Published in final edited form as:

Free Radic Biol Med. 2011 December 15; 51(12): 2150–2157. doi:10.1016/j.freeradbiomed.2011.09.037.

Nitric oxide-cyclic GMP signaling in stem cell differentiation

Kalpana Mujoo^{*1}, Joshua S. Krumenacker², and Ferid Murad³

¹Brown Foundation Institute of Molecular Medicine, Texas Therapeutics Institute, UT Health Science Center at Houston, Houston, TX 77030

²Indiana University School of Medicine, Department of Family Medicine, Indianapolis, IN 462002

³Department of Biochemistry and Molecular Biology, George Washington University, Washington DC, 20037

Abstract

The nitric oxide-cyclic GMP (NO-cGMP) pathway mediates important physiological functions associated with various integrative body systems including the cardiovascular and nervous systems. Furthermore, NO regulates cell growth, survival, apoptosis, proliferation and differentiation at the cellular level. To understand the significance of the NO-cGMP pathway in development and differentiation, studies have been conducted both in developing embryos and stem cells. Manipulation of the NO-cGMP pathway by employing activators and inhibitors as pharmacological probes and/or genetic manipulation of NO signaling components has implicated the involvement of this pathway in regulation of stem cell differentiation. This review will focus on some of the work pertaining to the role of NO-cGMP in differentiation of stem cells into cells of various lineages particularly into myocardial cells and stem cell based therapy.

Keywords

Nitric oxide; cyclic GMP; stem cells; differentiation

Introduction

Nitric oxide (NO) is a diatomic free radical and one of the most important signaling molecules in mammalian physiology. NO mediates a host of physiological events such as smooth muscle relaxation, vasodilatation, neurotransmission, inhibition of platelet aggregation, and immunomodulation [1-3]. Furthermore, at the cellular level, NO regulates cell growth, survival, apoptosis, proliferation and differentiation [3]. NO is also involved in the pathophysiology of several diseases such as diabetes, cancer, cardiovascular disease and various inflammatory conditions due to its reduced bioavailability, production or due to overproduction of NO [2]. NO is synthesized by a group of enzymes known as nitric oxide synthases (NOS) by oxidation of a guanidino nitrogen of L-arginine into L-citrulline and NO [3]. There are 3 isoforms of NOS (NOS-1, NOS-2 and NOS-3) which utilize a complicated array of co-factors and co-substrates such as molecular oxygen, NADPH, tetrahydrobiopterin (BH4), flavin mononucleotide, flavin adenine dinucleotide, heme and

^{*}Correspondence and request for reprints: Kalpana Mujoo, Ph.D., Brown Foundation Institute of Molecular Medicine, SRB 530B, UT Health Science Center at Houston, Houston, TX 77030, Phone: 713-500-2428, FAX: 713-500-2447, Kalpana.Mujoo@uth.tmc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

calcium/calmodulin for the synthesis of NO [2-3]. NOS-1 (neuronal NOS) and NOS-3 (endothelial NOS) are constitutive NOS enzymes regulated by calcium/calmodulin. NOS-2 enzyme (inducible NOS) is induced in response to inflammatory stimuli and is activated independent of calcium as the enzyme is always bound to calcium/calmodulin once it is synthesized. The three isoforms of NOS are encoded by three different genes on different chromosomes and they exhibit 50-60% identity with each other and cytochrome p450 enzymes [4-7] while the identity of a particular isoform between species can be between 85-90%. The NOS isoforms have a heme prosthetic group and are active as homo-dimers consisting of two identical monomers which can be divided into a C-terminal reductase carboxy domain and an N-terminal oxygenase amino domain [5, 8].

NO mediates its downstream effects either through cyclic GMP (cGMP) dependent or independent mechanisms. The cGMP dependent mechanism is mediated through its receptor soluble guanylyl cyclase (sGC). cGMP independent effects of NO are mediated through its interaction with metal complexes, oxygen (O_2) and superoxide ($O_2^{\cdot -}$) that mediate various downstream events. Interaction of NO with oxygen species leads to nitrosation and nitration of proteins. These posttranslational modifications particularly occur during an inflammatory response and in cases where superoxide dismutase is not able to scavenge excess superoxide ($O_2^{\cdot -}$) [1].

This review is particularly focused on demonstrating how manipulation of the NO/cGMP pathway is involved in regulation of stem cell differentiation. In addition to discussing our contributions to the stem cell field pertaining to NO/cGMP, we have included major findings wherever possible. We do apologize to some investigators for the omissions made, as it is not possible to include more than 120, 000 publications on the NO-cGMP pathway.

Guanylyl cyclase and cGMP

The second messenger cGMP is synthesized by a family of enzymes known as guanylyl cyclases that share amino acid sequence with the catalytic domain of the adenylyl cyclases which synthesize another second messenger, cyclic AMP [9]. Two classes of guanylyl cyclases (soluble and particulate) synthesize cGMP from intracellular guanosine triphosphate (GTP). Particulate cyclases (pGC) are membrane bound receptors that bind to ligands such as natriuretic peptides, guanylin peptides and *E. coli* heat stable enterotoxin (STa).

Soluble guanylyl (sGC) is the only known physiological receptor of NO. sGC is a heme containing obligatory hetero-dimer composed of α (~ 80 kDa) and β (~ 70 kDa) subunits which make up the active enzyme. To date, four sGC isoforms (α_1 , α_2 and β_1 , β_2) encoded by four different genes have been identified. However, only sGC α_1/β_1 and sGC α_2/β_1 heterodimers are activated by NO [2-3, 10]. sGC α_1/β_1 are ubiquitously expressed in adult mammalian tissue with the highest levels in the brain, lung, heart, kidneys, muscle and spleen [11]. Furthermore, vascular smooth muscle and endothelial cells also predominantly express α_1 and β_1 subunits [12]. However, in addition to sGC α_1/β_1 the brain exhibits the highest expression of sGC α_2/β_1 form as well. The formation of α_2/β_1 heterodimer was also identified in the human placenta [10].

Each sGC subunit consists of three domains which make up its structure and function: a) an N-terminal heme-binding domain; b) a dimerization domain and c) a C-terminal catalytic domain. At the functional level, NO binds to the heme prosthetic group of its physiological receptor sGC, which catalyzes the conversion of GTP to the second messenger cGMP. NO mediates most of its physiological effects as a cell signaling molecule through the production of cGMP by sGC. cGMP directly interacts with its downstream effectors such as cGMP dependent kinase (cGKI or PKG), cyclic nucleotide gated channels (CNG) and

cGMP dependent phosphodiesterase (PDEs) to mediate physiological effects such as vascular smooth muscle tone and motility, phototransduction, and maintaining fluid and electrolyte homeostasis [13-15]. The genomic structures and promoters of sGC α_1 and sGC β_1 subunits and transcriptional regulation of sGC expression have been previously described [16-17].

In response to NO, sGC activity increases more than 200 fold; however, this stimulated activity is quickly removed by phosphodiesterase (PDE) 5A enzyme [18-19]. Previous studies suggest that gene expression of sGC α_1 and β_1 subunits decrease during aging and hypertension and the mRNA levels of all subunits vary during the embryonic development [20]. In addition to NO donors [21], estrogen [17], cAMP elevating compounds, [22-23] and cytokines [24] also modulate the expression of sGC. Alternative splicing of sGC α and β subunits may reveal another regulatory mechanism of sGC activity. For instance, the sGC α_2j splice variant carries an insertion in the catalytic domain. This variant was detected in some human tissues and it was shown to exhibit a dominant negative function [25]. Alternative splice forms of the β_1 and β_2 subunit have also been reported [26-28]. Furthermore, three new sGC α_1 splice variants (N1-sGC α_1 , N2- sGC α_1 and C-sGC α_1) in different human tissues were identified. Functional analysis revealed that N1-sGC α_1 protein demonstrated a dominant negative effect on the activity of sGC where as C-sGC α_1 produced a fully functional NO sensitive enzyme [29].

Allosteric activators of sGC

Previous studies suggest that the loss of NO bioavailability and/or responsiveness to endogenous NO has been implicated in the pathogenesis of cardiovascular and other diseases. Although NO donors and organic nitrates provide symptomatic improvement in patients with cardiovascular disease, there is no evidence that such treatment can reduce mortality. Therefore, it is proposed that NO independent heme dependent sGC stimulators and NO independent and heme independent sGC activators might provide an advantage over the currently employed therapies [30]. Previous studies suggest that sGC stimulators such as YC-1, BAY41-2272, and BAY41-8543 can be used as pharmacological tools to stimulate the activity of sGC under reduced heme moiety in many cell systems. Furthermore, combination of NO donors and sGC stimulators induce cGMP accumulation in a synergistic manner [30]. Allosteric sGC stimulators, BAY41-2272 and YC-1 are reported to also inhibit PDE 5 activity, an enzyme which degrades cGMP. The second group comprise of NO independent and heme independent sGC activators (BAY 58-2667 and HMR-1766) which demonstrate potent action on the oxidized form of sGC enzyme [30, 31].

