INTRODUCTION

There are several clinical situations where stem cells could be employed to recover proper function of central nervous system (CNS). The most important are i) stroke, ii) traumatic brain injury, iii) spinal cord injury and iv) neurodegenerative disorders (Alzheimer’s disease, Parkinsonism, amyotrophic lateral sclerosis and Huntington’s disease). Thus, one of the goals of regenerative medicine is to ameliorate irreversible destruction of brain tissue by harnessing the power of stem cells in the process of neurogenesis. Several types of stem cells, including mesenchymal stem cells, hematopoietic stem cells, as well as neural cells differentiated from embryonic stem cell lines, have been proposed as potential therapeutic vehicles. In this review paper we will discuss a perspective of stem cell therapies for neurological disorders with special emphasis on potential application of cells isolated from adult tissues. In support of this our group found that murine bone marrow contains a mobile population of Oct-4+CXCR4+SSEA-1+Sca-1+lin–CD45– very small embryonic-like stem cells (VSELs) that are mobilized into peripheral blood in a murine stroke model. The number of these cells in circulation increases also after pharmacological mobilization by administration of granulocyte colony stimulating factor (G-CSF). Recently we found that VSELs are present in various non-hematopoietic adult organs and, interestingly, our data indicate that the brain contains a high number of cells that display the VSEL phenotype. Based on our published data both in human and mice we postulate that VSELs are a mobile population of epiblast/germ line-derived stem cells and play an important role as an organ-residing reserve population of pluripotent stem cells that give rise to stem cells committed to particular organs and tissues - including neural tissue. In conclusion human VSELs could be potentially harnessed in regenerative medicine as a source of stem cells for neurogenesis.

Key words: brain regeneration, central nervous system, neurogenesis, stem cells, stroke, very small embryonic-like stem cells
STEM CELLS RESIDING LOCALLY IN BRAIN AND CIRCULATING IN PERIPHERAL BLOOD (PB) AND THEIR POTENTIAL ROLE IN AMELIORATING STROKE

As mentioned above, in mammalian brain neural stem cells have been identified in the subventricular zone (SVZ) of the lateral ventricles and olfactory bulb as well as in the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (4-6). As reported these anatomical areas contain so-called “neurovascular niches” composed of neuroblasts, astrocytes and neural stem cell/neuronal precursors cells that reside in proximity to a rich microvascular network (9). It has been postulated that an integral component of these niches is vasculature and neuro- and angiogenesis are tightly coupled and thus co-regulated by reciprocal signaling (9). This signaling is crucial to ensure proper balance of stem cell number in “neurovascular niches” that is regulated by ratio of symmetric vs. asymmetric divisions of neural stem cells. An important role in effective neurogenesis plays also a balance in formation of neurons and glia cells. It is known for example that excessive differentiation of neural cells into glia (e.g., astrogliosis and glial scar formation) may be detrimental to recovery (14).

Whether neurogenesis also occurs in other areas of the mammalian brain as result of proliferation and differentiation of migrating pluripotent stem cells requires further studies (15). Interestingly, mice with cyclin D2 deficiency have a severely reduced number of stem cells in the subventricular zone, olfactory bulb, and hippocampus, yet possess normally developed brains, and brain-derived cells from these animals are able to grow neurospheres (16). This suggests the involvement in brain development and neural tissue homeostasis of stem cells that reside in other locations. These cells could reside in other brain areas or translocate to the brain via PB from other organs or tissues, most likely from the bone marrow (BM) (12-17). To support participation of external cell sources in homeostasis of brain tissue, it is for example acknowledged that microglia in brain are derived from BM-derived macrophagic precursors (18). However, an alternative hypothesis has been also proposed that microglia cells are derived from neuroectodermal matrix cell – that are able to differentiate into microglia locally (19). It is also known that brain vessels contain pericytes that, as recently postulated, are related to the population of mesenchymal stem cells (MSCs) (20) and as postulated MSC may differentiate into neural cells. The neural differentiation of MSC into neural cells however, has been recently put into the question and we will discuss this issue latter on in this review.

