Cardiac Progenitor Cell Commitment Is Inhibited by Nuclear Akt Expression

Kimberlee M. Fischer, Shabana Din, Natalie Gude, Mathias H. Konstandin, Weitao Wu, Pearl Quijada, Mark A. Sussman

Rationale: Stem cell therapies to regenerate damaged cardiac tissue represent a novel approach to treat heart disease. However, the majority of adoptively transferred stem cells delivered to damaged myocardium do not survive long enough to impart protective benefits, resulting in modest functional improvements. Strategies to improve survival and proliferation of stem cells show promise for significantly enhancing cardiac function and regeneration.

Objective: To determine whether injected cardiac progenitor cells (CPCs) genetically modified to overexpress nuclear Akt (CPCeA) increase structural and functional benefits to infarcted myocardium relative to control CPCs.

Methods and Results: CPCeA exhibit significantly increased proliferation and secretion of paracrine factors compared with CPCs. However, CPCeA exhibit impaired capacity for lineage commitment in vitro. Infarcted hearts receiving intramyocardial injection of CPCeA have increased recruitment of endogenous c-kit cells compared with CPCs, but neither population provides long-term functional and structural improvements compared with saline-injected controls. Pharmacological inhibition of Akt alleviated blockade of lineage commitment in CPCeA.

Conclusions: Although overexpression of nuclear Akt promotes rapid proliferation and secretion of protective paracrine factors, the inability of CPCeA to undergo lineage commitment hinders their capacity to provide functional or structural benefits to infarcted hearts. Despite enhanced recruitment of endogenous CPCs, lack of functional improvement in CPCeA-treated hearts demonstrates CPC lineage commitment is essential to the regenerative response. Effective stem cell therapies must promote cellular survival and proliferation without inhibiting lineage commitment. Because CPCeA exhibit remarkable proliferative potential, an inducible system mediating nuclear Akt expression could be useful to augment cell therapy approaches. (Circ Res. 2011;108:960-970.)

Key Words: cardiac progenitor cell ■ proliferation ■ differentiation ■ Akt

Stem cell therapies are being explored as a novel way to treat heart failure.1–5 Unfortunately, to date, relatively modest improvements in cardiac structure and function have been observed, in part, because of poor stem cell proliferation and viability after delivery. To improve benefits of stem cell therapy, mechanisms promoting proliferation and survival of the stem cell population without inhibiting lineage commitment have become an area of intense research focus. Enhanced efficacy of genetically modified stem cells mediating myocardial regeneration following infarction has been demonstrated using Pim-1, a cell survival and proliferation kinase downstream of Akt/PKB.6

Akt/PKB is a pivotal regulatory kinase with various roles regarding growth, metabolism, and survival.7–13 In the heart, Akt is among the most well-studied cardioprotective kinases with well-documented capacity to prevent cardiomyopathic injury.6,19–26 Activation of Akt is initiated by growth factor dependent stimulation of receptor tyrosine kinases, which in turn stimulate a cascade of signaling events beginning with the activation of PI3 kinase (PI3K) at the plasma membrane. Subsequent activation of PDK1/2 (phosphoinositide-dependent kinase-1/2) phosphorylates and activates Akt. Downstream targets of Akt are numerous and include proproliferative and antiapoptotic substrates.10,14,18,20,27–29

To investigate mechanisms governing cardioprotective effects of Akt, a variety of systems including cardiac-specific overexpression and viral infections have been used. Numerous studies attribute short-term Akt activation to the profound protective effects seen in posts ischemic injury models, whereby Akt induces secretion of paracrine factors, drastically increases cell cycle and inhibits apoptosis in cardiomyocytes.14,19–22,24–26,30 Additionally, Akt activation stimulates neoangiogenesis and vasculogenesis, in part accounting for the dramatic improvements seen in pathologically chal-
lenged Akt transgenic mice. However, constitutive activation of Akt can have detrimental effects on the myocardium, including hypertrophic growth and abnormal vascular remodeling34; the majority of protective effects afforded by Akt have been found to be governed through its nuclear localization.14,21,24,35 Previous studies by our group demonstrate cardiac specific overexpression of nuclear Akt allows for expansion of the progenitor cell pool, as well as enhanced protection of the myocardium against pathological injury without induction of hypertrophic remodeling.14

