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Cytoprotection and Preconditioning for Stem Cell Therapy

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1. Introduction

Coronary heart disease is the leading cause of morbidity and mortality worldwide. To date, management of myocardial infarction (MI) has been limited to timely revascularization and drug therapy aimed to restore coronary blood flow and to reduce myocardial workload. When disease progresses to life-threatening end-stage heart failure, heart transplantation is the only effective therapeutic option available. However, its usage is very much restricted by the severe shortage of heart donors and the complications associated with enduring immune suppressive drug treatments (Miniati & Robbins, 2002). Therefore, innovative treatment strategies are clearly needed to improve patient outcomes. Recent advances in stem cell medicine have shed new light on potential MI therapies by exploiting the pluripotency of stem cells for cardiac repair and regeneration. Although immense progress has been made on the choice of cells and optimizing transplantation conditions, these remain critical issues when translating into the clinical setting of MI. In particular, poor survival of transplanted cells in the hostile microenvironment of the ischaemic myocardium and hence lack of significant engraftment in the heart has been a major impediment for achieving an effective stem cell therapy for MI (Pagani et al., 2003). Various cytoprotection strategies have been developed over the past decade to circumvent this limitation and the non-genetic approach of preconditioning has emerged as one of the most promising clinically adaptable strategies to promote stem cell survival and function under various ischaemic conditions. Although genetic enhancement of stem cells has been very successful in pre-clinical studies, the technical complexity and safety concerns (oncogenicity and mutagenesis) associated with this alternative approach have precluded its application in clinical translation (Bonaros et al., 2008; Penn & Mangi, 2008). This review will focus on current pre-clinical development of non-genetic preconditioning approaches to improve the therapeutic potential of stem and progenitor cells for repair of the heart after MI.

2. History of ischaemic and pharmacological preconditioning

The protective phenomenon of preconditioning was first described by Murry *et al.* in 1986 whereby exposure to brief cycle(s) of sub-lethal ischaemia with intermittent reperfusion, which in itself does not induce injury, render the heart more resistant to subsequent lethal ischaemic insults; this phenomenon was termed ischaemic preconditioning (IPC) (Murry et

al., 1986). Subsequent studies by various laboratories have quickly established IPC as the most powerful and effective means of endogenous protection against ischaemic injury. Although this protective intervention can be easily reproduced in various pre-clinical studies, successful translation into clinical practice has been limited by the safety consideration of needing to manipulate the already injured heart. To circumvent this limitation, effort has been concentrated on clarifying the underlying molecular mechanisms governing the cardioprotective effect of IPC which have lead to the discovery of various pharmacological agents that can directly activate the protective signalling pathways to achieve myocardial protection without ischaemia, an intervention called pharmacological preconditioning. Despite extensive research, the mechanism(s) underlying the protective effect of preconditioning remain to be fully elucidated. It is believed to involve multiple intricate endogenous signalling pathways (Yellon & Downey, 2003; Huffmyer & Raphael, 2009) including agonists of G-protein coupled receptors (adenosine, bradykinin, opioids, etc), growth factors (IGF, TGFβ, VEGF, etc), phosphodiesterase inhibitors, mitochondrial K_{ATP} channel openers, cytokines (TNFα, IL-1β, IL-6, etc), nitric oxide (NO), and others. Some of these have been promoted into the clinical arena, for example adenosine in AMISTAD I (Mahaffey et al., 1999) and II (Ross et al., 2005) trials. In general, non-genetic preconditioning strategies employed by current pre-clinical studies to improve survival and function of stem and progenitor cells can be categorized into ischaemic/hypoxic and pharmacological preconditioning.

3. Ex vivo ischaemic and hypoxic preconditioning of stem cells

The ischaemic conditions used to simulate IPC *in vitro* are quite diverse. The majority of studies have experimented with hypoxia or anoxia alone, termed hypoxic preconditioning (HPC), while others include nutrient deprivation. In some studies, hydrogen peroxide (H_2O_2) was used to simulate the ischaemic condition of oxidative stress (Li et al., 2009; Sharma et al., 2008). Furthermore, different HPC protocols, from the classical multiple cycles of brief hypoxia with intermittent reoxygenation to a single long-term exposure to hypoxia, have been employed to demonstrate the cytoprotective effect of HPC on stem and progenitor cells *in vitro*. It is also interesting to note that subjecting stem and progenitor cells to heat shock, as a form of sublethal cell stress, also capable of promoting their survival and *in vivo* engraftment (Laflamme et al., 2005; Maurel et al., 2005; Suzuki et al., 2000). Despite these differences and the lack of an optimal protocol definition, the beneficial effects of HPC on stem and progenitor cell function were unequivocally demonstrated in all these studies and involved multiple signalling molecules (Table 1) (Figure 1).

3.1 Survival

The poor survival and retention of transplanted stem and progenitor cells has driven the investigation towards effective cytoprotective strategies which aim to enhance their survival in the ischaemic environment. The extent of retention of the delivered cells was documented to be rather low with recent studies suggesting that more than 90% are lost partly because of necrosis and apoptosis in the ischaemic myocardium following their delivery by intramyocardial, retrograde transvenous, intracoronary or systemic routes (Terrovitis et al., 2010; Aicher et al., 2003; Goussetis et al., 2006). One means of overcoming this limitation would be to increase the survival of transplanted cells, thus avoiding the impractical and costly alternative of delivering large excesses of stem cells into the injured myocardium.

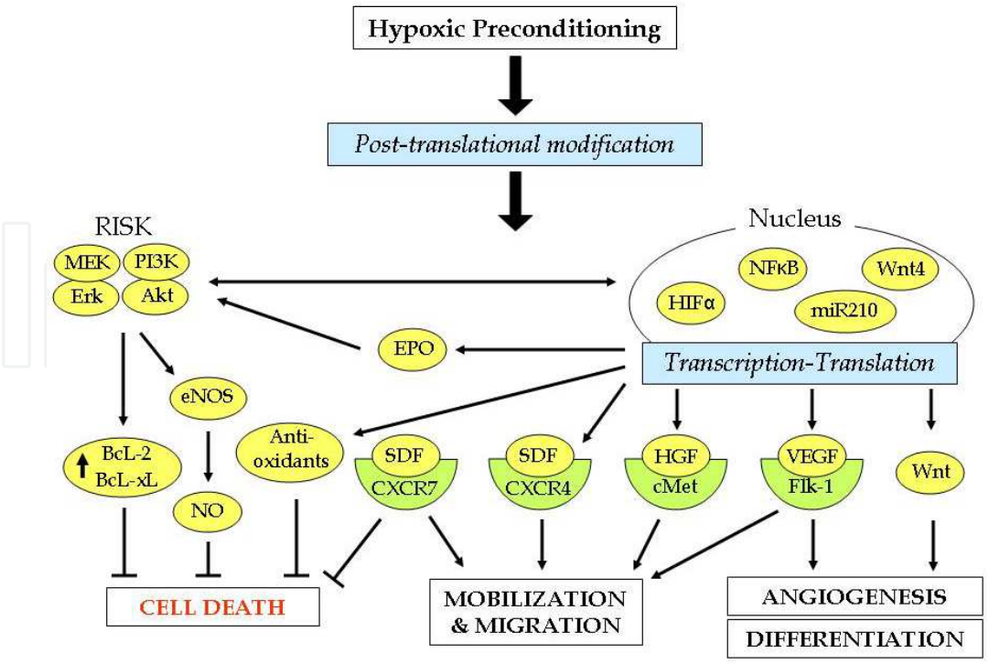

Fig. 1. Mechanisms underlying the cytoprotective effect of hypoxic preconditioning in stem and progenitor cells.

