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Strategies to promote donor cell survival: combining preconditioning approach with stem cell transplantation

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Abstract

Stem cell transplantation has emerged as a potential modality in cardiovascular therapeutics due to their inherent characteristics of self-renewal, unlimited capacity for proliferation and ability to cross lineage restrictions and adopt different phenotypes. Constrained by extensive death in the unfriendly milieu of ischemic myocardium, the results of heart cell therapy in experimental animal models as well as clinical studies have been less than optimal. Several factors which play a role in early cell death after engraftment in the ischemic myocardium include; absence of survival factors in the transplanted heart, disruption of cell-cell interaction coupled with loss of survival signals from matrix attachments, insufficient vascular supply and elaboration of inflammatory cytokines resulting from ischemia and/or cell death. This article reviews various signaling pathways involved in triggering highly complex forms of cell death and provides critical appreciation of different novel anti-death strategies developed from the knowledge gained from using an ischemic preconditioning approach. The use of pharmacological preconditioning for up-regulation of pro-survival proteins and cardiogenic markers in the transplanted stem cells will be discussed.

1. An overview of heart cell therapy

During the last decade, widespread experimental studies in animal models and clinical studies have shown the safety, feasibility and efficacy of cell based therapies for myocardial regeneration. Starting with the use of unipotent myogenic cells including skeletal muscle derived stem cells and cardiomyocytes, heart cell therapy has progressed to assess the feasibility of multipotent and pluripotent stem cells for cardiac repair [1,2]. Despite recent reports for their successful cardiomyogenic differentiation in vitro [3], failure of skeletal myoblasts to electromechanically integrate and synchronously contract with the host myocytes post engraftment have restricted their use as the choice donor cells in clinical settings [4]. Similarly, cardiomyocytes are an excellent option for use as donor cells but their limited availability remains an issue of concern in clinical application [5,6]. Engraftment of non-myogenic fibroblasts and smooth muscle cells with contractility characteristics different from cardiomyocytes have also been shown to improve cardiac function [7,8]. Nevertheless, a repertoire of stem and progenitor cells in the bone marrow with differing plasticity and differentiation potentials have emerged as the most extensively studied cells for their myocardial regenerative potential in experimental animals and clinical studies [9,10]. In case of bone marrow derived stem cell engraftment, the mainstay of mechanisms for cardiac repair is

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their differentiation to adopt cardiac phenotype together with angiogenesis [11–13]. More recently, a paracrine hypothesis has been proposed which conceives that grafted bone marrow cells produce cytokines, growth factors and other signaling moieties to promote infarct healing process with an as yet undefined mechanism [14,15]. Despite encouraging results in experimental animals and clinical studies, there is a lingering controversy regarding the ability of bone marrow cells to transdifferentiate into cardiomyocytes [16]. Additionally, some safety concerns have been raised regarding bone marrow cells multipotentiality which might lead to their uncontrolled differentiation into undesired lineage [17,18].

Embryonic stem cells have emerged as promising candidates for cardiac repair and may adopt different phenotypes [19]. However, their use is afflicted with several difficulties including technical limitations of undifferentiated propagation in vitro and purification of spontaneously differentiated cardiomyocytes from cell culture. Despite these difficulties, recent progresses in stem cell biology have paved the way to exploit embryonic stem cells as a clinically relevant and renewable source to generate cardiomyocytes and endothelial cells for engraftment [20,21]. The cardiomyocytes and endothelial cells thus generated formed stable grafts in animal hearts without tumor formation. Nevertheless, availability and immune status as cell graft, and teratogenic nature remain debatable issues for their progress in clinical applications [22,23]. More recently, the existence of resident cardiac stem cells has added a new dimension to the approach of stem cell therapy and implies a paradigm shift in the long standing dogma about the heart being unable to repair itself in the event of injury [24]. The myocardial progenitor populations include cells expressing the c-kit, stem cell antigen-1 (Sca-1), side population (SP cells), MDR1, and cells expressing the transcription factor islet-1 (isl-1) [25,26]. The roles of these cells in tissue maintenance, repair, and possible therapeutic applications will be an exciting area of investigation in future.

Of all these available options, choice of donor cell is a matter of the required outcome of the procedure [27]. Whereas the engrafted skeletal myoblasts form neofibers and provide a tenacious support for the weakened myocardium [28], improve oxygenation in the ischemic myocardium [29] and enhance diastolic cardiac function [30], bone marrow cell transplantation mainly improves systolic function and induces angiogenesis. A more relevant approach would be co-transplantation of the two cell types in order to achieve benefits associated with the use of each cell type [31]. Moreover, their transplantation needs to be combined with other strategies in order to generate donor cells which have been “pre-formatted” to survive and engraft in the ischemic heart to support the inadequate intrinsic repair mechanisms.

2. Massive cell death as an impediment of successful heart cell therapy

Besides choice of donor cells, their engraftment is confronted with the problem of poor survival in the host myocardium. A significantly high percentage of the donor cells die within hours after transplantation, thus compromising the optimal outcome of the procedure [32]. In some studies, more than 70–80% of the cells die within 3 days whilst others have shown that 93% of skeletal myoblasts were lost in 2 days after transplantation [33]. Toma and colleagues have reported less than 0.44% mesenchymal stem cell survival by day 4 after engraftment in an immunodeficient mouse heart model [34]. As meager as 1% survival of the transplanted cells have been reported in patient hearts [35]. Although the underlying cause of unusually high and accelerated cell mortality generally remains undefined, these disconcerting reports related with the problematic high attrition of donor cells have implicated multiple factors. Besides other mechanisms, the dynamics of early donor cell death incriminates pathological processes including local immune and inflammatory responses, loss of trophic factors and local tissue ischemia as the prime factors responsible for massive cell death [36]. Hence, it is imperative to reinforce the donor cells to withstand the rigors of the microenvironment of the infarcted heart

incurred from ischemia, inflammatory response, and pro-apoptotic factors in order to develop heart cell therapy into an effective therapeutic modality.