Direct evidence however, is still missing on whether stem cells for neurons, macroglia, and oligodendrocytes could be derived from a population of BM-derived primitive stem cells circulating in blood (i.e., VSELs). There are, in fact, some data that support this notion: a detectable level of donor-derived chimerism in brain tissue has been observed in patients after hematopoietic transplantation (21). To explain these data we suggest that cells identified in those patients could be descendants of primitive pluripotent stem cells (PSCs) that were infused into the patients along with the hematopoietic graft.

It is well demonstrated that in different stress situations related, for example, to organ damage, the numbers of various types of stem cells increases in PB - both in experimental animals and, more importantly, in patients (22). This phenomenon was demonstrated for hematopoietic stem/progenitor cells (HSPCs) (23), MSCs (24) and endothelial progenitor cells (EPCs) (25). These stem cells are mobilized into PB from BM and probably other non-hematopoietic niches as well, perhaps to contribute to regeneration (22). Accordingly, the number of these cells is elevated in PB in both mice and humans after granulocyte-colony stimulating factor (G-CSF) administration, heart infarct, and - what is of significance for this review - also in stroke (22, 26). An important question remains: are these cells merely a sign of tissue damage or are they being mobilized to contribute to the regeneration processes? We envision that stem cells, including VSELs circulating in PB, are involved in regeneration of minor tissue damage: for example, small ischemic foci after transient brain hypoxia (12, 13). In an extension of this notion, our team has recently postulated a potential role for these cells in brain recovery from hypoxia related to sleep apnea (27 –).

However, since brain damage after stroke still leads to irreversible brain damage, these cells circulating in PB are obviously ineffective in repairing more extensive tissue damage. We can assume that in case of larger tissue injuries (e.g., resulting from massive stroke) a local highly proteolytic environment is created (as the result of attracting granulocytes, which secrete proteolytic enzymes) that disrupts chemotaxis and homing of circulating PB cells to the damaged organ. A more robust contribution of circulating stem cells in brain regeneration is also probably hampered by the presence of the blood-brain barrier, which in some less damaged areas of central nervous system (CNS) could still be not efficiently permeable for circulating stem cells. Thus, it will be of primary importance to increase the effectiveness of delivery of appropriate stem cell candidates by i) increasing the number of circulating stem cells in PB (i.e., after administration of mobilizing agents such as G-CSF and/or CXCR4 antagonists), ii) preventing occurrence of a proteolytic environment in damaged brain that may disrupt “homing” of circulating stem cells (e.g., by local administration of inhibitors of metalloproteinases), iii) increasing permeability of the blood-brain barrier, or iv) by local delivery of appropriate stem cells that are able to participate in effective regeneration of the CNS (1-3, 28).

POTENTIAL SOURCES OF STEM CELLS FOR BRAIN REPAIR

There is no doubt that identification of the most efficient source of stem cells that can contribute to regeneration of damage brain is crucial for development of stem cell-based therapies for the central nervous system (CNS). Stem cells could be delivered locally into damaged tissues, infused systematically or delivered in combination with organic or synthetic scaffolds. Scaffolds ensure formation of tridimensional network in regenerating tissues where growing cells have better accessible to newly formed vessels. This ensures more efficient access of nutrients and trophic factors to the cells as well as provides better conditions for metabolic exchange.

Overall there are two major types of stem cells that are under investigation in laboratory animals and humans: stem cells isolated from embryos and stem cells isolated from adult tissues (29). Both of these populations of cells with the potential for neuro-regeneration will be discussed below.

**Stem cells isolated from embryos**

The advantage of embryo-derived stem cells is that they are pluripotent. PSCs, according to their definition, are able to differentiate into cells from all three germ layers, including neural tissue. Various potential sources of embryo-derived PSCs are available and their potential ethical and scientific limitations are described in Table 1. First, such cells can be obtained from the developing morula or blastocyst using, for example, frozen embryos stored by in vitro fertilization clinics. From such embryos were derived the currently existing immortalized human embryonic stem cell lines. However, in addition to
ethological concerns related to the use of donated embryos for therapeutic purposes, there is still the major problem of histoincompatibility between embryo-derived PSCs and the recipient of these cells. Such cells will differentiate into mature cells that express donor embryo-derived HLA antigens, and thus will be recognized and subsequently destroyed by the immune system of the recipient (29, 30).