Although the cardioprotective effects of nuclear Akt are clear within myocytes of transgenic animals, protective effects of nuclear Akt expression on the cardiac progenitor cell population have not been investigated. The present study evaluates the ability of cardiac progenitor cells (CPCs) modified with nuclear Akt (CPCeA) to mediate cardioprotection in infarcted hearts.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Lentiviral Vectors and Generation of Lentivirus**

Bicistronic lentiviral vectors were generated as previously described.6

**CPC Isolation, Cell Culture, and Lentiviral Infection**

CPCs isolated from 10 to 12-week-old male FVB mice, cultured in cardiac stem cell media and infected with lentivirus as previously described.6 Cells were incubated with Akt kinase inhibitor-V (AIV) (10\(\mu\)mol/L) for 7 days with and without dexamethasone (Dex) at 10\(\mu\)mol/L where indicated.

**Trypan Blue and CyQuant Assays**

Uninfected, CPCe, and CPCeA cells were plated in quadruplicate (10,000 cells/well) in 24-well plates. To determine proliferation rate cells were harvested at 96-hours and stained 1:10 with trypan blue. Viable cells determined by trypan blue exclusion were counted. CPCe and CPCeA were plated in quadruplicate in 96-well plates (4000 cells/well), and CyQuant (Invitrogen) reagent was added at indicated time points, incubated for 45 minutes, and read at 530 nm.

**Immunoblots, Immunohistochemistry, and Confocal Microscopy**

Immunoblots, immunohistochemistry, and confocal microscopy were performed as previously described6 with additional details in the Online Methods section.

**C-kit\(^+\) Cell Quantification**

Sections from CPCe- or CPCeA-injected animals were immunolabeled with antibodies against c-kit, GFP, and tropomyosin. Tropomyosin was used to measure infarct area using Leica confocal software. Total c-kit\(^+\) cells, c-kit\(^+\)GFP\(^+\) and c-kit\(^+\)GFP\(^-\) CPCs were quantified in the infarct area.

**Myocardial Infarction, Injections, Echocardiography, and Hemodynamics**

Infarctions, echocardiography, and hemodynamics were performed as previously described,6 with additional details provided in the Online Methods section. Animals were injected at 5 sites surrounding the border zone with PBS, CPCe, or CPCeA (total of 100,000 cells per heart).
SuperArray and Quantitative Real-Time Polymerase Chain Reactions

For quantitative real-time polymerase chain reaction (qRT-PCR), RNA was harvested as per the protocol of the manufacturer (Zymo Research, R1055). cDNA was obtained using iScript cDNA synthesis kit (Bio-Rad, 170 to 8891). qRT-PCR was run using the iQ SYBR Green Supermix (Bio-Rad, 170 to 8882) because cDNA was synthesized. Cell proliferation array (SuperArray, PAMM-020) was performed according to the protocol of the manufacturer. Primers for qRT-PCR were designed using PubMed Primer-blast. Sequences provided in the Online Methods section.

Enzyme-Linked Immunosorbent Assay

Cells were plated at 3000 cells/well in 150 mL of stem cell media and cell culture supernatant harvested 24-hours later. SDF-1 ELISA was run according to the protocol of the manufacturer (RayBiotech, ELM-SDF1alpha-001).

Statistics

Statistics were calculated using Prism software. One-way ANOVA and 2-way repeated-measures ANOVA analysis for echocardiography with Tukey post hoc test were calculated. Values with $P<0.05$ were considered statistically significant.

Animal Studies

All animal studies were performed in accordance with IACUC approved protocols.

