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REVIEW

ABC transporters, neural stem cells and neurogenesis – a different perspective

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Stem cells intrigue. They have the ability to divide exponentially, recreate the stem cell compartment, as well as create differentiated cells to generate tissues. Therefore, they should be natural candidates to provide a renewable source of cells for transplantation applied in regenerative medicine. Stem cells have the capacity to generate specific tissues or even whole organs like the blood, heart, or bones. A subgroup of stem cells, the neural stem cells (NSCs), is characterized as a self-renewing population that generates neurons and glia of the developing brain. They can be isolated, genetically manipulated and differentiated *in vitro* and reintroduced into a developing, adult or a pathologically altered central nervous system. NSCs have been considered for use in cell replacement therapies in various neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. Characterization of genes with tightly controlled expression patterns during differentiation represents an approach to understanding the regulation of stem cell commitment. The regulation of stem cell biology by the ATP-binding cassette (ABC) transporters has emerged as an important new field of investigation. As a major focus of stem cell research is in the manipulation of cells to enable differentiation into a targeted cell population; in this review, we discuss recent literatures on ABC transporters and stem cells, and propose an integrated view on the role of the ABC transporters, especially ABCA2, ABCA3, ABCB1 and ABCG2, in NSCs' proliferation, differentiation and regulation, along with comparisons to that in hematopoietic and other stem cells.

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Introduction

Taking advantage of the natural potential of self-renewal of the living body, "regenerative medicine" is sure going to play an essential role in providing an innovative way to treat many human disorders. Stem cells are the engines that drive the renewal of adult mammalian tissues. They divide continuously, throughout life, to produce new progeny cells that undergo a robust development program towards differentiation and maturation to replace old expired tissue cells. They are a unique population of cells capable of self-renewal and differentiation into different cell types. A major effort in regenerative medicine is the search for suitable renewable sources of cells that can be used as means to treat human diseases. Stem cells, capable of eluding detection by the host's immune system and with the potential of expansion in culture, strike as a very promising source of cells for therapeutic applications [1].

Stem cells can be categorized as either embryonic stem (ES) or adult stem cells. Adult stem cells exist as undifferentiated cells interspersed among differentiated ones in a tissue or organ, and exhibit the intrinsic ability to self-renew and to potentially differentiate into the major specialized cell types of the tissues or organs. However, they cannot give rise to all cell types of an organism and can only differentiate into restricted lineages. Therefore, in contrast to totipotent ES cells, these tissue-specific stem cells are considered multipotent or pluripotent. A variety of stem cell populations have been discovered residing within adult tissues like the brain, bone marrow, blood, liver, pancreas and others [2, 3]. Of these, the hematopoietic stem cells (HSCs) from bone marrow can differentiate

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into all the different immune cells and have been routinely used to treat leukemia, lymphoma and immune deficiencies [4, 5]. A certain class of HSCs from blood and bone marrow called the "side population" (SP) is described also as CD34-negative (-), c-Kit-positive (+) and Sca-1$^+$ cells, according to their specific surface antigens [6, 7]. These bone marrow repopulating SP cells are probably among the best-characterized examples of pluripotent adult stem cells to date [8, 9].

We now know that different types of stem cells exist, but they are found in minute populations in the human body. Hence, the use of biologically unique stem cell markers is required to stringently identify and isolate these cells. The consensus working definition of stem cells is that they can both replenish their own population and differentiate to form committed daughter cells [10]. The candidate stem cell must also demonstrate the ability to proliferate, self-renew over an extended period of time (and not just once or a few times as observed with the progenitor cells), and generate a large pool of progeny that can differentiate into the primary cell types of the tissue from which it is obtained [11]. In addition to their ability to self-renew and differentiate, they are usually quiescent, dividing infrequently [12, 13]. To accurately define a cell as a stem cell, it is necessary not only to confirm the renewal capability of the founding cell or population of cells over an extended period of time but also to demonstrate the generation of progeny cells several orders of magnitude more numerous than the starting population.

The essential role of tissue stem cells is then to maintain and repair the tissue in which they are found. They also require the presence of a tightly regulated environmental niche comprising of other cell types, stroma and growth factors for survival [14, 15]. Due to the above-mentioned characteristics of stem cells, they are especially attractive candidates in neuroregenerative therapy applications. For instance, their self-renewing ability would be useful for the treatment of neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD). Since stem cells and their differentiation products are potentially of broad therapeutic uses, efforts that would enable the supply of a continual source of primitive stem cells *in vitro* will be vital in revolutionizing the treatment of many diseases [16-18].

The most remarkable changes in the brain that occur with aging may be the alterations in cognition and plasticity. Aging of the central nervous system (CNS) is associated with a progressive loss of neural functions, which is exacerbated in neurodegenerative disorders such as AD [19-21]. Two major cell replacement strategies are considered for the therapy of neurodegenerative disorders such as AD, and involve transplantation of exogenous tissue/cells and activation of proliferation of endogenous cells, respectively [22-25]. Transplanted tissue/cells are used to either directly replace the lost tissue or to deliver genetically engineered neural stem cells (NSCs) that can secrete factors which promote cell survival and proliferation. However, successful application of any cell replacement therapy likely will require the understanding of the complex relationships between NSCs and the more restricted neural and glial progenitor cells, as well as the underlying biology of their renewal and differentiation programs.

Neural stem cells

The anatomical location and lineage specificities of NSCs were only established when they were finally identified in the subependymal region and in the hippocampal dentate gyrus (DG), where they divide to generate progenitors that migrate along the rostral migratory stream to differentiate in the olfactory bulb or to integrate into the surrounding hippocampal neural circuitry, respectively [26-28]. Similar to HSCs, these nestin+ NSCs may be defined operationally as cells that can continuously self-renew and have the potential to generate intermediate and mature cells of both glial and neural lineages [29]. Furthermore, NSCs have also been reported to differentiate into hematopoietic cells [30].

