

Cardiopoietic programming of embryonic stem cells for tumor-free heart repair

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Embryonic stem cells have the distinct potential for tissue regeneration, including cardiac repair. Their propensity for multilineage differentiation carries, however, the liability of neoplastic growth, impeding therapeutic application. Here, the tumorigenic threat associated with embryonic stem cell transplantation was suppressed by cardiac-restricted transgenic expression of the reprogramming cytokine TNF- α , enhancing the cardiogenic competence of recipient heart. The *in vivo* aptitude of TNF- α to promote cardiac differentiation was recapitulated in embryoid bodies *in vitro*. The procardiogenic action required an intact endoderm and was mediated by secreted cardio-inductive signals. Resolved TNF- α -induced endoderm-derived factors, combined in a cocktail, secured guided differentiation of embryonic stem cells in monolayers produce cardiac progenitors termed cardiopoietic cells. Characterized by a down-regulation of oncogenic markers, up-regulation, and nuclear translocation of cardiac transcription factors, this predetermined population yielded functional cardiomyocyte progeny. Recruited cardiopoietic cells delivered in infarcted hearts generated cardiomyocytes that proliferated into scar tissue, integrating with host myocardium for tumor-free repair. Thus, cardiopoietic programming establishes a strategy to hone stem cell pluripotency, offering a tumor-resistant approach for regeneration.

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Abbreviations used: BMP, bone morphogenetic protein; CFP, cyan fluorescence protein; EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; MAPK, mitogen-activated protein kinase; MEF, myocyte enhancer factor; NGF, nerve growth factor; TACE, TNF- α converting enzyme; TAK, TGF- β -activated kinase; VEGF, vascular endothelial growth factor.

Stem cell-based therapeutic modalities are increasingly considered for organ repair (1–5). Current clinical trials use adult stem cells, addressing their safety and efficacy (6–9). While optimization of adult cytotypes is ongoing (10, 11), recognition of the vigorous regenerative capacity of embryonic stem cells has created a distinct opportunity in cardiovascular medicine (12–16). Isolated from the inner cell mass of blastocysts, embryonic stem cells have a unique aptitude to form ectoderm, mesoderm, and endoderm (17), revealing this population as a renewable cell source with a reliable potential for cardiogenesis (18–22). However, use of embryonic stem cells carries a latent threat for neoplastic transformation caused by the inherent propensity associated with pluripotency for unguided differentiation upon transplantation (23–25). This risk particularly transpires when embryonic stem cells are removed from the inner cell mass of a differentiating blastocyst and established *in vitro* before implantation in a foster environment, such as that of the host myocardium (26).

During embryogenesis, as embryonic stem cells differentiate into the three germinal layers, markers of pluripotency decline with tissue specification (27). Cardiac differentiation in the embryo proceeds within the anterolateral mesoderm and requires the instructive guidance of the adjacent endoderm (28–30). Cardiogenic instruction induces expression of the homeobox gene *Nkx2.5*, the earliest molecular marker of the cardiac fate (31). *Nkx2.5* then activates transcription of the myocyte enhancer factor *MEF-2C* to promote cardiomyocyte differentiation and cooperates with zinc finger transcription factors of the *GATA* family to initiate cardiac gene expression (32, 33). Though the heart can mimic the endoderm in guiding stem cell cardiogenesis, its capacity to secure cardiogenic transformation is limited compared with the embryo itself (34), creating a threat for unguided growth that impedes safe embryonic stem cell use.

Here, the aptitude of recipient heart to prevent unguided differentiation of transplanted embryonic stem cells was enhanced through

transgenic expression of TNF- α , a reprogramming cytokine (35) and an integral component of the myocardial homeostatic response (36). As the heart mimics the endoderm in guiding cardiogenesis, the embryoid body and isolated endodermal cells were used to dissect TNF- α -mediated induction of cardiac differentiation. The identity of TNF- α -induced

proteins in the endodermal secretome was delineated through a subtractive proteomic approach. Combinatorial application of identified factors on a monolayer of embryonic stem cells was found to be sufficient in deriving a cardiac predetermined progenitor population. Cardiopoietic programming eliminated embryonic stem cell susceptibility for multilineage

