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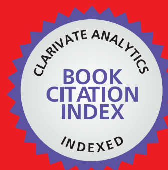

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The Promise of Hematopoietic Stem Cell Therapy for Stroke: Are We There Yet?

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1. Introduction

Stroke is the leading cause of permanent disability in industrialized nations (Lloyd-Jones et al, 2010). Ischemic stroke occurs secondary to blood flow interruption to the brain, typically secondary to the occlusion of an intra- or extra-cranial artery. This lack of blood supply to the brain results in a paucity of nutrients, glucose, and oxygen, which leads to cerebral ischemia and infarction (Wardlaw et al, 2003). However, intracranial artery occlusion results in varied rates of tissue injury depending on the local anatomy, as well as numerous still-to-be-deciphered physiologic factors. Generally, the ischemic core of the occluded vascular territory rapidly infarcts and becomes unsalvageable tissue. However, the surrounding region of ischemia, known as the penumbra, often receives enough collateral supply that it may be saved, providing adequate perfusion is reestablished in a timely fashion. The concept of restoring normal perfusion to the penumbra, thereby rescuing crucial brain tinssue and hence neurological function, is fundamental.

To date, the significant majority of stem cell stroke research has focused on evaluating the potential of Neural Stem Cells in cerebral ischemic repair (Garzon-muvdi et al, 2009; Miljan et al, 2009; Bersano et al, 2010; Locatelli et al, 2009; Burns et al, 2009). However, the field of Hematopoietic stem cell (HSC) research in stroke is not barren, as a small volume of literature has recently emerged. HSC have recently been shown to mobilize to the peripheral circulation from bone marrow in response to stroke (Hennemann et al, 2008), and increasing circulating HSC levels correlate with improved neurological function following stroke, suggesting a potentially critical role for HSC in limiting stroke injury and/or facilitating stroke recovery (Yip et al, 2008; Taguchi et al, 2009). Moreover, post-ischemic intravascular administration of exogenous HSC has recently been shown to ameliorate ischemic stroke in mice (Schwarting et al, 2008). Additionally, well established therapeutics that are known to mobilize HSC have shown very exciting preliminary results in animal models and are currently undergoing clinical evaluation for other modes of central nervous system injuries (Luo et al, 2009). Increasing levels of circulating HSC have recently been demonstrated to correlate with improved neurological function following stroke, suggesting a potentially critical role for HSC in limiting stroke injury and/or facilitating stroke recovery (Yip et al, 2008; Taguchi et al, 2009).

2. What are hematopoietic stem cells?

A stem cell has the capacity for self-renewal and the ability to differenretiate into multiple cell types (potentcy) (Melton et al, 2004). A progenitor cell has similar characteristics to a stem cell, however, it has limited potential for differentiation (it has limited self renewal capacity and can only differentiate into limited types of cells (Melton et al, 2004). Hematopoietic Stem Cells (HSC) are circulating bone marrow derived mononuclear cells that promote repair in areas of injury (Baum et al, 1998). HSC travel thru peripheral blood from the fetal liver to the bone marrow and seed the bone marrow with immature and maturing cells (Melton et al, 2004); the bone marrow then remains the main site of hematopoiesis in adult life (Melton et al, 2004). During embryogenesis the three embryonic germ layers partition into 3 embryonic layers: ectoderm, mesoderm and endoderm (Hall et al, 2000). The ectoderm gives rise to skin and neural cells and tissues (Hall et al, 2000); the mesoderm gives rise to the blood cells, bone, fat, cartilage and muscle and the endoderm gives rise to the respiratory system and digestive tract (Wells et al 1999). The resulting tissues and organs from these three layers retain their original specification throughout adulthood. The neural crest is the only exception to that rule; it is of ectoderm origin and gives rise to neural, muscle and bone cell lineages. Based on the three germ layers, it would seem that stem cells generate mature cells corresponding to the tissue of that origin only. However, stem cells can transdifferentiate into cells of a completely different lineage. Some tissues in the adult, have been shown to respond very well to re-generation by HSC, for example liver (Varga et al, 2010), and others have been shown to respond poorly, for example, heart (Rumyantsev et al 1987). This may indicate the presence of stem cells within these tissues The brain used to be thought of as a non renewing organ, however, it has now been shown to have a high cell turnover (Kajsutra et al 1999; Altman et al, 1965; Lois et al, 1993).

HSC can self renew themselves at a single cell level and can differentiate to mature progeny of non-renewing and terminally differentiated cells (Seita et al, 2010). In contrast totipotent cells (can give rise to all embyonic and extraembyonic cell types), pluripotent (can give rise to all cell types of the embryo), oligopotent (can give rise to limited cell lineages), or unipotent cells (can give rise to a unique mature cell type), HSC have multipotent developmental potential (can give rise to a subset to cell lineages) (Seita et al, 2010). The HSC in the bone marrow proliferate and differentiate into erythroid, lymphoid and myeloid lineages (Figure 1) (Kondo et al, 2003). Commitment to each lineage is dictated by several growth factors such as VEGF, EGF, IGF, FGF and PDGF. HSC are recruited to the peripheral circulation from bone marrow in response to stress or injury such as stroke (Paczkowska et al, 2005; Machalinski et al, 2006; Henneman et al, 2008). Bone marrow HSC give rise to the hemangiobalst (Urbick 2004; Hristov et al, 2004; Rumpold et al, 2004), which in turn give rise to mature endothelial cells (Hristov et al, 2004).

Bone marrow derived HSC contribute to hematopoietic tissues (tissues which can stimulate the bone marrow) such as: skin (krause et al, 2001), kidney (Kale et al, 2003), central nervous tissue (Brazelton et al, 2000, Weimann et al, 2003) and have also been found to contribute to non hematopoietic tissues such as: myocardium (Orlic et al, 2001) and Skeletal muscle (Ferrari et al, 1998). This phenomenon may be due to circulating HSC lodging in non-hematopoietic tissues or due to lineage conversion of HSC. HSC can either transdifferentiate

(alter their lineage specificity by activation of alternate genes), de-differentiate and re-differentiate (HSC can de-differentiate to a more primitive state (multipotent state) and then re-differentiate along a different lineage pathway), come from a homogenous starting population of cells or be a result of cell-cell fusion. Most HSC studies are done by injecting or implanting a large number of cells, many of these cells may be contaminating impurities resuting from harvesting or enriching techniques (described below). The presence of HSC in non-hematopoietic tissues may be due to the impurities present in the starting population (Kanof et al, 2001). Another contributing phenomeon for the presence of HSC in non-hemaoptopoietic tissues may be due to cell-cell fusion (Anderson et al, 2000). Cell-cell fusion is a natural occurence in skeletal myofibres (Anderson et al, 2000) but may also be pathologic, for excample in HIV infection of T-lymphocytes (Mccune et al, 1998). Terada et al (2002) were the first group to show that stem cells may fuse with cells of the central nervous system without commiting to the parent cell lineage. These fusion cells take on the phenotype of the parent cell without complete differentiation to their specific lineage. Alvarez-Dolado et al (2003) also showed that bone marrow derived HSC contribution to non-hematopoietic tissue repair was due to cell-cell fusion rather than transdifferentiation of the HSC.