HPC by sublethal hypoxia has been shown to enhance the survival of stem and progenitor cells isolated from various species including humans *in vitro* (Table 1). Similarly, the cytoprotective effect of HPC was demonstrated in a number of *in vivo* studies using clinically relevant ischaemic models (Table 2). In these studies, *ex vivo* HPC rendered stem and progenitor cells more resilient to cell death when transplanted into the infarcted myocardium (He et al., 2009; Hu et al., 2008; Tang et al., 2009; Uemura et al., 2006), ischaemic limb (Akita et al., 2003; Kubo et al., 2008; Li et al., 2002; Rosova et al., 2008) or ischaemic brain (Theus et al., 2008), and this finding was significantly correlated with improved functional recovery of the ischaemic tissues. For instance, transplantation of hypoxic preconditioned mesenchymal stem cells (MSCs) into the ischaemic myocardium showed enhanced therapeutic benefits in terms of infarct size reduction, increased angiogenesis, improved ventricular function and less adverse cardiac remodelling (Hu et al., 2008). These beneficial effects of hypoxic preconditioned stem cells are attributed to enhanced pleiotropic paracrine activities instead of transdiffentiation and cell fusion, which occurred at insignificantly low frequency. Convincing evidence in support of the paracrine paradigm were provided by *in vitro* studies with conditioned media, where media from hypoxia-conditioned MSCs was shown to be cytoprotective in cultured human aortic endothelial cells (Hung et al., 2007a) and primary rat cardiomyocytes (Gnecchi et al., 2006) subjected to hypoxic injury.

Mechanistic evaluations of the cytoprotective effect of HPC in stem and progenitor cells have implicated the up-regulation of a diverse array of soluble survival proteins such as growth factor VEGF (Akita et al., 2003; Potier et al., 2007), anti-apoptotic proteins Bcl-2 and Bcl-xL (Francis & Wei, 2010; Hu et al., 2008; Theus et al., 2008; Wang et al., 2008a), antioxidants heme oxygenase-1, hexokinase-2, catalase and superoxide dismutase (Kubo et

Stem cells	PC stimulus	End points	Mechanisms	References
Hypoxia				
MSCs (mouse)	<0.1% O_2, 4 h	↑ survival of co-cultured adult CMs	Akt; VEGF; SDF-1; eNOS	Uemura et al., 2006
MSCs (mouse)	0.5% O_2, 24 h	↑ survival	HIF-1α; Ang-1; VEGF/Flk1; Bcl-2; Bcl-xL; p105; EPO; NFκB	Hu et al., 2008
MSCs (mouse)	0.5% O_2, 24 h	↑ survival	HIF-1α; EPO; Bcl-2; Bcl-xL	Theus et al., 2008
MSCs (mouse)	1% O_2, 36 h	Conditioned media ↑ cell migration	Wnt4	Leroux et al., 2010
MSCs (mouse)	3% O_2, 3-24 h	↑ survival; ↑ cell migration; ↑ cell adhesion	Akt; HIF-1α; CXCR4; CXCR7	Liu et al., 2010
MSCs (rat)	<0.1% O_2 & serum-free, 3 h	↑ survival of co-cultured neonatal CMs	-	He et al., 2009
MSCs (rat)	0.5% O_2, 12 h	Conditioned media ↑ adult CMs survival	VEGF; HGF; bFGF; Thymosin β4	Gnecchi et al., 2006
MSCs (rat)	0.5% O_2, 24 h	↑ survival	Akt; VEGF; HIF-1α	Chacko et al., 2010
MSCs (rat)	1% O_2, 24 h	↑ survival	Catalase, Mn-SOD, p38MAPK, Bcl-2	Peterson et al., 2011
MSCs (rat)	2% O_2, 4-48 h	↑ angiogenic factors secretion	VEGF/Flk1; VE-cadherin	Li et al., 2002
MSCs (rat)	8% O_2, 10-30 min	↑ survival	Akt; ERK1/2; Bcl-2; Bax; VEGF	Wang et al., 2008a
MSCs (rat)	<0.1% O_2, 10/30 min x 1-3	↑ survival	Akt; ERK1/2; Bcl-xL; HIF-1α; miR-210	Kim et al., 2009
MSCs (human)	1% O_2, 22 h	↑ cell migration	HIF-1α; CX3CR1; CXCR4	Hung et al., 2007b
MSCs (human)	1% O_2, 2 d	↑ angiogenic factors secretion ↓ osteogenic diff.	VEGF	Potier et al., 2007
MSCs (human)	1% O_2 & serum-free, 2 d	Conditioned media ↑ endothelial cell survival & angiogenesis	Akt; IL-6; eNOS	Hung et al., 2007a
MSCs (human)	2% O_2, 3d	Restore hypoxia-induced ↓ of osteogenic diff.	-	Volkmer et al., 2010
MSCs (human)	1-3% O_2, 16 h	↑ cell migration	Akt; HGF/cMet	Rosova et al., 2008
PB-MNCs (mouse)	2% O_2, 24 h	↑ survival	HO-1, autocrine motility factor, hexokinase-2	Kubo et al., 2008

PB-MNCs (mouse)	2% O_2, 24 h	↑ cell migration	VEGF; NOS	Ii et al., 2005
PB-MNCs (human)	pO_2 of 35mmHg, 7 d	↑ endothelial diff.; ↑ cell migration	VEGF; KDR tyrosine kinase	Akita et al., 2003
ESNPCs (mouse)	1% O_2, 4-12 h + 24 h Reoxy.	↑ survival; ↑ neuronal diff.	HIF-1α; EPO; Bcl-2	Theus et al., 2008
ESNPCs (human)	0.1% O_2, 12 h + 0-5 d Reoxy.	↑ survival; ↑ neuronal diff.	HIF-1α; HIF-2α; EPO; VEGF; Bcl-2; Bax; Akt	Francis & Wei, 2010
ASCs (human)	1% O_2, 24 h + 24 h Reoxy.	↑ survival of co-cultured NSCs	-	Oh et al., 2010
CLK (mouse)	0.1% O_2, 4-24 h	↑ cell migration	HIF-1α; SDF-1/CXCR4	Tang et al., 2009
Bone marrow CD133+ (human)	1.5% O_2, 24 h + 2 d Reoxy.	↑ endothelial diff.; ↑ angiogenic-related genes	-	Ong et al., 2010
NSCs (mouse)	0.5% O_2, 3 h	↑ functional engraftment	Connexin-43	Jaderstad et al., 2010
Hydrogen peroxide				
MSCs (rat)	20 μM H_2O_2, 24 h	↑ survival ↑ cell migration	SDF-1/CXCR4; ERK1/2	Li et al., 2009
NPCs (mouse)	0.05-0.5 μM H_2O_2, 24 h	↑ survival	-	Sharma et al., 2008
Heat shock				
Skeletal Mb (rat)	42^0C, 1 h	↑ survival	HSP72	Suzuki et al., 2000
Skeletal Mb (rat)	42^0C, 70 min	↑ survival	HSP70	Maurel et al., 2005
ESCM (human)	43^0C, 30 min	↑ survival	HSP60, HSP70, HSP90	Laflamme et al., 2005

Table 1. Effect of ischaemic or hypoxic preconditioning on stem and progenitor cells *in vitro*. CLK (cardiosphere-derived Lin-c-kit+ progenitor cells), CMs (cardiomyocytes), diff. (differentiation), ESCM (embryonic stem cell-derived cardiomyocytes), ESNPCs (embryonic stem cell-derived neural progenitor cells), HO-1 (heme oxygenase-1), HSP (Heat shock protein), KDR (kinase insert domain receptor), Mb (myoblasts), NPCs (neural progenitor cells), NSCs (neural stem cells), PB-MNCs (peripheral blood mononuclear cells), Reoxy (reoxygenation), Wnt4 (wingless-related MMTV integration site 4).

al., 2008; Peterson et al., 2011) , erythropoietin (EPO) (Hu et al., 2008; Theus et al., 2008) and NO (Uemura et al., 2006; Ii et al., 2005) as the contributing factors. Other potential cytokines and growth factors that have been suggested are basic fibroblast growth factor (bFGF) (Gnecchi et al., 2006), hepatocyte growth factor (HGF) (Gnecchi et al., 2006), IL-1β (Kubo et al. 2008), IL-6 (Hung et al., 2007a) and thymosin β4 (Gnecchi et al., 2006), though more supporting evidence for these factors in mediating the pro-survival effect of HPC in the context of stem cell preconditioning are warranted. In addition, HPC has also been shown to activate several transcription factors and signal transduction cascades that are known to be