Apoptosis is a highly orchestrated physiological process for programmed killing of the damaged cells. This has been implicated as one of the key pathologic features in acute myocardial infarction and heart failure [37,38]. Depending upon the nature of both intrinsic as well as extrinsic death signals, separate self-regulated signaling pathways get activated to coordinately execute apoptosis by means of common death effector machinery. A schematic representation of the mechanism of apoptotic cell death is given in Figure 1. Initiated by extrinsic or intrinsic stimuli such as the loss of trophic factors, detachment from extracellular matrix, ischemia reperfusion injury or stimulation of death receptors, apoptosis is characterized by morphological alterations including membrane blebbing, chromatin clumping, nuclear condensation and cell shrinkage. These characteristic features of apoptosis are a direct consequence of caspase activation [39]. During extrinsic apoptosis signaling, ligand binding with cell surface death receptors such as TNF-α or Fas/CD95 stimulates recruitment of adaptor proteins to form death inducing signaling complex (DISC) and activation of caspase-8 which ultimately triggers the downstream caspase cascade [40]. Alternatively, intrinsic initiation of apoptosis has been well documented and involves a connection between destabilization of mitochondria by reduction of the mitochondrial transmembrane potential and concomitant release of apoptogenic factors cytochrome-*c*, endonuclease G and AIF, and caspase-3 activation [41]. In summary, the released cytochrome *c* thus initiates the formation of the poptosome, a complex comprising Apaf-1, cytochrome *c*, dATP and pro-caspase-9 to activate caspase-9 which in turn activates pro-caspase-3. Although, cell death receptor and mitochondrial pathways appear separate, a cross-talk between the two is possible and has been reported as the responsible mechanism for the massive host myocyte and donor cell death in the infarcted heart.

3. Strategies to enhance donor cell survival

Cell survival may greatly enhance the effectiveness of transplantation therapy. Therefore, development of a strategy to alleviate apoptotic cell death may be of prime consequence in the treatment of infarcted heart [42]. Concisely, the survival of transplanted cells requires adoption to unfriendly milieu in ischemic myocardium. Several remedial approaches have been recommended to overcome the menace of apoptotic cell death albeit with less than the desired results (Table-1). Exposure to brief hypoxia or anoxia may precondition the cells and make them resistant to subsequent lethal ischemic injury [43]. The approach of therapeutic transgene delivery to the heart either by direct injection of the transgene encoding vector or by engraftment of the genetically modified donor stem cells has given encouraging results [11, 44,45]. Growth factor gene delivery to the heart combined with cell transplantation has given encouraging results in terms of donor cell survival [46]. Ischemic myocardium primed with recombinant growth factor protein treatment prior to transplantation of the donor cells allows better survival of the cell graft [47]. Loss of adherence to the matrix is one of the main initiator of apoptotic cascade. Suspension of donor cells in fibrin glue for intramyocardial delivery significantly improves their retention in the infarcted heart. Moreover, subsequent to intramyocardial injection, fibrin also contributes towards increased survival of the cell graft by providing temporary extracellular matrix for the transplanted cells [48]. Alternatively, injection of fibrin glue into ischemic myocardium increases regional blood supply via neovascularization and provides better oxygenation for the cells. In the same context, collagen as a natural extracellular matrix component has been shown to support cell survival and growth both in cell culture and in vivo after transplantation into ischemic hearts and leads to improved left ventricle contractile function [49]. These strategies will be discussed in length in the later part of the review.

3.1 Combining preconditioning approach with stem cell engraftment

A more effective approach in this regard, however, would be to combine the powerful cytoprotective effects of preconditioning with stem cell engraftment [50,51]. Since the pioneering work of Murry and colleagues which showed that cyclic episodes of brief ischemia and reperfusion, referred to as ischemic preconditioning, rendered the heart more tolerant to subsequent lethal ischemia, preconditioning is considered as the most potent and effective mean of cytoprotection [52]. These authors showed that four 5-minute cycles of ischemia with intermittent reperfusion protected the heart from a subsequent episode of sustained ischemia and infarct size was reduced up to 75%. The cytoprotective effects of ischemic preconditioning can be mimicked in multiple ways such as pharmacological treatment, heat shock intervention, genetic modification of cells for overexpression of growth factors and survival pathway molecules, and ultrasound treatment of cells etc. [53–55].

3.1.1 Ischemic preconditioning for cytoprotection—Ischemic preconditioning improves ischemic tolerance in various body tissues including skeletal and cardiac muscles [56,5758]. In the experimental settings for cardioprotection, ischemic preconditioning significantly attenuated myocardial *infarct size* by reducing both necrotic and apoptotic myocyte death [59,60]. Nevertheless, the protective mechanism of ischemic preconditioning remains contentious despite more than two decades of research. To date, the reported signal transduction pathways initiated by ischemic preconditioning involve the activation of a diverse array of survival proteins including protein kinases A, C, and G, members of the MAPK family (Erk1/2, p38, JNK and BMK1), the phosphatidylinositol`3 -kinase (PI3K) PI3K-Akt cascade, and the JAK-STAT pathway [56,61–63]. In addition to initiation of survival signaling, induction of iNOS in host cardiomyocytes in response to ischemic preconditioning has been implied as a possible alternative mechanism underlying cardioprotection. Subsequent studies have shown that endothelial progenitor cells which act as reservoirs of cytokines get mobilized to the heart in response to ischemic preconditioning and express an array of potentially cardioprotective cytokines including nitric oxide synthase to impart cardioprotection [64]. Further studies confirmed the role of growth factors which are upregulated in response to ischemic preconditioning. In the mice subjected to ischemic preconditioning, myocardial SDF-1α mRNA increased within hours after preconditioning and attenuated the infarct size [65]. Similar observations were made in vitro in cardiomyocytes and resulted in phosphorylation of both ERK1/2 and Akt, decreased phosphorylation of JNK and p38 thus increasing their resistance to hypoxia/ reoxygenation damage. Ischemic preconditioning also enhances vascular endothelial growth factor (VEGF) in the heart and the cardioprotective effects of ischemic preconditioning were abolished in heterozygous Flt-1 knockout mice [66]. These studies show a clear relationship between cardioprotection and growth factor expression.

3.1.2 Pharmacological preconditioning—Activation of mitochondrial pathways which promote cell survival is endogenously occurring process and constitutes an integral part of the homeostatic mechanism [52]. These effects have been mimicked via pharmacological intervention using mitochondrial potassium channel openers [67,68]. A chemically diverse group of compounds such as nicorandil, bimakalim, minoxidil and diazoxide share the property of promoting K^+ current through ATP sensitive K^+-channels [69–72]. Amongst these, the prototype mitoK_{ATP} channel opener diazoxide has been widely demonstrated to suppress cell apoptosis and promote cell survival [50,73]. Although their exact mechanism of cytoprotection remains undefined, these pharmacological agents act via multiple mechanisms. This may include succinate dehydrogenase inhibition, mitochondrial depolarization, protein kinase-C (PKC) activation and involvement of a potassium conductance-independent pathway for cellular protection [74–77]. Our group has shown that cardiac protection from mitoKATP channels is dependent on Akt translocation from cytosol to mitochondria [78].