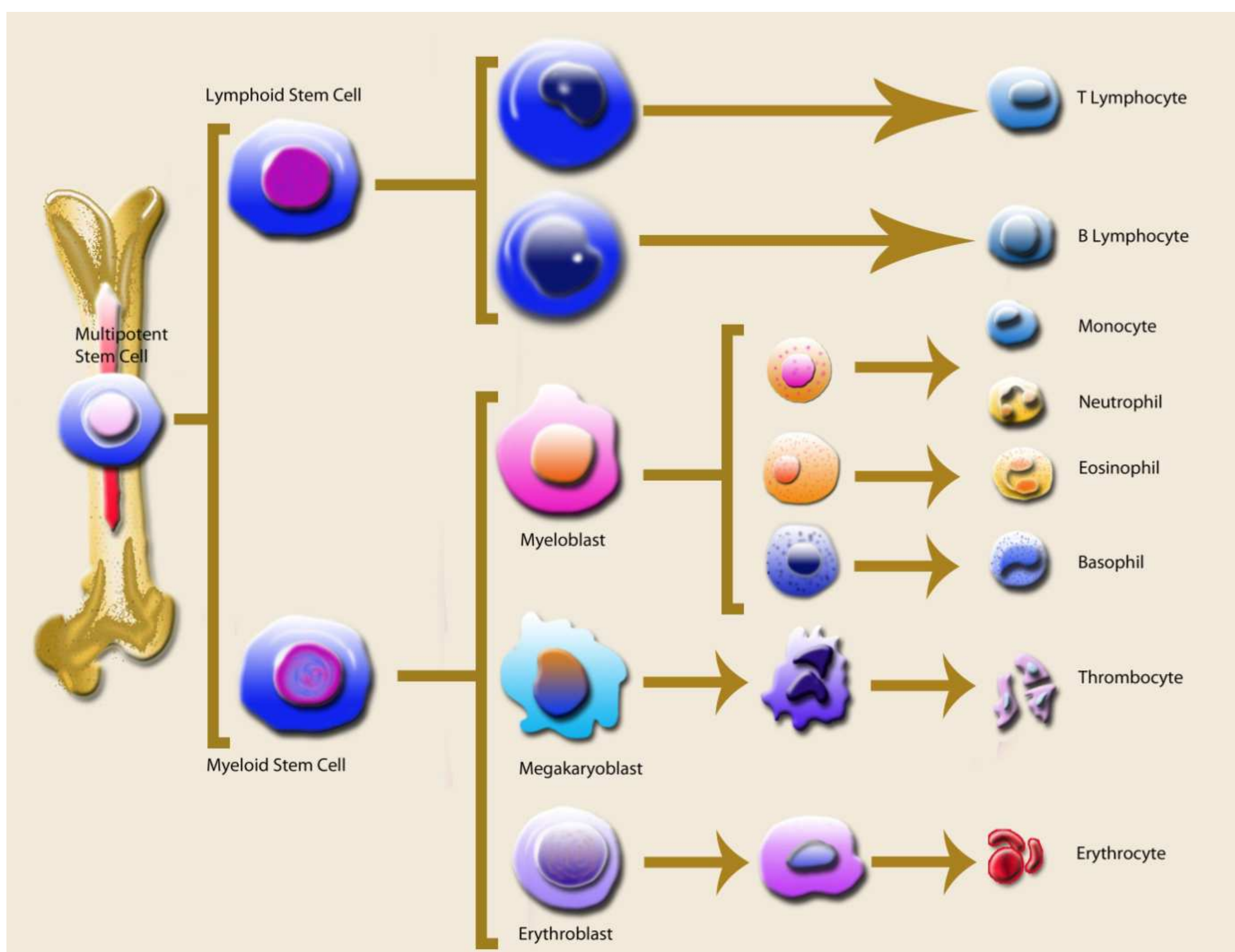

Fig. 1. Multipotent stem cells from the bone marrow can give rise to erythroid, lymphoid and myeloid lineages.

2.1 Mobilization and neovascularization

Mobilization of HSC to the blood occurs via trans-endothelial migration thru the bone marrow. Proteinases, such as elastase, cathepsin G, and matrix metalloproteinases, cleave and release the HSC from the bone marrow stroma and surrounding cells (Heissig et al, 2002). The HSC leave the bone marrow in response to several growth factors, such as SDF1-A, VEGF, EPO and G-CSF. All of these growth factors have been shown to increase levels of circulating HSC in the blood. Neovascularization is the de novo synthesis of blood vessels and it differs from Angiogenesis which refers to sprouting of capillaries from existing blood vessels (Carmeliet et al, 2005). Vasculogenesis/vascularization refers to differentiation of HSC into endothelial cells and was thought to occur only in the embryo (Shi et al, 1998). Bone marrow derived HSC have now been showed to home to a site of neovascularization, proliferate and differentiate into endothelial cells (Masuda et al, 2003). Several groups have shown that neovascularization occurs in response to ischemia in the heart (Hur et al, 2007; Cook et al, 2009; Shintani et al, 2001; Sanganalmath et al 2011). HSC injected into myocardial infarction patients showed an increase in blood flow and an improvement in heart function. In addition, HSC injected into a hind limb ischemia model also results in increased neovascularization in the ischemic limb. Cohorts with ischemic hind limbs were also injected autologous bone marrow which resulted in reduced chest pain and an augmentation of the ankle-brachial index. These studies show that HSC contribute to ischemic rescue, however, the mechanism of this rescue is unclear. If HSC are injected without an injury, there is very little incorporation of the cells and in the presence of ischemia, the rate of incorporation of the HSC is dependent on degree of ischemia (Shintani et al, 2001; Sanganalmath et al, 2011). However, even in the presence of a large ischemic injury, very few HSC have been detected at the site of injury. So then how can a few HSC contribute to blood vessel repair in the presence of a large ischemic injury such as a stroke? This may be accomplished by the paracrine release of growth factors by the few HSC that home to the site of injury. Growth factors secreted by these cells at the site include IGF-1 and FGF, which increase proliferation of HSC, MCP-1, which increases migration of the HSC towards the ischemic core, and TGFb, which promotes differentiation of the cells into mature endothelial cells.

Hematopoietic stem cells mobilize from the bone marrow to the blood in response to injury (Kucia et al, 2004). The HSC are associated with bone marrow stromal cells and exist as quiescent cells in the bone marrow. The HSC in the bone marrow must transform from this quiescent state to an active proliferative state before they can be mobilized to the peripheral blood. Proteinases, for example, elastase, cathpsin G and MMP's cleave the extracellular matrix which anchors the HSC to the bone marrow stroma (Heissig et al, 2002). MMP-9, secreted by the bone marrow stromal cells cleaves the membrane bound receptor mKitL (Heissig et al, 2002). Cleavage of the receptor converts it to the soluble KitL which can bind to the cKit receptor present on HSC (Figure 2). Binding of the sKitL to the cKit receptor activates signalling cascades enabling proliferation and mobilization of the cells to the peripheral blood (Heissig et al, 2002). While MMP-9 -/- mice were shown to have a reduced recruitment o the peripheral blood, MMP-9+/+ mice treated with SDF or VEGF showed a marked increase in mobilization of the HSC from the bone marrow to the peripheral blood (Heissig B, 2002). Presence of HSC in the peripheral blood was first shown in the 1960's and 70's (Korbling et al, 1994). Since then, peripheral blood HSC counts have been used as

biomarkers in diseases such as: diabetes (Fadini et al, 2006), Hyperhomocystenemia (Zhu et al, 2006), Aging (Heiss et al, 2005), Hypertension (Pirro et al, 2007), Systemic Scelrosis (Del Papa et al, 2006), Chronic smoking (Kondo et al, 2004) and Coronray Artery Disease (Kunz et al, 2006).

