Evaluation of neural plasticity in adult stem cells

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The role of stem cells has long been known in reproductive organs and various tissues including the haematopoietic system and skin. During the last decade, stem cells have also been identified in other organs, including the nervous system, both during development and in post-natal life. More recently, evidence has been presented that stem cells thought to be responsible for the generation of mature differentiated cells of one organ, such as haematopoietic stem cells, may have the ability to also differentiate across lineages and contribute to tissues other than haematopoietic cells, including neuronal tissue, suggesting that easily accessible stem cells sources may one day be useful in the therapy of ischaemic (stroke) and also degenerative diseases of the nervous system. Here, we will evaluate the validity of such claims based on a number of criteria we believe need to be fulfilled to definitively conclude that certain stem cells can give rise to functional neural cells that might be suitable for therapy of neural disorders.

Keywords: neural plasticity; mesenchymal stem cell; differentiation

1. INTRODUCTION

Scientific and public enthusiasm surrounding the promise of stem cells relates to the potential they hold to one day change medical therapies for a number of congenital or acquired disorders, some of which affect large proportions of the aging population. For instance, neurodegenerative diseases such as Parkinson's disease are characterized by more or less selective degeneration of specific neurons leading to functional impairment. Currently, most therapies for these neurodegenerative disorders are based on delaying the deleterious effects from the loss of neurons or other neural cell types. Based on current insights in the biology of neural stem cells (NSCs; Palmer *et al.* 1997; Takahashi *et al.* 1999; Song *et al.* 2002), it may one day be possible to replace the lost neural cells, and hence not only delay functional impairment, but also completely restore functional neural circuits and associated function. As NSCs are difficult to harvest from the adult brain, other stem cell sources that would be more readily available are being evaluated, including haematopoietic stem cells (HSCs; Till & McCulloch 1961; Becker *et al.* 1963; Till & McCulloch 1980), present in bone marrow (BM) and umbilical cord blood (UCB; Sanchez-Ramos *et al.* 2001; Forraz *et al.* 2002); or other stem cell populations present in BM such as mesenchymal stem cell or marrow stromal cells (MSCs; Caplan 1991; Pittenger *et al.* 1999), and more rare populations such as multipotent adult progenitor cells (MAPCs; Jiang *et al.* 2002), marrow-isolated adult multilineage inducible (MIAMI) cells (D'Ippolito *et al.* 2004), BM-derived multipotent stem cells (BMSCs; Yoon *et al.* 2005b) or unrestricted

somatic stem cells (USSCs; Kogler *et al.* 2004), for all of which claims have been made that they can differentiate to neuronal cell types. Such apparent lineage switch has been termed stem cell plasticity. However, many questions remain as to the validity of a number of these studies, in a large part owing to loose criteria, which have been used to define 'neural differentiation'.

One type of stem cells that definitively differentiate into mature terminally differentiated and functional neural cells *in vitro* are NSCs. The NSCs are present in the brain and spinal cord even in post-natal life and their ability to differentiate into neurons, astrocytes and oligodendrocytes has been shown both *in vitro* and *in vivo* (Palmer *et al.* 1997; Takahashi *et al.* 1999; Song *et al.* 2002; Seaberg & van der Kooy 2003). Criteria used to define differentiation of NSCs to mature neural cells should be used as the gold standard to gauge whether somatic stem cells of non-neuronal origin, such as HSCs or MSCs, can give rise to neural progeny. Another stem cell that has the definite potential to generate all mature neurons and glial cells is the embryonic stem (ES) cell, which is pluripotent, i.e. giving rise to all somatic cell types, including all neural tissue cells as well as germ cells. A separate chapter will address the ability of ES cells to generate neural cells.

To evaluate claims that HSCs, MSCs or other non-neuronal stem cells have the ability to differentiate to neuronal or glial cells, we will employ a series of criteria that are summarized in figure 1 that, in general, are fulfilled when evaluating the ability of foetal or adult brain derived NSCs to generate functional neuronal cells. First, presumed differentiated progeny should express neuronal- or glia-specific markers at the RNA and protein level. Double and triple staining is required to demonstrate that expression of certain markers is not random or spurious, resulting in co-expression of, for

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Figure 1. Criteria for the evaluation of neural plasticity.