In vitro cellular models are being studied in order to elucidate the underlying molecular mechanisms of tolerance and anti-apoptotic effects after preconditioning [68,79,80]. Based on these observations, it is hypothesized that preconditioning initiates survival signaling in the donor cells and set these cells into a "primed state" to encounter the rigorous harsh microenvironment of the ischemic myocardium. The preconditioned cells also release biologically active factors to act in a paracrine manner to exert cytoprotective effects on host myocytes. Depending upon the preconditioning stimulus, signaling pathways thus induced increase proliferation and differentiation of the implanted cells. In a recent study, we have shown that preconditioning of skeletal myoblasts using diazoxide was effective in promoting their survival in the infarcted heart post engraftment [81]. Elucidating the possible underlying mechanism, we observed that diazoxide preconditioning of the cells significantly favored Akt phosphorylation; a step which is crucial in survival signaling cascades. We also observed explicit increase of IL11 protein levels in the preconditioned myoblasts which occurred in PI3K/Akt and p42/44 MAPK-STAT3 dependent fashion, thus suggestive of its involvement in survival signaling of preconditioned myoblasts. We thus proposed that the secreted IL11 acted in autocrine and paracrine fashion to promote survival signaling in the preconditioned myoblasts and enhanced their survival under oxidant stress in vitro as well as post engraftment in a rat heart model of acute myocardial infarction (Figure 2). In order to demonstrate the versatility of this approach, we have successfully preconditioned bone marrow derived mesenchymal stem cells to promote their resistance to oxidant stress and achieve improved survival in the infarcted heart [53]. Interestingly we observed that preconditioning promoted mesenchymal stem cells survival and proliferation via activation of NFκB downstream of PI3K/Akt signaling. The preconditioned mesenchymal stem cells showed higher activity of phospho Akt as was evident from Akt activity assay and upregualted expression of RelA (member of NFκB). Treatment of cells with wortmannin prior to preconditioning abolished the effects of preconditioning and significantly reduced their survival post engraftment. Our results clearly depict that preconditioning of cells with diazoxide involves more than just K+ channel activity and multiple other molecular mechanisms are involved in the preconditioned cells which improve their resistance to oxidant stress. Moreover, pharmacological preconditioning of stem cells before transplantation will be highly effective in overcoming the problem of donor cell death post engraftment.

3.2 Heat shock preconditioning

Among other mechanisms, preconditioning involves stressor factors or heat-shock proteins (Hsps) to act as molecular chaperones to regulate newly synthesized proteins to be folded and transported across the membrane, or prevent incorrect protein folding and transportation thus enhancing the ability of stressed cells to cope with increased concentrations of denatured proteins [82,83]. Hsps are recognized as molecules that maintain cellular homeostasis during changes in the environment and their role under various pathological conditions has also been elucidated based on their altered gene expression [84]. Hsps can be induced under stressful conditions especially by physiologically relevant hyperthermia and are responsible for survival and antiapoptotic signaling in cells. The genomic response of mammalian cells to thermal stress however, is considerably more complex than previously recognized. It is therefore difficult to perform large-scale analysis of the effects of heat shock on normal cells. Hsps encompass a plethora of proteins that range in molecular weight from 10 to 150 kDa and are present in most cells albeit with variable pattern of distribution and expression [85]. Of all the Hsp families, Hsp70 family is considered to be induced as the first response to non-physiological conditions and includes the constitutively expressed Hsp73 in normal cells and the highly stress-inducible Hsp72. Hsp70 is positively related with thermotolerance of the cells and has been well studied for its role in stress related cytoprotective effects in general and cardioprotective actions in particular. The potential mechanism responsible for the cytoprotective effects of Hsp70 on stress-induced injury remains undefined. Molecular studies have shown p38 MAPK and c-jun

N-terminal kinase (JNK) increase levels of Hsp70 in the heart in response to hypoxia [86]. The role of cellular c-jun to promote cytoprotective activity of Hsp has also been studied in relation with Hsp90 [87]. In addition, induction of Fas-mediated signaling rapidly decreases inducible Hsp70 expression, and activation of Hsp70 suppresses Fas-mediated apoptosis [88]. These data imply that the inducible Hsp70 possibly acts as a negative regulator of Fas-mediated apoptosis. More so, the protective effects of Hsps are partly due to prevention of pro-inflammatory cytokine production and down-regulation of NF-κB in several cell types [89].

Keeping in view the cytoprotective nature of Hsps, the approach of heat shock preconditioning may be exploited to promote donor cell survival under oxidant stress in vitro and post engraftment in heart cell therapy. Due to the variable and complex nature of heat shock response from different cells, the preconditioning process needs to be extensively optimized prior to cell engraftment. In a study involving cardiac myoblasts, exposure of the cells to physiologically relevant hyperthermia improved their subsequent resistance to oxidant stress [90]. The temperature and duration of exposure are crucial determinants of the quality and the extent of resistance developed in the cells. Whereas chronic exposure to 39°C produced no discernible change in proliferation and intrinsic ATP levels in the cells, it successfully promoted their resistance to subsequent oxidant stress via constitutive upregulation of Hsp70 which served as a first line of defense. Thermal shock also promoted cell division and proliferation during acute phase post engraftment but there was no evidence that such a treatment contributed towards long term improvement in donor cell engraftment [55,91]. Studies have shown that the genetically modified myocytes overexpressing Hsp72 were more tolerant to hypoxic stress as compared with their normal counterparts [92]. Similar results were later duplicated in Hsp70 transgenic animals in the heart which conferred attenuated infarct size and significant functional recovery subsequent to experimental ischemia and reperfusion injury [93]. These results clearly demonstrate a possible role of Hsps as an effector molecule during preconditioning. To date, there is only meager published data investigating the direct consequences of thermic shock to induce differentiation pathways. Short-term chemical stimulation of rat mesenchymal stem cells using 2 mM β-mercaptoethanol for neuronal differentiation induced Hsp72 synthesis which simultaneously increased their survival under oxidant stress [94]. Heat shock treatment of telomerase-immortalized human mesenchymal stem cells prior to culture in differentiation medium induced osteoblastic differentiation of the cells [95].