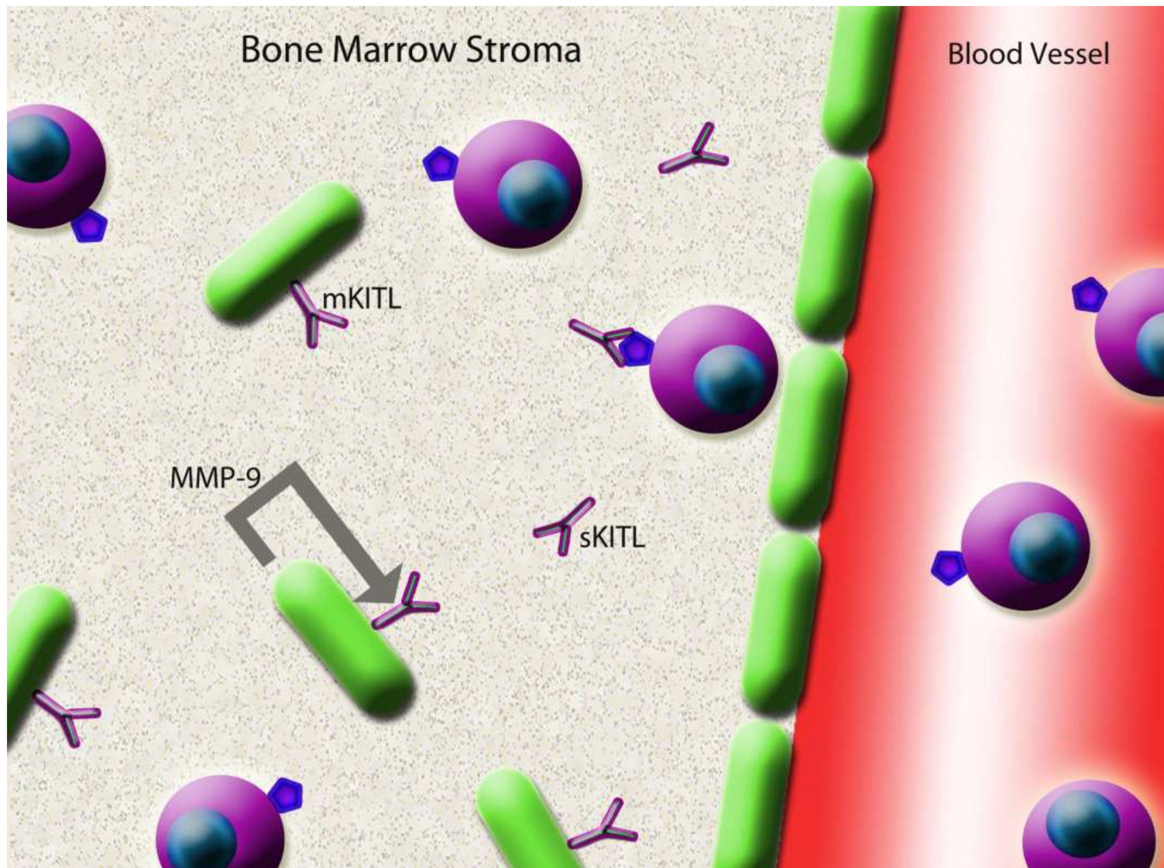

Fig. 2. Bone marrow stromal cells (green) secrete MMP-9, which cleaves the membrane bound cKIT receptor present on HSC (purple). Cleavage of the receptor converts it to the soluble KitL which can bind to the cKIT receptor present on the HSC. Binding of the KitL to the receptor enables proliferation and migration of the HSC to the peripheral blood.

2.2 Growth factors in HSC mobilization

Growth Factors which have remained in the forefront of HSC research include Vascular Endothelial Growth Factor (VEGF) (Leung et al, 1998) and Stromal Derived Growth Factor-1 A (SDF1-A) (Ma Q et al, 1998). VEGF is produced by many cells, not only HSC (Leung et al, 1989) and is formed by alternate splicing of a single gene (Leung et al 1989). Alternate splicing of the parent gene leads to the formation of VEGF (Leung et al 1989), VEGFB (Olofesson et al, 1996), VEGFC (Chilov et al, 1997), VEGFD (Latitinen et al, 1997) and VEGFE (Ogawa et al, 1998), however, VEGF is by far the most studied growth factor (Leung et al, 1998). Carmeliat et al (1996) and Ferrara (1996) both showed that homozygous knockouts of VEGF (VEGF +/-) die in utero due to impaired hematopoiesis (Ferrara et al, 1996) and anigogenesis (Carmeliat et al, 1996). VEGF can bind to 2 receptors: VEGFR1 (flt) (Ortega et al, 1997; Shalaby et al, 1995) and VEGFR2 (kdr) (Terman et al, 1992). Binding of VEGF to VEGFR1 contributes to vascular remodelling (Fang et al, 1996) and binding to VEGFR2

initiates proliferation, migration and differentiation of HSC (Ortega et al, 1997; Matthews et al, 1991) through the PI3K/AKT/NFkb pathway (Byrne M, 2005). Oxygen levels in the bone marrow are typically lower than the peripheral blood (Harrison et al, 2002) thus leading to lower oxidative stress and higher HSC survival and proliferation (Jang et al, 2007).