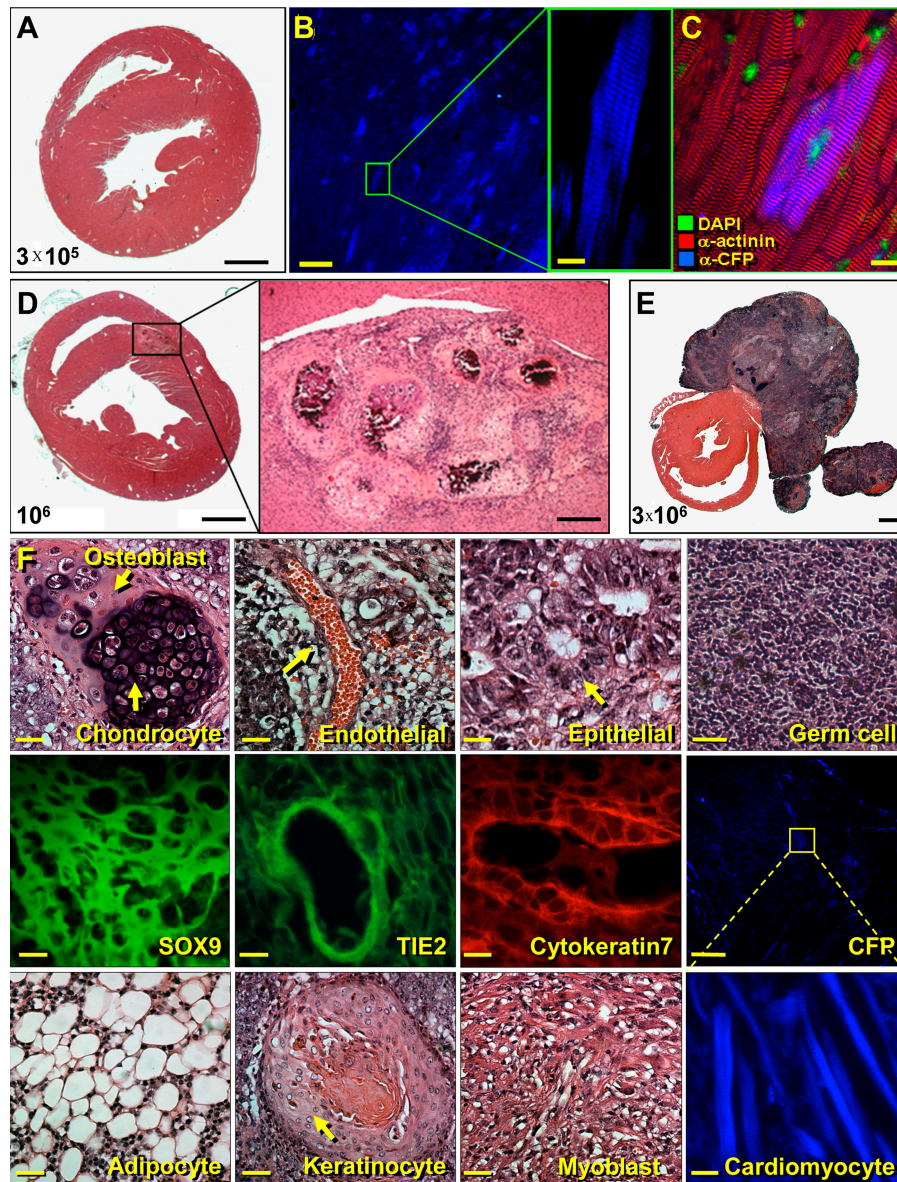


Figure 1. Tumorigenic risk of embryonic stem cell therapy.

(A–C) Transplantation of 3×10^5 embryonic stem cells into normal heart (A, transverse section) generated α -actinin-positive cyan fluorescent embryonic stem cell-derived cardiomyocytes (B and inset) that properly integrated into host myocardium (C). Bars: (A) 2 mm; (B) 70 μ m; (B, inset and C) 10 μ m. (D and E) Embryonic stem cells injected at 10^6 – 3×10^6 cells per heart harbored a risk for uncontrolled growth with formation of teratoma that remained encapsulated (D and inset) or protruded into the thoracic cavity (E). Bars: (D and E) 2 mm; (D inset) 300 μ m. (F) On histology with hematoxylin-eosin staining, diverse embryonic stem cell-derived phenotypes were documented. These included osteoblasts, chondrocytes,