It has been found recently that both endogenous and transplanted NSCs seem to be attracted to various experimental brain lesions of disparate etiologies, such as tumors or areas of neurodegeneration [31]. For instance, NSCs have shown tropism toward gliomas and toward degenerating spinal cord motor neurons in a transgenic mouse model of amyotrophic lateral sclerosis [32-34]. Tumor-tropic NSCs have also been observed in peripheral malignancies apart from those primary brain malignancies. It is still challenging to understand the fate of NSCs in brain lesions [35]. In certain pathologies, such as in stroke lesions, transplanted cells appear to form astrocytes and neurons [36]. Sometimes, a glial fate, even if not ideal, may still be preferred over neural differentiation, as the latter might form abnormal and possibly damaging circuits. Reactive astrocytosis induced by inflammatory cytokines released by microglia in response to a pathological process is characterized by an increase in glial fibrillary acidic protein (GFAP), showing that GFAP is a marker in the differentiation of NSCs into astrocytes [37, 38].

Neurosphere cultures are regularly used as a source of primitive neural cells. Such cultures have been characterized by Ruud Hulspas and Peter J Quesenberry as the means to purify neural progenitor cells, and several subpopulations of cells have been defined [39]. Neurospheres are useful to evaluate NSC multipotentiality (through the character-

ization of cell phenotypes that arise from a differentiating sphere) and to analyze NSCs' self-renewal capacity using clonal secondary neurosphere assays that assume isolated true stem cells can generate new spheres [40].

Neurospheres are floating structures that can be obtained by exposing dissociated embryonic or adult CNS tissue to growth factors [41-43]. These heterogeneous spheroid structures contain NSCs/progenitors and differentiated cells embedded in a complex extracellular matrix and organized three dimensionally (3D) with a core of differentiating $GFAP^+$ and β-tubulin III^+ cells surrounded by nestin+, epidermal growth factor receptor $(EGFR)^+$ and β1-$integrin^+$ undifferentiated cells [40]. Thus, neurospheres consist predominantly of committed progenitors mixed with differentiated astrocytes as well as neurons, with relatively few stem cells [44]. Histotypical 3D neurospheres in suspension culture are thus characterized as heterogeneous clusters containing unequal stem cell subtypes where $nestin^+$ dividing cells surround a core of differentiated $GFAP^+$ and β-tubulin III^+ cells. Cell divisions can be found mainly at the edge of the neurosphere where NSC markers such as notch1 and nestin are also expressed [41]. This pattern suggests that NSCs may be a rare subgroup of $nestin^+$ cells that simultaneously express a combination of markers, such as LeX/ssea1/β1-integrin/notch1 [40, 41, 45]. Furthermore, some adult astrocyte-like $GFAP^+$ cells are actually NSCs, which are probably derived from an embryonic glial lineage [46]. Therefore, the 3D neurosphere organization could give some indication of whether a neurosphere is generated by a true stem cell or by a non-stem cell population when the neurospheres are characterized by staining with a stem cell marker like nestin or musashi-1 [47, 48]. The DNA-binding dye Hoechst-33342 has been used as a method to identify potential stem cells in a host of tissues, including the bone marrow, heart, lung, muscle, eye and pancreas [9]. The dual emission of the Hoechst dye generates a distinct SP from the whole population of cells. This unique segregation is conferred by the ATP-binding cassette (ABC) transporter proteins, such as ABCB1, which actively pump out the Hoechst dye. In fact, when the whole-cell population is treated with verapamil, an inhibitor of these transporters, the SP phenotype is lost [9]. Interestingly, it was observed that neurosphere cultures contain a relatively higher number of stem cells that stain weakly with Hoechst-33342 when cultured in presence of EGF and basic fibroblast growth factor (bFGF); however, when cultured separately either with EGF or bFGF, they give rise to neurosphere populations with characteristics of freshly isolated embryonic mouse brain stem cells but with much fewer stem cells that stain weakly with Hoechst-33342 [41, 49].

Single-cell studies have provided new insights into NSC biology by demonstrating the role of symmetric and asymmetric divisions in cell fate determination [40]. Asymmetric divisions are crucial to generate diversity, whereas symmetric divisions allow for maintenance of the stem cell pool or for the expansion of a progenitor population. The "feature" of NSCs may be a complex state that can be acquired or lost [50]. Both the survival capacity and proliferation potential of cells are fundamental for stem cell maintenance and renewal, or alternatively may be essential for the generation of a cell population that can subsequently acquire stem cell properties. The environmental niche where the cells reside may play a fundamental instructive role in NSC development [51]. The term NSC may therefore need to include the capacity of a cell to survive and to depend on a niche to acquire or maintain stem cell status [52].