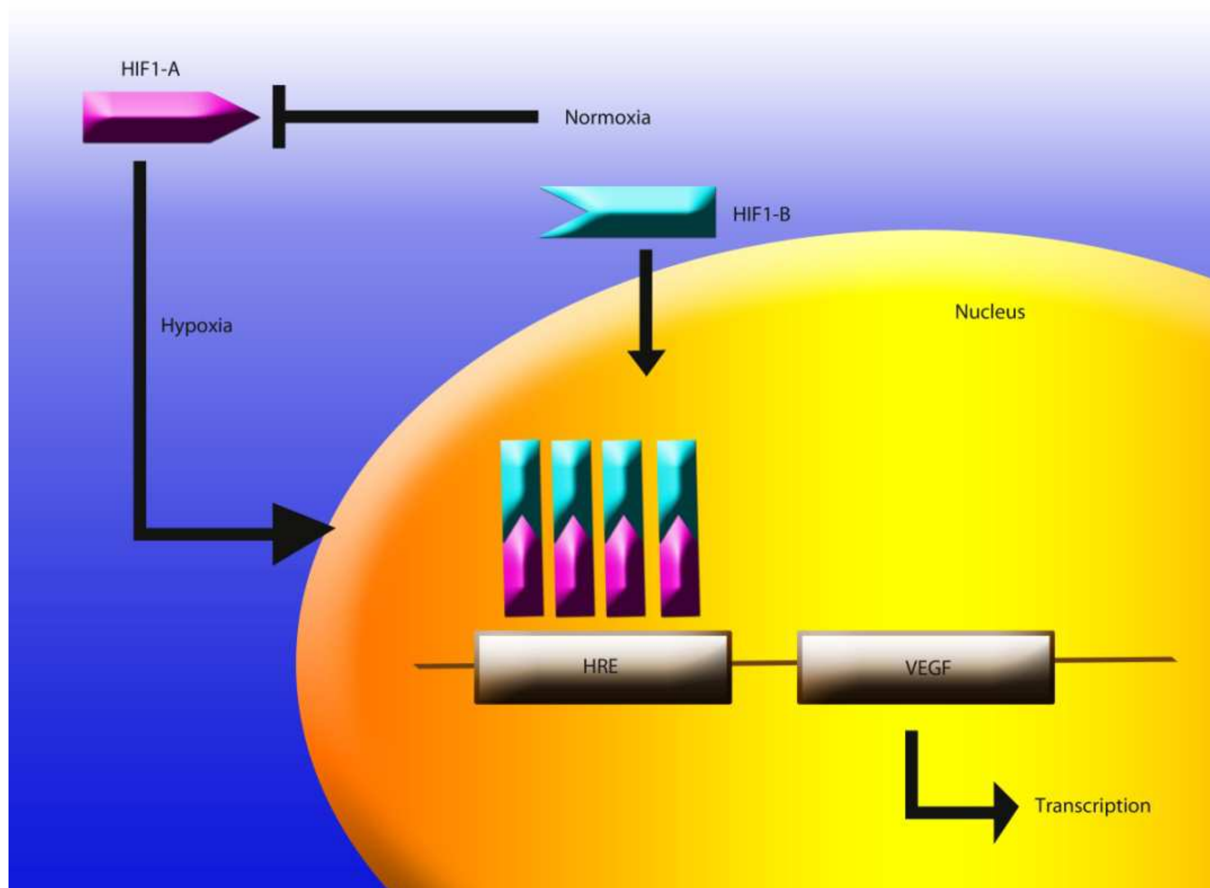

Fig. 3. Hypoxia Inducible Factor (HIF) is composed of two subunits: HIF1-A and HIF1-B. Normoxia leads to degradation of HIF1-A and hypoxia causes it to translocate to the nucleus for activation. In the nucleus, HIF1-A binds with HIF1-B to form heterodimers and bind to hypoxia Response Elements (HRE) to activate transcription of VEGF.

Hypoxic environments activate hypoxia Induced Factor-1 (HIF-1) (Wang et al, 2005). HIF1 is composed of 2 subunits: HIF-1A (the oxygen sensing domain) and HIF-1B (Wang et al, 2005). Under conditons of normoxia, HIF1-A is degraded by proteasomes (Jaakkola et al, 2001) and under hypoxia both units form heterodimers (Figure 3) and bind to Hypoxia Response Genes to activate transcription of VEGF (Wang et al, 1995). Shuweiki et al (1992) showed that hypoxia increases VEGF production which is necessary for hematopoietic activity in the bone marrow (Carmeliat et al, 1992; Gerber et al, 2002). Gerber et al (2002) also showed bone marrow engraftment failure if VEGF negative HSC were injected into lethally irradiated mice. In addition, Hooper et al (2009) showed that impaired VEGFR2 on the HSC also failed to engraft lethally irradiated mice (Hooper et al, 2009). Rehn et al (2011) recently futher confrimed that loss of VEGF expression in VEGF knockout mice increased impaired HSC which were not able to engraft secondary lethally irradiated mice (Rehn et al, 2011).

Stromal Derived Growth Factor-1 Alpha (SDF1-A) is released by cells following stress, injury or hypoxia (Muller et al, 2001). SDF1-A is localized to chromosome 10q11.1 and is highly conserved between species. SDF1-A belongs to the CXC family of chemokines and was originally described as a pre B cell growth stimulating factor. SDF1-A is a ligand for CXCR4, a G protein coupled receptor, and their interaction mediates a chemotactic response followed by cell migration. The receptor for SDF1-A is a 7 transmembrane receptor, CXCR4 (Kucia et al, 2004). Binding of the SDF1-A to its receptor, initiates the PI3K-AKT and NfkB Pathway. This pathway leads to the phosphorylation of MAPK, an intracellular calcium efflux and a subsequent adhesion of the cells to fibronectin (Kucia et al, 2004). SDF1-A and its receptor CXCR4 have been shown to regulate trafficking of HSC in response to injury (Ma et al, 1998; Lapidot O et al, 2002; Pituch-Noworolska et al, 2003). Increasing SDF1-A expression in a hind limb ischemia model has been shown to increase mobilization of HSC and increased angiogensis in the hind limb (Hiasa et al, 2004). An increase in SDF1-A levels in the blood leads to an increase in CXCR4 positive cells to the injured/hypoxic area (Kucia et al, 2004). Following injury, bone marrow levels of SDF1-A decrease (Petit et al, 2002), while those in the peripheral blood increase (Morris et al, 2003). Reduced levels of SDF1-A in the bone marrow mediates secretion of MMP-9 which facilitates mobilization of the cells from the bone marrow to the peripheral blood (Janowska-Wieczoveka et al, 2000). Once in the circulation, the HSC can differentiate into myeloid cells, lymphocytes, erythrocytes, platelets or endothelial progenitor cells (Kondo et al, 2003). Increased levels of SDF1-A help to retain recruited bone marrow HSC in close proximity to angiogenic blood vessel growth (Grunewald et al, 2006).

Eventhough SDF1-A leads to an increase in mobilization of the HSC from the bone marrow, it has also been shown to cultivate a protease rich environment in the bone marrow, which can be both beneficial (Janowska-Wieczoveka et al, 2000; Hiasa et al, 2004) and harmful (McQuibban et al, 2001). An upregulation in SDF1-A levels also leads to an increase in MMP-2 expression which cleaves SDF1-A to a toxic fragment which is incapable of binding to its receptor (CXCR4) and has been shown to be neurotoxic (McQuibban et al, 2001; Zhang et al, 2003). To overcome this unwelcomed cleavage, Segers et al (2011) designed deliverable SDF1-A with mutations which made it resistant to MMP-2 cleavge. This mutated form of SDF1-A sustained local SDF1-A levels, increased SDF1-A levels in the ischemic limb and increased vascular density (Segers et al, 2011). However, the role of SDF1-A in generation of mature vessels is unknown. Therapies using angiogenic growth factors leads to unstable vessel formation which regress following cessation of the therapy (Gounis et al, 2005). In contrast to VEGF, SDF1-A prevents tortuos blood vessel formation (due to extensive proliferation of endothelial cells) which are hyper-permeable (Segers et al, 2010). A study by Moore et al (2001) looked at the synergistic effect of SDF1-A and VEGF; IV delivery of replication incompetent adenovectors expressing the SDF1-A gene incresed plasma SDF1-A levels and increased mobilization of bone marrow derived cells which were positive for VEGFR2 (Moore et al, 2001). Delivery of replication incompetent adenovector expressing VEGF increased plasma VEGF levels and increased moblization of HSC which were positive for VEGFR1. In addition these authors looked at the possibility of combining VEGF with other growth factors such as Angiopoietin-1. The combination treatment prolonged HSC mobilization and increased proliferation of capillary beds (Moore et al, 2001).