Results

Overexpression of Nuclear-Akt in CPCs

cDNA from murine Akt was fused to a 3× nuclear localization sequence (NLS) targeting Akt to the nuclear compartment of the cell and a myc-tag to facilitate detection of the engineered protein. Bicistronic lentiviral vectors (Online
Figure 3. Intramyocardial injection of CPCeA does not improve cardiac function. A through C, Electrocardiographic assessment of anterior wall dimension (AWD) (A), fractional shortening (FS) (B), and ejection fraction (EF) (C) in sham (●) (n=4), PBS (✩) (n=7), CPCe (▲) (n=8), and CPCeA (◆) (n=7) 12 week after infarction (means±SEM). D through F, Cardiac function of sham (●) (n=4), PBS (✩) (n=5), CPCe (▲) (n=6), and CPCeA (◆) (n=5) were evaluated using in vivo hemodynamic measurements of left ventricular developed pressure (LVEDP) (D), left ventricular end diastolic pressure (LVEDP) (E), and dP/dT (F) 12 weeks after intramyocardial injection (means±SEM). Two-way ANOVA analysis was run for echocardiography and 1-way ANOVA for hemodynamics. Where appropriate, Tukey post hoc test was performed: #P<0.05, ##P<0.01, ###P<0.001 compared with sham; *P<0.05, **P<0.01, ***P<0.001 compared with CPCe; +P<0.05, ++P<0.01, +++P<0.001 compared with PBS; P<0.05, ++P<0.01, +++P<0.001 compared with CPCeA.

CPCeA Increase Proliferation and Alter Expression of Cell Cycle Genes

The proliferation rate of CPCeA was significantly (P<0.01) increased relative to CPCe, over a 96-hour time course as determined by Trypan blue exclusion measuring total number of viable cells (Figure 1A). Additionally, CPCeA have a significant (P<0.05) increase in proliferation compared with CPCe over a 96-hour time course as determined by CyQuant assay (Figure 1B). Proliferation was attenuated in CPCeA and CPCe by 96-hours with addition of AIV (Figure 1B). Altered RNA expression for several cell cycle genes was confirmed in CPCeA compared with CPCe controls by cell cycle array (Online Figure II, A). Specifically, cyclin-D1 protein is
markedly attenuated (Online Figure II, B) and protein expression of Chk1 and CDC2 is significantly increased in CPCeA (Online Figure II, C) compared with CPCe controls.

**Increased Number of C-kit⁺ Cells in Hearts Receiving CPCeA**

To assess whether protective benefits are gained from intramyocardial injection of CPCeA, 12-week-old female mice were subjected to infarction and CPCe or CPCeA were adoptively transferred. The number of c-kit⁺ cells was quantified within the infarct region of animals receiving PBS, CPCe or CPCeA. At 12 weeks, CPCeA-injected hearts had a significant ($P<0.01$) 2.3-fold increase in total c-kit⁺ cells compared with CPCe-injected controls (Figure 2A). Additionally, CPCeA-injected hearts had a significant ($P=0.005$) 2.7-fold increase in c-kit⁺ eGFP⁺ cells (Figure 2B) and a 1.8-fold increase in c-kit⁺ eGFP⁻ cells ($P<0.05$) (Figure 2C) compared with CPCe-injected controls. There was no statistical difference ($P>0.05$) in the number of c-kit⁺ eGFP⁻ cells between CPCe and saline-injected hearts. Although a significant number of c-kit⁺ GFP⁺ CPCs were identified in hearts receiving CPCeA after 12 weeks, there was a noticeable lack of GFP⁺ CPCs expressing markers consistent with cardiac lineage commitment as evidenced by the absence of colocalization between GFP and desmin or sarcomeric α-actin (Figure 2D and 2E). In comparison, control CPCe acquire markers consistent with cardiogenic lineage commitment after infarction (Online Figure III and previously published data⁶).

**CPCeA Do Not Improve Function or Structure of Infarcted Myocardium**

Cardiac function, after infarction and injection, was assessed by echocardiography and in vivo hemodynamics. Hearts of animals receiving CPCeA did not show a statistically significant improvement over CPCe-injected hearts in anterior wall dimension (Figure 3A), fractional shortening (Figure 3B), or ejection fraction (Figure 3C), at 12 weeks as assessed by 2-way ANOVA statistical analysis. Hemodynamic assessment further confirmed deterioration of cardiac function in CPCeA-injected animals as assessed by left ventricular developed pressure (Figure 3D), left ventricular end diastolic pressure (Figure 3E), and dP/dT maximum and minimum (Figure 3F). In fact, as early as four weeks postinjection, cardiac function in CPCeA-injected hearts was not statistically different ($P>0.05$) from PBS-injected controls. CPCe-injected hearts show a statistically significant ($P<0.05$) improvement in cardiac function at early time points (4 weeks), but beneficial effects were not sustained and were indistinguishable from PBS-injected controls by 12 weeks (Figure 3). Additionally, at 12 weeks after infarction, CPCeA-injected animals did not have a statistically significant reduction in infarct size compared with CPCe-injected controls (Online Figure IV).