Thus, taking into consideration the fact that PSCs received from histoincompatible embryos would differentiate into tissues that would be rejected, an alternative strategy known as “therapeutic cloning” has been proposed (Table 1) (29, 31). Therapeutic cloning is based on the creation of a cell in vitro that is comparable to the fertilized oocyte (zygote) in its developmental potential. This is accomplished by replacing the nucleus of the oocyte with a nucleus taken from a somatic cell of the patient (31). Following transfer of the nucleus, such an oocyte (clonocyte) may develop into a blastocyst and all cells derived from this blastocyst will be histocompatible with the donor of the nucleus. This strategy to generate “customized histocompatible embryos” for therapeutic purposes was developed in mice and so far the experiment has not been reproduced with human cells (31). Thus, besides ethical and religious concerns, the main obstacles to broader usage of therapeutic cloning as a strategy to obtain PSCs for regenerative medicine are 1) the ineffectiveness of this strategy in obtaining human clonotes, 2) the necessity of having access to human oocytes and, most importantly, 3) the finding that PSCs obtained from embryos may give rise to teratomas (Table 1). Because of all these concerns, the strategy of therapeutic cloning to obtain PSCs from normal human embryos for clinical purposes was rightly abandoned.

An alternative strategy for obtaining PSCs has been recently proposed, which is based on genetic modification of normal somatic cells (i.e., fibroblasts and hematopoietic cells) (29, 32). In this approach, somatic cells are forced by genetic manipulation to express several genes that regulate the developmental potential of embryonic stem cells (i.e., Oct-4, Nanog, Klf4, and c-Myc). The PSCs created by this technology are called induced PSCs (iPS). However, these immortalized/transformed cells are very problematic, because after transplantation into mice they also give rise, in a similar manner as PSCs isolated from embryos, to teratomas and other primitive tumors. This precludes their clinical application in the foreseeable future. Thus, besides their utility as a useful model to study neural differentiation or to evaluate teratocytotoxicity of drugs, it is unlikely, due to their “neoplastic” character, that iPS cells will find application in regenerative medicine.

In experimental settings there are also employed some established immortalized neural cell lines isolated from neural tissues (33) or umbilical cord blood (UCB) (34). However, again because of their neoplastic character (i.e., an unlimited in vitro expansion that exceeds a physiological Hayflick number of cell divisions) and because of the problem of histoincompatibility, they have no realistic chance of being employed in the clinic.

### Stem cells isolated from adult tissues

During the era of excitement about stem cell plasticity, several other types of cells isolated from adult tissues (e.g., MSCs and HSCs) have been proposed as potential sources of stem cells for regeneration of the CNS. However, the rationale for employing these non-neuronal stem cells is somewhat unclear.

Mesenchymal stem cells (MSCs) are isolated from BM, PB, or umbilical cord blood (UCB) by expansion of an adherent population of fibroblast-like cells (35), and it is widely accepted that MSCs contribute to the regeneration of mesenchymal tissues (i.e., bone, cartilage, muscle, ligament, tendon, adipose, and stromal support). MSCs could be also isolated form several other tissues (e.g., PB, adipose tissue and even dental pulp) (36). An unexpected discovery from a few years ago suggested that MSCs are able to give rise to neuronal cells (37). However, this phenomenon was recently called into question by the possibility of an in vitro artifact (38, 39). Accordingly, it has been demonstrated that morphological changes may be induced in MSCs during culture by the addition of components that are included in neural-differentiating media, such as beta-mercaptoethanol, dimethylsulfoxide, and butylated hydroxyanisol. Fibroblasts cultured under these conditions could shrink, elongate, and mimic neurons, but this proved to be only an in vitro morphological artifact (38, 39). Thus, the rationale behind applying MSCs in brain regeneration is not well supported. Similarly, there is no significant rationale for using BM-, PB-, or UCB-derived hematopoietic stem cells (HSCs) for regeneration of neural tissues. Old data that postulated stem cell plasticity of HSCs, such that these cells may become neural stem cells (40), have been not confirmed in recently published studies. This phenomenon is currently explained as a transient change in HSC phenotype induced by neural tissue-derived, spherical membrane fragments called microvesicles (or exosomes) that may transfer neural cell-surface receptors, mRNA, and miRNA to the HSCs employed for regeneration (41).