ABC transporters

ABC transporters constitute one of the largest known superfamilies of proteins. These evolutionary highly conserved multispan transmembrane molecules use the energy of ATP hydrolysis to translocate a broad spectrum of molecules across the cell membrane. To date, 48 members of the human family of ABC transporters have been identified which, based on their structural relatedness, are subdivided into seven families, designed ABC A-G. [53-58]. These ABC transporters participate in diverse cellular processes, including drug resistance and metabolism, transport of lipids and organic anions, and iron metabolism [54, 55]. The various transporters exhibit different catalytical properties (for instance substrate specificity, mode of transport, transporter vs channel, protein-protein interactions), and additional substances that are transported by the ABC molecules include peptides, amino acids, carbohydrates, vitamins, glucoronide, glutathione conjugates and xenobiotics [59-63]. In eukaryotes, the ABC transporters are located in the plasma membrane and the membranes of intracellular compartments such as the Golgi, endosomes and in the mitochondria [61]. When comparing various ABC transporter subfamilies – one major difference between the transporters is the presence of the additional N-terminal extension in a transmembrane domain (called TMD0) in some members of the ABCC-subfamily [64]. In contrast, the order of the TMDs and nucleotide binding domains (NBDs) in the two-dimensional structure of most of the transporters is identical. Membrane-associated ABC transporters have been found in two forms, the full-length transporters that are characterized by two identical halves each containing the NBD and the half-transporters that function as homo- or heterodimers (Figure 1) [53-55,64]. As one of the best studied ABC transporters, the multidrug resistance (MDR1) P-glycoprotein (P-gp), also known as ABCB1, is a glycosylated membrane-associated enzyme

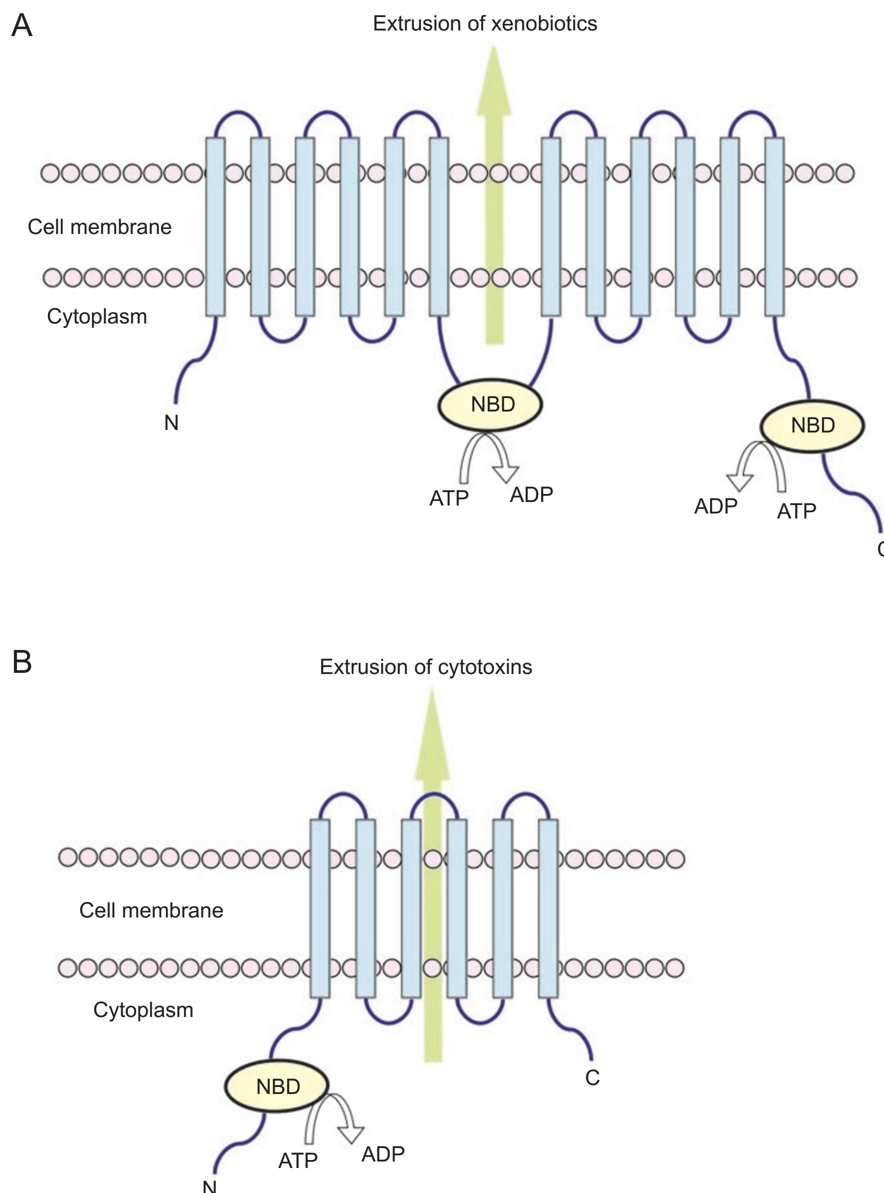

Figure 1 Structural features of ABC transporters. Most of the transporters are membrane-associated with six membrane-spanning regions, and are characterized by the presence of the ABC region. **(A)** ABCB1 typically is a full-length transporter of two identical halves as shown, with two NBDs that contain conserved sequences of the ABC. **(B)** ABCG2, on the other hand, is a half-transporter consisting of one NBD containing an ABC, followed by a six membrane-spanning domain.

of the full-length form comprising 1 280 amino acids (with a molecular weight of 170 kDa), and is characterized by two identical halves, each with one NBD; it exports a wide range of diverse substrates [54, 55, 57]. Another member of the ABC transporter family that is intensively studied is ABCG2 (also known as MXR/BCRP1/ABCP), which is a half-transporter and requires dimerization for its functional activity [65, 66]. Unlike other ABC half-transporters, which are localized to intracellular membranes at numerous compartments within the cell, ABCG2 is expressed exclusively in the plasma membrane [65, 66]. ABCG2 has been identified to confer resistance to anthracycline anticancer drugs, and is expressed in both malignant and normal tissues [67-70]. It is a membrane-associated 663 amino-acid transporter that is highly expressed in placenta and HSCs (SP/CD34$^-$) [71-73]. ABCG2 exhibits transporter

activity for several exogenous substrates such as cytotoxic drugs, fluorescent dyes and endogenous substrates such as folic acid, sulfated conjugates of steroids, bile salts and porphyrins [74]. However, the precise physiological role for ABCG2 has not yet been clearly defined despite extensive research.