instance, oligodendrocyte and neuron markers. Second, expression of these markers should occur in a temporal fashion consistent with development. Third, differentiated cells should display functional characteristics consistent with neurons and glia, such as voltage-gated sodium channels, depolarizing responses to neurotransmitters and release of neurotransmitters in response to depolarization. Finally, the stem cells themselves or more committed progeny should be able to acquire a fully differentiated phenotype *in vivo* in a location-appropriate manner. Similar to *in vitro* differentiation, *in vivo* differentiated cells should express the correct complement of neuronal or glial markers, function appropriately, and integrate in the neural circuitry. The last criterion is the most difficult to attain, as many *in vivo* studies using NSCs have not fully proven true function of NSC-derived neurons *in vivo*, and it remains to be determined in many cases whether functional benefit is the result of the integration of donor neural cells in the brain or spinal cord, versus trophic effects elaborated by the donor cells that elicit endogenous neural cells to repair the deficit (Chopp & Li 2002; Lu *et al.* 2003; Taguchi *et al.* 2004; Yan *et al.* 2004). For instance, when grafted in the striatum, dopaminergic committed cells mature further and appear to be able to provide functional benefit, even though most of these papers do not demonstrate functional integration (Carvey *et al.* 2001; Tai & Svendsen 2004; Wang *et al.* 2004). Other papers have, however, shown evidence of functional synapses of *in vitro* generated dopaminergic neuronal cells or precursors within the central nervous system (CNS; Kim *et al.* 2002). Likewise, when grafted in a mouse model of demyelination, NSCs can generate oligodendrocytes that remyelinate neurons (Brustle *et al.* 1999; Yandava *et al.* 1999).

Other caveats exist that need to be kept in mind when assessing the ability of non-neural stem cells to generate neuronal or glial cells. The most conclusive way of demonstrating that a donor cell has engrafted, thus allowing for further determination of an acquired neural fate, is by using donor cells that have been genetically modified to express β -galactosidase or a fluorescent protein gene under the control of a neural specific promoter (Roy *et al.* 2000). The second best option is cells in which the fluorescent gene or the β -galactosidase gene is expressed from a ubiquitous promoter. Less desirable are methods that passively label cells, such as membrane intercalating dyes like PKH26, as these may be recycled following death of the donor cells in microglia among other cells (Yoon *et al.* 2005a).

Commonly used for engraftment studies in the brain is bromodeoxyuridine (BrdU) or tritiated thymidine labelling of the DNA of proliferating cells, where donor cells can be followed by staining with anti-BrdU antibodies or by autoradiography (Kopen *et al.* 1999; Shihabuddin *et al.* 2000; Cao *et al.* 2001; Li *et al.* 2001; Lu *et al.* 2001; Mahmood *et al.* 2001; Reubinoff *et al.* 2001; Ben-Hur *et al.* 2003; Chu *et al.* 2004; Munoz-Elias *et al.* 2004; Surendran *et al.* 2004). Recently, it has been demonstrated that oligonucleotides from dying cells can be recycled in endogenous proliferating cells, or cells undergoing simple DNA repair, and therefore controls in which labelled but dead cells are transplanted, should be done (Burns *et al.* 2006).

An additional caveat that has come to light in a series of studies over the last 5 years is that perceived plasticity might be caused by the stem cell or its progeny fusing with host cells of a different lineage leading to the apparent lineage switch, which is not direct but owing to the acquisition of genetic information of the differentiated cell with which the stem cell fused (Wagers *et al.* 2002; Alvarez-Dolado *et al.* 2003; Weimann *et al.* 2003a). This phenomenon underlying the heterokaryon technique was described many decades ago, and was more recently demonstrated to occur between ES cells and somatic cells (Terada *et al.* 2002; Ying *et al.* 2002), including neural cells. In addition, similar events have been documented *in vivo* for multiple cell types. As differentiation *in vitro* sometimes is achieved by coculture with mature neurons or glial cells, and as differentiation *in vivo* occurs near endogenous neuronal cells, it is important to assess whether presumed differentiation could be due to fusion between stem cells and already differentiated cells rather than cell intrinsic differentiation. Owing to the latter possibility, careful examination of the presumed donor cell with neural characteristics is needed. Fused cells will likely have two sets of sex chromosomes, hence fluorescence *in situ* hybridization for the X and Y chromosomes may aid in determining whether the putative donor-derived neural cell is the result of intrinsic differentiation of the donor cell or due to fusion (Wang *et al.* 2003). However, this analysis is not full proof as the Y-chromosome can be lost, and because reduction of chromosomal material may occur (Wang *et al.* 2003). An alternative method to demonstrate whether putative lineage switch has occurred due to fusion is the Cre-*lox* recombinase system (Alvarez-Dolado *et al.* 2003; Harris *et al.* 2004). Here, Z/EG Cre-reporter mice are used as marrow donors for transplantation into mice that ubiquitously express Cre. In this model, any cell resulting from fusion of a BMDC with a host cell should express enhanced green fluorescent protein (EGFP), provided that the Cre recombinase gene is expressed (Harris *et al.* 2004).