Besides Hsp70, all other Hsps in general and the sub-family of small Hsps in particular are involved in cytoprotection [96]. Small Hsps constitute a large family of proteins with monomeric molecular weight of 10–30 kDa. Unlike the high molecular weight Hsps which are involved in protein folding *in vivo*, small Hsps play an important role in protecting the organism from stress. Hsp27 is highly expressed in the heart and acts as an endogenous cytoprotective stress response protein, eliciting cardioprotection via its role as a molecular chaperone. By serving as the terminal substrate of the p38 MAPK cascade, Hsp27 affords a functional link between p38 MAPK and the actin cytoskeleton [97]. It also interacts with Akt to maintain the kinase in an active conformation state [98]. Transgenic overexpression of Hsp20 in cardiomyocytes was associated with improved contraction and protection against beta-agonist-induced apoptosis. The extent of infarction and apoptotic cell death was significantly reduced in the transgenic hearts with an associated increase in protein ratio of Bcl-2/Bax and reduced caspase-3 activity.

3.3 Cytokine preconditioning of stem cells

Deprivation of trophic factors induces apoptosis in the cells which are dependent on these factors for survival. Treatment of stem cells with growth factor proteins which orchestrate improved survival, differentiation and proliferation may significantly improve their effectiveness in cardiac repair process [99]. Cytokine preconditioning basically involves growth

factor interaction with growth factor receptors present on the surface of cells to activate downstream signal transduction for cell survival.

Unlike other chemokines which interact with multiple G-protein coupled receptors, SDF-1α mediates its effects through its only known specific receptor CXCR4. The SDF-1α/CXCR4 ligand/receptor system is distributed in different tissues and cell types and modulates several biological functions through signal transduction pathways. These include increased cell growth, proliferation, survival and anti-apoptosis, emigrational and transcriptional activation. We have recently shown that chemokine preconditioning of mesenchymal stem cells by treatment with the recombinant SDF-1α protein enhanced their survival and proliferation under anoxic conditions in vitro and after engraftment in the infarcted heart [51]. The pro-survival effects of SDF-1α were abolished by pretreatment of the cells with CXCR4 blocker AMD3100 and were found to involve activation of PI3K/ Akt pathway. The preconditioned mesenchymal stem cells also released antiapoptotic and angiogenic cytokines for paracrine consequences. The cumulative effect of preconditioning on myocyte regeneration, angiogenesis and cell survival in the ischemic heart resulted in reduced infarct size and left ventricle remodeling. Similar results have also been observed with other growth factors. The addition of VEGF2 to endothelial progenitor cell cultures resulted in significant and dose-dependent decreases in endothelial progenitor cell apoptosis. Incidentally, the cells treated with VEGF-2 showed elevated expression of phosphorylated Akt [46]. In an interesting study, H9c2 cardiomyoblasts grown on collagen matrix supplemented with VEGF and basic fibroblast growth factor (bFGF) showed improved survival and growth characteristics. The bio-artificial graft thus generated was later used for myocardial restoration [49]. The use of bio-artificial graft approach may also promote retention of the transplanted cells as it will overcome the problem of cellular washout which generally accounts for significantly high loss of cells after intramyocardial injection.

Besides anti-apoptotic effects, cytokine preconditioning may stimulate the release of growth factors for paracrine influence on the host myocytes and for paracrine/ autocrine cues to direct the stem cells to adopt certain specific phenotype. Different growth factors act as key mediators of cellular differentiation which is regulated by the multiple factors. Besides other factors, the developmental fate of stem cells is determined by their inherent plastic nature and the extracellular micro environmental cues emanating from growth factors constituting the cardiac milieu. Conversely, an optimal combination of growth factors for directed differentiation of stem cells into the desired lineage remains a challenge. Many of these growth factors exert non-specific effects on stem cell differentiation through the activation of multiple intracellular signaling pathways. Culture of CD133+ cells in $VEGF_{165}$ and brain-derived nerve growth factor, either alone or together, promoted their myo-endothelial co-differentiation [100]. In the same context, we have also performed transduction of embryonic stem cells for $VEGF_{165}$ over expression which caused their endothelial differentiation besides incurring apoptotic resistance [101]. Several growth factor including bone morphogenetic protein-2 and bFGF, have been implicated as critical components of this early cardiomyogenic inductive signaling [102]. Pretreatment of bone marrow cells with a cocktail of growth factors promotes their cardiomyogenic differentiation [103]. Transforming growth factor (TGF) family includes 30 growth and differentiation factors including TGF-β, activins, inhibins, and bone morphogenetic proteins. TGF-β is important for progression of differentiation in most cells. There is an evidence that TGF-β family proteins are able to redirect differentiation of stem cells that have fully differentiated or are engaged in differentiation along a particular lineage [104].

Most of the above-mentioned studies reflect the cytoprotective effects of growth factors and their ability to direct their differentiation. We have recently shown that IGF-1 preconditioning of stem cells has dual effects of cytoprotection as well as improved integration of the preconditioned cells with the host myocytes post engraftment via upregulated expression of connexin 43. A wide range of studies have shown that IGF-1R has specific anti-apoptotic

signaling capacity which enables IGF-1 to mediate intracellular signaling. IGF-1/IGF-1R ligand/receptor interaction causes sequential activation of signal transduction pathways which prevents cell apoptosis [105]. Two major pathways activated by IGF-1/IGF-1R interaction included PI3K/Akt and mitogen-activated protein kinase (MAPK)/Erk1/2. The Akt pathway is initiated by the interaction of IGF-1R with one of its major substrates, insulin receptor substrate-1 that activates PI3K and Akt. The MAPK (p44/p42) is another anti-apoptotic pathway of the IGF-1R system and gets activated by mitogenic signals, DNA damage, or oxidative stress. Furthermore, this particular pathway has been implicated in the protection from apoptosis by IGF-1R [106]. In one of our recently concluded studies, we performed ex vivo preconditioning of bone marrow derived Sca-1+ cells with IGF-1 which markedly promoted their survival and proliferation in the infarcted heart. Elucidating the mechanism of IGF-1 preconditioning, we found that the preconditioned cells had higher phosphorylation of IGF-1R which activated 5 fold more expression of connexin 43 in the preconditioned cells in a PI3K/Akt dependent manner. Interestingly, when connexin 43 gene silencing studies were performed in the IGF-1 preconditioned cells using connexin 43 specific siRNA, we observed that the survival of the cells was significantly compromised under anoxia in vitro and after engraftment in the infarcted rat heart. These observations clearly depicted a role for connexin 43 in Sca-1 + cell survival downstream of PI3K/Akt. Interestingly, the preconditioned cells continued to overexpress connexin 43 after engraftment which helped these cells to engraft better than their non-preconditioned counterparts. Thus it is clear that IGF-1 preconditioning of stem cells concomitantly met the two fundamental requirements for heart cell therapy; donor cell survival and their engraftment, by upregulated expression of connexin 43 which played a dual role of cytoprotection as well as engraftment of the transplanted cells.