3. Sources of hematopoietic stem cells

Very little information is available on human fetal tissue research, which is done mostly in Europe. Gallacher et al (2000) first reported the presence of HSC in aborted human fetuses and subsequent work focused on fetal tissues of vertebral animals (Dzierzak et al, 1999). Embryonic stem cells have also been shown to have high proliferative capacity (Rolletschek et al, 2004) along with the ability of differentiating into several types of blood cells (Hole et al, 1999). Embryonic tissues are a rich source of HSC; however, due to the lack of a source for obtaining these cells, very little data is available on their use in humans. Human cord blood cells (HUCB), produced by the placenta, support a developing fetus and are discarded upon delivery. HUCB are multipotent (able to generate multiple germ layers) and a rich source of HSC which have been used in research studies. The first successful cord blood transplant was done in children with Fanconi's anemia (Laughlin et al, 2001) and more recently have been used in clinical trials for autoimmune disorders, cerebral palsy and Type I diabetes(Laughlin et al, 2001). HUCB offer an ethical source of HSC (McGuckin et al, 2008), have a low risk of host versus graft disease and are readily available from bio banks (Forraz et al, 2011).

Bone marrow stem cells (BMSC) are obtained from a donor from the hip bone (Diefenderfer et al, 2011). The donor is anesthetized and bone marrow drawn up in a syringe (Lee et al, 2004). This procedure is painful and may require a hospital stay (Lee et al, 2004). BMSC contain HSC, stromal and progenitor cells (Diefenderfer et al, 2011). Peripheral blood also has circulating HSC, which can also be induced to mobilize from the bone marrow into the blood in response to GCSF administration (Elfenbein et al, 2004). This procedure is easier on the donor and can be used for autologous and allogeneic administration (Brown et al, 1997; Lickliter et al, 2000). Peripheral blood stem cells have also been used successfully in patients (Verbik et al, 1995; Brown et al, 1997). The biggest difference in obtaining HSC from different sources is the quantity of cells. While HUCB may be a viable source for HSC, very few HSC can be obtained, thereby limiting use to children and not adults. BMSC offers the best viable option, for autologous HSC administration and higher yields.

4. Isolation of hematopoietic stem cells

HSC isolation can be a multistep process requiring the use of Ficoll (Fuss et al, 2009), FACS (Schlenke et al, 1998), Magnetic microbeads (Woywodt et al, 2005), or culturing of Early and late outgrowth cells (Asahara et al, 1997). Depending on the source of sample and the species, protocols may need to be adjusted accordingly. HSC vary in size and density compared to other cells present in the bone marrow or the blood (Fuss et al, 2009). Therefore, they move through specialized density media such as Ficoll (Fuss et al, 2009), at a specific rate when a centrifugal force is applied. The red blood cells and granulucytes are more dense than the HSC and can easily be separated from them following the centrifugation (Kanof et al, 2001; Jaatinen et al, 2007). HSC are isolated from the monouclear cell layer, however, since this layer has impurities, such as lymphocytes (which have overlapping densities with the HSC) (Kanof et al, 2001; Jaatinen et al, 2007), further purification is needed to obtain a pure HSC fraction. In addition to using a density medium, several groups (Schlenke et al, 1998; Ruitenberg et al, 2006) have used a cell preparation tube (CPT) which contains a cell separater solid at the bottom of a vaccutainer tube. The CPT tube requires fewer steps to obtain the mononuclear layer and Ruitenberg et al (2006)

reported that there was no significant difference in using either the Ficoll density medium or the CPT tube.

The HSC can be further purified from the bone marrow or peripheral blood and depleted for lineage markers by using either flow cytomtery (Schlenke et al, 1998) or microbeads coated with antibodies (Woywodt et al, 2005). Flow cytometery can be used to select for the presence or absence of markers simultaneously, however, this method cannot process a large number of cells quickly (Schlenke et al, 1998). Column based methods offered a much quicker way of enriching HSC, however, an additional step had to be included to elute cells from the columns (Jaatinen et al 2007). Consequently, magentic bead technology was developed which allowed for either a negative or positive selection of selected markers (Horrocks et al, 1998; Woywodt et al, 2005). A positive selection (an antibody is used to target an antigen which is expressed on the surface of desired cells) yields a higher purity of HSC due to the use of one antibody and a negative selection (use of antibodies to target antigens not expressed on desired cells) requires several antibodies to remove unwanted cells (Woywodt et al, 2005). In contrast to using freshly isolated cells, Asahara et al (1997) first isolated HSC and cultured them in specific media on fibornectin coated dishes. The cells that grew in these cultures were called either early or late endothelial progenitor cells. Both types of cells were positive for acetylated LDL and lectin binding, however, only the Late endothelial progenitor cells were positive for VE-Cadherin, von Willebrand factor and CD31 (all markers of mature endothelial cells) (Asahara et al, 1997).

In addition to a variety of techniques which can be used to isolate the HSC, an array of cell surface markers can be used to enrich for the desired HSC fraction. Asahara et al (1997) were the first to report that mononuclear cells enriched in CD34+ cells can also mature to endothelial cells. They used the peripheral blood monouclear cells to isolate progenitors using magnetic microbeads. The progenitors wre plated onto fibronectin coated plates and confirmed to have endothelal characteristics. Subsequently, cells expressing CD133, CD34 and VEGFR2 were defined as HSC (Asahara et al, 1997; Hristov et al, 2004). CD133 is a membrane glycoprotein which is lost after differentiation and VEGFR2 is present on all endothelial cells. Hemangioblasts in the bone marrow express CD133, CD34 and VEGFR2; once these cells are activated and committed to an endothelial lineage, they gain expression of CD31 and retain CD133, CD34 and VEGFR2. Fully differentiated endothelial cells express CD34, Von Willebrand Factor, VEGF, CD31, VE- Cadherin and E-Selectin (Figure 4). In mice, lineage depletion includes removal of CD5, CD45R, Cd11b, Ly6G and TER119 (Spangrude et al, 1998; Horrocks et al, 1998) and subsequent selection of the SCA and cKIT positive cells (Uchida et al, 1992; Osawa et al, 1996; Lois et al, 2001).

5. Stroke

Chen et al (2001) were the first to report the use of Human cord blood cells (HUCB) following stroke (Chen et al, 2001). The authors used a middle cerebral artery occlusion (MCAO) model in Wistar rats. The HUCB were injected at either day 1 or 7 post stroke. Behavior (rotarod) analysis and neurological score were recorded on days 1, 7, 14, 21, 28 and 35 days post stroke. Behavior was significantly improved in animals which received the HUCB at 1 day post stroke whereas day 7 only showed an improvement in the neurological score and not the behavior testing. The lesion volume was determined by H&E staining on day 35 post stroke and no significant differences were observed in animals which received