2. *IN VITRO* NEURONAL DIFFERENTIATION FROM NON-NEURAL STEM CELLS

The majority of studies suggesting that non-neural stem cells can differentiate to neuronal or glia *in vitro* have been focused around MSCs. The MSCs have been

isolated from BM (Caplan 1991; Pittenger *et al.* 1999), adipose tissue (De Ugarte *et al.* 2003), skeletal muscle (Jankowski *et al.* 2002) and UBC (Hou *et al.* 2003). MSCs are typical mesodermal cells that, at the single cell level, differentiate into cartilage, fat, bone and skeletal muscle cells (Pittenger *et al.* 1999). A number of studies have suggested that, aside from these mesodermal cell lineages, MSCs may also be capable of differentiating to neurons and glia under specific conditions *in vitro*. Agents used to induce neural differentiation included non-specific inducers such as isobutylmethylxanthine (Deng *et al.* 2001), dimethylsulphoxide (DMSO) and/or butylated hydroxyanisole (BHA; Woodbury *et al.* 2000). Others used specific cytokines known to induce neural development *in vivo*, or neuronal and glia differentiation from NSCs and ES cells *in vitro*, such as basic fibroblast growth factor (bFGF), and neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial-cell-line-derived neurotrophic factor (GDNF; Sanchez-Ramos 2002; Barry & Murphy 2004). In most instances, differentiation was shown only at the morphologic level, i.e. neural cell-like morphology and expression of a few neuron- or glia-specific proteins or transcripts. Surprisingly, several of the published reports indicated that such lineage switch occurred already after 5–12 h (Woodbury *et al.* 2000), significantly faster than what is seen for NSCs, posing the question whether induction of proteins and transcripts reflect simple activation of gene transcription under the influence of non-specific inducers rather than true cell differentiation. In one study, it was postulated that changes in morphology upon addition of DMSO-containing medium were caused by rapid disruption of the actin cytoskeleton, leading to morphological changes resembling neuritis (Neuhuber *et al.* 2004). It should also be noted that in many instances low-level expression of presumed neuron- or glia-specific markers can already be detected in undifferentiated cells (Tondreau *et al.* 2004).

Only recently studies have started to address whether putative neural progeny from MSCs have functional characteristics consistent with neurons. Wislet-Gendebien *et al.* (2005) reported that nestin positive cells, present in MSC cultures maintained for 25 population doublings cocultured with cerebellar granule neuron cultures, started to express either glial fibrillary acidic protein (GFAP) or neuronal nuclei (NeuN). Undifferentiated nestin positive MSCs had a low resting membrane potential, which increased to -57 mV by day 15, at which time they also acquired functional sodium voltage-gated channels. In comparison with the normal maturation of neurons as described by Carleton *et al.* (2003), the phenotype reported by Wislet-Gendebien *et al.* (2005) is an intermediate phase of neural development still lacking the ability to fire trains of action potentials and evidence of strong synaptic activity. Although differentiation required coculture of MSCs with mature neurons and could therefore be due to fusion, the investigators also cocultured MSCs with paraformaldehyde fixed cerebellar granule neurons with similar results, suggesting directed differentiation.

A second study provided further evidence that MSCs may generate neuronal cells through genetic

manipulations. Dezawa *et al.* (2004) overexpressed the intracellular domain of Notch in MSCs, to induce constant Notch activation signalling. They demonstrated that this resulted in expression of the glutamate transporter GLAST, 3-phosphoglycerate dehydrogenase (3-PGDH), and nestin, and upon addition of the trophic factors, forskolin (FSK), bFGF and ciliary neurotrophic factor (CNTF), in more than 95% of MSCs that expressed microtubule-associated protein-2ab (MAP-2ab), a known marker for post-mitotic neurons. MAP-2ab co-stained with neurofilament-M and β -tubulin isotype 3 (Tuj1), but not glial markers. Electrophysiological analysis demonstrated the presence of outward rectified K^+ currents in more than 50% of the cells, but no voltage-gated fast sodium currents suggesting that cells at that stage were not fully mature neurons. Addition of GDNF resulted in more than 40% of cells expressing tyrosin hydroxylase (TH) and increased expression of transcription factors Nurr-1, Lmx1b, En1 and Ptx3, suggestive of dopaminergic neuron commitment. Further evidence for dopaminergic-like neuron differentiation came from studies in which K^+ depolarization resulted in the release of 1.1 pmol dopamine/ 10^6 cells and improvement of the rotational behaviour of 6-hydroxy dopamine (6-OHDA) treated animals to a similar extent as has been described for foetal midbrain transplantations.