**CPCeA Express Paracrine Factors Induced by Akt Activity**

Increase numbers of endogenous (eGFP⁺, c-kit⁻) stem cells within the infarct (Figure 2C) suggests CPCeA release paracrine factors promoting recruitment of resident CPCs to the site of injury. Thus, mRNA expression of paracrine factors known to be induced by Akt activity was assessed by quantitative RT-PCR (qRT-PCR) on CPCeA in vitro. CPCeA express significantly ($P<0.001$) more transcripts for FGF-2, FST-1, SDF-1 and VEGF (Figure 4A). CPCeA exhibit a significant 3.5-fold increase in SDF-1 protein expression compared with CPCe controls by ELISA assay (Figure 4B). Interestingly, SDF-1 is a potent chemoattractant, previously demonstrated to attract stem cells to sites of injury.³⁶,³⁷

**CPCeA Abrogate In Vitro Differentiation**

Absence of in vivo structural and functional improvement, combined with the observation that injected CPCeA did not appear to acquire markers consistent with cardiac lineage commitment, prompted assessment of CPCeA capacity for differentiation in vitro. CPCe and CPCeA were treated with dexamethasone (Dex) for seven days to induce differentiation and evaluated for c-kit protein expression by immunocytochemistry. CPCeA maintained c-kit expression after treatment with Dex in contrast to CPCe controls whereby c-kit expression was lost (Figure 5, right). Both CPCe and CPCeA expressed c-kit before Dex treatment (Figure 5, left). In addition to retention of c-kit expression on differentiation.
stimulation, CPCeA also fail to induce cardiac troponin (cTnT), Mef2C, or Gata4 transcripts, markers consistent with cardiogenic lineage commitment (Figure 6A through 6C). In contrast, CPCeP express significantly ($P<0.001$) more Mef2C and Gata4 transcript than CPCeA when treated with Dex (Online Figure V).

Elevated Levels of Phosphorylated CREB in CPCeA
cAMP response element binding protein (CREB) is a downstream target of Akt and has been previously demonstrated to promote progenitor cell proliferation.\(^{12,38}\) CPCe and CPCeA were treated with and without Dex for seven days and immunoblotted to assess CREB phosphorylation status. Undifferentiated CPCeA had a statistically significant ($P=0.007$) 3.3-fold increase in the level of phospho-CREB compared with CPCe (Figure 7A and 7B). The differential in CREB phosphorylation was even greater after differentiation, with Dex treated CPCeA showing a 19-fold significant ($P=0.0003$) increase in the level of phospho-CREB compared with Dex treated CPCe controls (Figure 7B). By comparison, increases in phospho-CREB after differentiation were not observed in CPCeP (Online Figure VI).

Attenuation of Akt Activity Increases Cardiac Lineage Commitment
CPCe and CPCeA were treated in vitro with inhibitor (AIV) to inhibit Akt kinase activity and subjected to Dex induced differentiation. CPCeA treated with AIV and Dex show a statistically significant ($P<0.002$) 2.7-fold reduction in phospho-CREB protein expression (Figure 8A) compared with Dex treated CPCeA without AIV treatment. Presumably nuclear Akt activity blocks the capacity of CPCeA to undergo lineage commitment on exposure to Dex. Therefore CPCeA and CPCe were treated with AIV and assessed for transcript levels of cTnT either with or without Dex exposure. CPCe and CPCeA do not express cTnT before Dex-induced differentiation (Figure 8B) as detected by qRT-PCR. In contrast, on induction of differentiation CPCeA express low levels of cTnT, although at significantly ($P<0.008$) reduced levels compared with CPCe controls (Figure 8B). Next, treatment of CPCeA and CPCe with AIV before Dex-induced differentiation was performed to confirm overexpression of nuclear Akt in CPCeA abrogates cardiac lineage commitment. Indeed, CPCeA treated with AIV and Dex had a significant reduction in phospho-CREB protein levels (Figure 8A) as well as a statistically significant ($P<0.008$) increase in cTnT transcript (Figure 8C), compared with CPCeA treated with...
Dex alone. CPCe also had significant increases in TnT transcript after treatment with AIV and Dex, compared with CPCe treated with Dex alone (Figure 8C), although the difference was not as dramatic as in CPCeA.