Several ABC transporters are also called multidrug resistance proteins (called MRPs or MDRs) because these membrane glycoproteins mediate the ATP-dependent export of organic anions, including cytotoxic and antiviral drugs from cells [64, 59, 75, 76]. For instance, the human ABC transporter subfamily C (symbol ABCC) consists of 12 members, nine of which comprise a group of multidrug resistance proteins (also known as MRP1-MRP9 or ABCC1-ABCC6, ABCC10-ABCC12) [64, 65, 76, 77]. Insofar, MRP1-MRP6 are the best-characterized members of the ABCC subfamily. Conjugates of lipophilic compounds with glutathione, glucuronate or sulfate are the preferred physiological substrates of MRP1 and MRP3 [78-83]. MRP6 is described to transport glutathione conjugates as well as the endothelin receptor antagonist BQ123 [78-82]. Substrates for MRP4 and MRP5 include cyclic AMP, cyclic GMP and nucleotide analogues [78-82]. Reduced glutathione (GSH) has been recognized as a co-substrate for MRP1, for MRP2 and most recently for MRP4 [78-82]. In addition to being important export pumps for physiological substances, MRP subfamily members are involved in the active efflux of toxic organic anions [78-82]. MRP transporters have been shown to confer resistance to harmful drugs, possibly by the above-mentioned ATP-dependent export of such molecules [54-57].

ABCB1 is widely expressed in normal tissues with excretory functions such as the liver, kidney and intestine, and is also involved in barrier functions such as in the blood-brain barrier (BBB) and the blood-testis barrier [54, 57, 84, 85]. Apart from being induced by chemotherapeutic drugs, ABCB1 expression and upregulation of its activity have been observed in primary rat hepatocyte culture by EGF [86]. Also, ABCB1 expression actively protects cells against cell death and ABCG2 defends against natural heme metabolites [70, 87-91]. By transporting a variety of exogenous and endogenous compounds out of cells, they are able to reduce the body load of potentially harmful substances. However, one side effect of this protective function is that they also eliminate various useful drugs from the body, causing drug resistance [55, 56, 92]. Interestingly, ABCB1 expression is also found to be induced in paradigms of tissue regeneration [92-100].

ABCB1 activation was studied *in vivo* mainly in the liver, and *in vitro*, in hepatocytes [86, 94-96]. In addition, transient ABCB1 upregulation *in vivo*, which is associated with the tissue stress response, has been described in neurons and glial cells in the brain [97-99]; EGF was found to have a stimulatory effect on ABCB1 phosphorylation and multidrug resistance activity in a breast cancer cell line [100].

ABC transporters in the brain

In the brain, the proteins MRP1, MRP4 and MRP5 (ABCC family) were clearly localized, by confocal laser scanning microscopy, to the luminal side of brain capillary endothelial cells [101]. The MRP4 and MRP5 proteins were also detected in astrocytes of the subcortical white matter [101, 102]. Notably, MRP5 protein was present in pyramidal neurons [101]. Another study has revealed that MRP1 and MRP5 are more abundant in various brain cells than the other family members though MRP3 and MRP4 could also be detected in astrocytes [103]. MRP proteins may thus contribute to the resistance of the brain to several cytotoxic and antiviral drugs [104].

A recent real-time reverse transcription-polymerase chain reaction (RT-PCR) assay has been used to investigate the specific expression pattern of the ABC subfamily-A transporters in the brain and has shown that neurons express predominantly ABCA1 and ABCA3; astrocytes express ABCA1, ABCA2 and ABCA3; microglia express ABCA1 and oligodendrocytes express ABCA2 and ABCA3 [105]. With its expression in liver and brain, ABCB1 – the prototype of the B subclass of transporters – and ABCA1 regulate the high-density lipoprotein levels in the plasma and cholesterol contents of several cell types in these organs [106-114]. Most interestingly, the ABCB1 transporter also shows strong expression in neurons of the hippocampus formation, particularly in the granule cells of the DG [85]. Volk *et al*. [99] have demonstrated neuronal upregulation of ABCB1 expression in the CA3/CA4 region and hilus of the hippocampus formation 24 h after inducing a status epilepticus in rat brains. In general, however, ABCB1 is predominantly localized in the apical membrane of capillary endothelial cells which form the BBB, and in epithelial cells of the blood-cerebrospinal fluid barrier, while other cell types in the brain show little or no expression under normal conditions [84, 85, 97-99].

ABC transporters and diseases

In humans, genetic defects of ABC transporters have been implicated in several diseases involving transport deficiencies. Dysfunctions of these proteins have been causally related to several pathological phenotypes, spanning from neurological or metabolic diseases to drug resistance [115-117]. For instance, the most common fatal autosomal recessive genetic disease affecting Caucasian populations,

cystic fibrosis, is caused by mutations in the ABCC7 transporter (or CFTR), which is a regulated ion channel [118]. Most of the naturally occurring mutations in the ABCC7 gene either induce an alteration in protein biosynthesis or lead to a defective channel function [60, 64, 119, 120]. Approximately 80% of all mutations found with the ABC transporters are located within the NBDs.

Recent findings have shown that ABCG2 is strongly expressed on progenitor cells/reactive ductules in human liver, which might protect them from cytotoxic agents [121]. This could explain why in almost all conditions of liver damage and cell loss, progenitor cells/reactive ductules are able to withstand this damage and even expand and contribute to the repair process by differentiating into hepatocytes and/or cholangiocytes [121-125]. A rather basolateral hepatocytic expression in chronic biliary diseases may be an adaptive mechanism to pump bile constituents back into the sinusoidal blood [120].

