics and multipotentiality as neurospheres from SVZ [3,85,86,87]. These *in vitro*-expanded neurogenic neurospheres were then demonstrated to restore function and plasticity after brain lesions in adult rodents [88]. This cell preparation would therefore represent a convenient alternative for clinical application of our cell therapy strategy for post-lesional repair of peripheral nerves. The long-term safety of this novel cell type should however be checked precisely in future assays, since cell therapies with mesenchymal stem cells were recently documented to elicit neuromas in human patients [89].

5.2. Alternative neuroguide

Another issue in our cell therapy strategy concerns the use of autologous venous shaft as a neuroguide. Although largely used in human clinics [57, 58], such procedure has indeed been reported to generate inflammatory or necrotic outcomes. This risk could be avoided by using a synthetic neuroguide which has already been shown to favor neurogenesis from transplanted neural stem cells [59,60,90,91]. The basic neuroguide structure can be manufactured by diverse methods: spinning mandrel technology, sheet rolling, injection-molding, freeze-drying, and electro-spinning.

It will be interesting to study this cell enhancement with FDA- and CE-approved neuroguides [60, 90]:

- Nerve tubes from biodegradable and biological materials:
- type 1 collagen =Integra NeuraGen ® ; Neuro-matrix and Neurolac [91,92,93] with retrospective studies [94];
- Nerve tubes from biodegradable and synthetic materials :
- polyglycolic acid PGA = **NEUROTUBE®** [95,96] ;
- poly-lactic-glycolic acid (PLGA) ;
- poly-L-lactide-caprolactone (PLCL) **Neurolac®** [97];
- polyvinyl alcohol hydrogel SaluBridge.
- Nerve guide with internal multitubular architecture, which is required for bridging long nerve gaps. If we take this point of view, acellular nerve graft Axogen'Avance ® is promissing and obtained by cryocongelation and chimical treatment from human nerves [93,98]. The graft can be handled like an autograft, held by epineurium and bridge gaps up to 50 mm.

With synthetic neurotube, three-dimensional scaffold can be obtained by electrospinning and progress with nanotechnologies. This approach could be optimized with extracellular matrix (ECM) proteins or peptides (laminin-1) and neurotrophic growth factors like basic fibroblast growth factor (bFGF), brain derived neurotrophic factor (BDNF) or nerve growth factor (NGF) [99].

Above all maybe, the use of acellular or totally synthetic conduits for nerve gap bridging is a *priori* likely to reduce the risk of post-operative neuromas. More systematic and extensive studies are required to evaluate properly the risk over benefit probability ratios of all available combinations. For instance, a novel cell therapy strategy which differs from ours merely by using an acellular vein graft, was recently reported to produce only 35-75% of neural cells, including glial and proliferative cells along with neurons [100]. The highly positive pro-repair impact of autologous vein wall, which we have empirically demonstrated, may prove difficult to mimic with man-controlled substitutes.

6. Conclusion

Thus, transplantation of neural stem cells from adult mammalian brain inside an autologous venous graft provides an efficient repair strategy, in the pig model. Our study provides the proof-of-concept for further study in human clinics. It also provides progress in basic cell biology, since the grafted exogenous neurospheres were shown to promote nerve regeneration through 2 distinct original mechanisms: i) indirect activation of intrinsic myelinating glial cells (as recently reviewed in 66), and ii) genesis of new neurons aligned in-between the two ends of the severed nerve. The latter one is totally novel in the field of post-lesional plasticity and repair of peripheral nerves. It deserves though further analysis, by deciphering the neurotransmitter phenotype of neurosphere-derived new neurons and their ultrastructural pattern of connectivity.

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