Discussion

For years, treatment of the damaged myocardium has had the major limitation of being unable to regenerate functional cardiac tissue. Pharmaceutical treatments prolong the life of many patients, but ultimately fail as a permanent “fix” for treatment of heart failure. Recently, the advent of stem cell research and tissue regeneration presents a potential long-term solution for the repair of damaged myocardium. Clinical trials whereby stem cells are delivered to the damaged myocardium are underway; however, results generally offer modest short-term improvements in cardiac function. A plausible biological explanation for the underwhelming outcomes of such adoptive transfer studies is that only a minority of delivered stem cells survives in damaged myocardium. These observations led to the hypothesis that increasing the ability of adoptively transferred stem cells to survive and proliferate will significantly improve efficacy of stem cell regeneration in the heart.

Genetic modification of stem cells with survival kinases, in particular Akt and associated downstream targets, improves the ability of progenitor cells to mitigate cardiac damage and improve regeneration.6,26,39 Although constitutive activation of Akt leads to hypertrophic growth and abnormal vascular remodeling, a plethora of studies have shown short-term Akt activation, as well as nuclear localized Akt, imparts protective benefits including growth, inhibition of cell death and increased angiogenesis to the pathologically challenged heart. However, successful modification of stem cells requires the ability to increase proliferation and survival without inhibiting lineage commitment following appropriate environmental stimulation. The majority of experiments involving Akt overexpression in stem cells do not measure the amount or the duration of this stimulation within the progenitor cell pool.19,22,26,40 Although protective benefits have been gained through Akt activation, several studies also demonstrate sustained overexpression can inhibit lineage commitment and terminal differentiation in various progenitor cell populations.12,41

Overexpression of nuclear Akt dramatically increases CPC proliferation and significantly expands the cell population in vitro (Figure 1), likely resulting from regulation of various cell cycle genes (Online Figure II). Although we previously reported activation of Pim-1 downstream of nuclear Akt in myocytes,20 Pim-1 expression in CPCs was not increased in CPCeA when compared with controls (Online Figure I, C). The basis for this differential action of nuclear Akt accumulation on Pim-1 expression is unknown at present, but presumably is tied to context-dependent cross-talk between...
nuclear Akt and Pim-1 depending on the cell type and possibly proliferation status.

CPCeA-injected hearts also retain significantly more c-kit⁺GFP⁺ cells after infarction compared with CPCe controls, indicating CPCeA had increased proliferation in vivo (Figure 2A, 2B, and 2E). Numerous studies also demonstrate modification of progenitor cells with Akt, induces secretion of paracrine factors,23,30,42–44 promoting survival of endogenous myocardium as well as the adoptively transferred population. Similarly, CPCeA secrete paracrine factors, most notably SDF-1 (Figure 4A and 4B), a chemottractant that promotes recruitment of stem cells to sites of injury. SDF-1 production by CPCeA may account in part for the observed increase of endogenous c-kit⁺GFP⁺ cells to the infarct. Although the majority of the c-kit⁺GFP⁺ population are presumably endogenous CPCs at the time of assessment, we cannot exclude the possibility that SDF-1 secretion also leads to increased homing of bone marrow stem cells to the infarct in CPCeA-injected hearts (Figure 2C) that may include mast cells. Regardless of cell origin, the increase in endogenous c-kit⁺ cells observed in CPCeA-injected animals, whether CPCs, bone marrow cells, mast cells, or a combination, does not mediate salutary action in the infarcted myocardium (Figure 3).

Although CPCeA persistence after adoptive transfer into infarcted hearts was significantly improved compared with CPCe controls there was no concurrent benefit for myocardial function (Figure 3) or structure (Figure 2B, 2D, and 2E; Online Figure IV), because overexpression of nuclear Akt antagonizes cardiogenic lineage commitment (Figure 2D and 2E; Figures 5 and 6; and Figure 8B and 8C). In contrast to CPCeA, CPCs overexpressing Pim-1, a downstream target of Akt, retain the capacity for cardiogenic commitment as demonstrated by increased gene expression of Gata4 and Mef2C when compared with CPCeA (Online Figure V). These effects are likely the consequence of Akt-mediated deregulation of cell cycle control and overriding normal differentiation programming. These results help to explain, at least in part, the stark contrast in protective benefits afforded by CPCeP6 but not from CPCeA when delivered to the infarcted heart.