endothelial, epithelial, and germ cell types (first row), and adipocytes, keratinocytes, and myoblasts (third row), reflecting embryonic stem cell pluripotency. Immunohistochemistry was used to verify the multiplicity of cell types, i.e., using SOX9 to confirm chondrocytes and cytokeratin 7 for acinar epithelium (second row). Moreover, tumors derived from embryonic stem cells programmed to express GFP driven by the TIE2 promoter visualized the endothelial phenotype (second row), whereas embryonic stem cells engineered to express CFP under the cardiac actin promoter revealed the presence of embryonic stem cell-derived cardiomyocytes within teratomas (second and third row). Bars: F (all panels except epithelial in first row) 10 μ m; (epithelial in first row) 5 μ m; (CFP, inset) 100 μ m.

differentiation averting the risk for tumorigenic outcome, removing a critical impediment in therapeutic application.

RESULTS

Tumorigenic risk of embryonic stem cells transgenically manipulated in vivo

To assess the tumorigenic risk of pluripotent stem cells differentiating outside of the natural embryonic program, embryonic stem cells were delivered at increasing loads into the myocardial parenchyma of wild-type mice. Delivery of embryonic stem cells at $\leq 1,000$ cells/mg of myocardial tissue ($\sim 3 \times 10^5$ stem cells/heart) resulted in incorporation of stem cell-derived cardiomyocytes in the area of transplantation ($n = 50$ mice), tracked by fluorescence emitted upon cardiac differentiation (Fig. 1, A and B). Autofluorescence and cell fusion were excluded through multiwavelength immunocytochemical and nuclear probing, indicating that embryonic stem cells undergo nonfusogenic cardiac trans-formation in the recipient heart (Fig. 1 C). Transplantation of 3,000 embryonic stem cells/mg ($\sim 10^6$ stem cells/heart) resulted in teratoma formation within the myocardial parenchyma in 18% of treated hearts (Fig. 1 D). Delivered at 10,000 per mg of myocardial mass ($\sim 3 \times 10^6$ stem cells/heart), embryonic stem cells escaped cardiogenic differentiation in 68% of treated animals, generating massive tumors emanating from the heart into the thoracic cavity (Fig. 1 E). Embryonic stem cell-derived teratoma consisted of a multigermlinal cellular heterogeneity, including osteoblasts, chondrocytes, adipocytes, keratinocytes, myoblasts, endothelial, epithelial tissue, and germinal cells (Fig. 1 F). Verification of cytotypes was made by immunostaining with SOX9 (chondrocytes) and cytokeratin7 (acinar epithelial cells) and through delivery of cells engineered to express cyan fluorescent protein (CFP) under control of the cardiac actin promoter (for cardiac cells) or GFP under control of the Tie2 promoter (for endothelial cells), revealing the narrow margin of safety associated with delivery of pluripotent stem cells in wild-type hearts (Fig. 1 F).

The propensity for tumorigenic outcome correlated with embryonic stem cell load (Fig. 2 A). The neoplastic threat associated with stem cell transplantation was eliminated by cardiac-restricted transgenic expression of the stress cytokine TNF- α (TNF- α -TG), averting tumorigenic outcome even at doses of 10,000 cells per mg of myocardial tissue ($\sim 3 \times 10^6$ stem cells/heart) that produced uncontrolled growth in wild-type hearts (Fig. 2 A). Up-regulated myocardial expression of TNF- α secured cardiac differentiation of implanted embryonic stem cells with proper integration within host myocardium occurring over an increasing range of stem cell loads (Fig. 2, A and B). Autofluorescence and cell fusion was ruled out by multiwavelength confocal visualization after immunohistochemical and nuclear probing (Fig. 2 C). The in vivo action of TNF- α was associated with myocardial up-regulation of the p38 mitogen-activated protein kinase and enhanced expression of the cardiogenic growth factor TGF- β (Fig. 2 D). Deletion of the TGF- β receptor kinase