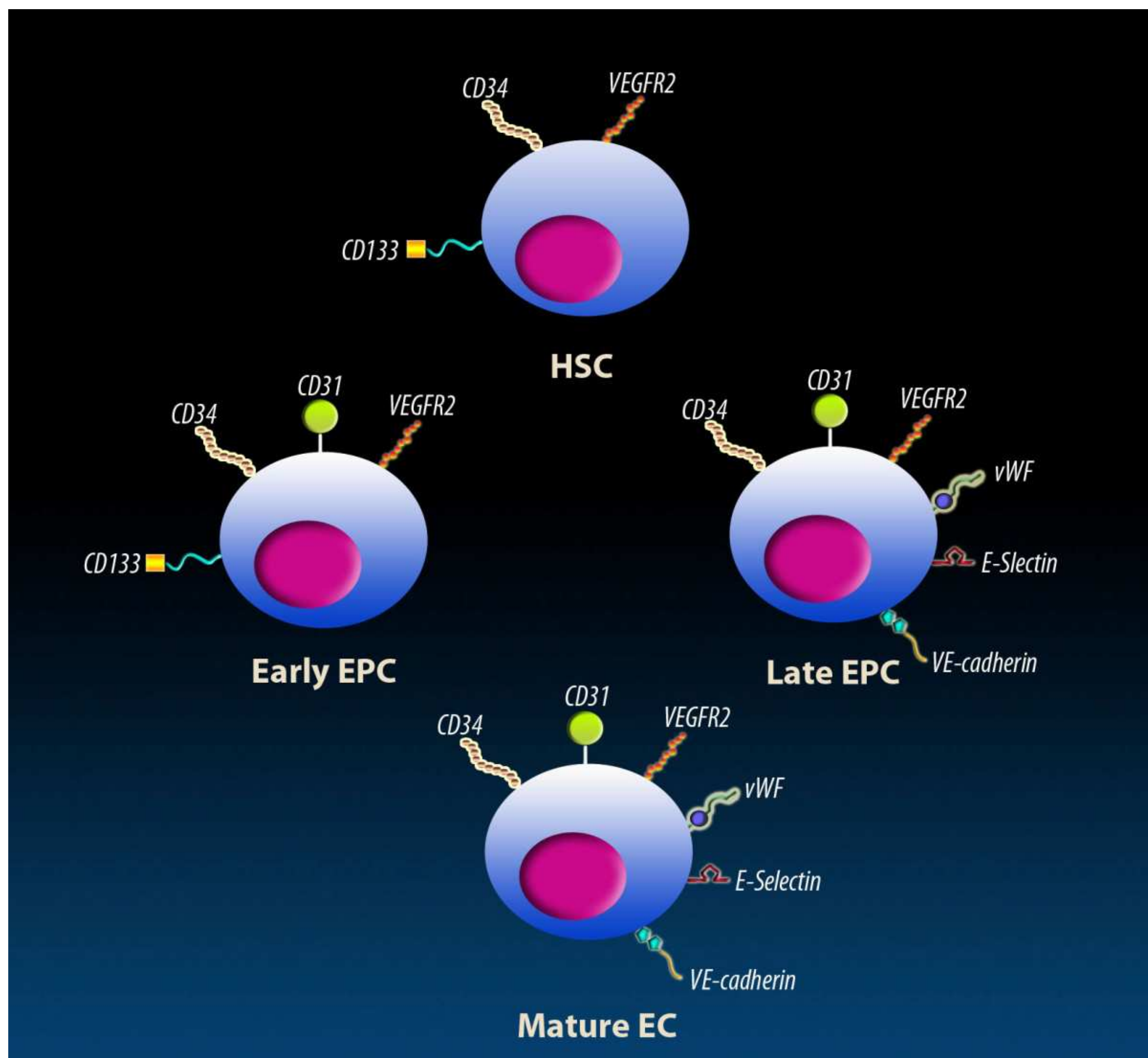

Fig. 4. Hematopoietic stem cells (HSC) express CD133, CD34 and VEGFR2. Mature endothelial cells (EC) express CD34, CD31, VEGFR2, Von Willebrand Factor, E-Selectin and VE-Cadherin. Early endothelial progenitor cells (EPC) express CD31 in addition to the markers carried by the HSC and late EPC express Von Willebrand factor, E-selectin and VE-cadherin in addition to the early EPC markers.

the HUCB and those that did not. HUCB were localized in the ischemic hemisphere by immunostaining for MAB1281 (an anti-human nuclei monoclonal antibody). Additionally co-staining was done for neurons (using micro-tubule-associated protein 2 and NeuN), astrocytes (using glial fibrillar acidic protein) and endothelial cells (using von Willebrand Factor). Animals which did not receive any HUCB showed no positive cells for MAB1281 and of the positive cells in the transplanted animals, 2% of the cells were positive for NeuN markers, 3% for neuronal markers, 6% for astrocytic markers and 8% for endothelial markers. The ability of HUCB to migrate towards an ischemic area was also tested in vitro using a chemotaxis assay. Ischemic brain extracts stimulated migration of HUCB in vitro. This study showed that IV transplantation of HUCB significantly improved behavior when

the cells were administered at day 1(Chen et al, 2001) , however, no explanations were offered for the mechanism of action for these cells other than the possibility of release of trophic growth factors from the HUCB.

A possible mechanism by which HUCB may be contributing to a reduction in behavior deficits was suggested by Regelsberger et al (2011). These authors postulated that the HUCB may be modulating delayed apoptosis in the ischemic hemisphere leading to the reduction in behavior deficit. Spontaneously hypertensive rats were used in this study for either a histological evaluation (at 25, 48, 72 and 96 hours) or a molecular biology study (at 6, 24, 36 and 48 hours) post stroke. The HUCB were injected intravenously and histological analysis was done for infarct volumes by assessing the loss of MAP-2 positive neutrophil staining and cleaved caspase-3 positive cells were also counted. The results of this study showed that the number of cleaved caspase-3 cells in the infarct area increased from days 1 to 4 and were not affected by the IV administration of the HUCB. Molecular analysis by real time PCR also showed no differences in the levels of caspase-3 mRNA or survivin (an anti-apoptotic protein) in groups that received or did not receive the HUCB. Unlike other studies using HUCB (Vendrame et al, 2006), the infarct volumes in this study were not affected by the administration of HUCB. The study could not provide any evidence that HUCB contributed to the reduced behavior deficits due to anti-apoptotic effects. Another study (Leonardo CC et al, 2010) suggested that HUCB may lead to a reduced recruitment of pro-inflammatory cells to the ischemic hemisphere and hence faster recovery. Sprague Dawley rats were used to induce an MCAO and HUCB were delivered intravenously. Brain sections were stained for CD11b (inflammatory cell marker) and MMP-9 (causes basement membrane degradation leading to blood brain barrier leakage). Animals which did not get any HUCB had a significantly higher number of CD11b staining cells in the ischemic hemisphere and these cells were also positive for MMP-9 (Leonardo et al, 2010).

In contrast to the previous studies, Makinen et al (2006) have reported that IV administration of HUCB does not contribute to the functional recovery of Wistar rats. These authors suggested that studies evaluating functional behavior outcome following stroke do not utilize a comprehensive battery of tests to evaluate sensorimotor, cognitive and histological tests for the animals. This study used the beam walking test (assesses forelimb and hind limb function), cylinder paw placement (use of impaired contralateral (to lesion) paw to assess forelimb asymmetry, water maze (for length, latency and swimming speed). The HUCB were administered at 24 hours post stroke to avoid an inflammatory response which can complicate recovery if the HUCB are administered immediately after stroke. Previously mentioned studies (Vendrame et al, 2006) had used 10^6 HUCB for IV administration and this study used $1\text{-}5x10^7$, to ensure that enough HUCB were injected to be detected. None of the behavioral tests showed any significant rescue with IV administration of HUCB. The infarct volumes did not show any difference in either group of animals, however, histological analysis showed the presence of a few HUCB in the ipsilateral hemisphere in close proximity to blood vessels in animals which received the HUCB. In addition to the histological study, Makinen et al injected ^{111}In-Oxine labeled HUCB for Bio-imaging of the live animals. The images revealed that the HUCB were trapped in the liver, lungs, spleen and kidneys. No signal was picked up in the brain. The authors argued that since other studies don't use a comprehensive evaluation method for the animals, they report the presence of HUCB in the brain and a functional recovery following HUCB

administration. However, a comprehensive battery of tests performed in this study revealed that HUCB do not contribute to behavior recovery following stroke (Maiken et al, 2006).