Figure 8. Nuclear Akt Attenuation increases lineage commitment. A, Immunoblot and quantitation of Dex-treated CPCe and CPCeA, incubated with and without Akt inhibitor. B and C, qRT-PCR quantitation of TnT transcript levels in CPCe and CPCeA treated with or without Dex treatment and no AIV (B) or with AIV (C). Values were normalized to CPCe treated with Dex (means±SEM, n=3).
The transcription factor CREB is phosphorylated and activated by Akt on serine 133.12,45 Phosphorylation of CREB induces proliferation and elevated levels of phosphorylated CREB are found in several forms of cancer.46 Chondrocyte progenitor cells overexpressing Akt and phospho-CREB were highly proliferative but also refractory to differentiation,12 consistent with our observations of CPCeA. Furthermore, terminal differentiation of chondrocyte progenitor cells was observed only after inhibition of Akt activity.12 Similarly in our studies, inhibition of Akt activity in CPCeA reduced phospho-CREB levels 2.7-fold (Figure 8A) and resulted in increased expression of cardiac TnT transcript, a marker consistent with cardiogenic differentiation (Figure 8B and 8C). In contrast to CPCeA, CPCeP do not overexpress phospho-CREB after differentiation (Online Figure VI) and are capable of cardiogenic lineage commitment (Online Figure V).6 Although CPCeA delivery enhances endogenous CPC recruitment that presumably are capable of lineage commitment,6 the increase in endogenous CPCs fails to provide functional improvements in the damaged myocardium. Thus, improving endogenous repair responses may modestly increase short-term benefits to infarcted myocardium but ultimately fail to bring about the magnitude of a response necessary for meaningful regeneration. Collectively, results presented here help to reconcile the argument that although paracrine factor delivery may provide minor benefits, it is not sufficient for long-term improvements in structure and function of the damaged myocardium.

Genetic modification of stem cell populations with cardioprotective genes has now been demonstrated in numerous studies as a legitimate approach to foster repair and regeneration in the pathologically damaged heart. Importantly, this study demonstrates that although paracrine factors may mitigate damage in early stages, CPC commitment is essential to the long-term regenerative response. Therefore, effective cardiac stem cell therapies must promote cellular survival and proliferation, as well as long-term engraftment and successful lineage commitment of the donated cell population.

Acknowledgments
We thank all members of the Sussman laboratory for helpful discussions and technical support.

Sources of Funding
K.M.F. is supported by the Rees-Stealy Foundation, an ARCS Foundation fellowship, and an Inamori fellowship. M.A.S. is supported by NIH grants 2R01HL067245, 1R37HL091102-01, RC1HL100891-02, 1R21HL102714-01, P01HL085577-05, R01HL105759-01, and 1R21HL104544-01. M.H.K. is supported by Deutsche Forschungsgemeinschaft (DFG) grant KO 3900/1-1.

Disclosures
None.

References
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28. Gneecchi M, He H, Liang OD, Melo LG, Morello F, Mu H, Noiseux N, Zhang L, Pratt RE, Ingwall JS, Dzau VJ. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med. 2005;11:367–375.


Novelty and Significance

What Is Known?

- Cell-mediated cardiac regeneration is a novel therapeutic modality for the treatment of heart disease.
- Existing stem cell therapies provide only modest structural and functional benefits to the damaged myocardium.
- Genetic modification of CPCs could potentially increase their therapeutic efficacy.

What New Information Does This Article Contribute?

- Nuclear Akt modification of CPCs inhibits lineage commitment.
- Paracrine factor secretion improves homing but does not contribute to long-term protective benefits in the structure or the function of the infarcted myocardium.
- CPC lineage commitment is essential for long-term structural and functional recovery in the pathologically challenged myocardium.