In vivo models	Stem cells	PC stimulus	End points	References
MI (mouse)	MSCs (mouse)	Anoxia, 4 h + 2 h reoxygenation	↓ infarct size & apoptosis; ↑ LV function	Uemura et al., 2006
MI (mouse)	CLK (mouse)	0.1% O_2, 6 hours	↓ infarct size; ↑ angiogenesis; ↓ LV remodelling; ↑ LV function	Tang et al., 2009
MI (rat)	MSCs (mouse)	0.5% O_2, 24 h + 2 h reoxygenation	↑ cell survival; ↓ infarct size; ↑ angiogenesis; ↑ LV function	Hu et al., 2008
MI (rat)	MSCs (rat)	Anoxia & serum-free, 3 h	↓ infarct size & apoptosis; ↑ LV function	He et al., 2009
MI (rat)	MSCs (rat)	10/30 min anoxia-reoxygenation	↑ cell survival	Kim et al., 2009
Limb ischaemia (mouse)	MSCs (mouse)	1% O_2, 36 h	↑ cell survival; ↑ skeletal muscle regeneration; ↑ limb perfusion; ↑ neovascularization	Leroux et al., 2010
Limb ischaemia (mouse)	MSCs (human)	1-3% O_2, 16 h	↑ limb perfusion	Rosova et al., 2008
Limb ischaemia (mouse)	PB-MNCs (mouse)	2% O_2, 24 h	↑ cell survival; ↑ limb perfusion; ↑ neovascularization	Kubo et al., 2008
Limb ischaemia (rat)	MSCs (rat)	2% O_2, 24 h	↑ limb perfusion; ↑ neovascularization	Li et al., 2002
Limb ischaemia (rat)	PB-MNCs (human)	pO_2 of 35mmHg, 7 d	↑ limb perfusion; ↑ neovascularization	Akita et al., 2003
Brain ischaemia (rat)	ESNPCs (mouse)	1% O_2, 8 h	↑ cell survival; ↑ recovery of sensorimotor function	Theus et al., 2008
Diabetic cardiomyopathy (rat)	MSCs (rat)	Anoxia	↓ apoptosis; ↑ angiogenesis; ↓ LV remodelling; ↑ LV function	Li et al., 2008
Spinal cord injury (rat)	ASCs (human)	1% O_2, 24 h + 24 h reoxygenation	↑ survival of co-transplanted NSCs	Oh et al., 2010

Table 2. Therapeutic potential of hypoxic-preconditioned stem and progenitor cells.

protective and functionally beneficial including the survival kinase Akt (Hung et al., 2007a; Kim et al., 2009), ERK1/2 (Wang et al., 2008a), p38MAPK and survivin (Peterson et al., 2011), SDF-1/CXCR4 and CXCR7 chemokine signalling pathway (Liu et al., 2010), microRNA(miR)-210 (Kim et al., 2009), transcription factors HIF-1α (Kim et al., 2009; Francis & Wei, 2010) and NFκB (Hu et al., 2008). It is also important to note that these mechanistic

pathways and paracrine factors interact with each other and are not mutually exclusive. For example, stabilisation of HIF-1α by HPC, possibly through activation of the PI3K/Akt pathway (Francis & Wei, 2010; Liu et al., 2010), allows its translocation into the nucleus to up-regulate the transcription and translation of various anti-apoptotic proteins such as CXCR7 (Liu et al., 2010), Bcl-2 (Francis & Wei, 2010) and miR-210 (Kim et al., 2009) in stem and progenitor cells. Furthermore, up-regulation of miR-210 has been demonstrated to down-regulate the expression of CAP8AP2, a pro-apoptotic protein that activates death-effector caspase-8 and promotes Fas-induced apoptosis (Kim et al., 2009).

3.2 Differentiation and engraftment

In addition to cell survival and retention, lack of significant functional cell engraftment of transplanted cells in the injured tissues has posed another significant challenge for cell-based therapy. In most studies, transplanted stem and progenitor cells do not appear to be trans-differentiated and incorporated into host tissues. Instead, the functional improvement of the ischaemic conditions is likely attributed to the paracrine activities of transplanted cells. Therefore, interventions that can promote stem cell differentiation and functional engraftment in the target tissues post-transplantation should deserve much attention. HPC has been shown not only to enhance stem cell survival but also to promote their differentiation and engraftment. Hypoxia is a potent differentiation inducer of stem cells and studies have demonstrated an acceleration of MSC differentiation when cultured under hypoxic conditions (5-8% O_2) compared with that in normoxic culture, possibly through stabilisation of the oxygen sensitive transcription factor HIF-1α (Lennon et al., 2001; Ren et al., 2006). In contrast, a number of studies have indicated that hypoxia strongly inhibits the differentiation capacity of human bone marrow-derived MSCs (Hung et al., 2007b; Potier et al., 2007; Salim et al., 2004; Volkmer et al., 2010) and adipose-derived mesenchymal stem cells (ASCs) (Malladi et al., 2006; Wang et al., 2005), without affecting the cell viability. Similar conflicting results on stem cell differentiation potential were demonstrated by studies on short-term exposure to hypoxia. Studies on mouse (Theus et al., 2008) and human (Francis & Wei, 2010) embryonic stem cells (ESCs) have indicated a favourable effect of HPC in promoting their neuronal differentiation. A recent study by Volkmer *et al.* has also reported that HPC can restore the osteogenic differentiation capacity of human MSCs which was otherwise compromised under hypoxic conditions (Volkmer et al., 2010). Conversely, Potier et al. showed that short-term exposure of human MSCs to hypoxia (<1% O_2) has a negative impact on their osteogenic differentiation under normal *in vitro* culture condition (21% O_2) (Potier et al., 2007). Although the reason for this discrepancy remains unclear, the differences in cell type and species, oxygen tension, duration of exposure to hypoxic conditions and culture conditions could be the answers to these contradictory results.

In terms of functional engraftment, a recent *in vitro* study by Jaderstad and associates has reported an increased in gap-junctional intercellular communication between hypoxic preconditioned neural stem cells and host cells *in vitro*, a consequence of increased expression of connexin 43 (Jaderstad et al., 2010). In support of this finding was a previous study reporting that HPC of human MSCs enhanced their xenografting efficiency into chick embryos, a model employed to examine the *in vivo* engraftment and differentiation potential of stem cells (Hung et al., 2007b). In addition, Xie *et al.* has reported that conditioned medium from rat neonatal cardiomyocytes subjected to 2 hours of hypoxia followed by overnight reoxygenation can induce MSC differentiation into cardiomyocyte lineage as

indicated by an increase in cardiac myosin heavy chain and troponin T expression (Xie et al., 2006). The latter study has also suggested that HPC may induce secretion of various soluble differentiation factors, whose identity remains to be determined and depend on the cell types. However, what remains unknown is whether these effects of HPC in promoting stem cell differentiation and *in vivo* engraftment can be translated when preconditioned stem cells are transplanted into adult tissues. To this end, early studies on peripheral blood mononuclear cells (Akita et al., 2003; Kubo et al., 2008) and MSCs (Leroux et al., 2010; Li et al., 2002) have indicated that HPC enhances not only their differentiation into endothelial progenitor cells (EPCs) *in vitro* but also promotes neovascularisation when transplanted into the ischaemic hindlimb. This effect was associated with improvement of blood perfusion and acceleration of tissue repair. Nevertheless, the lack of detailed histological analysis of angiogenesis in the host tissues, i.e. quantifying the blood vessels derived from implanted cells, has cast doubt on the enhanced functional integration of these transplanted cells as the contributing mechanism (Akita et al., 2003). Instead, the improvement in overall neovascularisation in these studies can be interpreted as a result of increased angiogenic cytokines released by preconditioned cells, such as VEGF (Akita et al., 2003; Leroux et al., 2010; Li et al., 2002), thus promoting intrinsic angiogenesis in the host. Supporting this notion is a study that showed MSCs subjected to *ex vivo* HPC expressed a higher level of VEGF mRNA and induced greater local VEGF production in the ischaemic hindlimb after implantation, possibly through activation of the canonical Wnt (wingless-related MMTV integration site) pathway (Li et al., 2002; Leroux et al., 2010).