On the other hand, there is no doubt that there have been slight improvements demonstrated in animal models and patients after treatment with MSCs or HSCs (1-3). However, this is not related to differentiation of these cells into neurons, because such cells are rapidly eliminated after local delivery (1-3). MSCs or HSCs employed for regeneration could, however, release growth factors or cytokines that promote neo-vascularization of damaged tissues and inhibit the process of apoptosis in damaged cells (42-44). On the other hand, as recently postulated by us, cells used as therapeutics and injected locally to the damaged area may also release microvesicles/exosomes that are enriched in bioactive lipids (e.g., sphingosine-1 phosphate) that may

<table>
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<th>Table 1: Pluripotent stem cells isolated from embryo. * This problem is viewed differently by the various major religions of the world. A number of religions potentially accept therapeutic cloning (for example, Islam, Buddhism, Judaism), but unquestionably the majority of them reject reproductive cloning.</th>
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<tr>
<td><strong>PSC isolated from embryos obtained by fertilization and stored in embryo banks</strong></td>
</tr>
<tr>
<td>Risk of developing teratomas</td>
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<tr>
<td>Histoincompatibility problem</td>
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<td>Ovum donor required</td>
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<td>Ethical reservations</td>
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promote both vascularization of damaged organs and inhibit cell apoptosis (45).

The potential application of neural stem cells isolated from olfactory bulb (46) or even retina (47) is also questionable, because of the problem of obtaining such cells from living donors. Even if it were technically possible to isolate these cells from cadaveric organ donors (e.g., from brain), the problem of histoincompatibility would remain a major limiting factor.

VERY SMALL EMBRYONIC-LIKE STEM CELLS (VSELS) AS FUTURE POTENTIAL THERAPEUTICS FOR DAMAGED BRAIN

As mentioned above, our team recently identified a population of very small cells (VSELS) that express several PSC markers (48). VSELS are present in a variety of adult organs and, interestingly, our data indicate that the brain contains a relatively high number of cells that display the VSEL phenotype (49). These small cells that are smaller than erythrocytes (3–5 µm in diameter in mice) were initially separated by us from murine BM (42), and subsequently a similar population of small cells (5–7 µm in diameter) were purified from human UCB and mPB. VSELS are i) CD45– (mouse and human) ii) Sca-1+ (mouse), iii) BM(42), and subsequently a similar population of small cells that are smaller than erythrocytes (3–5 µm in diameter). Interestingly, our data indicate that the brain contains a relatively high number of cells that display the VSEL phenotype (49). These small cells that are smaller than erythrocytes (3–5 µm in diameter in mice) were initially separated by us from murine BM (42), and subsequently a similar population of small cells (5–7 µm in diameter) were purified from human UCB and mPB. VSELS are i) CD45– (mouse and human) ii) Sca-1+ (mouse), iii) BM(42), and subsequently a similar population of small cells that are smaller than erythrocytes (3–5 µm in diameter) were purified from human UCB and mPB.

Our most recent research indicates that VSELS express several markers characteristic of epiblast germ line stem cells (51). The expression of the pluripotency transcription factor Oct-4 in these cells was confirmed by demonstrating the unmethylated state of the Oct-4 promoter and its epigenetic histone code that is characteristic for transcriptionally active DNA (52). Furthermore, we recently observed that most of the homeodomain-containing developmental transcription factors in VSELS are repressed by specific epigenetic marks, called bivalent domains that represent a state of the DNA structure characteristic of PSCs, where transcriptionally antagonistic histone codes physically co-exist within the same promoter. We observed that murine Oct-4 VSELS do not proliferate spontaneously in vitro if cultured alone and that the quiescence of these cells is regulated by genomic imprinting through DNA methylation, which is an epigenetic program that ensures the parent-specific monoallelic transcription of some developmentally important genes (52).