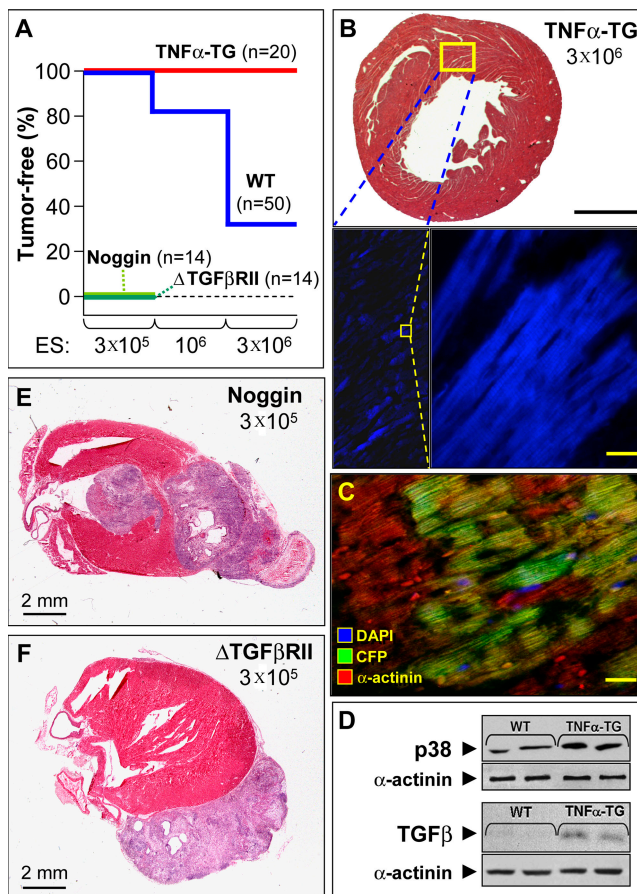


Figure 2. Neoplastic risk of embryonic stem cell therapy averted with transgenic cytokine overexpression. (A) Host heart (WT) demonstrated a finite capacity to secure tumor-free stem cell transplantation, i.e., 100% tumor-free outcome at 3×10^5 cells/heart versus 82% and 32% tumor-free outcomes at 10^6 cells/heart and 3×10^6 cells/heart, respectively (blue). Transgenic cardiac-restricted overexpression of TNF- α (TNF-TG) increased the capacity of host heart to guide stem cell cardiogenesis nullifying uncontrolled growth, i.e., 100% tumor-free outcome at 3×10^6 cells/heart (red). Conversely, deletion of the kinase domain of the TGF- β receptor (Δ TGF β RII) or overexpression of the BMP inhibitor Noggin, both disrupting cardiogenic signaling in embryonic stem cells (ES), precipitated tumorigenesis after transplantation, i.e., 0% tumor-free outcomes at 3×10^5 cells/heart (light and dark green). For each condition, n indicates the number of treated animals. (B and C) TNF- α priming achieved tumor-free incorporation of transplanted stem cells (3×10^6 cells/heart; top panel), with embryonic stem cell-derived cardiomyocytes integrating with host myocardium as determined by CFP expression (B, bottom and C). Bars: (B, top) 2 mm; (B, bottom and C) 10 μ m. (D) Transgenic TNF- α overexpression (TNF-TG) up-regulated myocardial p38 MAPK and TGF- β expression with α -actinin used as a control for protein loading. (E and F) Blockade of the TGF- β superfamily through overexpression of Noggin or Δ TGF β RII precipitated tumor formation because of uncontrolled growth of implanted embryonic stem cells even at 3×10^5 cells/heart.

domain (Δ TGF- β RII) in embryonic stem cells or disruption of the stem cell ability to recognize members of the TGF- β superfamily, i.e., BMP through overexpression of the BMP inhibitor, noggin, prevented cardiac differentiation leading to