Effectors of sGC

sGC mediates many of its physiological effects through production of cGMP. One of the major targets of cGMP is the cGMP dependent protein kinase, PKG. The binding of cGMP to PKG leads to its activation as a serine/threonine kinase [13]. Activated PKG can act on many substrates including the IP3 receptor, phospholamban, vimentin, the phosphatase inhibitor G substrate and subunits of myosin light chain phosphatase [32-37]. There are two distinct genes that encode PKG (I&II) with PKGI resulting into two isoforms (PKGI α and PKGI β) through splicing. These isoforms are differentially expressed in various tissues and are activated by various stimuli [38-42]. Studies have shown that PKGI α directly phosphorylates myosin phosphatase, an enzyme that regulates vascular smooth muscle tone [37].

Cyclic nucleotide gated channels (CNG) are another target of cGMP. CNGs regulate Na⁺ and Ca⁺ influx into cells. Activation of CNG by cGMP is produced by retinal guanylyl

cyclase (particulate enzymes) which regulates phototransduction and neurotransmission in the retina [13-14].

The cyclic nucleotide phosphodiesterases (PDE) are a family (PDE1-11) of enzymes that either hydrolyze second messengers, cAMP and cGMP and/or are regulated by cGMP [43]. cGMP regulates the activity of PDE-2,-3,-5 and -9. Out of these, PDE-2, -3 and -5 are expressed in cardiac myocytes, PDE-3 and -5 in vascular smooth muscle cells, and PDE-2,-3 and -5 in vascular endothelial cells [43-50]. PDE-2 can hydrolyze both cAMP and cGMP, whereas PDE-5 selectively hydrolyzes cGMP. PDE-5 is a cytosolic protein with a particular localization in Z-bands of cardiac myocytes suggesting its association with other Z-band proteins such as PKG [51-52].

Role of Nitric Oxide-cyclic GMP in Development

Previous studies clearly demonstrate the crucial role of NO during development. This role has been mainly identified by the creation of NOS knockout animals in all combinations of the three isoforms [53]. Previously, the ability of all single NOS knockout models to develop rather normally, although with heart malformations, was attributed to compensation by the other NOS isoforms [54-55]. Double knockout animals in all cases exhibited decreased viability and the inability to produce offspring with other double knockouts. However, since those double knockouts were able to reproduce with wildtype animals, a defect in development was evident. Triple NOS knockout animals which completely lack endogenous production of NO also demonstrate low survival rate and significant reduction in number of offspring [56]. The animals present with nephrogenic diabetes insipidus and arteriosclerosis. These results provide the first evidence that the systemic deletion of all three NOSs can cause a variety of cardiovascular diseases in mice, thereby demonstrating the critical role of the endogenous NOS system in maintaining cardiovascular and volume homeostasis.

Further significance of NO-cyclic GMP pathway in development has emerged from generation of knockout animal models of other components of the NO signaling pathway. Previous work using chemical inhibitors also demonstrated a role of NO/cGMP in preimplantation embryo and apoptosis in the mouse [57]. Similarly, the functional significance of sGC α_1/β_1 heterodimer was demonstrated by conducting studies with sGC α_1 knock out animals. sGC α_1 knockout mice of both genders exhibit significantly decreased relaxing effects of major vasodilators such as acetylcholine, NO, YC-1 and BAY 41-2272 [58]. Similar results were demonstrated with cGMP dependent protein kinase I (cGKs) and sGC β_1 knockout animals [59-60]. The significance of PKG in development can be evaluated by studying PKG knock out (cGKI) animals, which show impaired NO/cGMP-dependent vasorelaxation and blunted cardiac negative inotropic response to cGMP along with a host of other problems in cardiovascular and neuronal systems [61]. sGC β_1 knockout animals exhibit the most severely developmental phenotype. About 70-80% sGC β_1 knockout animals died within two days after birth and 90% of survivors died within two weeks of severe gastrointestinal abnormalities [59]. These findings confirm the essential role of sGC-cGMP-cGKI in mediating various effects of NO signaling components *in vivo*.

Stem cells

Adult stem cell transplantation to treat cardiovascular, neurodegenerative and other diseases have been studied for many decades [62]. Although adult stem cells have been successfully used for allogenic transplantation for treating patients with hematological malignancies and a variety of inherited or acquired immune-hematological diseases [63], their limited proliferative capacity and plasticity limit their use in regenerative medicine. Furthermore, studies suggest that improved cardiac function due to bone marrow transplant has been

mainly attributed to the paracrine action from transplanted cells [64]. Therefore, use of embryonic and induced pluripotent stem cells could potentially revolutionize the field of regenerative medicine albeit with some disadvantages. Embryonic stem (ES) cells derived from the inner cell mass of blastocysts exhibit two unique properties 1) self-renewal which allows cells to grow continuously. (2) Pluripotency is a unique signature property which allows ES cells to differentiate into the derivatives of all the three germ layers; ectoderm, mesoderm and endoderm [64-65]. ES cells have been successfully differentiated into cells of many lineages including cardiac lineage by directed differentiation, embryoid body (EB) directed differentiation, and stromal cell co-culture methods [64].

Induced pluripotent stem (iPS) cells were initially generated by Yamanaka and colleagues by identifying a set of key transcription factors (OCT4, SOX2, KLF-4 and c-myc) which can reprogram somatic cells such as fibroblasts to a pluripotent state when over-expressed [66]. Further studies indicated the possibility of re-programming mouse or human somatic cells using a different set of 4 factors (OCT4, SOX2, NANOG and LIN 28) [67-73]. The recent discovery of a new pluripotency factor, RONIN, may also facilitate the production of iPS cells from human extraembryonic tissues through its co-expression with KLF-4 and c-myc [74-75]. Somatic cells have also been induced to a pluripotent state by introduction of only two transcription factors (OCT4, SOX-2) and valproic acid (VPA), a small molecule histone deacetylase inhibitor (HDAC) which increases the efficiency of reprogramming primary human fibroblast to pluripotent state [76]. Earlier studies also indicate that iPS cells can be differentiated into cells of various lineages such as myocardial and neuronal cells with similar approaches as ES cells. [77-79]. Although, iPS cells appear to be similar to ES cells with respect to morphology, global gene and protein expression and differentiation potential; more recent studies suggest that iPS cells do differ from ES cells in gene expression, DNA methylation state and miRNA expression [80-82].

Role of NO in Stem Cell Differentiation

The significant role of the NO-cGMP in regulation of stem differentiation is beginning to emerge from our studies [83-89] and that of other investigators. The significance of the NO-cGMP pathway in the differentiation of adult stem cells is indicated by the work of other investigators which show that alteration of intracellular NO-cGMP levels can modulate the differentiation of bone marrow derived progenitor cells [90-94]. Previous studies indicate that the exposure of CD34+ bone marrow derived cells to NO donors shows inhibition of erythroid but induction of myeloid colony formation [90]. Furthermore, NO production by murine bone marrow cells was shown to inhibit the colony formation ability of isolated Sca-1 and Thy-1 positive cells [91]. The importance of NO in regulation of stem cell number and function *in vivo* was demonstrated by exposure of mice to NOS inhibitors which showed an increase in the number of stem cells compared to the untreated mice [92]. Further studies conducted in eNOS knockout mice demonstrate impaired mobilization in BM stem and progenitors cells [93] that may be related to impaired neo-vascularization. Moreover, eNOS knockout mice exhibit markedly reduced capacity to produce endothelial progenitor cells from hematopoietic stem cells. Therefore, these mice are unable to re-vascularize the tissue subjected to ischemic injury [94].

The role of NO in heart development has been implicated by studies in both developing embryos and ES cells. For instance, both NOS-2 and NOS-3 were detected during the early stages of cardiomyogenesis in mouse embryos. ES cell-derived cardiomyocytes, in the same study, were also shown to express NOS-2 and NOS-3, similar to that seen *in vivo*. Incubation of these cultures with the NOS inhibitor L-NAME resulted in a profound differentiation arrest of these cardiomyocytes [95]. It was further demonstrated that NO can facilitate ES cell-derived cardiomyogenesis through both exogenous stimulation with NO donors or by introduction of the NOS-2 gene. Interestingly, the role of endogenous NO in

the differentiation of ES cells into cardiomyocytes from this study appeared to differ from the previous, since the differentiation was only slightly compromised in the presence of L-NAME [96].