Since ABC transporters exert their functions as membrane associated multi-unit complexes by binding to other important functional proteins such as ion-channels [126, 127], this may explain the association of ABC transporter mutations with a wide spectrum of inherited diseases. Because ABC transporters likely exert their biological function in cooperation with possibly varying interacting partners, the observed range of clinical phenotypes associated with mutations in one ABC molecule may also reflect genetic variations of proteins that specifically interact with ABC transporters. Moreover, in molecular terms, such functional pleiomorphism is related to the wide range of compounds that ABC family members can transport. Thus, the genotype/phenotype diversity in individuals with mutated ABC transporters may primarily reflect the functional complexity of these large multi-span polypeptides [60, 64, 119, 120].

ABC transporters and stem cells

It was first demonstrated by Chaudhary and Robinson that the ABCB1 transporter is highly expressed on CD34+ hematopoietic cells, suggesting that the efflux pump activity could be responsible for the low retention of the fluorescent dye rhodamine 123 in these primitive subsets of cells. Rh123 is another fluorescent dye whose efflux can be used to enrich for potential stem cells. In bone marrow, the percentage of cells in the Rh123-effluxing subset is similar to that in the Hoechst-dye-effluxing SP cells (i.e. about 10%). However, the former does not segregate into a population as distinct as the Hoechst-dye-effluxing SP cells and, thus, must be used in combination with other surrogate stem cell markers for stem cell isolation [9,128]. Later Goodell *et al*. [9] demonstrated that a highly enriched stem cell fraction termed SP could be isolated following Hoechst-33342 staining. Recently, ABCA3, ABCB1 and ABCG2 were found in the primitive stem cells of different tissues [129-136]. In particular, ABCB1 is expressed in human CD34+ stem cells, which can be identified by their ability to transport fluorescent dyes like Rh123 and Hoechst-33342 [128, 129, 137-143]. Interestingly, the SP phenotype in rodent and human tissues often appears to be specifically determined by the expression of an ABC transporter: for example, both ABCB1 and ABCG2 transporters are highly expressed in the SP of stem cells from different tissues such as brain, bone marrow, pancreas, liver and others, all of which can be isolated based on the cells' ability to promote the efflux of the Hoechst-33342 fluorescent dye [9, 129-136, 142-150]. Moreover, different research teams have demonstrated that ABCA3 and ABCG2 were expressed at higher levels in SP cells than in non-SP cells in human, rhesus monkey and mouse hematopoietic tissues; and microarray analysis indicated that several genes related to stem cells were substantially upregulated in the SP cells in comparison to non-SP cells [130, 151, 152]. This SP phenotype is present in several kinds of stem cells from different tissues, including the hematopoietic, meschenchymal, heart, liver, and pancreatic stem cells; it disappears with verapamil treatment thus indicating that the SP phenotype might result from the expression of ABC transporters in a primitive subset of stem cells in mammals [140, 142, 131, 145, 146, 148, 153, 154]. Since SP stem cells show high repopulating activity and ABCA3, ABCB1 and ABCG2 expression, it has been hypothesized that the special phenotype of SP cells might be controlled or regulated by the expression of ABC transporters [142, 144, 145, 149, 155, 156].

In the acute-myeloid-leukemia-derived AML-SP1 cell line, an increased expression of the ABC transporters MDR1, MRP, ABCG2 and ABCA3 was found in SP cells [142]. The detection of ABCA3 in leukemic progenitor cells merits further investigation with regard to the role in intracellular drug transport in AML blast cells. *In vivo* propagation of leukemias, such as AML-SP1, is a model system of maintaining the population heterogeneity of the AML disease, especially the unique characteristics of leukemic SP cells [142]. In another study, Chiba *et al*. [131] reported that the SP phenotype of stem-like cells isolated from hepatocellular carcinoma seemed not to be related to ABCG2 (Bcrp1), but rather to several other ABC transporters, notably ABCB1 (MDR1), ABCB2, ABCC7 and ABCA5, as they found a several fold upregulation of expression of these genes in "PLC/PRF/5"-SP cells as compared to non-SP cells.

A recent paper by Israeli *et al*. [93] summarizes the work on the ABCB1- and ABCG2-deficient mice, sug-

gesting that ABCB1 and ABCG2 are expressed abundantly in stem cells and the extrusion of xenobiotics may not be their only function. While humans have only one ABCB1 gene (mdr1), mice have two, mdr1a (also called mdr3) and mdr1b (also called mdr1), together probably fulfilling the same function(s) as the single human mdr1 (ABCB1) [157-160]. To determine whether expression of ABC-like genes was needed for the SP phenotype, mice with targeted disruptions of the mdr1a/mdr1b genes were analyzed, and no difference in the number of SP cells relative to wild-type mice was observed, demonstrating that ABCB1 is not required for the SP phenotype. In the same study, it was found that cells transfected with a ABCG2 cDNA readily expelled Hoechst dye but not Rho123, and that efflux activity was abolished by reserpine (an inhibitor of multidrug transporters [161]), and these findings are consistent with the Hoechst-low, Rho-bright phenotype of bone-marrow SP cells from ABCB1-negative mice [162, 163]. However, studies by Zhou *et al*. [147, 155, 164] have demonstrated that loss of ABCG2 gene expression leads to a significant reduction in the number of SP cells in the bone marrow and that ABCG2 expression is necessary for the SP phenotype in HSCs and they hypothesized that ABCG2 defines the SP stem cell phenotype and it provides protection from cytotoxic substrates. Taken together, these results indicate a link between ABCG2 expression and the SP phenotype. Apart from that, the intensity of ABCG2 expression increased distally from the non-SP cells towards the SP cells, leading to the conclusion that the expression of ABCG2 transporter can directly confer the SP phenotype in transduced primary bone-marrow cells both *in vitro* and *in vivo* [162-165].