3.3 Cell migration

In cell-based therapy, effective treatment also relies on the ability of transplanted stem and progenitor cells to migrate to the site of injured tissues to exert reparative and regenerative effects. Short-term exposure to hypoxia has been shown to enhance the migratory capacity of stem and progenitor cells *ex vivo* by modulating the expression of various chemokines and cytokines receptors. Hung *et al.* reported an upregulation of CXC3R1 and CXCR4 expression on both mRNA and protein levels when MSCs were cultured under hypoxic condition compared with normoxia, resulted in an increased cell migration in response to the fractalkine/CX3CL1 and SDF-1α/CXCL12, respectively (Hung et al., 2007b). A recent study also showed that HPC enhances MSC adhesion, an important step during cell trafficking *in vivo*, through upregulation of CXCR4 and CXCR7 (Liu et al., 2010). The induction of these chemokine receptors has been shown to be driven mainly by transcription factor HIF-1α (Liu et al., 2010; Hung et al., 2007b; Tang et al., 2009). Using low dose of H_2O_2 as a preconditioning stimulus, Li *et al.* also showed that the enhanced chemotaxis of preconditioned MSCs was attributed to the up-regulation of CXCR4 in an ERK-dependent manner (Li et al., 2009). Translating these *in vitro* findings into an *in vivo* setting, Tang *et al.* has shown that short-term exposure of murine cardiac progenitor cells to hypoxia not only enhanced their migratory activity *in vitro* but also *in vivo* recruitment to the ischaemic myocardium when administered intravenously, through a CXCR4-dependent manner (Tang et al., 2009). In addition to the chemokine mechanisms, other studies have suggested that HPC enhanced migratory function of stem and progenitor cells through regulation of cytokine signalling (Akita et al., 2003; Rosova et al., 2008; Ii et al., 2005). For instance, hypoxia enhanced the migratory function of human EPCs in response to VEGF, possibly through up-regulation of KDR/VEGFR2 expression (Akita et al., 2003). In another study,

Rosova and colleagues showed that HPC increased the expression of the tyrosine kinase receptor, c-Met, in preconditioned MSCs rendered the cells more responsive to HGF (Rosova et al., 2008). Interestingly, HPC can also induce the secretion of chemo-attractants from preconditioned MSCs to promote endothelial cell migration through Wnt4-dependent signalling pathway (Leroux et al., 2010).

3.4 Cell proliferation

Stem and progenitor cells self-renew and this is one of the properties that make them an attractive autologous cell source for cell-based therapy and tissue engineering, where success is highly dependent on abundant cell supply. *Ex vivo* cell expansion is traditionally performed under ambient oxygen concentration of 20% O_2, which is considered to be hyperoxia compared to their physiological niches (2-7% O_2). Thus, it is imperative to simulate various aspects of the stem and progenitor cells' endogenous microenvironment, including hypoxia, in order to maintain their native characteristics and to comprehend how they respond to a hypoxic environment in injured ischaemic tissues. Studies investigating the effect of hypoxia on stem cell proliferation potential have yielded contradictory results, possibly due to the differences in hypoxic conditions, cell type, serum concentration and culture duration (Das et al., 2010). Compared to the routine normoxic culture of 20% O_2, long-term culture of human MSCs in 1% O_2 has been shown to reduce their proliferative potential (Hung et al., 2007b). Conversely, bone marrow-derived MSCs (D'Ippolito et al., 2006; Grayson et al., 2007; Lennon et al., 2001; Ren et al., 2006), but not ASCs (Wang et al., 2005), cultured under hypoxic conditions with slightly higher oxygen tension (≥2% O_2), showed increased cell proliferation. Importantly, short-term exposure to hypoxia did not negatively affect the proliferative potential of stem cells (Francis & Wei, 2010; Leroux et al., 2010; Rosova et al., 2008), an observation that will alleviate the safety concerns of HPC when clinical applications are being considered.

4. Ex vivo pharmacological preconditioning of stem cells

While ischaemic or hypoxic preconditioning has been shown to regulate multiple stress-responsive mechanisms that promote stem and progenitor cell survival under various ischaemic conditions, preconditioning with specific pharmacological agents seems to target a more linear signalling pathway. This has been explored in various studies on stem and progenitor cells (Table 3).

4.1 Diazoxide

The mitochondrial ATP-sensitive potassium (mitoK$_{ATP}$) channel is an important mediator of cardioprotection (Yellon & Downey, 2003; O'Rourke, 2004) where opening of the channels has been shown to induce protection by preventing calcium overload, inhibiting mitochondrial permeability transition pore (mPTP) opening, preserving ATP production, uncoupling of mitochondrial oxidative phosphorylation, succinate dehydrogenase inhibition, reducing detrimental reactive oxygen species (ROS) production at reperfusion and PKC activation. Using the mitoK$_{ATP}$ opener diazoxide, Baines and colleagues were the first to demonstrate that opening of mitoK$_{ATP}$ channels prior to ischaemia could mimic the infarct-limiting effect of IPC in the setting of myocardial ischaemia-reperfusion injury (Baines et al., 1999). Recently, diazoxide has also featured in a number of studies by Ashraf's group to precondition stem and progenitor cells. They have shown that *ex vivo* preconditioning with diazoxide can promote skeletal myoblasts (Haider et al., 2010; Niagara

PC stimulus	Cells	In vitro	In vivo	Mechanisms	References
Diazoxide (200 μM), 30 min	Skeletal Mb (rat)	↑ survival	(MI) ↑ survival; ↑ angiomyogenesis; ↑ LV function	Akt; bFGF; HGF	Niagara et al., 2007
Diazoxide (200 μM), 30 min	Skeletal Mb (rat)	↑ survival	(MI) ↑ survival & proliferation; ↑ angiomyogenesis; ↑ LV function	Akt; ERK1/2; STAT3; IL-11; miR-21	Haider et al., 2010
Diazoxide (200 μM), 30 min	MSCs (rat)	↑ survival	(MI) ↑ survival; ↓ infarct size; ↑ angiomyogenesis; ↑ LV function	Akt; GSK3β; NFκB; HGF; IGF; FGF-2; Ang-2	Afzal et al., 2010
Diazoxide (200 μM), 1-3 h	MSCs (rat)	↑ survival	-	NFκB; Fas; miR-146a	Suzuki et al., 2010
Diazoxide (200 μM), 30 min	MSCs (rat)	↑ survival	(MI) ↑ survival; ↓ infarct size; ↑ LV function	Akt; bFGF; HGF	Cui et al., 2010
SDF-1 (50 ng/mL), 1 h	MSCs (rat)	↑ survival; ↑ proliferation	(MI) ↑ survival; ↓ infarct size; ↑ angiomyogenesis; ↑ LV function	Akt; CXCR4; VEGF	Pasha et al., 2008
SDF-1 (10-100 ng/mL), 24 h	MSCs (rat)	↑ survival	-	CXCR4	Chen et al., 2009
IGF-1 (100 nM), 30 min	BM Sca-1$^+$ (mouse)	↑ survival; ↑ myogenic diff.	(MI) ↑ survival; ↓ infarct size; ↑ myogenic diff.; ↑ angiogenesis; ↑ LV function	Akt; Cx43	Lu et al., 2009
IGF-1 (100 nM), 30 min	BM Sca-1$^+$ (mouse)	↑ survival	-	ERK1/2; Cx43	Lu et al., 2010
TGFα (0.25 μg/mL) 24 h	MSCs (mouse)	-	(MI) ↓ apoptosis; ↑ LV function	VEGF; p38MAPK	Herrmann et al., 2010b
TGFα (0.25-1 μg/mL), 24 h	MSCs (human)	↑ VEGF production	-	PI3K; MEK	Wang et al., 2008b
TGFα (0.01-1 μg/mL), 24 h	MSCs (human)	↑ HGF production	-	PI3K; MEK; TNFR; p38MAPK	Wang et al., 2009b
PDGF (0.4 nM), 5 d	MSCs (rat)	↑ adipogenic diff.	-	-	Tamama et al., 2006
PDGF (0.4 nM), 5 d	MSCs (human)	↓ adipogenic-; ↑ osteogenic- diff.	-	-	Tamama et al., 2006
BMP-2 + bFGF + IGF-1 (10, 50 & 2 ng/mL), 1-7 d	MSCs (rat)	↑ survival; ↑ myogenic diff.	(MI) ↑ survival; ↓ infarct size; ↑ LV function	Cx43; Akt; CREB	Hahn et al., 2008
IGF-1 + bFGF (50 & 50 ng/mL), 1 h	MSCs (mouse)	↑ survival & proliferation; ↑ angiogenic potential	-	Akt; SOD; Ang-1; Bax; Bak; 16^{INK4a}; $p66^{shc}$; p53	Khan et al., 2011