Role of sGC in stem cell differentiation

Our studies with mouse and human embryonic stem (ES) cells demonstrate differential expression and function of NOS isoforms, sGC subunits and PKG during differentiation of stem cells into cells of myocardial lineage (Table 1). Our studies indicate that the NOS-1 isoform is expressed in both mouse and human ES cells and mRNA and protein levels fall to basal level as ES cells differentiate. On the contrary, we observed a temporal induction of both NOS-2 and NOS-3 isoforms during various stages of ES cell differentiation in murine and human ES cells. Furthermore, our studies indicate that ES cells do not express enzymatically active NO receptor sGC. However, sGC α_1 , α_2 and β_1 levels increase during cell differentiation that coincides with a robust increase in NO-inducible intracellular cGMP levels in ES-derived differentiated cells and cardiomyocytes [83-84]. In contrast, sGC β_2 gene expression in human ES cells declines as cells progress through differentiation, indicating that this subunit may have some role in the pluripotency of stem cells.

We have also demonstrated that stem cells express low levels of protein kinase G. However, differentiated cells show robust temporal induction of PKG protein levels [83]. These results collectively point to the involvement of NO signaling components in the differentiation of stem cells. Furthermore, we demonstrated for the first time that in addition to NO donors, allosteric sGC stimulators BAY 41 2272 and YC-1 markedly increased the expression of cardiac specific transcription factors and cardiac specific proteins [85]. A combination of various NO donors and sGC stimulators further enhanced the expression of cardiac specific markers with a concomitant increase in the expression of sGC subunits at mRNA and protein level. cGMP analysis in undifferentiated cells revealed a lack of stimulation with NO donors. However, ES-derived differentiated cells acquired the ability to be stimulated with NO donors and sGC activators. These studies therefore demonstrate that NO donors and various sGC activators alone or in combination may facilitate differentiation of stem cells into myocardial cells with the robust increase in second messenger cyclic GMP (cGMP) accumulation [85], thereby implicating an important role of NO/cGMP pathway in differentiation of stem cells. Our unpublished studies suggest that in contrast to ES cells, there may be an aberration in the NO/cGMP pathway in iPS cells. We are further characterizing this in current studies.

The NO receptor sGC is regulated at multiple levels. sGC has been shown to be regulated at transcriptional, post transcriptional, and post translational levels [29,97-100]. At the post-transcriptional levels alternative splicing produces a number of sGC splice variants [25-27, 29, 101]. Previous studies have established the presence of sGC α_1 splice variants, which encode proteins with deletions in both the C- and N-termini [29]. One such characterized splice form (sGC- α_1 N1) exhibited dominant negative properties while another splice variant (sGC- α_1 C) formed a fully active heterodimer with the β_1 subunit. Since sGC alternative splicing has been shown to regulate sGC function, we reasoned that it might also play a role in stem cell differentiation by modulating sGC activity. Accordingly, our recent studies demonstrate that sGC α_1 subunit undergoes alternative splicing during ES cell differentiation [86]. The C-type sGC α_1 splice variant is highly expressed in differentiating cells; however, its intracellular distribution varies from the canonical sGC α_1 subunit [86].

NO-cGMP pathway has also been implicated in the differentiation of stem cells into cells of various lineages in response to various plant compounds. Zhu et al [102] demonstrated that icariin (a constituent of *Epimedium*, a traditional Chinese medicine) induced differentiation of mouse ES cells into cardiomyocytes by elevation of cAMP/cGMP ratio in ES cells as

well as upregulation of the endogenous generation of NO during the early stages of cardiac development. Similarly, the plant compound genistein has been shown to stimulate osteoblastic differentiation in bone marrow culture via the NO-cGMP pathway [103]. Our most recent studies indicate that polyphenol curcumin induces differentiation of stem cells via modulation of the NO pathway [104].

In order to study the mechanism of NO induced differentiation of stem cells, a recent study [105] demonstrated that the NO donor DETA-NO induced differentiation of mouse and human ES cells by downregulation of two master genes Nanog and Oct-4. Exposure of cells to DETA-NO along with valproic acid further enhances the differentiation of stem cells into cells of endodermal lineage [105]. Another study suggests that exposure of mES cells to low concentrations of NO promotes their survival by the inhibition of pro-apoptotic and up regulation of anti-apoptotic genes [106].

Role of PKG in stem cell differentiation

The significance of PKG in development can be evaluated by studying PKG knock out (cGKI) animals, which show impaired NO/cGMP-dependent vasorelaxation, blunted cardiac negative inotropic response to cGMP, along with a host of other problems in cardiovascular and neuronal systems [61]. Studies from our laboratory indicate absence of PKG expression in mouse stem cells. However, PKG levels increased as cells went through differentiation process [83] suggesting a possible role of PKG in differentiation. Our previous studies [85] have also shown that cGMP analog 8-bromo-cGMP, which activates PKG, can induce differentiation of stem cells. Studies from other investigators have also indicated that PKG can positively regulate the migration of neural precursor cells [107] and survival of OP9 progenitor cells [108]. A recent study demonstrated that cGMP modulates the fate of neural stem cells *in vivo* as higher GMP levels differentiate the neural stem cells to neuronal cells [107]. In contrast, downregulation of PKG and PKC has been shown to enhance cardiomyocyte production during ES stem cell differentiation [109]. Therefore, it seems that PKG might play an important role in regulation of stem cell differentiation and possibly in proliferation and survival of stem cell-derived cardiomyocytes.

Role of PDEs in stem cell differentiation

The number of circulating blood progenitor cells is reduced in patients with cardiovascular risk. Previous studies have shown that PDE5 inhibitor Vardenafil increases circulating progenitor cells in humans possibly due to increased cGMP levels [110]. Another study has shown that PDE7 may play an important role in osteoblastic differentiation [111]. Administration of PDE inhibitor Sildenafil has been shown to differentiate neural stem cells into neurons in brain *in vivo* [107]. Cyclic GMP has been shown to prevent heart failure induced by hypertrophy and pathological remodeling. Earlier studies have shown that (by blocking the catabolism of cGMP) PDE5A inhibitor Sildenafil Citrate suppresses chamber and myocyte hypertrophy in mice suggesting that PDE5A inhibition may provide a novel treatment strategy for cardiac hypertrophy and remodeling [112].

Role of Nitric oxide-cGMP in heart and in stem cell based therapy

The role of NO in regenerative potential of ESC cells was recently shown in a mouse model of hindlimb ischemia where NO treated ESC injected in the cardiac left ventricle selectively localized in the ischemic hindlimb and contributed to the regeneration of muscular and vascular structures [113]. Transplantation therapy using stem cells has a promise to revolutionize regenerative medicine. ES cell-derived cardiomyocytes show great promise because a) Unlimited self-renewal properties of ES cells could theoretically provide an unlimited supply of cardiomyocytes [65], b) ES cells can reliably be differentiated into cardiomyocytes *in vitro*, avoiding the plasticity controversy of adult stem cells [114-116],

and c) ES cell-derived cardiomyocytes may escape xenograft rejection, even in the absence of immunosuppressive therapy [117-120]. It has been previously demonstrated that ES cells delivered to the heart after damage do engraft and improve heart function [117,120]. Furthermore, when engrafted ES cells were engineered to express vascular endothelial growth factor (VEGF), heart improvement was enhanced which may be due to increased formation of capillaries and blood delivery [121].

NO signaling plays an integral role in heart function, including ventricular compliance, myocardial preload, afterload, reperfusion injury and angiogenesis [122-125]. Following myocardial infarction, NOS-2 expression is increased, which may contribute to myocardial dysfunction and injury [126-127]. In contrast, production of NO by NOS-1 and NOS-3 could be beneficial for the heart following myocardial infarction [128-129]. It seems that these beneficial effects of NO occur through a cGMP-mediated pathway, since NO and cGMP can protect cardiomyocytes in hypertrophy models [130-131]. This protection was later suggested to occur through activation of PKG [132]. Therefore, understanding NO signaling in the context of the derivation and delivery of ES cell-derived cardiomyocytes and transplantation may further optimize the conditions for repair following myocardial infarction.

Notably, eNOS-derived NO has been shown to increase the beneficial production of VEGF, implicating a novel therapy for cardiovascular diseases [133]. Previous studies have shown that hyperstimulation of cGMP synthesis via activation of PKG or genetic upregulation of natriuretic peptide receptor signaling blunts hypertrophy *in vitro* and *in vivo* despite sustained pressure load [32,134-136], whereas inhibition of this signaling worsens hypertrophy [137]. Patients with myocardial infarction (MI) leading to cardiomyopathy have poor prognosis despite pharmacological and other treatment modalities [138]. Cell transplantation studies have demonstrated formation of cardiomyocytes and other heart cells from transplanted bone marrow stem cells, cardiac stem cells and ES cells and migration of primitive cells to the heart [139-140]. Although, Islet-1+ (LIM homeodomain transcription factor; Isl1+) cardioblast (endogenous cardiac progenitors that contribute substantially to the embryonic heart) in very low numbers have been identified, and shown to fully differentiate into myocardial lineage [141], it is unlikely these endogenous progenitors will significantly contribute towards the replacement of myocardial cells. In contrast, transplantation of different types of progenitor and stem cells have shown beneficial effects in improving cardiac damage after MI in animal models and many clinical trials [138,142]. A previous study has demonstrated that eNOS in the host myocardium promotes MSC migration to the ischemic myocardium and improves cardiac function through cGMP-dependent increases in SDF-1 α expression [143]. Similarly, a recent study indicated that early combination of PDE5 inhibitor sildenafil (which prevents the degradation of cGMP) and adipose-derived MSC exhibited greater preservation of left ventricular (LV) function in rat dilated cardiomyopathy model than untreated animals [144] thereby implicating the role of eNOS and cGMP in mediating such effect.