Besides growth factors, delivery of the pro-survival factor cocktail has been attempted to achieve improved survival of the donor cells [107]. The full pro-survival factor cocktail containing 50% (vol/vol) growth factor–reduced Matrigel, supplemented with ZVAD (100 mM, benzyloxycarbonyl-Val-Ala-Asp(O-methyl)-fluoromethyl ketone, Calbiochem), Bcl-XL BH4 (cell-permeant TAT peptide, 50 nM, Calbiochem), cyclosporine A (200 nM, Wako Pure Chemicals), IGF-1 (100 ng/ml, Peprotech) and pinacidil (50 mM, Sigma). The constitution of the cocktail was based on an attempt to protect the cells by addressing the most prevalent multiple parallel processes responsible for cell death.

Despite promising results, one impediment in the use of growth factors or proteins from survival signaling pathways is the short biological half-lives of most of the protein factors which necessitates multiple administrations of growth factors to have stable therapeutic effects. This problem may be overcome by genetic modification of stem cells for growth factor expression.

3.4 Genetic modulation of stem cells

Ex-vivo genetic modulation of stem cells prior to transplantation has been extensively studied for delivery of transgenes encoding for growth factors and anti-apoptotic factors to confer apoptotic resistance and enhance cell viability of the donor cells and host myocytes [108]. There is evidence in literature that the cells overexpressing angiogenic growth factors show better survival and neovascularization after engraftment and improve cardiac function by limiting the remodeling process in the scar and/or decreasing apoptosis of the hypertrophied myocytes in peri-infarct region [11,44,45]. Moreover, the genetically modified cells serve as a reservoir of the therapeutically active transgene expression product which may function in autocrine and paracrine manner to confer therapeutic effects.

3.4.1 Genetic modulation of stem cells for growth factor expression—The delivery of angiogenic growth factors by genetic manipulation has given encouraging results [44,109].

Transplantation of ex-vivo genetically modified cells for VEGF grants early phase and late phase effects. Firstly, survival signaling is activated in the cells which reduces apoptotic index of donor cells and host myocytes. Secondly, there is improved regional blood flow in the ischemic myocardium through VEGF induced vasodilatation thus leading to preservation of the cell graft as well as myocardial architecture in the ischemic area during the early phase after engraftment. This is followed by late phase angiogenic effect which salvages the host myocardium, in conjunction with the functional benefits of cellular cardiomyoplasty from the donor cell derived neomyogenesis [110].

Heme oxygenase-1 (HO-1), the rate-limiting enzyme of heme catabolism, is known to modulate various cellular functions, including cytokine production, cell proliferation, and apoptosis, in stress-related conditions. HO-1 plasmid modification of graft mesenchymal stem cells protected the cells from subsequent hypoxia injury in vitro when subjected to 1% oxygen for 24 h [111]. Engraftment of the HO-1 gene modified cells showed 5-fold increase in cell graft survival in ischemic heart in vivo as compared with the cells without gene modification. The increase in cell survival was attributed to anti-inflammatory and anti-apoptosis activities of HO-1 which reduced infiltration of mononuclear cells and down-regulated the expression of pro-inflammatory cytokines. These findings underscore the role of angiogenic growth factor gene delivery for protection of the cell graft from ischemia/inflammation induced death. Similar results have also been reported with other angiogenic growth factors [112].

In an attempt to enhance cytoprotection with angiogenic gene delivery, multimodal gene therapy approach has been devised in which multiple growth factor genes are concomitantly delivered to the heart for their combined effects. Nevertheless, an optimal combination of multiple genes is required to be determined for their supplemental and synergistic interaction to achieve the desired therapeutic outcome. Yau and colleagues delivered VEGF and IGF-1 encoding transgenes to the infarcted rat heart using mesenchymal stem cells [113]. Although, independent expression of VEGF and IGF-I was cytprotective, the combined VEGF and IGF-1 delivery synergistically contributed towards survival of the cell graft. On the same note, we have reported a novel bicistronic vector encoding for VEGF and angiopoietin-1 (Ang-1) which was used for simultaneous delivery of the two genes to infarcted heart in a porcine model of coronary artery ligation [114]. The transfected cells co-overexpressed VEGF and Ang-1 at the site of cell graft with resultant attenuation of infarct size expansion and improved regional blood flow in the infarcted myocardium.

IGF-I is an important extracellular effector molecule which is produced in the heart and other tissues and acts in both autocrine as well as paracrine manner for pleiotropic effects. IGF-1 gene delivery has also been combined with heart cell therapy to promote donor cell survival, engraftment and differentiation in the host myocardium [115,116]. In a recently concluded study, we have carried out mesenchymal stem cell based delivery of IGF-1 to the infarcted rat heart and exploited IGF-1/IGF-1 ligand/receptor signaling for distinct beneficial effects on the carrier cells as well as host myocytes to promote their survival and proliferation (our unpublished data). IGF-1 overexpressing mesenchymal stem cells showed significantly higher survival when subjected to 8h anoxia as compared with the non-transfected cells. Molecular studies showed that IGF- 1 overexpression exerted its biological effects by binding to its transmembrane receptors and stimulated Akt phosphorylation at Thr308 and Bcl2 via PI3K signaling. Upon transplantation into infarcted rat heart, the transplanted cells continued to overexpress IGF-1 and protected the host myocytes as was evident from lower TUNEL positivity. Histochemical examination of tissue sections at 7 days post engraftment of the cells showed numerous islands of the surviving host myocytes in the centre of the infarct as a result of IGF-1 overexpression on host myocytes (Figure 3).