The imprinted genes play a crucial role in embryogenesis, fetal growth, maintaining the totipotential state of the zygote, and maintaining the pluripotency of developmentally early stem cells (52). The expression of imprinted genes is regulated by DNA methylation on differentially methylated regions (DMRs), which are CpG-rich cis-elements present within their loci (52). We observed that VSELS freshly isolated from murine BM erase the paternally methylated imprints (i.e., at the Igf2-H19 and Rasgrf1 loci); however, they hypermethylate the maternally methylated imprints (i.e., at Igf2 receptor (Igf2R), Kcnq1-p57KIP2, and Peg1 loci). Further studies are required to determine whether a similar phenomenon occurs in human VSELS, as well as for cells isolated from non-hematopoietic tissues (e.g., the CNS) that display the VSEL phenotype.

Because paternally expressed imprinted genes (Igf2 and Rasgrf1) enhance embryo growth, while those that are maternally expressed (H19, p57KIP2, and Igf2R) inhibit cell proliferation (52), the unique genomonic imprinting pattern observed in VSELS demonstrates a growth-repressive program in this developmentally early stem cell. Based on the unique pattern of imprinting, VSELS highly express growth-repressive genes (H19, p57KIP2, and Igf2R) and downregulate growth-promoting genes (Igf2 and Rasgrf1), which explains their quiescent status at the molecular level (51, 52). Importantly, we observed that the quiescent pattern of genomic imprinting could be progressively changed during the formation of spheres by VSELS in co-cultures with myoblastic C2C12 cells (53), in which VSELS proliferate and differentiate. These results suggest, on the other hand, that the epigenetic reprogramming of genomic imprinting should maintain quiescence of the most primitive pluripotent adult stem cells (i.e., Oct-4+ VSELS) deposited in the adult body and protect them from premature aging and tumor formation. On the other hand, they show clearly that some external micro-environmental signals can change the epigenetic state of this imprinting and force VSELS to differentiate (54).

We envision that VSELS are epiblast-derived PSCs deposited early during embryonic development in developing organs as a potential reserve pool of precursors for tissue-committed stem cells (TCSCs) and thus this population has an important role in physiological tissue rejuvenation and regeneration. Since, as demonstrated in murine in vitro models, VSELS have the potential to differentiate into neurons, oligodendrocytes and macrophages, we propose that these cells could be harnessed for regeneration of a damaged CNS. The most accessible source of VSELS for neuroregeneration would be i) the patient’s own BM, ii) patient-stored UCB, or iii) patient-mobilized PB. We also propose that VSELS could be isolated from histocompatible-related or un-related donors as is currently done with HSCs in hematopoietic allo-transplants (54).

However, before this strategy can be adopted in the clinic, we need to develop a more efficient ex vivo expansion strategy to generate neural stem cells from VSELS. We are aware that reestablishment of the proper imprinting state in VSELS to somatic type 1 will be crucial in obtaining these cells for regeneration purposes. Therefore, we are focused in our laboratory on this important task.

Interestingly, our data generated in experimental animals indicate that number of VSELS decreases with the age. Therefore, it is likely that the better regeneration potential of young individuals may be correlated with higher number of these cells deposited during embryogenesis in the adult tissues (55). Our most recent data indicate that one of detrimental factors in depletion of VSELS over time is calorie uptake-related increase in plasma level of insulin and insulin-like growth factors (56).

FACS-BASED IDENTIFICATION OF VSELS IN PATIENT PERIPHERAL BLOOD

In our laboratory, we have established well-validated protocols for VSEL identification in human PB using flow cytometric methods. Similarly to mice, human VSELS are mobilized into PB of patients as the result of several tissue and cytometric methods. Similarly to mice, human VSELS are mobilized into PB of patients as the result of several tissue and mobilization protocols for VSEL identification in human PB using flow cytometric methods.