PC stimulus	Cells	In vitro	In vivo	Mechanisms	References
Sevoflurane (2%), 30 min x 3	EPCs (human)	↑ CFC	-	VEGF	Lucchinetti et al., 2009
Isoflurane (0.5 mM), 10 min	ESCM (human)	↑ survival	-	mitoK$_{ATP}$; ΔΨm; ROS	Sepac et al., 2010
CsA (0.5-5 μM), 30 min	MSCs (rat)	↑ survival	-	Bcl-2; BAD; ΔΨm	Wang et al., 2008a
LPS (1 μg/mL), 12 h	MSCs (mouse)	↑ survival	-	TRL-4; Akt; NFκB	Wang et al., 2009c
LPS (1 μg/mL), 2 d	MSCs (mouse)	-	(MI) ↑ survival; ↓ cardiac fibrosis; ↑ angiogenesis; ↑ LV function	TRL-4; Akt; VEGF; NFκB	Yao et al., 2009
Melatonin (5 μM), 24 h	MSCs (rat)	↑ survival ; Conditioned media ↑EPCs proliferation & angiogenesis	(Renal IR) ↑ survival; ↑ renal function; ↑ renal cell proliferation; ↑ angiogenesis	Catalase; SOD, bFGF; HGF	Mias et al., 2008
Trimetazidine (10 μM), 6 h	MSCs (rat)	↑ survival	(MI) ↓ infarct size; ↑ LV function	Akt; HIF-1α; survivin; Bcl-2	Wisel et al., 2009
Fucoidan (10 μg/mL), 36 h	EPCs (human)	↑ cell migration; ↑ angiogenic potential	-	-	Zemani et al., 2005
LPA (10 μM), 1 h	MSCs (rat)	-	(MI) ↑ survival; ↑ angiogenesis	VEGF	Liu et al., 2009
Lithium Chloride (5-20 mM), 24 h	Skeletal Mb (rat)	↑ survival & proliferation; ↑ gap junction formation	-	Cx43; VEGF; β-catenin; GSK-3β	Du et al., 2009
rhHsp90α (0.1-10 μM), 24 h	MSCs (rat)	↑ survival	-	Akt; ERK1/2; NO; Bcl-2; Bcl-xL; Bax	Gao et al., 2010
β-met (2 mM), 1 h	MSCs (rat)	↑ survival	-	HSP72	Cizkova et al., 2006
rhEPO (10 U/mL), 24 h	ESNPCs (mouse)	↑ survival	-	Bcl-2	Theus et al., 2008
Carbamylated EPO (100 ng/mL), 30 min	ESCM (human)	-	(MI) ↑ survival	Akt	Robey et al., 2008
Simvastatin (25 μM), 24 h	EPCs (human)	↑ survival	-	-	Henrich et al., 2007

Ang (angiopoietin), β-met (β-mercaptoethanol), CFC (colony forming capacity), CREB (cAMP response element binding protein), Mb (myoblasts), rh (recombinant human), ΔΨm (mitochondrial membrane potential).

Table 3. Pharmacological preconditioning of stem and progenitor cells.

et al., 2007) and MSCs (Afzal et al., 2010; Suzuki et al., 2010) survival both *in vitro* and *in vivo* post-transplantation. Furthermore, transplantation of these preconditioned cells into the infarcted myocardium was associated with smaller infarct size, improved LV function, myogenic differentiation and angiogenesis (Afzal et al., 2010; Haider et al., 2010; Niagara et al., 2007). The potential mechanisms responsible for the cytoprotective effect of diazoxide in skeletal myoblasts include enhanced release of paracrine growth factors such as bFGF and HGF, and activation of survival kinase PI3K/Akt (Niagara et al., 2007). A later study by the same group expanded the mechanistic finding to include IL-11, the ERK1/2-STAT3 signalling pathway and up-regulation of miR-21 (Haider et al., 2010). In MSCs, Afzal *et al.* reported NFκB activation as another important underlying mechanism of diazoxide-induced protection especially during the late phase preconditioning (Afzal et al., 2010). The activation of NFκB in diazoxide preconditioned MSCs was subsequently implicated to regulate the expression of miR-146a, which in turn acts as a negative regulator of the Fas gene, a death receptor of apoptosis (Suzuki et al., 2010).

4.2 Stromal cell-derived factor-1 (CXCR12)

SDF-1 or CXCL12 is a cytokine belonging to the CXC chemokine subfamily. Specific binding of SDF-1 to CXCR4 induces dimerization of the receptor and activates multiple signalling pathways to regulate trafficking and differentiation of stem and progenitor cells (Chen et al., 2011; Kucia et al., 2004). In addition, activation of the SDF-1/CXCR4 axis has been shown to promote cell survival and proliferation (Broxmeyer et al., 2003; Kucia et al., 2004; Hu et al., 2007). The therapeutic potential of SDF-1 was later illustrated in an experimental model of myocardial infarction (Takahashi, 2010). For example, SDF-1 administration conferred cardioprotection through the PI3K/Akt signalling pathway in the setting of acute ischaemia-reperfusion injury (Hu et al., 2007) and chronic ischaemic heart failure (Saxena et al., 2008). In the setting of hindlimb ischaemia, intramuscular injection of SDF-1 increased angiogenic factor VEGF expression, and enhanced the retention and neovascularisation efficacy of transplanted EPCs (Yamaguchi et al., 2003). Other delivery methods for SDF-1 include direct gene delivery with adenoviral vector (Abbott et al., 2004) and plasmid DNA (Hiasa et al., 2004) encoding for SDF-1, or transplantation of genetically modified cardiac fibroblasts (Askari et al., 2003), MSCs (Zhang et al., 2007) or skeletal myoblasts (Elmadbouh et al., 2007) that over-expressed SDF-1 into the ischaemic tissues. Therefore, it is not surprising that SDF-1 has been exploited as a potential preconditioning agent to enhance stem and progenitor cells survival and function without the long-term concern of genetic manipulation. In this regard, pre-treatment with SDF-1 has been shown to enhance MSC survival both *in vitro* (Chen et al., 2009; Pasha et al., 2008) and following intramyocardial transplantation in the infarcted myocardium through activation of PI3K/Akt signalling pathway and is dependent on CXCR4 (Pasha et al., 2008). In this study, the authors also demonstrated that SDF-1 preconditioning enhances paracrine activities of transplanted MSCs in the infarcted myocardium and contributes to a smaller infarct size, improved cardiac function and promoted revascularization. Interestingly, the cytoprotective effect of SDF-1 appears not to be limited to pre-treatment. Treatment of EPCs with SDF-1 at the end of lethal serum deprivation also significantly reduced apoptotic cell death (Yamaguchi et al., 2003). However, *in vivo* application of SDF-1 requires vigilant safety evaluation before translating into the clinical setting because of the potentially detrimental side effects of its cleavage

products. For instance, SDF-1(5-68), a toxic product of SDF-1 cleavage by exopeptidases and metalloproteinase-2, has been implicated in neuroinflammation and neuronal death (Zhang et al., 2003). In which case, SSDF-1(S4V), a modified SDF-1 that is resistant to endogenous proteases cleavage, may have a better clinical safety profile (Segers et al., 2007).