Intrinsically produced SDF-1α protein level in the heart remains elevated for 5–7 days after infarction thus signifying its specific role in the repair process [117]. Protein and gene delivery of human SDF-1α (hSDF-1α) targeted to the infarcted heart, has been employed for cytoprotection of myocytes in order to attenuate infarct size expansion [51,118]. In our subsequent work, overexpression of hSDF-1α supported cell survival during the initial phase after transplantation [119]. Our results depicted successful and efficient non-viral vector transfection of skeletal myoblasts with hSDF-1α using FuGene™6 in the presence of $ZnCl_2$ which was effective for indirect gene therapy after myocardial infarction.

3.4.2 Transgenic overexpression of pro-survival signaling molecules—Targeting of pro-survival genes is a novel modality to modulate cell survival signaling for the desired therapeutic effects. Transgenic overexpression of pro-survival signaling molecules promotes resistance of the donor cells under ischemia and supports their survival. PI3-kinase is a critical component in a signal transduction pathway which mechanistically acts through activation of Akt [120]. Transduction of cardiomyocytes with PI3-kinase encoding adenoviral vector for overexpression of PI3-kinase significantly enhanced their survival upon subsequent exposure to hypoxia [121]. This was attributed to the activation of various protein factors including Akt, a major player in survival signaling cascade in many systems, and its downstream pro-survival signaling molecules [122]. Therefore, Akt gene modification of stem cells has been demonstrated to significantly enhance their survival post engraftment in the infarcted heart [45]. Intramyocardial injection of 5×10^6 Akt overexpressing MSCs reduced collagen deposition and attenuated infarct size in rat heart model by inhibition of cardiomyocyte apoptosis in the peri-infarct area. There was significant inhibition of the remodeling process and complete normalization of systolic and diastolic functions. Later studies from the same research group showed that Akt-modified mesenchymal stem cells released paracrine factors which immensely contributed towards host myocyte survival and functional recovery of the infarcted heart [14]. Using a comprehensive functional genomic strategy, secreted frizzled related protein 2 (Sfrp2) was found as the paracrine factor that mediated myocyte survival and repair of the infarcted heart after ischemic injury via modulation of Wnt signaling [123]. However, we argue that engraftment and longer term growth of donor cells may be less meaningful if regional blood flow in the ischemic myocardium is not restored. We opted to genetically modify mesenchymal stem cells for concomitant overexpression of Akt and Ang-1 [11]. Ang-1 has a pivotal role as the modulator of vascular development by promoting formation of stable and leak resistant blood vessels [124]. More importantly, Ang-1 enhances endothelial cell survival via PI3K/Akt pathway [125]. A major role has been shown for Akt downstream of Ang-1/Tie2 signaling pathway and is the primary mediator of endothelial cell survival via Akt/FKHR transcription factor [126]. Our choice of Ang-1 and Akt transgene combination achieved maximum beneficial effects on cell survival and angiogenesis. When subjected to anoxia for 8h, we observed that cells co-overexpressing Akt and Ang-1 were more resistant to anoxic injury as compared with their cell counterparts transduced with null vector or with either of Akt or Ang-1 alone. We also observed that mesenchymal stem cells co-overexpressing Akt and Ang-1 were able to engraft better after transplantation in an infarcted rat heart and were able to adopt myogenic and endothelial phenotype.

Alternatively, the 26 kD antiapoptotic protein Bcl-2 belongs to the Bcl-2 family of proteins and serves as a critical regulator of pathways involved in apoptosis. Bcl-2 regulates the metabolic functions of mitochondria during ischemic conditions and contributes to cytoprotection [127]. Mesenchymal stem cells genetically modified to over express Bcl-2 can delay the onset of cell death and modestly augment viable cell growth in the first 48 hours of apoptosis. Cardiomyoblasts overexpressing Bcl-2 showed increased resistance to apoptosis and survived better after engraftment [128]. Interestingly, overexpression of Bcl-2 in transgenic mice improved the heart function and inhibited cardiomyocytes apoptosis [129]. Bcl-2 gene is known not only for regulating apoptosis but also controls non-apoptotic cell death [130].

Recently, transgenic cell lines derived from embryonic stem cells expressing Bcl-2 were found to self-renew continuously, even under serum-free and feeder cell-free conditions [131]. A comparison of Bcl-2 overexpression and heat shock treatment for anti-apoptotic effects on smooth muscle cells showed significantly reduced cell loss after transplantation and improved cardiac function after myocardial infarction. Still, Bcl-2 modified cells showed better survival and reduced inflammatory response [132]. Despite encouraging results from the studies which involved targeting of survival signaling molecules in the cells, the approach is not without some undesired effects. The downside of this approach is that overexpression of Akt and Bcl-2 genes may predispose the cells to neoplasia and hence, increase the risk of tumorigenesis. Therefore, the level of expression and the time duration for it's continuation should be optimized to avoid undesired outcome.

4. Concluding remarks

Improvement in cardiac function subsequent to stem cell transplantation is directly related with the number of cells injected and retained at the site of cell graft. The loss of cells due massive cell death and cellular washout is generally compensated by a very high number of cell engraftment. Optimization of cell engraftment conditions with special emphasis on cell survival strategies may significantly enhance the functional outcome of the procedure. Cell death is a multi-factorial phenomenon and may warrant multi-prong strategy to improve cell survival. Addressing one single factor responsible for cell death may not be an effective strategy. Defining approaches to reduce the harsh microenvironment of the host ischemic myocardium and priming the cells to a state of 'readiness' by stimulation of their survival signaling pathways may significantly address this issue. Ischemic preconditioning is a powerful protective phenomenon against lethal ischemic injury. Signaling pathways of ischemic preconditioning can be successfully employed for anti-apoptotic measures in cell based therapies. Cytokine treatment prevents left ventricular remodeling and dysfunction after myocardial infarction through increased neovascularization and reduced apoptosis in the border area [133]. Cytokine therapy combined with stem cell transplantation for priming the heart prior to stem cell transplantation makes the host myocardial milieu more hospitable for the cells. Alternatively, preconditioning of stem cells with cytokines may upregulate pro-survival proteins and transcription factors to facilitate their directed differentiation to adopt specific cardiac phenotype. Pharmacological preconditioning may be more appropriate to take advantage of molecular mechanisms responsible for protection against ischemic injury during stem cell transplantation and further activating endogenous cellular machinery for cellular regeneration. With the emergence of paracrine factor hypothesis, combining multiple cell types with inherent ability to release paracrine factors, co-administration of cell types dedicated to varying potentials and different functions may help the donor cells survive better [134]. The success of this approach however, will be determined by the choice of the donor cells being employed for simultaneous engraftment. Combining cells with angiogenic as well as myogenic potential may provide an ideal combination to develop a biological bypass via neovascularization to restore regional blood flow in the ischemic myocardium and improve contractile function of left ventricle by undergoing myogenic differentiation. Additionally, these cells may be preconditioned to fortify their resistance to ischemia through stimulation of their survival signaling pathways.