However, most of work has been done with adult stem cells which have limited plasticity. Previous studies demonstrate that transplantation of human ES-derived cardiomyocytes (in coronary ligation model) have shown better improvement of mouse cardiac function up to 4 weeks compared to ES-derived non-cardiomyocytes. However, this effect diminishes after 12 weeks of transplantation [145]. An additional study using ES-derived cardiomyocytes indicated that improved mouse cardiac function in a mouse model correlated with vascularity, not the graft size [146]. Although not implicated in the paper, it might suggest the involvement of NO in mediating such effect.

Cardiomyocytes derived from human iPS cells also show great potential in regenerative medicine and drug discovery [147]. Unfortunately the effect of NO-cGMP pathway using ES or iPS-derived cardiomyocytes in animal models of cardiac injury has not been reported as of yet. However, based on our *in vitro* studies and that of other investigators, we propose that NO-cGMP signaling should play a key role in the capacity of stem cell derived cardiomyocytes to engraft, home, and repair damaged myocardium.

Summary and Perspectives

Previous important studies using NOS, sGC and PKG knockout models clearly demonstrate the significant role of NO-cGMP signaling in development. Furthermore, studies conducted by us and others suggest that manipulation of NO-cGMP pathway contributes to cell fate determination of stem cells. Our studies for the first time demonstrated that NO-independent heme-dependent allosteric sGC stimulators such as BAY41 2272 and YC-1 induced differentiation of stem cells. Combination of NO donors and sGC activators further enhanced the differentiation with robust increase in cGMP accumulation. These studies may have strong implications as these mediators can be used as a cocktail to enhance the capacity of stem cell derived cardiomyocytes to improve myocardial, microvascular, regenerative (replacement) and cardiovascular function. However, the effects of the NO-cGMP signaling pathway most likely involve temporal, compartmental/spatial, and localized concentration-dependent autocrine and paracrine responses. Based on our previous studies and those of others, in defining the fundamental role of nitric oxide and cyclic GMP in numerous physiological processes, we suggest that these mediators also participate in stem cell proliferation and differentiation.

Acknowledgments

This work was supported in part by National Institutes of Health (GM076695) and grants from the Dunn foundation, the Welch foundation, Mathers foundation, NASA and the University of Texas. KM would like to thank Balraj Singh and Vivek M Singh for critically reading this review article.

References

1. Beckman JS, Koppenol WH. Nitric oxide, superoxide and peroxynitrite: the good, the bad and the ugly. *Am J Physiol.* 1996; 271:C1424–C1437. [PubMed: 8944624]
2. Bryan NS, Bian K, Murad F. Discovering of nitric oxide signaling pathways and targets for drug development. *Front Biosci.* 2009; 14:1–18. [PubMed: 19273051]
3. Murad F. Shattuck Lecture: Nitric oxide and cyclic GMP in cell signaling and drug development. *New Engl J Med.* 2006; 355:2003–2011. [PubMed: 17093251]
4. Moncada S, Palmer RMJ, Higgs J. Nitric Oxide; physiology, patho-physiology and pharmacology. *Pharmacol Rev.* 1991; 43:109–142. [PubMed: 1852778]
5. Xia H, Bredt DS. Cloned and expressed nitric oxide synthase proteins. *Methods Enzymol.* 1996; 268:427–436. [PubMed: 8782609]
6. White KA, Marletta MA. Nitric oxide synthase is a cytochrome-P450 type heme protein. *Biochemistry.* 1992; 31:6627–6631. [PubMed: 1379068]
7. Abu-Soud HM, Yoho LL, Stuehr DJ. Nitric oxide synthases reveal a role for calmodulin in controlling electron transfer. *Proc Natl Acad Sci USA.* 1993; 90:10769–10772. [PubMed: 7504282]
8. Liu Q, Gross SS. Binding sites of nitric oxide synthases. *Methods Enzymol.* 1996; 268:311–324. [PubMed: 8782597]
9. Krumenacker JS, Hanafy KA, Murad F. Regulation of nitric oxide and soluble guanylyl cyclase. *Brain Res Bull.* 2004; 62:505–515. [PubMed: 15036565]
10. Russwurm M, Behrends S, Harteneck C, Koesling D. Functional properties of a naturally occurring isoform of soluble guanylyl cyclase. *Biochem J.* 1998; 335:125–130. [PubMed: 9742221]

11. Budworth J, Meillerais S, Charles I, Powell K. Tissue distribution of the human guanylate cyclases. *Biochem Biophys Res Commun.* 1999; 263:696–701.
12. Mergia E, Russwurm M, Zoidl G, Kosling D. Major occurrence of the new alpha2beta1 isoform of NO-sensitive guanylyl cyclase in brain. *Cell Signal.* 2003; 15:189–195. [PubMed: 12464390]
13. Lucas KA, Pitari GM, Kazerounian S, Ruiz-Stewart I, Park J, Schulz S, Chepenik KP, Waldman SA. Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol Rev.* 2000; 52:375–414. [PubMed: 10977868]
14. Biel M, Seeliger M, Pfeifer A, Kohler K, Gerstner A, Ludwig A, Jaissle G, Fauser S, Zrenner E, Hofmann F. Selective loss of cone function in mice lacking the cyclic nucleotide-gated channel CNG3. *Proc Natl Acad Sci U S A.* 1999; 96:7553–7557. [PubMed: 10377453]
15. Boolell M, Allen MJ, Ballard SA, Gepi-Attee S, Muirhead GJ, Naylor AM, Osterloh IH, Gingell C. Sildenafil: an orally active type 5 cyclic GMP-specific phosphodiesterase inhibitor for the treatment of penile erectile dysfunction. *Int J Impot Res.* 1996; 8:47–52. [PubMed: 8858389]
16. Sharina IG, Krumenacker JS, Martin E, Murad F. Genomic organization of alpha₁ and beta₁ subunits of the mammalian soluble guanylyl cyclase genes. *Proc Natl Acad Sci USA.* 2000; 97:10878–10883. [PubMed: 10984516]
17. Krumenacker JS, Hyder SM, Murad F. Estradiol rapidly inhibits soluble guanylyl cyclase expression in rat uterus. *Proc Natl Acad Sci USA.* 2001; 98:717–722. [PubMed: 11209068]
18. Humbert P, Niroomand F, Mayer B, Koesling D, Hinsch KD, Gausepohl H, Frank R, Schulz G, Bohme E. Purification of soluble guanylyl cyclases from bovine lung by a new immunoaffinity chromatographic method. *Euro J Biochem/FEBS.* 1990; 190:273–278.
19. Lee YC, Martin E, Murad F. Human recombinant soluble guanylyl cyclase: expression, purification and regulation. *Proc Natl Acad Sci USA.* 2000; 97:10763–10768. [PubMed: 10995472]
20. Pyriochou A, Papapetropoulos A. Soluble guanylyl cyclase; more secrets revealed. *Cell Signal.* 2005; 17:407–13. [PubMed: 15601619]
21. Papapetropoulos A, Go CY, Murad F, Catravas JD. Mechanisms of tolerance to sodium nitroprusside in rat cultured aortic smooth muscle cells. *Brit J Pharmacol.* 1996; 117:147–55. [PubMed: 8825356]
22. Klöss S, Srivastava R, Mülsch A. Down-regulation of soluble guanylyl cyclase expression by cyclic AMP is mediated by mRNA-stabilizing protein HuR. *Mol Pharmacol.* 2004; 65:1440–51. [PubMed: 15155837]
23. Papapetropoulos A, Marczin N, Mora G, Milici A, Murad F, Catravas JD. Regulation of vascular smooth muscle soluble guanylate cyclase activity, mRNA, and protein levels by cAMP-elevating agents. *Hypertension.* 1995; 26:696–704. [PubMed: 7558233]
24. Papapetropoulos A, Abou-Mohamed G, Marczin N, Murad F, Caldwell RW, Catravas JD. Downregulation of nitrovasodilator-induced cyclic GMP accumulation in cells exposed to endotoxin or interleukin-1 beta. *Br J Pharmacol.* 1996; 118:1359–66. [PubMed: 8832057]
25. Behrends S, Harteneck C, Schultz G, Koesling D. A variant of the alpha 2 subunit of soluble guanylyl cyclase contains an insert homologous to region within adenylyl cyclases and function as a dominant negative protein. *J Biol Chem.* 1995; 270:21109–13. [PubMed: 7673142]
26. Behrends S, Vehse K. The beta(2) subunit of soluble guanylyl cyclase contains a human-specific frameshift and is expressed in gastric carcinoma. *Biochem Biophys Res Commun.* 2000; 271:64–9. [PubMed: 10777682]
27. Chhajlani V, Frändberg PA, Ahlner J, Axelsson KL, Wikberg JE. Heterogeneity in human soluble guanylate cyclase due to alternative splicing. *FEBS Lett.* 1991; 290:157–158. [PubMed: 1680753]
28. Okamoto H. Molecular cloning of a novel variant of the rat soluble guanylate cyclase beta 2 subunit. *Int J Biochem Cell Biol.* 2004; 36:472–80. [PubMed: 14687925]
29. Sharina IG, Jelen F, Bogatenkova EP, Thomas A, Martin E, Murad F. Alpha1 soluble guanylyl cyclase (sGC) splice forms as potential regulators of human sGC activity. *J Biol Chem.* 2008; 283:15104–15113. [PubMed: 18381288]
30. Evgenov OV, Pacher P, Schmidt PM, Hasko G, Schmidt HHHW, Stasch JP. NO-independent stimulators and activators of soluble guanylyl cyclase: discovery and therapeutic potential. *Nature Rev.* 2006; 5:755–768.