Finally, the same authors also found that the ABCG2 transporter was expressed in a highly regulated manner, with the highest expression in primitive cells and subsequent downregulation following commitment to differentiation. Another research group could show that enforced expression of ABCG2 also inhibited hematopoietic development, and resulted in less progeny in the bone marrow and peripheral blood [149]. Therefore, it is suggested that ABCG2 expression may play a role in the self-renewal of the early stem cell by partially blocking differentiation. For instance, it is possible that the ABCG2 transporter is expelling a substrate capable of inducing differentiation, or that the ABCG2 transporter plays a role in mediating extracellular signals influencing stem cell interactions with the microenvironment. In contrast, overexpression of ABCB1 in bone marrow cells led to proliferation of SP cells, resulting in prolonged survival in culture and enhanced repopulation after transplantation into mice [144]. Therefore ABCB1 expression may characterize proliferating stem cells, while ABCG2 expression may distinguish quiescent ones. Thus, in addition to the possible role of ABCB1 and ABCG2 as a determinant of the SP phenotype, they could potentially serve as novel stem cell markers and may confer required functional properties to these cells, as suggested by the conserved SP phenotype in a wide variety of different types of stem cells. Further studies on mice deficient in or over-expressing both transporters will be required to understand their precise roles in stem cells and to clarify the relationships between ABCG2/ABCB1 expression, the SP phenotype and expression of other markers such as CD34 and CD133 [140, 143, 148].

ABC transporters and NSCs

Hulspas and Quesenberry initially hypothesized that the SP phenotype in mouse NSCs was probably due to the expression of ABCB1 [39]. Recent results show that NSCs from mouse forebrain are contained in a population distinct from the SP [134]. Moreover, other research data show that the ABCA2 transporter is widely expressed in early neural progenitors developed *in vitro* from ES cells [166]. ABCA2 expression in the adult mouse and rat brains seems to be region-dependent because it is limited to the oligodendrocytic lineage – unambiguously excluded from astrocytes – and to a subset of cortical GABAergic inter-neurons and pyramidal glutamatergic neurons where it could be localized to lysosomal-related organelles [166]. ABCA2 has also been suggested to be a marker of neural progenitors as it is expressed in the subventricular zone of the lateral ventricle and the DG of the hippocampal formation, sites of continual neurogenesis in the adult brain, and in nestin+ cells differentiated *in vitro* from ES cells [166].

It was only very recently that the distribution and functional properties of the transporters were studied in human neural stem/progenitor cells (hNSPCs). It was found that more than half of the hNSPCs within neurospheres expressed nestin, an NSPC marker [167, 168]. Furthermore, all nestin+ cells simultaneously expressed ABCB1 [167, 168]. Moreover, when the hNSPCs were isolated by fluorescence activated cell sorting (FACS) using the ABCB1 antibody, there was an increase in nestin+ cells compared to cells separated by control IgG (Islam *et al*., unpublished observation). Taken together, these results suggest that this ABC transporter may contribute to neural stem/progenitor cell expansion *in vitro*.

Further study revealed that cultured hNSPCs expressed functional ABCB1 as well as ABCG2 at the cell surface, and that their expression was downregulated during differentiation of hNSPCs, similar to the downregulation of ABCG2 in HSCs at the stage of lineage commitment [143, 167, 168]. It was observed that both ABC transporters were downregulated during hNSPC differentiation, together with nestin downregulation and GFAP upregulation (Figures 2

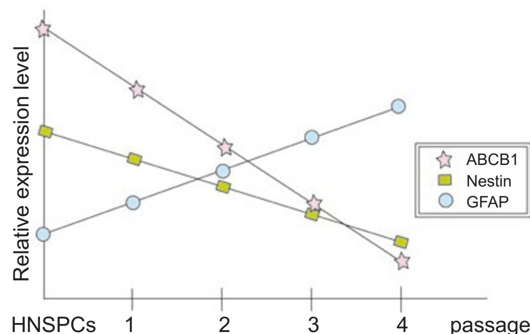

Figure 2 Expression of ABCB1 during hNSPCs differentiation. hNSPCs (neurospheres) were induced to differentiate into glia and were then passaged four times. The graph shows the relative time course expression of ABCB1, nestin and GFAP in these hNSPCs over the four passages of differentiation. (adapted with permission from Islam *et al*. [76]).

and 3). The downregulation of nestin and upregulation of GFAP are considered to be indicators of stem/progenitor differentiation or maturation, and therefore it was proposed that ABCB1 and ABCG2 may be markers of neural stem/progenitor cells, and may have a functional role in upholding the undifferentiated status of hNSPCs [167, 168]. Consistent with this idea, it was found that ABCG2 had a high substrate-stimulated ATP-hydrolysis activity in these cells, further suggesting that the ABCG2 transporter was functionally active in hNSPCs and may play a regulatory role in the maintenance of the undifferentiated state, possibly through modulating the uptake of small hydrophobic molecules involved in differentiation [147, 155, 168].

Because an intrinsic property of neurospheres is the heterogeneity of the few stem/progenitor cells within the neurospheres, this renders it difficult to directly identify and purify these stem cells [169]. Usually, the objective of transplanting NSCs is to generate the maximum number of neurons from the neurosphere cultures. The key issue thus lies in the isolation of such proliferated stem/progenitor cells from the neurospheres; and cell sorting with ABC transporter-specific antibodies may provide a new strategy for stem cell purification from neurospheres.