In addition to MSCs, populations of rare cells, such as MAPCs (Jiang *et al.* 2002), BMSCs (Yoon *et al.* 2005b) and MIAMI (D'Ippolito *et al.* 2004), have been isolated from BM, and USSCs (Kogler *et al.* 2004) from UCB, that may have greater multilineage differentiation potential. *In vitro* studies have shown that MAPCs and BMSCs can acquire the phenotype of astrocytes (GFAP), neurons (neurofilament-200 (NF-200), Tuj1 and NGF), and oligodendrocytes (galactocerebroside (GalC)), and staining using multiple antibodies has shown specific expression for NF-200, GFAP or GalC. Jiang *et al.* (2003) reported that gene expression assessed by RT-PCR (Otx2, Otx1, Pax2, Pax5 and nestin) occurs in a temporal manner consistent with midbrain development followed by mature expression of either dopa decarboxylase (DDC) and TH positive cells, serotonin-containing (serotonin positive) neurons or aminobutyric acid (GABA)-containing (GABA positive) neurons when MAPCs were stimulated sequentially with bFGF, Shh + FGF8 and BDNF (Jiang *et al.* 2002, 2003). Differentiated MAPCs also had outward rectified K^+ currents and voltage-gated fast sodium currents. Little information regarding function of presumed neural cells was provided for MIAMI cells, USSCs and BSSCs.

3. IN VIVO GRAFTING AND FUNCTION

(a) Engraftment and functional effects of MSCs

A number of studies have tested whether undifferentiated MSCs grafted *in vivo* in the brain commit to the neural lineage and affect the function of the CNS (Kopen *et al.* 1999; Hofstetter *et al.* 2002). Although some studies have suggested that MSCs can differentiate spontaneously into neural cells *in vivo* (Satake *et al.* 2004), these conclusions were in general

based solely on the identification of neuron- (nestin) or glia- (GFAP; Kopen *et al.* 1999) specific markers in rare donor cells that could be found. However, as there is significant evidence that undifferentiated MSCs express such markers (nestin, Tuj-I, neuron-specific enolase (NSE), Neu-N, MAP-2 and GFAP; Deng *et al.* 2001; Tondreau *et al.* 2004), this does not constitute proof that MSCs differentiated *in vivo* into neural cells, and to our knowledge no study has definitively proven that grafting of undifferentiated MSCs into the brain results in differentiation to neurons or glia. In one study, however, where MSCs were grafted in embryonic rat brain, widespread distribution of donor cells throughout the brain was seen, with acquisition of region-specific marker expression within the BrdU-labelled donor cells (Munoz-Elias *et al.* 2004). No functional assessment was done and no control studies were done where dead MSCs were grafted to assure that the BrdU label was not transferred to the rapidly proliferating endogenous neural progenitor and stem cell population in the developing brain. Moreover, none of these studies have definitively addressed whether the presumed neural differentiation could be caused by fusion between donor cells and mature neurons/glia or with endogenous NSCs.

However, it is clear from a number of studies that injection of undifferentiated MSCs in different injury models may have beneficial effects. For instance, MSCs themselves, and even more so when engineered to express neurotrophic factors, promote functional recovery and decrease the infarct size following middle artery occlusion (Behrstock & Svendsen 2004; Kurozumi *et al.* 2004). Mechanisms underlying these beneficial effects are in part via improved angiogenesis (Chen *et al.* 2003, 2004; Borlongan *et al.* 2004) and induction of endogenous neurogenesis (Chen *et al.* 2004). Likewise, MSCs grafted in spinal cord lesions support axonal growth of the resident neurons, form guiding strands for neural outgrowth, leading to functional improvement (Ankeny *et al.* 2004; Neuhuber *et al.* 2005). It should be noted that all these effects are trophic, engaging the endogenous neural cells in the repair process, rather than stem cell-based differentiation.