31. Martin E, Lee YC, Murad F. YC-1 activation of human soluble guanylyl cyclase has both heme-dependent and heme-independent components. *Proc Natl Acad Sci USA*. 2001; 98:12938–12942. [PubMed: 11687640]
32. Aszódi A, Pfeifer A, Ahmad M, Glauner M, Zhou XH, Ny L, Andersson KE, Kehrel B, Offermanns S, Fässler R. The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function. *EMBO J*. 1999; 18:37–48. [PubMed: 9878048]
33. Endo S, Suzuki M, Sumi M, Nairn AC, Morita R, Yamakawa K, Greengard P, Ito M. Molecular identification of human G-substrate, a possible downstream component of the cGMP dependent protein kinase cascade in cerebellar Purkinje cells. *Proc Natl Acad Sci U S A*. 1999; 96:2467–2472. [PubMed: 10051666]
34. Komalavilas P, Lincoln TM. Phosphorylation of the inositol 1, 4, 5-trisphosphate receptor by cyclic GMP-dependent protein kinase. *J Biol Chem*. 1994; 269:8701–8707. [PubMed: 8132598]
35. Pryzwansky KB, Wyatt TA, Lincoln TM. Cyclic guanosine monophosphate-dependent protein kinase is targeted to intermediate filaments and phosphorylates vimentin in A23187-stimulated human neutrophils. *Blood*. 1995; 85:222–30. [PubMed: 7803796]
36. Raeymaekers L, Eggermont JA, Wuytack F, Casteels R. Effects of cyclic nucleotide dependent protein kinases on the endoplasmic reticulum Ca²⁺ pump of bovine pulmonary artery. *Cell Calcium*. 1990; 11:261–268. [PubMed: 2163283]
37. Surks HK, Mochizuki N, Kasai Y, Georgescu SP, Tang KM, Ito M, Lincoln TM, Mendelsohn ME. Regulation of myosin phosphatase by a specific interaction with cGMP-dependent protein kinase I alpha. *Science*. 1999; 286:1583–1587. [PubMed: 10567269]
38. Jarchau T, Häusler C, Markert T, Pöhler D, Vanderkerckhove J, De Jonge HR, Lohmann SM, Walter U. Cloning, expression, and in situ localization of rat intestinal cGMP-dependent protein kinase II. *Proc Natl Acad Sci U S A*. 1994; 91:9426–9430. [PubMed: 7937783]
39. Lohmann SM, Vaandrager AB, Smolenski A, Walter U, De Jonge HR. Distinct and specific functions of cGMP-dependent protein kinases. *Trends Biochem Sci*. 1997; 22:307–312. [PubMed: 9270304]
40. Orstavik S, Solberg R, Taskén K, Nordahl M, Altherr MR, Hansson V, Jahnsen T, Sandberg M. Molecular cloning, cDNA structure, and chromosomal localization of the human type II cGMP-dependent protein kinase. *Biochem Biophys Res Commun*. 1996; 220:759–65. [PubMed: 8607838]
41. Tamura N, Itoh H, Ogawa Y, Nakagawa O, Harada M, Chun TH, Suga S, Yoshimasa T, Nakao K. cDNA cloning and gene expression of human type Ialpha cGMP-dependent protein kinase. *Hypertension*. 1996; 27:552–557. [PubMed: 8613202]
42. Uhler MD. Cloning and expression of a novel cyclic GMP-dependent protein kinase from mouse brain. *J Biol Chem*. 1993; 268:13586–13591. [PubMed: 8514791]
43. Tsai EJ, Kass DA. Cyclic GM signaling in cardiovascular pathophysiology and therapeutics. *Pharmacol Ther*. 2009; 122:216–238. [PubMed: 19306895]
44. Lugnier C, Keravis T, Eckly-Michel A. Cross talk between NO and cyclic nucleotide phosphodiesterases in the modulation of signal transduction in blood vessel. *J Physiol Pharmacol*. 1999; 50:639–652. [PubMed: 10639014]
45. Maurice DH. Cyclic nucleotide phosphodiesterase-mediated integration of cGMP and cAMP signaling in cells of the cardiovascular system. *Front Biosci*. 2005; 10:1221–1228. [PubMed: 15769620]
46. Netherton SJ, Maurice DH. Vascular endothelial cell cyclic nucleotide phosphodiesterases and regulated cell migration: implications in angiogenesis. *Mol Pharmacol*. 2005; 67:263–72. [PubMed: 15475573]
47. Pauvert O, Salvail D, Rousseau E, Lugnier C, Marthan R, Savineau JP. Characterisation of cyclic nucleotide phosphodiesterase isoforms in the media layer of the main pulmonary artery. *Biochem Pharmacol*. 2002; 63:1763–72. [PubMed: 12007579]
48. Phillips PG, Long L, Wilkins MR, Morrell NW. cAMP phosphodiesterase inhibitors potentiate effects of prostacyclin analogs in hypoxic pulmonary vascular remodeling. *Am J Physiol Lung Cell Mol Physiol*. 2005; 288:L103–115. [PubMed: 15377497]

49. Sadhu K, Hensley K, Florio VA, Wolda SL. Differential expression of the cyclic GMP-stimulated phosphodiesterase PDE2A in human venous and capillary endothelial cells. *J Histochem Cytochem.* 1999; 47:895–906. [PubMed: 10375378]
50. Thompson WJ, Ashikaga T, Kelly JJ, Liu L, Zhu B, Vemavarapu L, Strada SJ. Regulation of cyclic AMP in rat pulmonary micro vascular endothelial cells by rolipram-sensitive cyclic AMP phosphodiesterase (PDE4). *Biochem Pharmacol.* 2002; 63:797–807. [PubMed: 11992650]
51. Takimoto E, Belardi D, Tocchetti CG, Vahebi S, Cormaci G, Ketner EA, Moens AL, Champion HC, Kass DA. Compartmentalization of cardiac beta-adrenergic inotropy modulation by phosphodiesterase type 5. *Circulation.* 2007; 115:2159–2167. [PubMed: 17420342]
52. Zhang M, Koitabashi N, Nagayama T, Rambaran R, Feng N, Takimoto E, Koenke T, O'Rourke B, Champion HC, Crow MT, Kass DA. Expression, activity, and pro-hypertrophic effects of PDE5A in cardiac myocytes. *Cell Signal.* 2008; 20:2231–2236. [PubMed: 18790048]
53. Tranguch S, Huet-Hudson Y. Decreased viability of nitric oxide synthase double knockout mice. *Mol Reprod Dev.* 2003; 65:175–179. [PubMed: 12704728]
54. Lee TC, Zhao YD, Courtman DW, Stewart DJ. Abnormal aortic valve development in mice lacking endothelial nitric oxide synthase. *Circulation.* 2000; 101:2345–2348. [PubMed: 10821808]
55. Feng Q, Song W, Lu X, Hamilton JA, Lei M, Peng T, Yee SP. Development of heart failure and congenital septal defects in mice lacking endothelial nitric oxide synthase. *Circulation.* 2002; 106:873–879. [PubMed: 12176963]
56. Tsutsui M, Shimokawa H, Morishita T, Nakashima Y, Yanagihara N. Development of genetically engineered mice lacking all three nitric oxide synthases. *J Pharmacol Sci.* 2006; 102:147–54. [PubMed: 17031076]
57. Chen HW, Jiang WS, Tzeng CR. Nitric oxide as a regulator in preimplantation embryo development and apoptosis. *Fert Steril.* 2001; 75:1163–1171.
58. Nimmegeers S, Sips P, Buys E, Brouckaert P, Van de Voorde J. Functional role of the soluble guanylyl cyclase alpha(I) subunit in vascular smooth muscle relaxation. *Cardiovasc Res.* 2007; 76:149–159. [PubMed: 17610859]
59. Friebe A, Mergia E, Dangel O, Lange A, Koesling D. Fatal gastrointestinal obstruction and hypertension in mice lacking nitric oxide-sensitive guanylyl cyclase. *Proc Natl Acad Sci U S A.* 2007; 104:7699–7704. [PubMed: 17452643]
60. Schlossmann J, Feil R, Hofmann F. Insights into cGMP signalling derived from cGMP kinase knockout mice. *Front Biosci.* 2005; 10:1279–1289. [PubMed: 15769624]
61. Hoffman F, Feil R, Kleppisch T, Schlossmann J. Function of cGMP dependent protein kinases as revealed by gene deletion. *Physiol Rev.* 2006; 86:1–23. [PubMed: 16371594]
62. Volarevic V, Ljubic B, Stojkovic P, Lukic A, Arsenijevic N, Stojkovic M. Human stem cell research and regenerative medicine-present and future. *Br Med Bull.* 2011 Jun 13. Epub ahead of print.
63. Brignier AC, Gewirtz AM. Embryonic and adult stem cell therapy. *J Allergy Clin Immunol.* 2010; 125:S336–S344. [PubMed: 20061008]
64. Dambrot C, Passier R, Atsma D, Mummery CL. Cardiomyocyte differentiation of pluripotent stem cells and their use as cardiac disease models. *Biochem J.* 2011; 434:25–35. [PubMed: 21269276]
65. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science.* 1998; 282:1145–1147. [PubMed: 9804556]
66. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* 2006; 126:633–676.
67. Okita K, Ischisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. *Nature.* 2007; 448:313–317. [PubMed: 17554338]
68. Maherali N, Sridharan R, Xie W, Utikal J, Eminli S, Arnold K, Stadtfeld M, Yachechko R, Tchieu J, Jaenisch R, Plath K, Hochedlinger K. Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. *Cell Stem Cell.* 2007; 7:55–70. [PubMed: 18371336]

69. Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R. *In vitro* reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature*. 2007; 448:318–324. [PubMed: 17554336]
70. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007; 131:861–872. [PubMed: 18035408]
71. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. Induced pluripotent stem cell lines derived from human somatic cells. *Science*. 2007; 318:1917–1920. [PubMed: 18029452]
72. Lowry WE, Richter L, Yachechko R, Pyle AD, Tchieu J, Sridharan R, Clark AT, Plath K. Generation of human induced pluripotent stem cells from dermal fibroblasts. *Proc Natl Acad Sci U S A*. 2008; 105:2883–2888. [PubMed: 18287077]
73. Park IH, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley GQ. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature*. 2008; 451:141–146. [PubMed: 18157115]
74. Dejosez, M.; Krumenacker, JS.; Zitur, LJ.; Passeri, M.; Chu, LF.; Songyang, Z.; Thomson, JA.; Zwaka, TP. *Cell*. Vol. 133. 2008. Ronin is essential for embryogenesis and the pluripotency of mouse embryonic stem cells; p. 1162-1174.
75. Nagata S, Toyoda M, Yamaguchi S, Hirano K, Makino H, Nishino K, Miyagawa Y, Okita H, Kiyokawa N, Nakagawa M, Yamanaka S, Akutsu H, Umezawa A, Tada T. Efficient reprogramming of human and mouse primary extra-embryonic cells to pluripotent stem cells. *Genes Cells*. 2009; 14:1395–404. [PubMed: 19912344]
76. Huangfu D, Osafune K, Maehr R, Guo W, Eijkelenboom A, Chen S, Muhlestein W, Melton DA. Induction of pluripotent stem cells from primary human fibroblast with only oct4 and sox2. *Nature Biotech*. 2008; 26:1269–1275.
77. Schenke-Layland K, Rhodes KE, Angelis E, Butylkova Y, Hyderkhan-Hagvall S, Gekas C, Zhang R, Goldhaber JJ, Mikkola HK, Plath K, MacLellan WR. Reprogrammed mouse fibroblasts differentiate into cells of cardiovascular and hematopoietic lineages. *Stem Cells*. 2008; 26:1537–1546. [PubMed: 18450826]
78. Narazaki G, Uosaki H, Teranishi M, Okita K, Kim B, Matsuoka S, Yamanaka S, Yamashita JK. Directed and systemic differentiation of cardiovascular cells from mouse induced pluripotent cells. *Circulation*. 2008; 118:498–506. [PubMed: 18625891]
79. Zhang J, Wilson GF, Soerens AG, Koonce CH, Yu J, Palecek SP, Thomson JA, Kamp TJ. Functional cardiomyocytes derived from human induced pluripotent stem cells. *Cir Res*. 2009; 104:e30–e41.
80. Chin MH, Mason MJ, Xie W, Volinia S, Singer M, Peterson C, Ambartsumyan G, Aimiwu O, Richter L, Zhang J, Khvorostov I, Ott V, Grunstein M, Lavon N, Benvenisty N, Croce CM, Clark AT, Baxter T, Pyle AD, Teitell MA, Pelegriani M, Plath K, Lowry WE. Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. *Cell Stem Cell*. 2009; 5:111–123. [PubMed: 19570518]
81. Doi A, Park IH, Wen B, Murakami P, Aryee MJ, Irizarry R, Herb B, Ladd-Acosta C, Rho J, Loewer S, Miller J, Schlaeger T, Daley GQ, Feinberg AP. Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. *Nat Genet*. 2009; 41:1350–1353. [PubMed: 19881528]
82. Deng J, Shoemaker R, Xie B, Gore A, LeProust EM, Antosiewicz-Bourget J, Egli D, Maherali N, Park IH, Yu J, Daley GQ, Eggan K, Hochedlinger K, Thomson J, Wang W, Gao Y, Zhang K. Targeted bisulfate sequencing reveals changes in DNA methylation associated with nuclear reprogramming. *Nat Biotechnol*. 2009; 27:353–360. [PubMed: 19330000]
83. Krumenacker JS, Katsuki S, Kots A, Murad F. Differential expression of genes involved in cGMP-dependent nitric oxide signaling in murine embryonic stem (ES) cells and cell-derived cardiomyocytes. *Nitric Oxide*. 2006; 14:1–11. [PubMed: 16188470]
84. Mujoo K, Krumenacker JS, Wada Y, Murad F. Differential expression of nitric oxide signaling components in undifferentiated and differentiated human embryonic stem cells. *Stem cells & Develop*. 2006; 15:779–787.