A model for ABC transporter expression in NSCs

Studies of the ABC transporters and SP phenotype have mainly focused on hematopoietic (including mesenchymal) stem cells, with some studies on pancreatic and liver stem

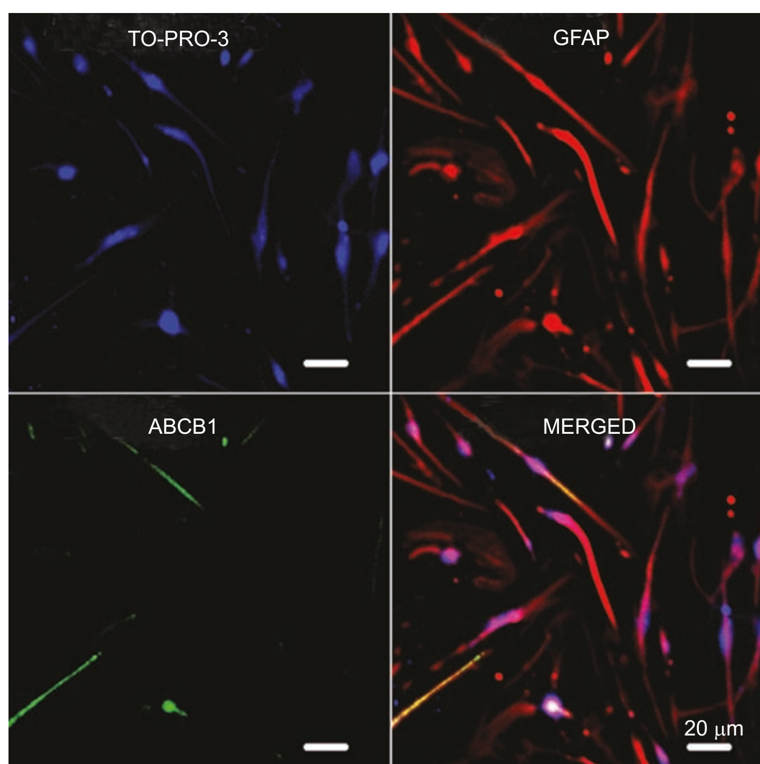

Figure 3 Immunocytochemical analysis of the ABCB1 and GFAP expression pattern in hNSPCs differentiated into glia cells. Nuclei were stained with TO-PRO-3. Scale-bar = 20 μm. (adapted with permission from Islam *et al*. [76]).