Cell-mediated cardiac regeneration has withstood concerns related to safety and is now undergoing scrutiny for efficacy and durability. Unfortunately, present stem cell therapies provide only modest functional and structural improvements to the damaged heart, lagging far behind the desired benefits necessary for justifiable widespread clinical implementation. To enhance the regenerative process, genetically altered stem cells capable of enhanced proliferation and survival have been shown to drastically improve structural and functional benefits to the pathologically challenged myocardium. This study demonstrates that increased proliferation and survival of CPCs is ineffective for enhancing reparative processes if lineage commitment is inhibited. CPCs modified with nuclear-targeted Akt kinase, a mediator of cell survival and proliferation, show enhanced expansion and persistence on adoptive transfer to the infarcted myocardium with increased secretion of protective paracrine factors. Despite short-term benefits to the infarcted heart, long-term cardiac repair fails because nuclear Akt-modified CPCs are incapable of cardiac lineage commitment. Therefore, to realize the full potential of CPC-mediated regeneration, cell-based therapy will require enhancement of cellular survival, proliferation, and long-term engraftment, together with successful lineage commitment of the donated cell population.
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_Circ Res._ 2011;108:960-970; originally published online February 24, 2011; doi: 10.1161/CIRCRESAHA.110.237156

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Data Supplement (unedited) at:
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SUPPLEMENTAL MATERIAL

Supplemental Methods:

Generation of Lentiviral vectors
Bicistronic lentiviral vectors were generated by introducing murine AKT cDNA fused to a 3X nuclear localization sequence as well as a myc tag. The control construct CPCe, expresses eGFP off an internal ribosomal entry site (IRES), while the nuclear targeted AKT construct termed CPCeA, expresses nuclear targeted AKT from a myeloproliferative sarcoma virus LTR-negative control region deleted (MND) promoter as well as eGFP off an IRES.

Cardiac Progenitor Cell Isolation, Cell culture, and Lentiviral Infection
CPCs were isolated from 10-12 week old male FVB mice and cultured in cardiac stem cell (CSC) media: DMEM F-12, 10% FBS, 1% PSG, 1X ITS (Lonza, 17-838Z), .4mg/ml EGF (Sigma, E9644), .02ng/ml bFGF (Peprotech, 100-18B), and 1000U/ml LIF (Chemicon #ESG1107). CPCs were plated in 96-well flat bottom plates and transduced with lentivirus (eGFP or nuclear AKT) at an MOI of 10 overnight. CPCs were washed 18 hours later and fresh CSC media was added. Cells were expanded and analyzed by flow cytometry to determine the percentage of eGFP+ cells. Cells were incubated with Akt inhibitor V (10mmol/L) for 7 days with and without Dexamethazone where indicated. Differentiation media consisted of aMEM, 10% FBS, 1% PSG, and 10^-8mol/L Dexamethazone.

Western blot:
CPCe and CPCeA were plated in 6-well dishes (50,000 cells/well), harvested in sample buffer, boiled, and sonicated before running on 4-12% Bis-Tris gels. Primary antibodies were incubated overnight at 4°C in 7% milk. Secondary antibodies were used at 1:4000 dilutions and incubated at room temperature for two hours. Membranes were washed in 1X (Tris Buffered Saline and Tween) TBST and scanned on a Typhoon.

Myocardial Infarction, Injections, Echocardiography, and Hemodynamics:
Ten to twelve week old female FVB mice were anesthetized under isoflurane, intubated, and ventilated. A thoracotomy was performed and the LAD ligated. Vehicle (n=18), CPCe (n=16), or CPCeA (n=18) were injected by blinded surgeon at five minutes post ligation around border zone in five sites with a total of 100,000 cells per heart. Sham animals (n=8) were used as controls. Infarction size was standardized by echocardiography performed on animals imaged along a parasternal short-axis view by M-mode recorded at 3 days post-infarction/injection. Lack of anterior wall motion in conjunction with at least 40% decrease in EF and FS were required for study inclusion. Hemodynamic performance assessed by echocardiography three days post-infarction was not statistically different between infarcted and injected groups (PBS, CPCe, and CPCeA). Closed chest hemodynamic assessment was performed on anesthetized mice (3% Chloral hydrate, 300mg/kg) prior to insertion of microtip pressure transducer (FT111B, Scisense) into the right carotid artery and advancement into left ventricle. The catheter was connected to an A/D converter (FV892A, Scisense) for data collection. After hemodynamic measurements, hearts were arrested in diastole and perfused with phosphate-buffered formalin. Hemodynamic assessment was performed under chloral hydrate sedation and heart rate of all animals was required to be between 400-500 beats
per minute for study inclusion. As a consequence of exclusion criteria, fewer animals were analyzed in hemodynamic study compared to echocardiography which accounts for minor discrepancies between end-point results between hemodynamic and echocardiography assessments.