4.3 Growth factors

Various growth factors have been employed to precondition stem and progenitor cells in order to enhance their paracrine activity, cell survival and differentiation (Abarbanell et al., 2009). For example, Lu and colleagues showed that preconditioning with IGF-1 improved bone marrow Sca-1$^+$ stem cell survival against simulated ischaemia *in vitro* and after *in vivo* transplantation. This pro-survival effect of IGF-1 was shown to be dependent on the activation of PI3K/Akt (Lu et al., 2009) and ERK1/2 (Lu et al., 2010) signalling pathways, and a down-stream mediator connexin-43. A subsequent study indicated that pre-treatment with IGF-1 also enhances Sca-1$^+$ cell cardiomyogenesis potential and transplantation of these preconditioned cells into infarcted heart mitigated myocardial infarction and ventricular dysfunction (Lu et al., 2009). Moreover, MSCs exposed to IGF-1 had an enhanced migratory response to SDF-1, a response dependent on the PI3K/Akt signalling pathway (Li et al., 2007).
It has been well documented that epidermal growth factor (EGF), the prototypical growth factor with intrinsic protein tyrosine kinase activity, plays an important role in the regulation of cell growth, proliferation and differentiation by binding to its receptor (Wells, 1999). In MSCs, EGF has been shown to promote cell proliferation and motility which are beneficial for *ex vivo* cell expansion prior to *in vivo* transplantation, but fail to rescue MSCs from low serum-induced apoptosis despite an elevation of Akt. Similar effects were observed with platelet-derived growth factor (PDGF) (Tamama et al., 2006). On the other hand, pre-treatment with TGFα, another member of the EGF superfamily and a potent activator of EGF receptor, has been shown to enhance the therapeutic potential of MSCs in an experimental model of acute myocardial ischaemia-reperfusion injury in terms of myocardial function recovery and inflammation (Herrmann et al., 2010b). These beneficial effects of TGFα have been attributed to the reduction in pro-inflammatory cytokine production and increased VEGF production. The latter effect was shown to be mediated by MEK and PI3K signalling pathways (Herrmann et al., 2010b). It is interesting to note that the effect of TGFα on VEGF production in MSCs has been demonstrated as bimodal, i.e. production of VEGF was suppressed by low concentrations of TGFα whereas high concentrations increased the secretion (Wang et al., 2008b). In addition to VEGF, TGFα treatment also increases the production of HGF from MSCs (Wang et al., 2009b). HGF is an important signalling factor in stem cell-mediated repair where it promotes stem cell adhesion, migration and survival (Vandervelde et al., 2005). Although the potential of HGF as a preconditioning agent to promote stem and progenitor cell survival is yet to be confirmed, existing studies have showed that short-term exposure of MSCs to HGF can activate Akt and ERK1/2 pro-survival kinases, and induce expression of anti-apoptotic protein, Bcl-2 and Bcl-xL (Forte et al., 2006). Additionally, HGF treatment also increased MSC motility function through up-regulation of c-Met receptors and induced their differentiation along a myogenic lineage, effects that can enhance their therapeutic potential in cardiac repair (Forte et al., 2006; Neuss et al., 2004; Rosova et al., 2008). Regarding cell proliferation, HGF treatment was reported to have a negative impact on MSC proliferation by blocking cells in the G_0-G_1 phase through p38 MAPK pathway and concomitant up-regulation of cell cycle progression inhibitors, $p21^{waf1}$ and $p21^{kip}$ (Neuss et al., 2004; Forte et al., 2006).

Although different growth factors may share some common downstream signalling pathways in modulating stem and progenitor cell functions, they also independently activate distinct signalling cascades (Abarbanell et al., 2009). This rationale that a combination of multiple growth factors may have additive and synergistic effects in promoting stem and progenitor cell survival and functional improvement, offers a strategic advantage over the approach involving the use of a single factor. In this respect, a recent study by Hahn *et al* has demonstrated the pro-survival effect of multiple growth factors in MSCs using a combination of IGF-1, bFGF and BMP-2 (Hahn et al., 2008). Treatment with this growth factor cocktail also enhanced MSC cardiac differentiation efficiency and incurred cytoprotection of co-cultured adult cardiomyocytes. This effect was dependent on gap-junction communication leading to phosphorylation of Akt and c-AMP response element binding protein (CREB) in cardiomyocytes. Moreover, the therapeutic efficacy of MSCs in infarcted myocardium was also significantly potentiated with this multiple growth factors preconditioning method (Hahn et al., 2008). Similarly, Khan *et al* preconditioned MSCs isolated from diabetic mice with a combination of IGF-1 and bFGF and showed enhanced cell survival, proliferation, motility and angiogenic potential compared to untreated cells or cells treated with single growth factor (Khan et al., 2011). Furthermore, a potent synergistic effect between TGFα and TNFα has been demonstrated to enhance the MSC-derived VEGF and HGF production that may be essential for cell survival, migration and angiogenesis (Herrmann et al., 2010a; Wang et al., 2008b; Wang et al., 2009b).

4.4 Anaesthetics

Anaesthetics, routinely administered in patients during surgery, are widely recognised to have preconditioning properties protecting the heart and many other organs against ischaemia and reperfusion injury in laboratory settings as well as in humans with coronary heart diseases (Huffmyer & Raphael, 2009; Yellon & Downey, 2003). The efficacy of anaesthetic-induced preconditioning has been shown to be similar to IPC in terms of infarct size reduction, increased collateral blood flow and attenuated inflammatory responses during ischaemia. Although the mechanisms underlying the protective effect of anaesthetic-induced preconditioning have been shown to resemble those responsible for IPC (Zaugg et al., 2003), Mullenheim *et al* showed that administration of sevoflurane combined with IPC can synergistically protect rabbit hearts against myocardial infarction indicating the existence of parallel protective mechanisms (Mullenheim et al., 2003). In ESC-derived cardiomyocytes, brief exposure to the commonly used volatile anaesthetic, **isoflurane**, significantly attenuated cell death against oxidative stress through opening of mitoK$_{ATP}$ channels, production of signalling ROS and inhibition of mPTP opening (Sepac et al., 2010). Other benefits include enhanced cell growth capacity and increased expression of pro-angiogenic VEGF as demonstrated in human EPCs preconditioned with **sevoflurane** (Lucchinetti et al., 2009).

4.5 Cyclosporin-A (CsA)

The mPTP is a non-specific channel of the inner mitochondrial membrane, whose opening at the onset of reperfusion is a critical mediator of lethal myocardial ischaemia-reperfusion injury. IPC exerts its cardioprotective effect by inhibiting the opening of the mPTP and pharmacological inhibition of mPTP with CsA has been shown to confer cytoprotection both *in vitro* and *in vivo* (Hausenloy & Yellon, 2003; Lim et al., 2007). In addition to inhibiting mPTP opening, CsA can also inhibit calcineurin-mediated dephosphorylation of the apoptogenic

protein BAD and uncouple the mitochondrial respiratory chain, which might, in itself, result in protection (Wilkins et al., 2004). Interestingly, mPTP can also open reversibly under basal conditions without causing cell death. This form of reversible non-pathological mPTP opening has been noted to contribute to the cardioprotection elicited by IPC through the mitochondrial generation of signalling ROS and the subsequent activation of the pro-survival kinases Akt and ERK1/2 (Hausenloy et al., 2010). In stem cells, pre-treatment with CsA has been shown to protect MSCs from hypoxia-reoxygenation induced apoptosis through stabilizing mitochondrial membrane potential and promoting Bcl-2 and phosphorylated BAD protein expression (Wang et al., 2008a). In line with this study, we recently showed that pre-treatment with 0.2 μM of CsA for 30 minutes effectively increased the resistance of human ASCs to subsequent simulated ischaemia-induced cell death (Figure 2, unpublished data).

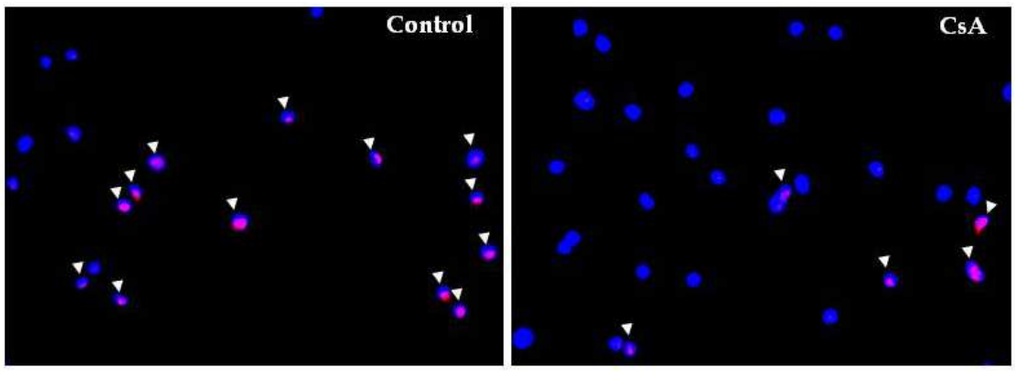

Fig. 2. Preconditioning with CsA confers cytoprotection in stem cells. Pre-treatment with CsA (0.2 μM, 30 min) reduced cell death (determined by propidium iodide staining, arrow heads) in hASCs subjected to 15 hours of simulated ischaemia (<1% O_2 and ischaemic buffer as described previously (Lim et al., 2008)) from 58.5±2.6% to 42.5±1.8% (n=4-5, $P < 0.05$).