85. Mujoo K, Sharin V, Bryan N, Krumenacker JS, Sloan C, Parveen S, Kots A, Murad F. Role of nitric oxide signaling components in differentiation of embryonic stem cells to myocardial cells. *Proc Natl Acad Sci USA*. 2008; 105:18924–18929. [PubMed: 19020077]
86. Sharin V, Mujoo K, Kots A, Martin E, Murad F, Sharina I. Nitric oxide receptor soluble guanylyl cyclase undergoes splicing regulation in differentiating human embryonic stem cells. *Stem Cells & Develop*. 2011; 20:1287–1293.
87. Krumenacker JS, Murad F. NO-cGMP signaling in development and stem cells. *Mol Gene & Metabol*. 2006; 87:311–314.
88. Madhusoodnan KS, Murad F. NO-cGMP signaling and regenerative medicine involving stem cells. *Neurochem Res*. 2007; 32:681–694. [PubMed: 17043768]
89. Madhusoodanan, KS.; Mujoo, K.; Murad, F. Expression and localization of guanylyl cyclases during the differentiation of embryonic stem cells into neural precursor cells using a novel and highly efficient method. Abstract in 8th ISSCR meeting; San Francisco, CA. June 16-19, 2010; page 215-poster session abstracts
90. Shami PJ, Weinberg JB. Differential effects of nitric oxide on erythroid and myeloid colony growth from CD34+ human bone marrow cells. *Blood*. 1996; 87:977–982. [PubMed: 8562969]
91. Bøyum A, Fjerdingsstad HB, Tennfjord VA, Benestad HB, Løvhaug D. Specific antibodies to mouse Sca-1- (Ly-6A/E) or Thy-1-positive haematopoietic progenitor cells induce formation of nitric oxide which inhibits subsequent colony formation of nitric oxide which inhibits subsequent colony formation. *Eur J Haematol*. 2004; 73:427–430. [PubMed: 15522065]
92. Michurina T, Krasnov P, Balazs A, Nakaya N, Vasilieva T, Kuzin B, Khrushchov N, Mulligan RC, Enikolopov G. Nitric oxide is a regulator of hematopoietic stem cell activity. *Mol Ther*. 2004; 10:241–248. [PubMed: 15294171]
93. Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, Zeiher AM, Dimmeler S. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med*. 2003; 11:1370–1376. [PubMed: 14556003]
94. Guthrie SM, Curtis LM, Mames RN, Simon GG, Grant MB, Scott EW. The nitric oxide pathway modulates hemangioblast activity of adult hematopoietic stem cells. *Blood*. 2005; 105:1916–22. [PubMed: 15546953]
95. Bloch W, Fleischmann BK, Lorke DE, Andressen C, Hops B, Hescheler J, Addicks K. Nitric oxide synthase expression and role during cardiomyogenesis. *Cardiovasc Res*. 1999; 43:675–684. [PubMed: 10690339]
96. Kanno S, Kim PKM, Sallam K, Lei J, Billiar TR, Shears LL II. Nitric oxide facilitates cardiomyogenesis in mouse embryonic stem cells. *Proc Nat Acad Sci USA*. 2004; 101:12277–12281. [PubMed: 15304656]
97. Kloss S, Furneaux H, Mülsch A. Post-transcriptional regulation of soluble guanylyl cyclase expression in rat aorta. *J Biol Chem*. 2003; 278:2377–83. [PubMed: 12441354]
98. Marro ML, Peiró C, Panayiotou CM, Baliga RS, Meurer S, Schmidt HH, Hobbs AJ. Characterization of the human alpha1 beta1 soluble guanylyl cyclase promoter: key role for NF-kappaB(p50) and CCAAT-binding factors in regulating expression of the nitric oxide receptor. *J Biol Chem*. 2008; 283:20027–20036. [PubMed: 18474600]
99. Sharina IG, Martin E, Thomas A, Uray KL, Murad F. CCAAT-binding factor regulates expression of the beta 1 subunit of soluble guanylyl cyclase gene in the BE-2 human neuroblastoma cell line. *Proc Natl Acad Sci USA*. 2003; 100:11523–11528. [PubMed: 14504408]
100. Zhou Z, Sayed N, Pyriochou A, Roussos C, Fulton D, Beuve A, Papapetropoulos A. Protein kinase G phosphorylates soluble guanylyl cyclase on serine 64 and inhibits its activity. *Arterioscler Thromb Vasc Biol*. 2008; 10:1803–1810. [PubMed: 18635821]
101. Cabilla JP, Ronchetti SA, Nudler SI, Miler EA, Quinteros FA, Duvilanski BH. Nitric oxide sensitive-guanylyl cyclase subunit expression changes during estrous cycle in anterior pituitary glands. *Am J Physiol Endocrinol Metab*. 2009; 296:E731–737. [PubMed: 19141686]
102. Zhu DY, Lou YJ. Icarin-mediated expression of cardiac genes and modulation of nitric oxide signaling pathway during differentiation of mouse embryonic stem cells into cardiomyocytes *in vitro*. *Acta Pharmacol Sin*. 2006; 27:311–320. [PubMed: 16490167]

103. Pan W, Quarles LD, Song LH, Yu YH, Jiao C, Tang HB, Jiang CH, Deng HW, Li YJ, Zhou HH, Xiao ZS. Genistein stimulates the osteoblastic differentiation via NO/cGMP in bone marrow culture. *J Cell Biochem.* 2005; 94:307–316. [PubMed: 15526288]
104. Mujoo, K.; Nikonoff, LE.; Kots, AY.; Murad, F. Curcumin: a plant derived compound induces differentiation of human embryonic stem cells into myocardial cells; possible role of nitric oxide pathway. Abstract presented at 8th ISSCR meeting; San Francisco, CA. June 16-19, 2010; held at page 38-poster session abstracts
105. Mora-Castilla S, Tejedo JR, Hmadcha A, Cahuana GM, Martin F, Soria B, Bedoya FJ. Nitric oxide repression of Nanog promotes mouse embryonic stem cell differentiation. *Cell Death Differ.* 2010; 17:1025–1033. [PubMed: 20075941]
106. Tejedo JR, Tapia-Limonchi R, Mora-Castilla S, Cahuana GM, Hmadcha A, Martin F, Bedoya FJ, Soria B. Low concentrations of nitric oxide delay the differentiation of embryonic stem cells and promote their survival. *Cell Death Dis.* 2010; 1:e80. [PubMed: 21368853]
107. Gomez-Pinedo U, Rodrigo R, Cauli O, Herraiz S, Garcia-Verdugo JM, Pellicer B, Pellicer A, Filipo V. cGMP modulates stem cell differentiation to neurons in brain *in vivo*. *Neuroscience.* 2010; 165:1275–1283. [PubMed: 19958812]
108. Wong JC, Ficus RR. Essential role of nitric oxide(NO)/cGMP/protein kinase G type - 1 α (PKG-1 α) signaling pathway and arterial natriuretic peptide (ANP)/cGMP/PKG-1 α autocrine loop in promoting proliferation and cell survival of OP9 bone marrow stromal cells. *J Cell Biochem.* 2011; 112:829–39. [PubMed: 21328456]
109. Mobley S, Shookhof JS, Foshay K, Park M, Gallicano GI. PKG and PKC are downregulated during cardiomyocyte differentiation from embryonic stem cells: Manipulation of these pathways enhances cardiomyocyte production. *Stem Cells Int.* 2010; 2010:701212. [PubMed: 21048852]
110. Foresta C, Cabrelle A, Ferigo M, Caretta N, Garolla A, Palu G, Ferlin A. PDE-5 inhibitor Vardenafil, increases circulating progenitor cells in humans. *Int J Impotence Res.* 2005; 17:377–380.
111. Pekkinen M, Ahlstrom ME, Riehel U, Huttunen MM, Lamberg-Allardt CJ. Effect of phosphodiesterase 7 inhibition by RNA interference on the gene expression and differentiation of human mesenchymal stem cell-derived osteoblasts. *Bone.* 2008; 43:84–90. [PubMed: 18420479]
112. Zhang M, Takimoto E, Hsu S, Lee DI, Nagayama T, Danner T, Koitabashi N, Barth AS, Bedja D, Gabrielson KL, Wang Y, Kass DA. Myocardial remodeling is controlled by myocyte-targeted gene regulation of phosphodiesterase type 5. *J Am Coll Cardiol.* 2010; 56:2021–2030. [PubMed: 20970280]
113. Spallotta F, Rosati J, Straino S, Nanni S, Grasselli A, Ambrosino V, Rotili D, Valente S, Farsetti A, Mai A, Capogrossi MC, Gaetano C, Illi B. Nitric oxide determines mesodermic differentiation of mouse embryonic stem cells by activating class IIa histone deacetylases: potential therapeutic implications in a mouse model of hindlimb ischemia. *Stem Cells.* 2010; 28:431–442. [PubMed: 20073046]
114. Liu Y, Song J, Liu W, Wan Y, Chen X, Hu C. Growth and differentiation of rat bone marrow stromal cells: does 5-azacytidine trigger their cardiomyogenic differentiation? *Cardiovasc Res.* 2003; 58:460–468. [PubMed: 12757880]
115. Reinecke H, Poppa V, Murry CE. Skeletal muscle stem cells do not transdifferentiate into cardiomyocytes after cardiac grafting. *J Mol Cell Cardiol.* 2002; 34:241–249. [PubMed: 11851363]
116. Wagers AJ, Sherwood RI, Christensen JL, Weissman IL. Little evidence for developmental plasticity of adult hematopoietic stem cells. *Science.* 2002; 297:2256–2259. [PubMed: 12215650]
117. Min JY, Yang Y, Converso KL, Liu L, Huang Q, Morgan JP, Xiao YF. Transplantation of embryonic stem cells improves cardiac function in postinfarcted rats. *J Applied Physiol.* 2002; 92:288–296. [PubMed: 11744672]
118. Min JY, Yang Y, Sullivan MF, Ke Q, Converso KL, Chen Y, Morgan JP, Xiao YF. Long-term improvement of cardiac function in rats after infarction by transplantation of embryonic stem cells. *J Thorac Cardiovasc Surg.* 2003; 125:361–369. [PubMed: 12579106]