**Histology and Embedding of heart tissue**
Briefly, hearts from saline, CPCe and CPCeA injected mice were retroperfused through the abdominal aorta with potassium chloride to arrest in diastole and fixed in 10% formalin for 24-hours, after which hearts were changed to 70% ethanol. Hearts were embedded in paraffin and 5 mm sections were cut.

**Deparaffinization**
Three changes for 5 minutes in xylene, followed by 3 changes for 3 minutes in 100% ethanol, followed by 2 changes for 3 minutes in 95% ethanol, followed by 1 change in 70% ethanol, followed by 5 minutes in deionized water.

**Antigen Retrieval**
Sections were placed in plastic coplin jars containing 10mmol/L citrate buffer (pH 6.0) and heated in the microwave on full power for 3 minutes. Power setting on microwave was then changed to 50% power for and additional 12 minutes. Every 3 minutes, microwave was stopped and coplin jars containing slides were refilled with citrate buffer to keep the level above the tissue sections. Extra citrate buffer was heated in the microwave alongside slides. Slides were allowed to cool and washed 3 times in deionized water, followed by equilibration in 1X Tris-NaCl (TN) buffer (150mM NaCl/100mM Tris pH 7.5). Proceed to blocking step (if not queching) with Tris-NaCl-Blocking buffer (TNB) (.1M Tris, pH 7.5+.15M NaCl + .5% Blocking reagent from Perkin Elmer catalog # FP1020).

**Quenching**
Quench endogenous tissue peroxidase activity in 3% H2O2/1X TN for 20 minutes. Wash again in three changes for 3 minutes each of 1X TN. Proceed to blocking.

**Immunohistochemistry**

<table>
<thead>
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<th>Antibody</th>
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<td>GFP</td>
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<td>Desmin</td>
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<td>Topro-3-iodide</td>
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<td>Molecular Probes</td>
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<td>Forward/Reverse</td>
<td>Sequence</td>
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<td>Forward</td>
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Supplemental Figure I: Overexpression of Nuclear Akt in CPCs

(A) Self-inactivating lentiviral vectors, termed Lv-egfp (GFP control) and Lv-Akt-nuc. (B) Representative immunoblot of CPCe and CPCeA, immunolabeled for myc-tag, Akt1/2, GFP, and GAPDH. (C) Representative immunoblot of CPCe and CPCeA, and CPCeP immunolabeled for Pim-1, Akt1/2, GFP, and GAPDH.
Supplemental Figure II: Nuclear Akt modified CPCs mediate gene expression of cell cycle proteins

(A) Total mRNA was extracted from CPCe (n=3) and CPCeA (n=3) and run on a cell cycle RT2 Profiler Cell Proliferation Array from SuperArray in triplicate. CPCeA and CPCe were normalized to GAPDH. Samples analyzed had ≥2-fold difference from control, with p<0.05, (mean ± SEM, n=3). (B-C) Protein expression of Cyclin D1 (B), Chk1 and CDC2 (C) were examined in CPCe and CPCeA (mean ± SEM). *p<.05, **p<.01 compared to CPCe.
Supplemental Figure III: CPCe acquire markers of cardiac lineage commitment

Hearts from infarcted animals injected with control CPCe were immunostained with GFP (green), Tropomyosin (red) and topro-3-iodide (blue) to mark nuclei.
Supplemental Figure IV: CPCeA and CPCe injected hearts have comparable infarct size.
Quantitation of infarction area in vehicle (n=3), CPCe (n=3), and CPCeA (n=4) treated hearts 12-weeks post injection (mean ± SEM).
Supplemental Figure V: CPCeA abrogate lineage commitment compared to CPCeP
Transcript levels of cardiac TnT (A), Mef2C (B), and Gata4 (C) were assessed by quantitative real time PCR in CPCe, CPCeP and CPCeA treated with and without Dex.
Supplemental Figure VI: CPCeP do not overexpress phosphorylated CREB after \textit{in vitro} differentiation

Immunoblot and quantitation of phosphorylated CREB in CPCeP and CPCeA treated with and without Dex for seven days.