4.6 Lipopolysaccharide

LPS is an antigenic component of the outer membrane of gram-negative bacteria and an agonist of Toll-like receptor-4 (TLR4) capable of eliciting immune responses in animals. The cardioprotective effect of LPS preconditioning was previously demonstrated in an *in vivo* murine model of myocardial ischaemia-reperfusion injury (Ha et al., 2008). Evidence supporting the pro-survival effect of LPS preconditioning in MSCs was recently provided by Wang and colleagues. The authors reported that activation of Toll-like receptor-4 (TLR4) with low dose of LPS can prevent MSCs from apoptotic cell death induced by oxidative stress and serum deprivation through PI3K/Akt- and NFκB-dependent mechanisms (Wang et al., 2009c). Furthermore, transplantation of these LPS-preconditioned MSCs into infarcted rat hearts significantly improved cardiac function, reduced apoptosis and fibrosis, and enhanced angiogenesis (Yao et al., 2009).

4.7 Other agents

Other drugs and hormones have also been employed as preconditioning agents to promote stem and progenitor cell survival and function. For example, pre-treatment with the pineal hormone **melatonin** can improve the therapeutic effectiveness of MSCs in the setting of acute renal ischemia-reperfusion injury by potentiating their survival and paracrine activity

(Mias et al., 2008). **Trimetazidine** is a cytoprotective anti-ischaemic agent that acts to reduce ischaemia-induced metabolic damage by shifting the energy substrate preference from fatty acid oxidation to glucose oxidation. It has been employed recently to precondition MSCs and showed increased cell survival and enhanced therapeutic potential in reducing myocardial ischaemic injury (Wisel et al., 2009). **Fucoidan**, a high molecular weight sulphated polysaccharide, also favourably enhanced the migratory potential of pre-treated EPCs *in vitro*. Although alone it did not promote angiogenesis, treatment with fucoidan potentiated the angiogenenic effect of bFGF in EPCs (Zemani et al., 2005). Du *et al.* preconditioned skeletal myoblasts with **lithium chloride** and showed enhanced cell survival and increased gap-junctional coupling with co-cultured neonatal cardiomyocytes, a result of increased Cx43 expression (Du et al., 2009). Similarly, pre-treatment with the endogenous phospholipid signalling molecule, **lysophosphatidic acid (LPA)**, can improve MSC survival *in vivo* in ischaemic myocardium and enhance their angiogenic effects (Liu et al., 2009). Interestingly, an *in vitro* study by the same group indicated that LPA failed to precondition MSCs (Chen et al., 2008). In this study, the anti-apoptotic effect of LPA was only evident when the drug was present throughout the hypoxia and serum deprivation insult but not when LPA was removed after the pre-treatment period. Nevertheless, the cytoprotective effect of LPA was demonstrated to be dependent on the activation of LPA receptor-1, and pertussis toxin-sensitive PI3K/Akt and ERK pathways (Chen et al., 2008).

Heat shock proteins (HSP) are known protective mediators of preconditioning induced by ischaemia, hypoxia, heat stress and oxidative stress (Das & Maulik, 2006) (Table 1). In MSCs, preconditioning with recombinant human HSP90α produced an anti-apoptotic effect via activation of PI3K/Akt and ERK signalling pathways (Gao et al., 2010). Prior to this study, genetic modification to over-express HSP20 had been shown to enhance the survival of MSCs against oxidative stress and improve their therapeutic potential in ischaemic rat heart (Wang et al., 2009a). Therefore, it is not surprising that drugs capable of increasing HSP expression are potential preconditioning agents. Indeed, short-term exposure to **β-mercaptoethanol** has been shown to protect MSCs from oxidative injury, a cytoprotective effect associated with an elevation of HSP72 expression (Cizkova et al., 2006).

The haematopoietic cytokine **erythropoietin** (EPO) also exerts cardioprotection in both animal and clinical studies with many intracellular signalling pathways implicated, including PI3K/Akt, ERK1/2, p38 MAPK, PKC, eNOS and guanylyl cyclase and the opening of mitochondrial K_{ATP} channels (Riksen et al., 2008). In MSCs (Hu et al., 2008; Theus et al., 2008) and embryonic stem cell-derived neural progenitor cells (NPCs) (Francis & Wei, 2010; Theus et al., 2008), EPO is already involved in the cytoprotective effect of HPC. As a preconditioning agent, recombinant human EPO mimicked the cytoprotective effect of HPC in protecting mouse NPCs against serum deprivation-induced apoptotic cell death (Theus et al., 2008). Similarly, ESC-derived cardiomyocytes survived better in the infarcted mouse hearts when they were preconditioned with carbamylated EPO prior to implantation (Robey et al., 2008). The hydroxyl-methylglutaryl coenzyme A reductase inhibitors, also known as statins, are effective for lowering serum cholesterol and have been widely prescribed for patients with coronary heart diseases as the primary and secondary preventive treatment of cardiovascular events. In addition to lipid-lowering effects, statins have been shown to exert multiple pleiotropic effects including protection from MI, improved endothelial function and reduced platelet adhesion and atherosclerotic plaque rupture (Ludman et al., 2009). In MSCs, treatment with **lovastatin** has been shown to improve cell survival when challenged

with hypoxia and serum deprivation, a protection mediated by PI3K/Akt and ERK1/2 pathways (Xu et al., 2008). Similarly, human EPCs pre-treated with **simvastatin** also exhibit higher resistance against TNFα induced apoptotic cell death (Henrich et al., 2007).

5. Ischaemic or hypoxic preconditioning in vivo mobilises endogenous stem and progenitor cells

Endogenous stem and progenitor cells can be mobilized from their niches in various organs and tissues, including bone marrow, skeletal muscle, heart, brain, skin, liver, adipose, blood vessels and others, and then home to target tissues. This property of stem and progenitor cells has fuelled investigations of potential clinically adaptable strategies to actively recruit endogenous stem and progenitor cells to serve as integrated participants in regenerating the injured tissues through stem cell trans-diffentiation and/or as supportive players via pleiotropic paracrine effects (Chen et al., 2011; Krankel et al., 2011). By subjecting rats to 3 weeks of chronic hypoxia, Rochefort *et al* have unveiled the potential of HPC in mobilising endogenous MSCs. In this study, circulating MSCs were higher in rats subjected to chronic hypoxia compared to the control normoxic cohort (Rochefort et al., 2006) (Table 4). Interestingly, this hypoxic condition did not affect the circulating level of haematopoietic stem cells (HSCs) indicating a possible cell-type specific effect of HPC (Rochefort et al., 2006). In line with this study, rats preconditioned with 6 hours of hypoxia daily for 6 weeks also have a higher level of $CD34^+CXCR4^+$ cells in their blood circulation and in the infarcted hearts with concomitant reduction in acute myocardial ischaemia-reperfusion injury (Lin et al., 2008). Using a relatively more invasive preconditioning protocol of transient coronary artery occlusion and reperfusion, Ii and colleagues have showed that IPC modulates endogenous EPC kinetic and increases their recruitment to the infarcted myocardium (Ii et al., 2005). This observation was associated with infarct size limitation, increased angiogenesis and cardiac function improvement, beneficial effects that were shown to be strongly dependent on the iNOS and eNOS activities of the EPCs (Ii et al., 2005). The mobilizing and homing effect of IPC was subsequently illustrated on other cell types such as MSCs and HSCs using a clinically relevant porcine myocardial ischaemia-reperfusion injury experimental model (Gyongyosi et al., 2010). Excitingly, Kamota *et al* showed that preconditioning applied on the abdominal aorta can also increase the accumulation of bone marrow-derived $sca\text{-}1^+$ and $c\text{-}kit^+$ stem cells in infarcted hearts through a SDF-1/CXCR4-dependent mechanism, thus protecting the hearts against injury (Kamota et al., 2009). This finding is clinically important as it supported the translation of the non-invasive strategy of remote IPC into clinical practise. Remote IPC is a clinically amenable strategy which can be induced by simple transient limb ischaemia. This cardioprotective strategy has been extensively trialled in patients undergoing cardiac surgeries such as coronary angioplasty and coronary artery bypass surgery, and has thus far showed tangible beneficial effects in reducing myocardial injury with no known adverse risks (Hausenloy & Yellon, 2008).