The absolute numbers of circulating VSELS in PB are exceptionally low (1–2 cells in 1µl of blood under steady-state conditions) and thus special flow cytometric protocols have to be applied for their identification. As shown in Fig. 1 panel A, we employ nuclear staining for detection of very small nucleated objects present in blood samples and for exclusion of anucleated cellular debris from further analysis. Such a defined fraction of very small nucleated cells (region P2) may be further analyzed for CD45 and CD133 antigens expression (Fig. 1 panel C) and two subpopulations, CD133+/Lin−/CD45− HSCs and CD133+/Lin−/CD45− VSELS, can be distinguished as shown in
Fig. 1. Identification of human VSELs circulating in patient blood by flow cytometric methods. Panels A–E: Analytical strategy for VSEL detection by classical flow cytometry. The total population of blood-derived leukocytes was stained with nuclear dye (DAPI) following the lysis of erythrocytes in ammonium chloride solution (panel A). Nucleated objects incorporating DAPI are included in region P1 and are further visualized in a dot-plot representing their FSC and SSC parameters, which are related to cellular size and granularity/complexity, respectively (panel B). Very small nucleated cells from region P2 are further analyzed based on the expression of the pan-leukocytic marker CD45 and human stem cell antigen CD133 (panel C). The population identified as CD133+/Lin–/CD45+ cells correspond to HSCs, while the CD133+/Lin–/CD45– cells correspond to the VSEL fraction. The size distribution of these stem cell populations are shown in panels D and E, respectively. VSELs may be clearly distinguished as a cluster of very small cells, as shown in Panel E (red box). Panel F: Representative multicolor image of VSELs circulating in the blood of a patient with gastric disease. Each photo presents the same cell stained for the following markers: Lin-FITC (green), CD45-APC-Cy7 (cyan), CD133-PE (yellow), and CD34-APC (magenta). Nuclei were stained with Hoechst (red). The scale represents 10 µm.
Fig. 1 panels D and E, respectively. VSELs may be clearly recognized among blood leukocytes as the fraction of cells exhibiting very small size, which we have previously established to be smaller than the diameter of the average human erythrocyte (58, 59).

Human VSELs shown in Fig. 1 panel E as a cluster of very small cells, may also be further characterized morphologically by flow cytometry imaging (ImageStream system; ISS). Fig. 1 panel F shows a representative image of CD133+/CD34−/Lin−/CD45− VSEL stem cells circulating in the blood of a patient with gastric disease detected by this technology. The ImageStream system, which combines the features of classical flow cytometry and fluorescence microscopy in one platform, is the first flow cytometric technology that enables certain identification of all acquired objects from their multicolor images (49). Thus, imaging cytometry based on utilization of ISS, which allows “decoding a dot”, found great application in VSEL identification in several animal and human specimens, including peripheral blood (49).

Based on such a multi-analytical flow cytometric approach, we are able, not only to identify and confirm the presence of VSELs among blood leukocytes, but also to quantitatively determine the absolute numbers of these rare cells circulating in the blood of patients with various tissue/organ injuries and disorders (13, 57). Recently other independent group reported that they successfully purified VSELs from human mobilized PB as well as BM (60).

Fig. 2. A rapid method for isolating VSELs from BM, mPB, or UCB. This novel strategy is based on: i) lysis of erythrocytes in a hypotonic ammonium chloride solution, ii) CD133+ cell selection by immunomagnetic beads, and iii) multiparameter sorting of CD45−/GlyA−/CD133+/ALDHhigh/low cells by FACS, with size-marker bead controls.

CELLS THAT EXPRESS VSEL MARKERS ARE MOBILIZED INTO PERIPHERAL BLOOD (PB) IN PATIENTS AFTER STROKE

In our studies of a few years ago, we became interested in the question of whether VSELs could be mobilized into PB in patients after stroke, in a similar way as patients after acute heart infarct (13, 57). To address this question we employed the staining strategy described in the previous paragraphs in a new study: a total of 44 patients afflicted with ischemic stroke who were admitted within 24 hours of onset of the first symptom and 22 healthy control subjects were enrolled. In each case, the stroke had been precisely documented in the clinic by computer tomography (CT) scan. Based on clinical examination and cranial CT findings, including volumetric analysis, patients were classified into the following four clinical subgroups: total anterior circulation infarcts (TACI), partial anterior circulation infarcts (PACI), posterior circulation infarcts (POCI), and lacunar infarcts (LACI). Additionally, we distinguished two subgroups of patients with different extensiveness of stroke (i.e., group A (small and medium: patients diagnosed as LACI and PACI) and group B (large: patients diagnosed as TACI)). The patients diagnosed as POCI, were included in group A or B on the basis of clinical examination and cranial CT analysis (13).