Nystedt et al (2006) took a different approach to the previous studies: instead of injecting whole HUCB, they used an enriched CD34+ population from the HUCB. CD34+ cells have been shown to promote angiogenesis (Shyu et al, 2006) and neurogenesis (Taguchi et al, 2004); therefore, the authors reasoned that injection of a pure population of targeted HSC would provide better functional rescue following stroke. Wistar rats were subjected to transient and permanent MCAO, CD34+ cells were injected IV 24 hours post-surgery. The animals were administered CD34+ cells obtained from 2 unrelated HUCB units for better engraftment; one unit acts as a 'fertilizer' and the other unit contributes to hematopoiesis. The animals were pre-trained on the beam walking and cylinder test and evaluated at days 4, 12 and 20; water maze was evaluated at days 22, 23, and 25 post stroke. There was no significant difference in infarct volumes in either model of MCAO and human nuclei specific MAB1281+ cells were not detected in ipsilateral or the contralateral hemisphere at day 25 post stroke. However, both models did show an upward trend in sensorimotor and cognitive performance in animals which received CD34+ cells in both models. Infarct volume did not change in either model following administration of CD34+ cells and these cells were not detected in the ipsilateral hemisphere, yet an improvement in functional recovery was observed. The authors reasoned that recovery may have been due to factors secreted by the CD34+ cells which contributed to the recovery or that the CD34+ cells were indeed present in the brain, but were below the detection limits of the antibodies used for detection. Taguchi et al (2004) also used an enriched CD34+ fraction to show that these cells contribute to new vessel formation following stroke. This study used SCID mice and Brdu labeling to visualize endothelial cell proliferation in the vasculature surrounding the penumbral region. The Brdu label was co-localized with CD31 (expressed by angiogenic endothelial cells) at day 1 and 3 post stroke. The highest density of newly formed vasculature was observed in the ischemic hemisphere of CD34+ transplanted animals. This effect was blocked with the administration of Endostatin (suppresses endothelial proliferation) and erythropoietin (a pro-angiogenic agent) increased the neovascularization. Functional recovery was also seen (at day 90 post transplant) in animals which received the CD34+ cells (Nystedt et al, 2006). These studies indicate that an enriched CD34+ fraction from HUCB may be better suited for regeneration of blood vessels in the brain rather than the whole HUCB fraction.

Use of autologous BMSC stem cells (BMSC) avoids ethical, infectious and immunological concerns. BMSC are abundant and therefore negate a need for cell expansion by culturing prior to administration. Iloshi et al (2004) used BMSC from rats, transfected with a LacZ reporter gene and administered IV following MCAO. The cells were administered at 3 or 72 hours post stroke. Animals which received BMSC had a significant reduction in behavior deficit (Morris water maze and treadmill stress test) at 2 weeks following stroke. Animals which received BMSC within 3 hours of stroke had an almost non-existent lesion and LacZ+ cells were detected in and around the ischemic zone. The authors suggested that the BMSC secrete angiogenic growth factors (e.g. VEGF and bFGF) (Chen et al, 2002; Kurozumi et al, 2005) which cause the functional recovery. Another mechanism suggested by the authors is that the BMSC integrate into the ischemic area and differentiate into mature cells rather than fuse with resident cells. Another study by Keimpema et al (2009) also evaluated the use of

BMSC following MCAO. This study, in contrast to the previous one, injected the BMSC intra-arterially (IA) at 3, 6, 12 and 24 hours post stroke. BMSC were observed in the blood vessels (when administered 3 hours post stroke), at the lesion (when administered at 6 hours post stroke) and phagocytic activity when BMSC were administered 24 hours post stroke. All time points evaluated showed a reduction in infarct volume. The authors defended their choice of arterial and administration of the BMSC at later time points by stating that signals from the lesion are relatively lower immediately following the stroke, thus IV injected BMSC get trapped in the spleen and are not observed in the ischemic regions of the brain. The authors also suggested that BMSC may release angiogenic growth factors which may also contribute to the reduction of infarct size. BMSC have also been engineered to overexpress potentially beneficial genes to the brain. For example, erythropoietin (EPO) is a known antioxidant, anti-apoptotic and anti-inflammatory (Chong et al, 2003). However, EPO cannot cross the blood barrier if injected IV and therefore its beneficial effect on the brain cannot be evaluated (Cho et al, 2010). Cho et al transduced BMSC to produce increased levels of EPO, and sterotaxically implanted $6x10^5$ cells. The transduced cells were shown to secrete higher levels of BDNF, SDF1-A and TGF1-b. Animals which received the transduced BMSC had improved neurological function, lower infarct volumes and higher levels of phosphorylated AKT (a downstream effector of EPO) (Cho et al, 2010).

6. Are HSC a viable avenue for stroke therapy?

It appears likely that a viable stroke therapy may be generated from HSC research, but much investigation is yet to be done. There are discrepancies in evaluation modalities currently being employed for studying the contribution of HSC to recovery following stroke. There is a lack of an overall consensus for which animal strain, stroke model, anesthetic, duration of occlusion, HSC markers, number of HSC to be injected, source of HSC, route of delivery, time of delivery, time for evaluation and kind of end point testing (for behavior), should be used for research. Several studies have either used Wistar rats (Nystedt et al, 2006) or Sprague Dawley (Leonardo et al, 2010) for the research. However, Bardutzky et al (2005) reported that the ischemic lesion evolution was substantially different between the Wister and Spargue Dawley strains. The authors used cortical blood flow and apparent diffusion co-efficient maps and 2,3,5-Triphenyltetrazolium chloride staining to arrive at that conclusion (Bardutzky et al, 2005). Other studies have also confirmed differences in the middle cerebral artery occlusion model in different strains (Fox et al, 1993; Sauter et al, 1995; Oliff et al, 1996). The rat model for middle cerebral artey was first described by Robinson et al in 1975 and has since been modified by several groups (Tamura et al, 1981; Bederson et al, 1986). As this model was refined over the years, researchers have chosen the best adaptation of this model for their research. For example, the anesthetic used for the rats varies from chloral hydrate (Bederson et al, 1986), ketamine hydrochloride (Longa et al, 1989), Halothane (Chen et al, 1992), and isoflurane (Belayev et al, 2009). While these anesthetic agents are used for the surgery, none of the publications report on the effect of the choice of the anesthetic on the injected HSC or the level of neuroprotection imparted by the choice of anesthetic. Culley et al (2011) showed that isoflurane does not kill stem cells, but does affect their proliferative capacity, which is an important feature for stem cell survival and engraftment into the area of injury. The mouse model of middle cerebral artery occlusion has been described by Connolly et al (1996) and has also been been modified and used by several researchers (Olsen et al, 1986; Clark et al, 1997; Belayev et al, 1999). Various

anesthetic gases have also been used for the mouse model of middle cerebral artery occlusion (Yanamoto et al, 2003) with no correlation on the effect of the choice of anesthetic to the engraftment potential of the HSC. The duration of occluison also varies from 45 minutes to 120 minutes (Campagne et al, 1999). Campagne et al (1999) showed that the time of occlusion affects the infarct size and hence would affect how quickly tissue can recover following occlusion. Li et al (2005) and Popp et al (2009) also showed that extent of infarct volume increases with occlusion time and leads to varying areas of confinement for neuronal and glial markers.