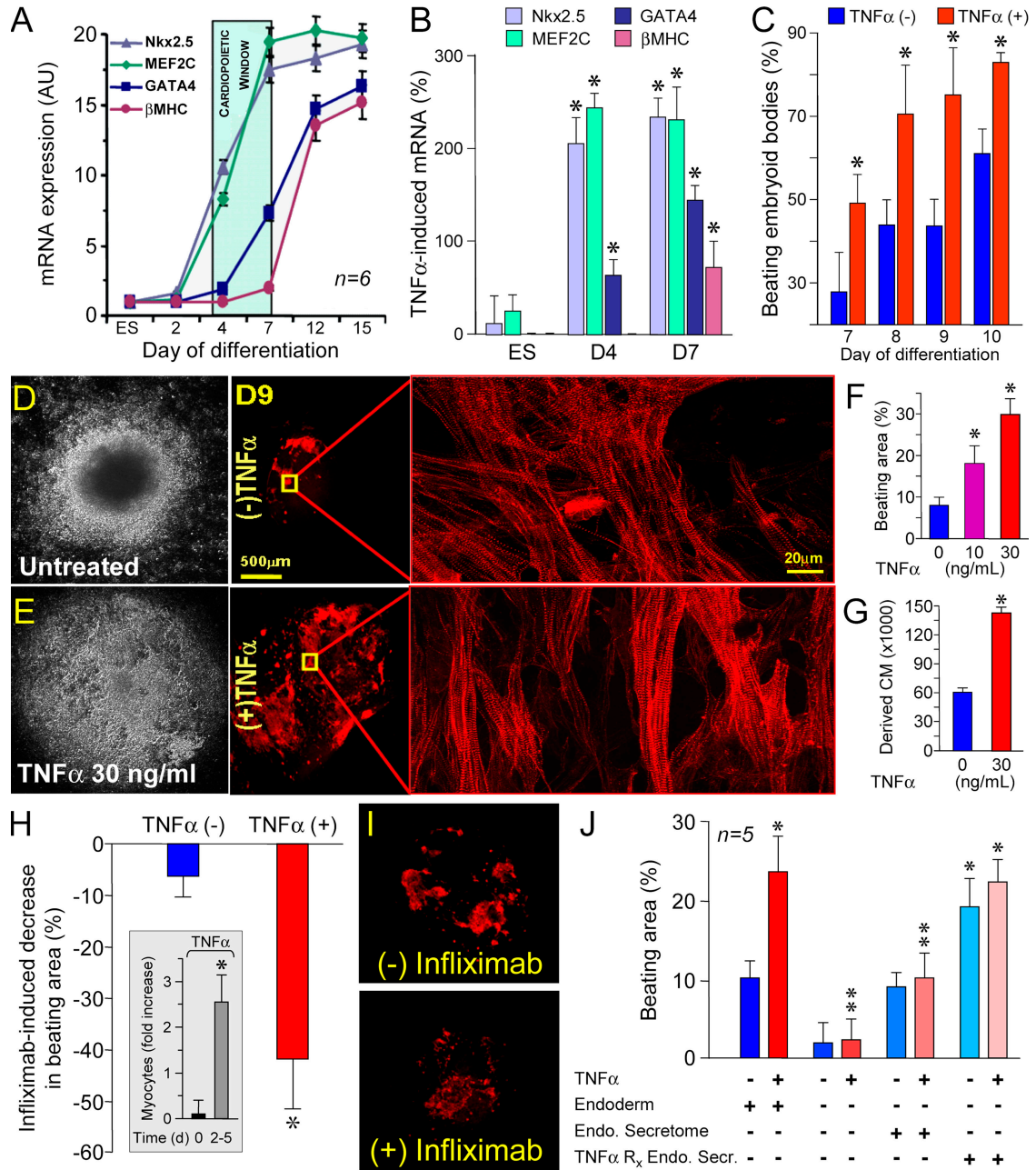


Figure 3. Procardiogenic TNF- α effects are mediated through the endoderm. (A) During embryoid body differentiation of embryonic stem cells (ES), mRNA expression profiling of cardiac transcription factors (Nkx2.5, MEF-2C, GATA4) revealed a window (days D4–D7) of exponential up-regulation (CARDIOPOIETIC WINDOW) 3 d before the appearance of sarcomeric mRNA (β MHC). Arbitrary units were calculated relative to the expression of β -tubulin used as denominator. (B and C) TNF- α (30 ng/ml) doubled the expression of cardiac transcription factors in comparison to untreated controls during the cardiopoietic window (B) and accelerated the formation of beating embryoid bodies (C). (D and E) TNF- α (30 ng/ml) increased sarcomeric content in treated ((+)TNF α) versus untreated ((-)TNF α) embryoid bodies determined by α -actinin immunofluorescence at day 9 (D9) of differentiation (middle) without altering sarcomeric organization (right). Bars in D apply also to E. (F) The percentage of embryoid body area beating was dependent on the TNF- α concentration. (G) TNF- α