As expected, we observed an increase in mRNA for both pluripotent (Oct-4 and Nanog) and neural (GFAP, Nestin, β-III-tubulin, Olig1, Olig2, Sox2, and Musashi-1) SC markers in
Peripheral blood-borne nucleated cells circulating in stroke patients (13). The increased expression levels of mRNA for Oct-4 and Nanog in PBMCNs in patients after stroke corresponded to previous observations made by us in an experimental murine model of stroke (13). However, maximally increased expression of neural stem cell markers in humans was delayed by two days (1 day for mice vs. 3 days for human). Interestingly, the kinetics of changes in expression of mRNA for early stem cells correspond to changes that we recently observed after heart infarct in humans and mice. Furthermore, we observed differences in VSEL mobilization between cases of POCI and PACI and other subtypes of stroke. Of note, patients with POCI have the best chance of recovery and PACI is associated with the highest risk of early recurrence of stroke (i.e., within 3 months), but it is not associated with high mortality and significant disability. Further studies will be required to see if the mortality of stroke could be related to position (distance) of the stroke area to "neurovascular niches" in SVZ of the lateral ventricles as well as in the SGZ of the dentate gyrus in the hippocampus (4-6, 9).

In sum, our studies demonstrate that the mobilization of VSELs and tissue-committed progenitor cells expressing early neural markers into PB occurs in patients with ischemic stroke and may perhaps be of prognostic value (13). It would be interesting to determine whether the number of these cells could be increased by administration of mobilization-promoting agents (e.g., G-CSF or AMD3100).

**ISOLATION OF VERY SMALL EMBRYONIC-LIKE STEM CELLS FOR POTENTIALLY THERAPEUTIC PURPOSES FROM BONE MARROW, UMBILICAL CORD BLOOD, AND PERIPHERAL BLOOD**

It is obvious that because of time constraints, it is not possible to purify these rare cells from bone marrow (BM) aspirates, umbilical cord blood (UCB) units, or mobilized peripheral blood (PB) by employing multiparameter staining and regular high-speed sorting only. We have calculated that by employing only one cell FACS sorter, isolation of all VSELs present in 100 ml of UCB would take up to 4 working days (58, 59).

Thus, to isolate these cells more efficiently, we propose a relatively short and economical three-step isolation protocol that allows recovery of ~60% of the initial number of Lin-/CD45-/CD133+ UCB VSELs (Fig. 2). This novel strategy is based on: i) lysis of erythrocytes in a hypotonic ammonium chloride solution, ii) CD133+ cell selection by immunomagnetic beads, and iii) sorting of Lin-/CD45-/CD133+ cells by FACS with size-marker bead controls. The entire isolation procedure takes 2–3 hours per UCB unit and isolated cells are highly enriched for an Oct-4+ and SSEA-4+ population of small, highly primitive Lin-/CD45-/CD133+ cells. This strategy could also be employed in sorting VSELs from BM aspirates and mobilized PB.

We propose that VSELs freshly isolated from BM, PB, or UCB or VSELs pre-committed to the neurological lineage in *ex vivo* cultures could be an optimal source of cells for regeneration of the CNS. Ongoing studies in our laboratories will address this attractive possibility.

**CONCLUSIONS**

Our data show that stress related to stroke triggers the mobilization of VSELs from BM, and perhaps other stem cell niches, into PB. We hypothesize that if these cells observed in mobilized PB in humans prove to be counterparts of the VSELs identified in mice, they could potentially be purified from the PB, expanded *ex vivo*, and employed for regeneration of damaged neural tissues. On the other hand, our data indicate that VSELs express several features of PSCs and could be potentially employed as a therapeutic alternative to the controversial use of stem cells isolated from embryos. Hence, while the ethical debate on the application of ESC in therapy continues, the potential of VSELs is ripe for exploration. Researchers must determine whether these cells could be efficiently employed in the clinic or whether they are merely developmental remnants found in the BM that cannot be harnessed effectively for regeneration. The coming years will bring definitive answers to these questions.

**Acknowledgments:** This work was supported by KBN grant (N N401 024536), EU structural funds, Innovative Economy Operational Program POIG.01.01.01-00-109/09-01 and the Henry M. and Stella M. Hoenig Endowment to MZR and N N401 0602 33 grant to EP.

Conflict of interests: None declared.

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Received: October 5, 2010
Accepted: January 31, 2011

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