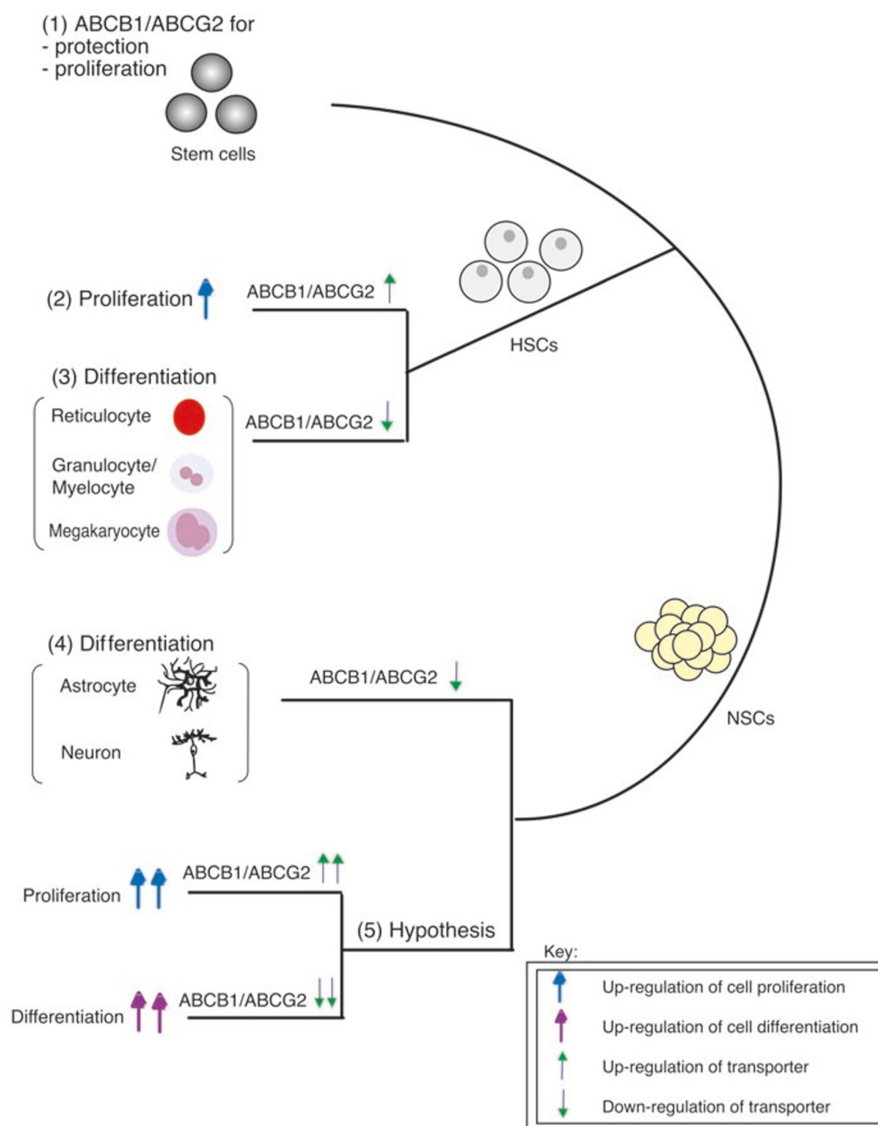

Figure 4 Schematic illustration of the effect of ABC transporters on stem cell proliferation and differentiation. (1) Expression of ABC transporters (especially ABCB1 and ABCG2) in stem cells like the NSCs, HSCs or pancreatic stem cells is thought to be essential for their *in vivo* proliferation and probably their self-renewal activity. (2) Enforced expression of ABCB1 or ABCG2 leads to enhanced proliferation in HSCs. (3) Downregulation of ABCB1 or ABCG2 in HSCs is observed with the differentiation of HSCs. (4) Downregulation of the ABC transporters in NSCs is observed with the differentiation into astrocytes or neurons. (5) The hypothesis suggests that expression of the ABC transporters in NSCs may have an effect on NSC differentiation or proliferation, such that significant upregulation of ABCB1 or ABCG2 expression may lead to an increase in self-renewal of NSCs, and correspondingly, a decrease in ABCB1 or ABCG2 expression may lead to increased differentiation of NSCs.

cells but very few on NSCs. Therefore, in this review, we intend to discuss the possible involvement of the ABC transporters, especially ABCB1 and ABCG2, in neural stem cell proliferation and differentiation.

The hypothesis put forth by Israeli *et al*. [93] is that the biological activities of ABCB1 and ABCG2 in stem cells are part of the normal tissue regeneration mechanism, probably conferring protection of the small stem cell population from cell death and enabling preservation of homeostasis under extreme stress conditions. Interestingly, the correlation between nestin and ABCB1 expression in hNSPCs, and inhibition of hNSCPs proliferation by an ABCB1

inhibitor (cyclosporine A) at a very low dose, suggested that the ABC transporter may contribute to neural stem/progenitor cell expansion [167, 168]. While ABCA2 shows higher expression in nestin+ mouse neural progenitors, at later developmental stages it undergoes a conspicuous downregulation, persisting only in limited subsets of differentiated neurons [166]. Based on these results and the observations that the ABC transporters are expressed at high levels in hNSPCs but are downregulated in differentiated hNSPCs (Figure 3), we hypothesize that these genes could potentially function as putative NSC markers in a similar way as p21CIP/WAF or musashi-1. For instance, while a high expression of ABCB1/ABCG2 reflects maintenance of proliferating NSCs in an undifferentiated state, low expression characterizes progenitors differentiating into neurons and astrocytes, which by themselves do not (or only at a very low level) express ABCB1/ABCG2 at the end of differentiation (Figure 4) [167, 168]. Chiba *et al*. [131] suggested that ABCB1 rather than ABCG2 is involved in their "PLC/PRF/5" SP cell phenotype . The published studies on NSCs support the hypothesis that ABCB1 (MDR1) might be more important than ABCG2 in controlling the specific phenotype of NSCs [166-168], in contrast to the HSCs where ABCG2 is involved in the SP phenotype and considered as its molecular determinant. In short, ABC transporters have emerged as an important new field of investigation in the regulation of stem cell biology, and manipulation of this system may promote stem cell amplification via a common defence mechanism adopted by these cells through their high expression of ABC membrane transporters [170].

Clinical relevance and Conclusions

The clinical implications of NSCs are aplenty. While their exact function and distribution is currently being assessed, they represent an interesting cell population, which may be used to study factors important for the differentiation and characterization of neurons, astrocytes and oligodendrocytes. Recently, there have been reports of NSC transplantations attempting to achieve functional recovery from CNS damage [171-176], and recent evidence suggests that NSCs may be a suitable source for the treatment of neurological diseases [22-27, 177-179]. Due to their proliferative and differentiation capacity, NSCs will be important in fighting numerous brain disorders like AD, PD and Huntington's disease, as well as spinal cord disorders. For instance, in the early stages of AD, a relatively discrete population of neurons is affected [19-21, 180], making it an ideal target for cell replacement therapy using endogenously activated NSCs. However, because curing AD requires the complete construction of the damaged neural circuitry, it will be an especially challenging task to induce NSCs to differentiate into a certain cell-type, highlighting a crucial need for developing the ability to control and predict NSC differentiation. It has already been shown that human NSCs transplanted into aged rat brains can differentiate into neural cells and significantly improve the cognitive functions of these animals, further pointing to NSCs as a promising candidate for neuro-replacement therapies [16,17, 181]. If new bio-imaging technologies could allow an early diagnosis of the onset of AD when the loss of cholinergic neurons has just begun, it might be possible to restore the generation of cholinergic neurons through the transplantation of NSCs, which would provide a potential remedy for the treatment of this disease.

Based on the evidence presented here, we would like to propose that while the ABC transporters are useful as universal stem cell markers and are functionally expressed in hNSPCs, they may also play a crucial role in hNSPC self-renewal by preventing differentiation, and hence will probably be vital in future NSC-based therapy in treating neurodegenerative disorders like AD and PD. In addition, these NSC markers are localized at the cell surface and thus could become a very useful tool, as antibodies raised against these transporters could be utilized for isolating NSCs from neurospheres through techniques like flow cytometry. In this respect, the ABC transporter family members may have provided a model for studying the regulation of NSC self-renewal and differentiation. Many new questions remain regarding how these molecules might function and what evolutionarily conserved substrates may be modulated. More experiments involving the analysis of hNSPCs transfected with genes for expression of the different ABC transporters will definitely provide more conclusive evidence, and hopefully may also facilitate future breakthroughs in the treatment of neurodegeneration, a field that has perplexed researchers for centuries.

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