Studies have also used a variety of sources for the HSC that are injected following stroke: HUCB (Vendrame M et al, 2006) , bone marrow (Iloshi et al, 2004) and Peripheral blood (Lickliter et al, 2000). In addition, there appears to be no consensus as to the number of cells injected for evalaution; Vendrame et al (2006) used $1x10^6$ and Makinen et al (2007) used $1\text{-}5x10^7$. Not only are the cell sources and numbers variable, but methods for enrichment before injection also vary (Iloshi et al, 2004; Taguchi et al, 2004). It seems logical that HSC fractions enriched for markers that are more committed towards an endothelial lineage (for example, CD34+ cells in humans) (Asahara et al, 1997) would offer a faster recovery than those that are whole enriched fractions. Since HSC have an intrinsic potential for proliferation in response to growth factor, such as VEGF (Ortega et al, 1997), it would seem that unnecessarily increasing the number of injected cells may not be needed. If injected cells can contribute to recovery directly and by paracrine secretion of tropic growth factors, then an exorbitant number of HSC may not be needed. Actual HSC numbers needed for adminitration in stroke patients for receovery is critical for those considering an autologus transplantation from their own bone marrow. The route of injection of HSC also varied from IV (Nystedt et al, 2006; Leonardo et al, 2010; Regelsberger et al, 2011), to IA (Keimpema et al, 2009) and direct implantation in the brain (Cho et al, 2010). Maikinen et al (2006) showed that IV injection of HSC lead to them being trapped in the spleen and kidneys and none of the cells were detected in the brain following the stroke. In contrast, Keimpura et al (2004) injected the HSC intra-arterially and showed that if the HSC were injected within 3 hours of the stroke, they would be found in the blood vessels in the brain. People have long focussed on an IV route of delivery of HSC for easier translation to human clincal trials. However, it is logical for the spleen to trap and remove cells which are not part of the normal circulation, hence reducing the number of cells which are available for contribution to recovery in the brain. Intra-arterial administration of the HSC is a viable alternate for the delivery of HSC since it places the cells in close proximity to the injury and avoids clearence of masses of cells by the spleen. Intra-arterial delivery may be challenging in smaller rodents, such as mice, however, it has been successfully used (Torrente et al, 2001).

End point testing after HSC administration has relied on immunohistochemical staining (Kramer et al, 2000) or behavioral outcome (Chen et al, 2001). Infarct volumes are evaluated using a variety of methods: 2,3,5-Tetrazolium Chloride staining (Kramer et al, 2000), Nissl staining (Popp et al, 2009), Cresyl violet staining (Tureyen et al, 2004), or MRI imaging (Lansberg et al, 2001). Immunohsitochemistry of tissues lends itself to artifacts from incomplete fixation, excessive dehydration and overstaining (Werner et al, 2000; Fowler et al, 2008), so the variability in these techniques is well known. However, some studies have used these stains to report no changes in infarct volumes following administration of HSC (Regelsberger et al 2011; Nystedt et al, 2006). This may be due to artifacts inherent in the nature of the staining methods rather than ineffective HSC contribution to reducing infarct

volume following stroke. MRI imaging offers the best option for in vivo evaluation of infarct volumes following stroke (Moseley et al, 2001). However, access to MRI imaging for reasearchers may not be as feasible as doing an imunohistochemical staining for infarct volume. Behavior testing has become a hallmark end point testing for stroke in rodents (Chen et al, 2001), however, Makinen et al (2006) were not incorrect in suggesting that the majority of studies do not utilize the full battery of tests to arrive at a conclusion. Even within the battery of tests recommended by Makinen et al (2006), there is plently of room for variability within studies. Behavior tests are not sensitive enough to forgive minor differences in testing protocols which may be due to availability of resources for the research. For example, studies have evaluated end point behavior testing in rodents as early as 24 hours post stroke (Nystedt et al, 2006) and as late as 25 days post stroke (Taguchi et al, 2004). Earlier time points for behavior may not have allowed sufficient times for the HSC to have an affect and the later time points may be too far away from the time of injury and thus allow for spontaneous recovery.

Studies have either reported the presence or absence of of HSC in the ipsilateral hemisphere following stroke (Makinen et al, 2006). However, few studies have addressed issues of cell-cell fusion (Terada et al, 2004) whereby the resident vascular endothelial cells and the injected HSC fuse together and the HSC do not take on the mature phenotype of the resident cell. While it may difficult to differentitate a maturing HSC from a resident endothelial cell, since a maturing cell would carry the same markers as the resident cell, it is important for future stroke HSC therapy to be able to differentiate whether certain treatments lead to integrating HSC or mere cell to cell fusion. HSC also have the ability to transdifferentiate, dedifferentiate and re-differentiate depending on the cues provided by the local environment. It is entirely possible that some HSC integrate into the vasculature and others transdifferentiate into neurons. However, the majority of studies focus either on vascular beds (Taguchi et al, 2009) or neuronal regeneration (Garzon-muvdi et al, 2009) rather than both.

In conclusion, it seems likely that HSC investigation may contribute critical knowledge to our constantly evolving understanding of stroke pathophysiology. However, this enthusiasm must be tempered with realistic expectations that this will only occur with detailed, diligent labortory investigation. It is our hope that with such critical investigation our field will achieve it's goal of providing a viable new therapy for stroke.

7. References

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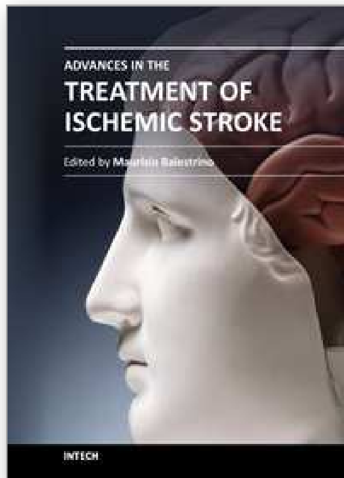

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In recent years research on ischemic stroke has developed powerful therapeutic tools. The novel frontiers of stem cells therapy and of hypothermia have been explored, and novel brain repair mechanisms have been discovered. Limits to intravenous thrombolysis have been advanced and powerful endovascular tools have been put at the clinicians' disposal. Surgical decompression in malignant stroke has significantly improved the prognosis of this often fatal condition. This book includes contributions from scientists active in this innovative research. Stroke physicians, students, nurses and technicians will hopefully use it as a tool of continuing medical education to update their knowledge in this rapidly changing field.

How to reference
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