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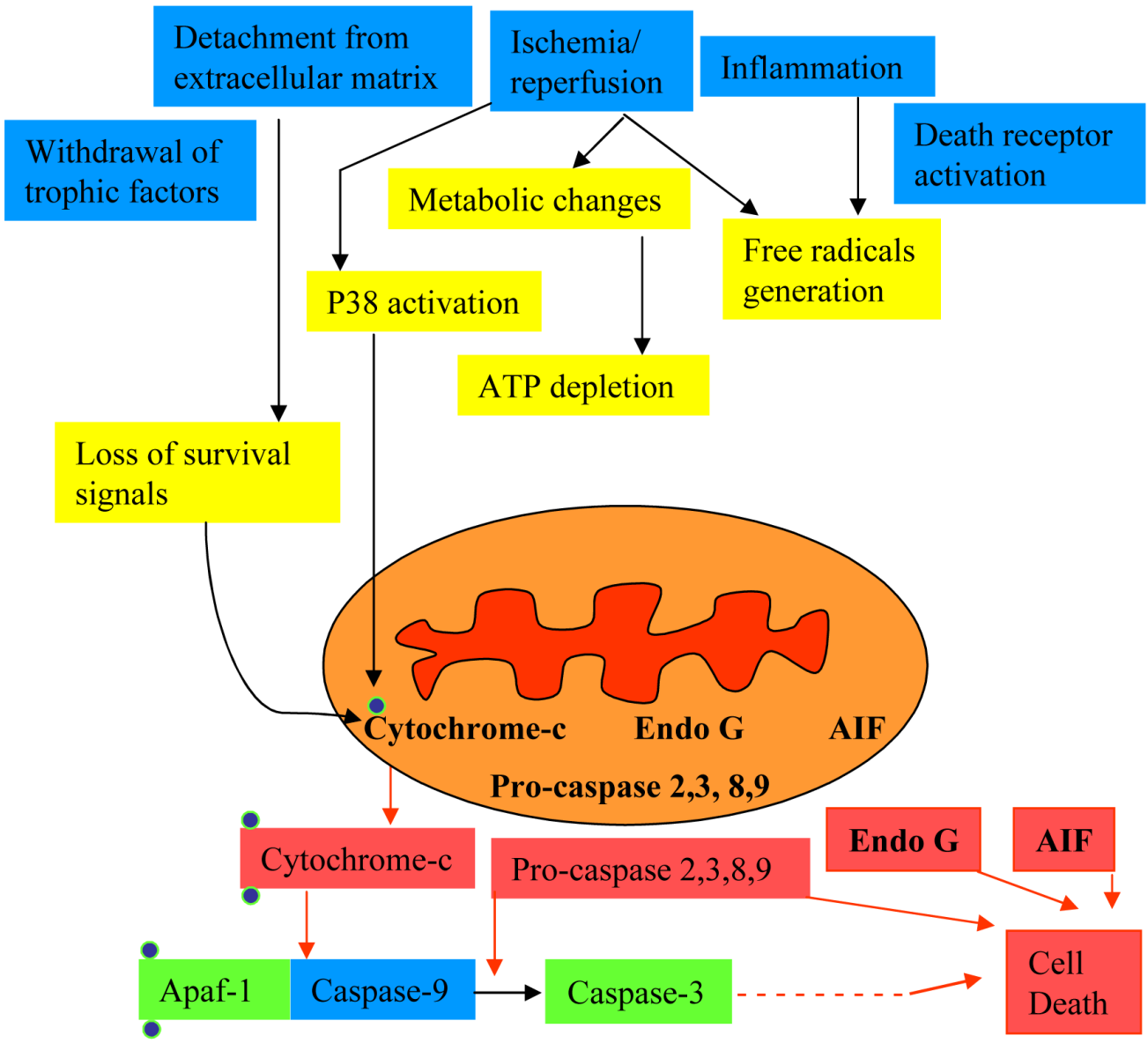

Figure 1.
Schematic representation of the key players in signaling pathways responsible for apoptotic cell death.
(*AIF= apoptosis inducing factor*; *Apaf= apoptosis protease activating factor*; *EndoG=endonuclease G*).