Although the precise molecular and cellular mechanisms governing the homing effect of ischaemic/hypoxic preconditioning on endogenous stem and progenitor cells remain to be fully addressed, evidence of a role for the chemokine axis SDF-1/CXCR4 (Kamota et al., 2009; Lin et al., 2008) and EPO (Lin et al., 2008) has been found in recent studies. Other possible stem cell homing factors include VEGF, colony-stimulating factor, monocyte

PC stimulus	Models	End points	Mechanisms	References
Hypoxia; 24 h of 50kPa O_2 daily for 3 weeks	Rat, Sham	↑ circulating MSCs	-	Rochefort et al., 2006
Hypoxia; 6 h of 10% O_2 daily for 6 weeks	Rat, MI	↑ circulating & heart $CD43^+CXCR4^+$ cells; ↓ infarct size; ↑ LV function; ↓ plasma CK-MB	SDF-1/CXCR4; EPO; VEGF	Lin et al., 2008
Ischaemia; 4 x 4 min/4 min coronary artery O/R	Mouse, MI	↑ circulating & heart EPCs; ↓ infarct size; ↑ LV function; ↑ angiogenesis	eNOS; iNOS; VEGF	Ii et al., 2005
Ischaemia; 2 x 5 min/5 min coronary artery O/R	Pig, MI	↑ circulating HSCs; ↑ heart MSCs & HSCs; ↓ infarct size & apoptosis; ↑ LV function	SDF-1α; VEGF; TNFα; IL-8	Gyongyosi et al., 2010
Ischaemia; 4 x 5 min/5 min abdominal artery O/R	Mouse, MI	↑ circulating $CD43^+flk\text{-}1^+$ cells; ↑ heart $Sca\text{-}1^+$ & $c\text{-}kit^+$ BMSCs; ↓ infarct size & apoptosis; ↑ LV function	SDF-1/CXCR4; VEGF	Kamota et al., 2009
Ischaemia; 25 min/7 d renal artery O/R	Mouse, RI	↑ renal EPCs	-	Patschan et al., 2006

Table 4. Effect of in vivo ischaemic/hypoxic preconditioning on endogenous stem and progenitor cells. BMSCs (bone marrow stem cells), O/R (occlusion/reperfusion), RI (renal ischaemia).

chemotactic protein-3, HGF, IGF-1, IL-8/growth regulated oncogene-1, stem cell factor, TGF-β3, Wnt antagonist and other chemokines (Krankel et al., 2011; Binger et al., 2009; Chen et al., 2011). However, whether these navigational factors govern the homing effect of *in vivo* IPC warrants further investigation. In perspective, a living body appears to host a great reservoir of various stem and progenitor cells ready to be recruited for regeneration and repair, and can be catalysed by external stimuli such as IPC. This is of great clinical importance because *in vivo* IPC can be readily and non-invasively achieved in patients. However, given the fact that majority of ischaemic disease sufferers are elderly patients, and endogenous stem and progenitor populations and functions are known to decline with age and are negatively affected by other co-morbidity such as diabetes and hypertension (Krankel et al., 2011), it is important to determine whether there are sufficient populations of functionally competent resident stem and progenitor cells to be mobilised and recruited to the target tissues to exert significant repair in older patient cohorts. Otherwise, a combination of exogenously administered cells and potent homing factors might be utilised to supplement the endogenous reservoirs for effective cell-based therapy. Therefore, detailed characterisation of various endogenous stem cell niches and deciphering the mechanisms governing endogenous stem cell repopulation, mobilisation and homing to target tissues are pivotal to the future development of clinically sound pharmacological interventions to harness fully the host's innate regenerative capacity.

6. Conclusion

Based on the existing literature, it is undeniable that non-genetic approaches of preconditioning techniques offer much promise as cytoprotective strategies for stem cell therapy, without the long-term concern of genetic manipulation. These pro-survival strategies are therefore well suited to clinical translation. Collectively, preconditioning of stem and progenitor cells elicits multiple beneficial effects including (1) promotion of cell survival in the hostile ischaemic environment, (2) enhancement of paracrine activity to create a supportive environment that is rich in trophic and angiogenic factors, (3) increase of cell motility and trafficking, (4) increase of cell proliferative potential, (5) promotion of cell differentiation allowing functional integration, and (6) enhancement of therapeutic efficacy in ischaemic tissues *in vivo*.

In cell based therapy for ischaemic diseases, the ability of preconditioning *ex vivo* to enhance stem and progenitor cell survival and function means more implanted cells will be available for tissue repair and thus fewer donor cells may be needed to achieve the same functional outcome. In parallel, *in vivo* preconditioning is capable of harnessing the host's inherent regeneration mechanisms through activation of various paracrine signalling cascades, and mobilizing and recruiting resident stem and progenitor cells for effective therapeutics (Figure 3). Therefore, it will be of great therapeutic interest to determine whether there is an additive or synergistic effect of *ex vivo* preconditioning of implanted cells and *in vivo* preconditioning of host tissues, which may provide an optimal regenerative environment for tissue repair and regeneration, and contribute to successful stem cell therapy and tissue engineering.

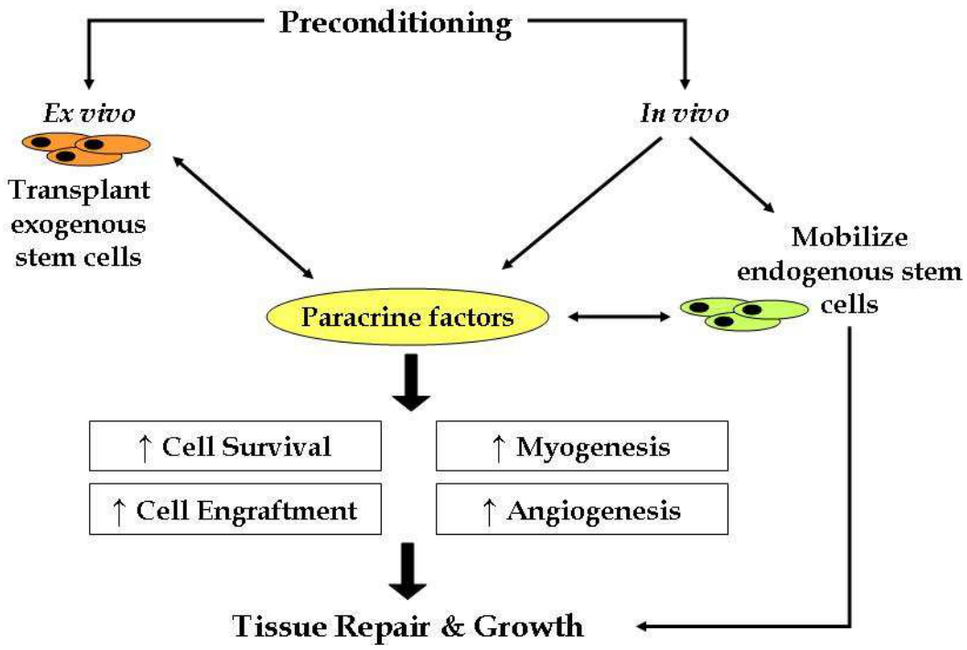

Fig. 3. Potential additive effect of *ex vivo* preconditioning (transplanted exogenous stem cells) and *in vivo* preconditioning (patients) to enhance tissue repair and tissue engineering with stem cells.

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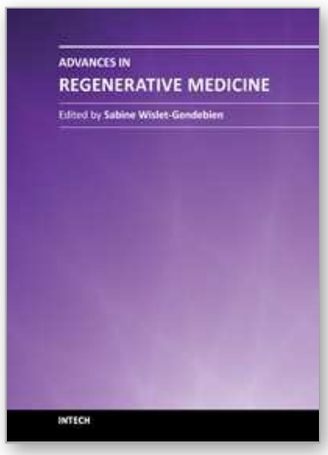

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Even if the origins of regenerative medicine can be found in Greek mythology, as attested by the story of Prometheus, the Greek god whose immortal liver was feasted on day after day by Zeus' eagle; many challenges persist in order to successfully regenerate lost cells, tissues or organs and rebuild all connections and functions. In this book, we will cover a few aspects of regenerative medicine highlighting major advances and remaining challenges in cellular therapy and tissue/organ engineering.

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