119. Naito H, Taniguchi S, Kawata T. Embryonic stem cell-derived cardiomyocyte transplantation into the infarcted myocardium. *Heart Surg Forum*. 2002; 6:1.
120. Behfar A, Zingman LV, Hodgson DM, Rauzier JM, Kane GC, Terzic A, Puceat M. Stem cell differentiation requires a paracrine pathway in the heart. *FASEB J*. 2002; 16:1558–1566. [PubMed: 12374778]
121. Yang Y, Min JY, Rana JS, Ke Q, Cai J, Chen Y, Morgan JP, Xiao YF. VEGF enhances functional improvement of postinfarcted hearts by transplantation of ESC-differentiated cells. *J Appl Physiol*. 2002; 93:1140. [PubMed: 12183512]
122. Kupatt C, Hinkel R, Vachenaue R, Horstkotte J, Raake P, Sandner T, Kreuzpointner R, Muller F, Dimmeler S, Feron O, Boekstegers P. VEGF165 transfection decreases postischemic NF- κ B-dependent myocardial reperfusion injury in vivo: role of eNOS phosphorylation. *FASEB J*. 2003; 17:705–707. [PubMed: 12586740]
123. Gyurko R, Kuhlencordt P, Fishman MC, Huang PL. Modulation of mouse cardiac function in vivo by eNOS and ANP. *Am J Physiol Heart Circ Physiol*. 2000; 278:H971–H981. [PubMed: 10710367]
124. Hoshida S, Yamashita N, Igarashi J, Nishida M, Hori M, Kamada T, Kuzuya T, Tada M. Nitric oxide synthase protects the heart against ischemia-reperfusion injury in rabbits. *J Pharmacol Exp Ther*. 1995; 274:413–418. [PubMed: 7542338]
125. Zhao X, Lu X, Feng Q. Deficiency in endothelial nitric oxide synthase impairs myocardial angiogenesis. *Am J Physiol Heart Circ Physiol*. 2002; 283:H2371–H2378. [PubMed: 12388304]
126. Wang D, Yang XP, Liu YH, Carretero OA, LaPointe MC. Reduction of myocardial infarct size by inhibition of inducible nitric oxide synthase. *Am J Hypertens*. 1999; 12:174–182. [PubMed: 10090345]
127. Wildhirt SM, Dudek RR, Suzuki H, Bing RJ. Involvement of inducible nitric oxide synthase in the inflammatory process of myocardial infarction. *Int J Cardiol*. 1995; 50:253–261. [PubMed: 8537149]
128. Janssens S, Pokreisz P, Schoonjans L, Pellens M, Vermeersch P, Tjwa M, Jans P, Scherrer-Crosbie M, Picard MH, Szeliid Z, Gillijns H, Van de Werf F, Collen D, Bloch KD. Cardiomyocyte-specific overexpression of nitric oxide synthase 3 improves left ventricular performance and reduces compensatory hypertrophy after myocardial infarction. *Circ Res*. 2004; 94:1256–1262. [PubMed: 15044322]
129. Damy T, Ratajczak P, Robidel E, Bendall JK, Oliviero P, Boczkowski J, Ebrahimian T, Marotte F, Samuel JL, Heymes C. Up-regulation of cardiac nitric oxide synthase 1-derived nitric oxide after myocardial infarction in senescent rats. *FASEB J*. 2003; 17:1934–1936. [PubMed: 12897062]
130. Calderone A, Thaik CM, Takahashi M, Chang DL, Colucci WS. Nitric oxide, atrial natriuretic peptide, and cGMP inhibit the growth-promoting effects of norepinephrine in cardiac myocytes and fibroblasts. *J Clin Invest*. 1998; 101:812–818. [PubMed: 9466976]
131. Ishigai Y, Mori T, Ikeda T, Fukuzawa A, Shibano T. Role of bradykinin-NO pathway in prevention of cardiac hypertrophy by ACE inhibitor in rat cardiomyocytes. *Am J Physiol*. 1997; 273:H2659–H2663. [PubMed: 9435601]
132. Wollert KC, Fiedler B, Gambaryan S, Smolenski A, Heineke J, Butt E, Trautwein C, Lohmann SM, Drexler H. Gene transfer of cGMP-dependent protein kinase I enhances the antihypertrophic effects of nitric oxide in cardiomyocytes. *Hypertension*. 2002; 39:87–92. [PubMed: 11799084]
133. Dulak J, Jozkowicz A, Guevara I, Dembinska-Kiec A. Gene transfer of vascular endothelial growth factor and endothelial nitric oxide synthase-implications for gene therapy in cardiovascular diseases. *Pol J Pharmacol*. 1999; 51:233–241. [PubMed: 10600037]
134. Kishimoto I, Rossi K, Garbers DL. A genetic model provides evidence that the receptor for atrial natriuretic peptide (guanylyl cyclase-A) inhibits cardiac ventricular myocyte hypertrophy. *Proc Nat Acad Sci USA*. 2001; 98:2703–2706. [PubMed: 11226303]
135. Zahabi A, Picard S, Fortin N, Reudelhuber TL, Deschepper CF. Expression of constitutively active guanylate cyclase in cardiomyocytes inhibits the hypertrophic effects of isoproterenol and aortic constriction on mouse hearts. *J Biol Chem*. 2003; 278:47694–47699. [PubMed: 14500707]

136. Fiedler B, Lohmann SM, Smolenski A, Linnemuller S, Pieske B, Schroder F, Molkentin JD, Drexler H, Wollert KC. Inhibition of calcineurin-NFAT hypertrophy signaling by cGMP-dependent protein kinase type I in cardiac myocytes. *Proc Nat Acad Sci USA*. 2002; 99:11363–11368. [PubMed: 12177418]
137. Knowles JW, Esposito G, Mao L, Hagaman JR, Fox JE, Smithies O, Rockman HA, Maeda N. Pressure-independent enhancement of cardiac hypertrophy in natriuretic peptide receptor A-deficient mice. *J Clin Invest*. 2001; 107:975–984. [PubMed: 11306601]
138. Leontiadis E, Manginas A, Cokkinos DV. Cardiac repair-fact or fancy? *Heart Fail Rev*. 2006; 11:155–170. [PubMed: 16937035]
139. Kofidis T, de Bruin JL, Yamane T, Tanaka M, Lebl DR, Swijnenburg RJ, Weissman IL, Robbins RC. Stimulation of paracrine pathways with growth factors enhances embryonic stem cell engraftment and host-specific differentiation in the heart after ischemic myocardial injury. *Circulation*. 2005; 111:2486–93. [PubMed: 15883216]
140. Kumar D, Kamp TJ, Lewinter MM. Embryonic stem cells: differentiation into cardiomyocytes and potential for heart repair and regeneration. *Coronary Artery Dis*. 2005; 16:111–116.
141. Laugwitz KL, Moretti A, Lam J, Gruber P, Chen Y, Woodard S, Lin LZ, Cai CL, Lu MM, Reth M, Platoshyn O, Yuan JX, Evans S, Chien KR. Post natal is11+ cardioblast enter fully differentiated cardiomyocyte lineages. *Nature*. 2005; 433:647–653. [PubMed: 15703750]
142. Smits AM, Vliet P, Hassink RJ, Goumans MJ, Doevendans PA. The role of stem cells in cardiac regeneration. *J Cell Mol Med*. 2005; 9:25–36. [PubMed: 15784162]
143. Li N, Lu X, Zhao X, Xiang FL, Xenocostas A, Karmazyn M, Feng Q. Endothelial nitric oxide synthase promotes bone marrow stromal cell migration to the ischemic myocardium via upregulation of stromal cell-derived factor-1alpha. *Stem Cells*. 2009; 27:961–970. [PubMed: 19353524]
144. Lin YC, Leu S, Sun CK, Yen CH, Kao YH, Chang LT, Tsai TH, Chua S, Fu M, Ko SF, Wu CJ, Lee FY, Yip HK. Early combined treatment with sildenafil and adipose-derived mesenchymal stem cells preserves heart function in rat dilated cardiomyopathy. *J Transl Med*. 2010; 8:88. [PubMed: 20868517]
145. van Laake LW, Passier R, Monshouwer-Kloots J, Verkleij AJ, Lips DJ, Freund C, den Ouden K, Ward-van Oostwaard D, Korving J, Tertoolen LG, van Echteld CJ, Doevendans PA, Mummery CL. Human embryonic stem cell-derived cardiomyocytes survive and mature in the mouse heart and transiently improve function after myocardial infarction. *Stem Cell Res*. 2007; 1:9–24. [PubMed: 19383383]
146. van Laake LW, Passier R, den Ouden K, Schreurs C, Monshouwer-Kloots J, Ward-van Oostwaard D, van Echteld CJ, Doevendans PA, Mummery CL. Improvement of mouse cardiac function by hESC-derived cardiomyocytes correlates with vascularity but not graft size. *Stem Cell Res*. 2009; 3:106–112. [PubMed: 19560991]
147. Braam SR, Passier R, Mummery CL. Cardiomyocytes from human pluripotent stem cells in regenerative medicine and drug discovery. *Trends Pharmacol Sci*. 2009; 30:536–545. [PubMed: 19762090]

Table 1
Regulation of nitric oxide signaling components during mouse and human embryonic stem cell differentiation

NO Signaling components	sGC α_1	sGC α_2	sGC β_1	sGC β_2	NOS-1	NOS-2	NOS-3	PKG1 α
Stem cells	-	-	-	+	+	-	-	-
EB stage	++	+	+	-	+/-	+	+	+
Differentiated cells	++++	+++	+++	-	-	+++	+++	+++

Symbols: - very low basal levels; + moderate increase in gene expression and protein levels; +++ robust increase in gene expression and protein levels.

Embryoid body (EB) stage represents mixture of undifferentiated and differentiated cells.