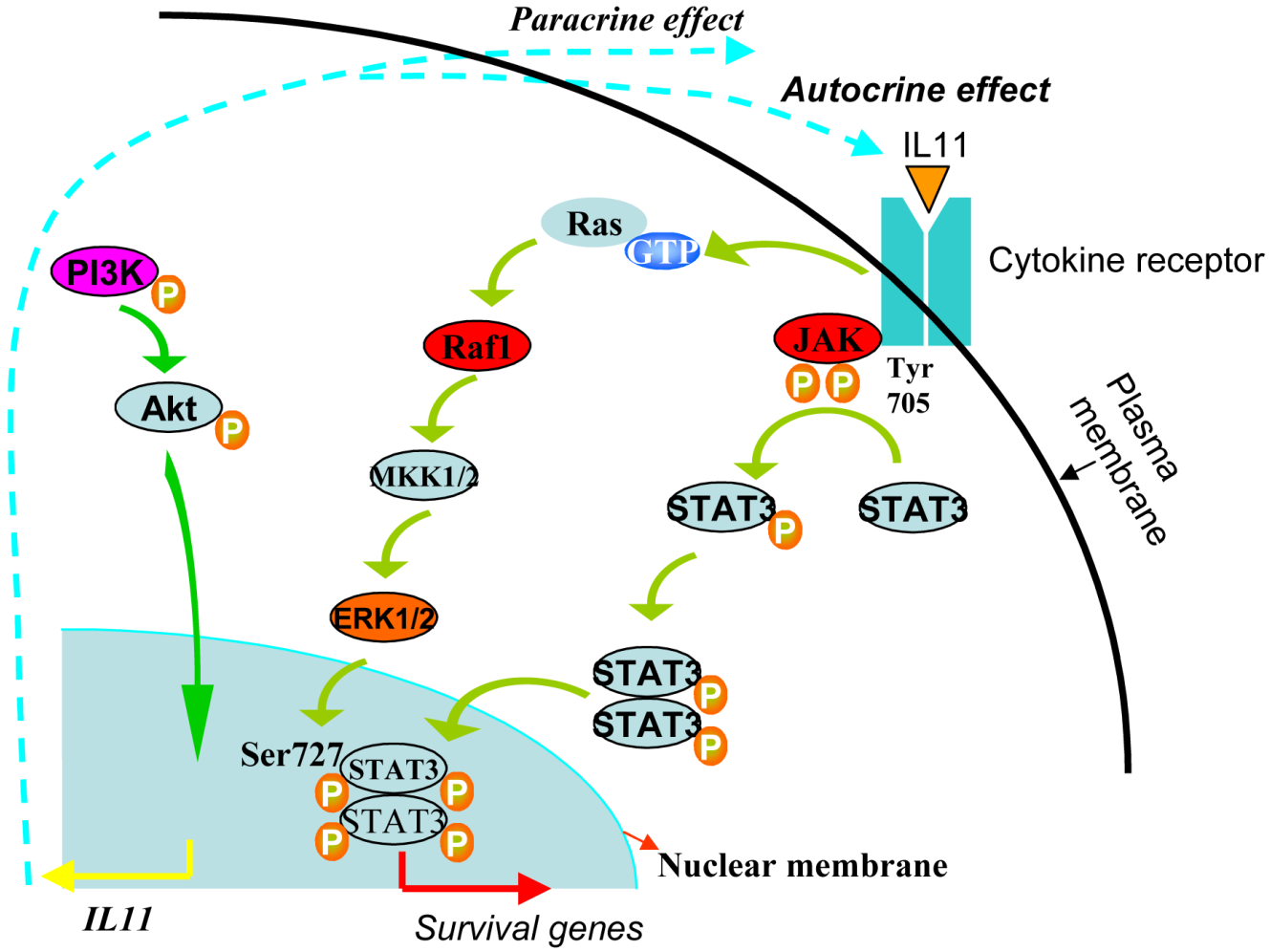

Figure 2.
Proposed mechanism involved in pharmacological preconditioning of skeletal myoblasts. Release of IL11 downstream of PI3K/Akt triggers autocrine and paracrine effects on the cells and involves ERK1/2 and STAT3 activation to promote cell survival.

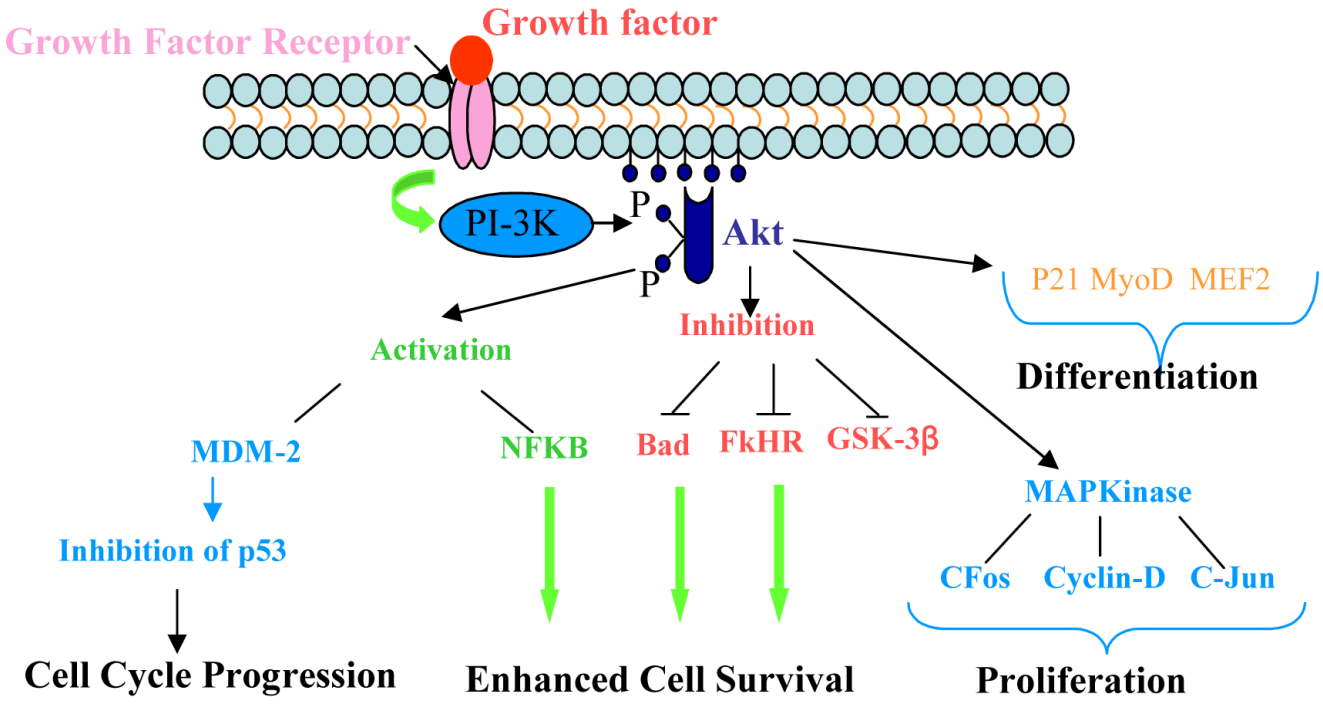

Figure 3.
Schematic representation of growth factor interaction with growth factor receptors on the cell surface to initiate PI3K/Akt pathway responsible for cell cycle progression, survival, and proliferation.

Table 1

Strategies to improve cell survival

Pro-survival Strategy	References
Anti-inflmmatory or immune treatment	
Anti-LFA-1	135,136 137
Interleukin-1 receptor antagonist	33,138,139
Depletion of C3 complement	140
Treatment of anti-CD154 antibody	141
CD4 and CD8 depletion	36
Natural killer cells depletion	36
Treatment of immunosuppression	142,143
Growth factors	
Fibroblast growth factor	144,145
Transforming growth factor-1	146,147
Erythropoietin	148
Insulin-like growth factor-I	149
Vascular endothelial growth factor	150,151
Others	
Purity of myoblasts (Desmin)	33
Donor and host myosin heavy chain	152
Heat shock treatment	55,153
Optimization of injection procedure	142
Fibrin glue	48