treatment increased the yield of cardiomyocytes after isolation from dissociated embryoid bodies. (H and I) The cardiogenic effect of TNF- α (30 ng/ml) on embryoid bodies was inhibited by infliximab (150 ng/ml), a neutralizing TNF- α antibody, as determined by beating area on video microscopy (H) and α -actinin staining (I). (H, inset) TNF- α is effective only after the initiation of embryoid body differentiation (days 2–5) producing a 2.5-fold increase in cardiomyocyte yield compared with TNF- α -treated embryonic stem cells (day 0). (J) TNF- α required an intact endoderm to induce cardiogenesis, as endodermal disruption prevented cytokine action. Addition of conditioned medium from isolated ventral endoderm (Endo. Secretome) rescued cardiogenesis but did not restore the TNF- α effect. Addition of condition medium from TNF- α -treated endoderm (TNF α R $_x$ Endo. Secr.) resulted in cardiogenesis at levels equal to that of TNF- α -treated embryoid bodies irrespective of the presence of TNF- α on the embryoid body itself. *, P < 0.05 versus untreated.

tumor formation even at low stem cell loads (Fig. 2 A, E, and F). Thus, the tumorigenic risk of embryonic stem cells can be blunted by TNF- α cardiac-restricted overexpression or exaggerated by removal of the TGF- β superfamily guidance of transplanted cells.

Cardiogenic TNF- α action recapitulated in embryoid body requires endoderm

The procardiogenic action of TNF- α revealed through in vivo transgenesis was validated and dissected in vitro through differentiation of embryonic stem cells in a controlled model of cardiogenesis (37). Within developing embryoid bodies, early cardiogenesis was characterized by a time window of exponential rise in markers of cardiac specification (Fig. 3 A). Between day 4 and 7 of differentiation, genes encoding cardiac transcription factors (Nkx2.5, MEF-2C, and GATA4) were reproducibly up-regulated before expression of sarcomeric genes (β -MHC) indicative of terminal differentiation ($n = 6$ differentiation experiments with 20 embryoid bodies for each time point; Fig. 3 A). Within this time window of early differentiation, embryoid bodies treated with TNF- α (30 ng/ml) demonstrated significant up-regulation of Nkx2.5, MEF-2C, and GATA4 ($n = 6$ differentiation experiments; Fig. 3 B) compared with untreated controls ($P < 0.05$). TNF- α treatment translated into an accelerated cardiac differentiation determined by an earlier increase in the number of beating embryoid bodies sustained throughout differentiation ($n = 80$ embryoid bodies; Fig. 3 C). TNF- α , in a concentration-dependent manner, increased cardiac content within each embryoid body as determined at day 9 of differentiation by α -actinin staining of sarcomeres ($n = 30$ embryoid bodies; Fig. 3, D and E), areas of beating activity ($n = 30$ embryoid bodies; Fig. 3 F), and quantification of cardiomyocyte yield ($n = 3$ differentiation experiments with 30 embryoid bodies for each isolation; Fig. 3 G). The cardiogenic effect of TNF- α was prevented by cotreatment with the TNF- α antagonist, infliximab (150 ng/ml, $n = 6$; Fig. 3, H and I). The action of TNF- α required that embryonic stem cells undergo trigeriminal differentiation into embryoid bodies as treatment of stem cells in the pluripotent state did not promote cardiogenesis (Fig. 3 I, inset). In fact, the cardiogenic effect of TNF- α was found to be dependent on the endoderm (Fig. 3 J). Although trigeriminal embryoid bodies displayed a vigorous response to TNF- α that augmented the beating area by >2 -fold above baseline (Fig. 3 J, first pair), embryoid bodies lacking endoderm after treatment with leukemia inhibitory factor (100 U/ μ l; 38) lost their cardiogenic response to TNF- α (Fig. 3 J, second pair). In endoderm-deficient embryoid bodies, rescue of baseline cardiogenesis was achieved by addition of conditioned medium derived from isolated visceral endoderm-like cells (Fig. 3 J, third pair). Endoderm-deficient embryoid bodies rescued by visceral endoderm-conditioned medium did not, however, demonstrate a cardiogenic response to TNF- α treatment, indicating that the cytokine must target the endodermal layer (Fig. 3 J, third pair). Rescue of TNF- α