Jiang *et al.* (2002) reported that, following micro-injection of a single ROSA26 MAPC into mouse blastocysts, MAPCs contribute to most if not all organs of the developing mouse. In these animals, MAPC-derived progeny were found throughout the entire brain, with β -gal positive neurons and astrocytes present in the striatum, hippocampus, thalamus, cortex, and the cerebellum at various levels, with normal developmental patterning (Keene *et al.* 2003). Thus, although the blastocyst studies may provide proof of principle that MAPCs can generate neurons and astrocytes *in vivo*, this needs to be confirmed in post-natal studies. However, when injected post-natally in a cortical brain ischaemia (stroke) model, functional improvement was seen without evidence that the MAPC progeny had integrated in the brain (Zhao *et al.* 2002), suggesting trophic effects underlying the improved function but not differentiation of MAPCs themselves to neural cells.

(b) *Engraftment and functional effects of haematopoietic stem cells*

A number of studies have also suggested that HSCs may differentiate to neural cells *in vivo*. The first studies suggesting this possibility date from 1999 to 2000. In two independent studies, investigators demonstrated that intravenous transplantation of BM cells either in newborn animals (PU.1 negative, which have failing haematopoiesis from birth) or in older animals following lethal irradiation, yielded a large number of BM-derived donor cells (Y-chromosome or GFP positive; Brazelton *et al.* 2000; Mezey *et al.* 2000) throughout the brain, which co-labelled with neuronal markers. In other studies, similar results were seen in humans that had undergone sex-mismatch BM transplantation (Mezey *et al.* 2003; Weimann *et al.* 2003a; Cogle *et al.* 2004). In these studies, the cell population used for transplantation was not pure and consisted of mononuclear BM cells. Hence, it is not possible to determine which cell in BM would have contributed to the brain. Subsequently, others have found that a large number of the presumed neuronal cells may be microglial cells, which are haematopoietic in origin (Vallieres & Sawchenko 2003). Furthermore, several groups have shown that some of the presumed donor cells detected in the brain that co-label with neural markers are the result of fusion between host brain cells and BM-derived cells (Wagers *et al.* 2002; Alvarez-Dolado *et al.* 2003; Weimann *et al.* 2003b).

As is the case with grafting MSCs in the brain, there is evidence that grafting BM cells or enriched HSCs in the brain may have therapeutic effects, independent of transdifferentiation of these cells to neural cells. In some of the studies, cells were administered intravenously shortly following a stroke, others grafted cells directly in the brain. Some studies have suggested a beneficial effect of systemically administered cells (Willing *et al.* 2003; Borlongan *et al.* 2004; Taguchi *et al.* 2004) on neural function. For instance, in a recent study, CD34⁺ cells from human UCB were infused in immunocompromised animals that had undergone a stroke 48 h earlier. The authors report significant increased neovascularization in the ischaemic zone, which provided a favourable environment for neuronal regeneration, mainly owing to endogenous neurogenesis (Taguchi *et al.* 2004). Similar effects have been described for animals that were treated with granulocyte colony stimulating factor (G-CSF), an agent that mobilizes haematopoietic progenitors from the BM into the blood (Shyu *et al.* 2004). However, other studies have suggested that such beneficial effects are limited and haematopoietic cell grafts may only be effective when administered within 24–48 h after the lesion, while intracerebral administration may have greater beneficial effects, even beyond 48 h after the stroke (Borlongan *et al.* 2005).

4. CONCLUSIONS

A large number of studies have been published over the last 5–10 years suggesting that non-neural stem cells may be capable of generating neurons or glia *in vitro* and *in vivo*. As discussed in this review, proof of neural differentiation has ranged from morphology and

immunophenotyping to electrophysiology and release of neurotransmitters. Clearly, only when functional characteristics of neurons or glia are demonstrated *in vitro* can the claim be made that a non-neural stem cell has differentiated into neural cell types, and only a few studies have attained this level of characterization. Likewise, studies in which MSCs, the more pluripotent MAPCs, BSSCs, USSCs or MIAMI cells as well as BM mononuclear cells or HSC enriched populations were transplanted *in vivo*, have not yet shown conclusively differentiation to functional neurons or glia. Hence, definitive proof that non-neural stem cells can differentiate down the neural lineage *in vivo* and *in vitro* is still not available.

Nevertheless, many *in vivo* studies demonstrate that non-neural stem cells may have significant functional impact in the setting of stroke and spinal cord injury, and perhaps certain neurodegenerative disorders, by inducing angiogenesis, recruiting endogenous stem cells, guiding axons or secreting neurotrophic factors. Clearly this may, as has been shown in animal models as well as clinical studies in cardiac ischaemic disorders (Assmus *et al.* 2002; Wollert *et al.* 2004; Gneccchi *et al.* 2005), have clinical relevance, despite the fact that the injected cell population did not itself generate neural cells.

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