action was achieved through treatment of endoderm-deficient embryoid bodies with condition medium derived from TNF- α -primed visceral endoderm-like cells (Fig. 3 J, fourth pair). The boost in the cardiogenic propensity of endoderm-derived conditioned medium after TNF- α stimulation was reproduced on pluripotent stem cells differentiating in monolayer. Visceral endoderm-like cells treated with TNF- α had a twofold increased capacity to induce cardiogenic differentiation of embryonic stem cells, with the total yield of cells demonstrating nuclear translocation of Nkx2.5 and MEF-2C increased from $34 \pm 4\%$ in the untreated condition to $73 \pm 6\%$ in the TNF- α -treated condition (data not shown). Thus, the procardiogenic action of TNF- α observed in vivo can be recapitulated in vitro with the action of the cytokine dependent on the endoderm.

Endodermal response to TNF- α reveals cardiogenic candidates

Proteomic two-dimensional gel analysis revealed that the TNF- α stimulation of isolated visceral endoderm-like cells induced secretion of proteins involved in sarcomerogenesis (profilin and cofilin), calcium signaling (calcyclin), myocardial reprogramming (nucleotide diphosphate kinase), and heart formation (FK506 binding protein FKBP12, cystatin, and ubiquitin) (39–42; Fig. 4 A). The identity of each protein was resolved by reconstruction of constitutive peptides using tandem mass spectrometry (Fig. 4 B) with the overall twofold increase in protein content after cytokine induction (Fig. 4 A, inset) confirmed at an individual protein level (Fig. 4 B, insets). Transcriptional profiling of total RNA revealed that 970 genes changed >1.5 -fold, of which 616 (64%) were up-regulated and 354 (36%) down-regulated in TNF- α -stimulated compared with untreated visceral endoderm (Fig. 4, C and D), underlying the cytokine induction of protein synthesis. Multidimensional liquid chromatography tandem mass spectrometric shotgun analysis, along with pathway analysis, was used to dissect the intracellular network downstream of TNF- α (Fig. 4 E), with protein identities established from individual mass spectra (Fig. 4 F). Subtractive analysis of the genomic and proteomic data obtained from TNF- α -treated versus untreated endodermal secretome resolved several cytokine-induced secreted growth factors including TGF- β 1, bone morphogenetic protein (BMP)-1, -2, and -4, vascular endothelial growth factor (VEGF)-A, IL-6, epidermal growth factor (EGF), fibroblast growth factor (FGF)-2 and -4, haploglobin, CSF-1, nerve growth factor (NGF)- β , and insulin-like growth factor (IGF)-1, and -2 (Fig. 4 E). Unbiased network analysis of the identified nodes in the endoderm secretome using the Ingenuity Pathway Knowledge Base ranked the “cardiovascular system development” function as the most overrepresented subnetwork following TNF- α induction, up from the twelfth rank in the untreated secretome and distanced by fivefold from the nearest function, identifying secreted factors as candidate cardiostrophs (Fig. 4 E).

Secreted factors IGF-1 and -2, CSF-1, BMP-1, and tissue inhibitor of metalloprotease-1 were initially resolved on

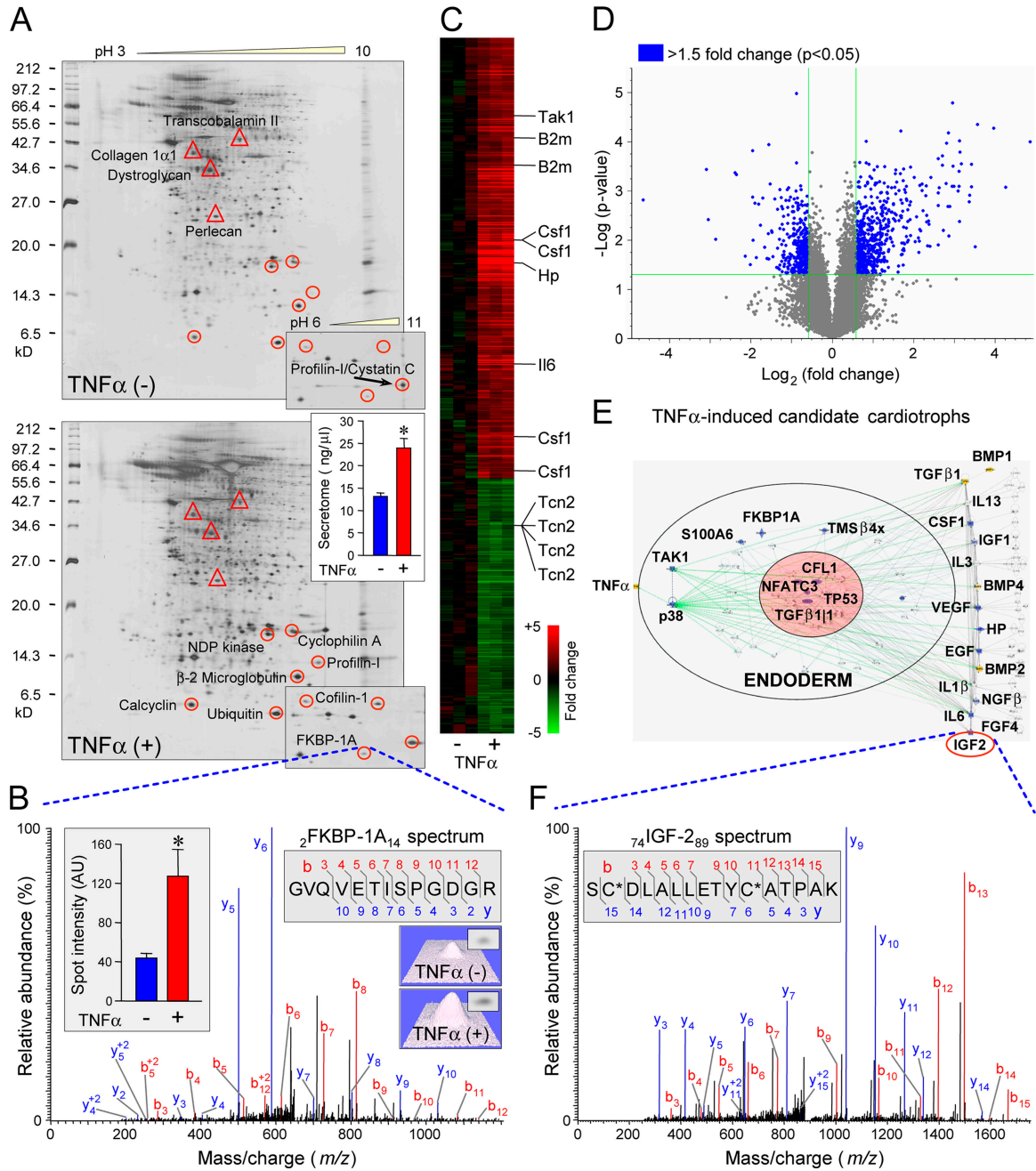


Figure 4. Proteomic and genomic dissection of TNF- α -primed endodermal secretome. (A) Two-dimensional gel electrophoresis (at pH 3–10 and 6–11) was used to resolve the endodermal secretome concentrated from 5-ml aliquots, with examples of TNF- α up-regulated proteins circled. Protein content was increased approximately twofold in TNF- α -primed versus naive secretome (inset). Triangles: examples of down-regulated or unchanged proteins. (B) Example LTQ tandem mass spectrum showing FKBP-1A peptide (amino acids 2–14). This approach was used to resolve protein identities by respective constituent peptide sequence signatures of individual peptide mass spectra, with relative abundance quantified (inset left) based on spot density (inset right). (C) Transcriptional profiling of total RNA from TNF- α -treated and untreated endoderm showing differential gene expression, including examples of genes encoding proteins identified by proteomics. (D) Volcano plots

revealed that 64% of genes were up-regulated and 36% down-regulated in TNF- α -treated versus untreated endoderm. (E) Proteomic shotgun analysis of the TNF- α -treated endodermal secretome and ELISA revealed the identity of secreted proteins, including FGFs, leukemia inhibitory factor, VEGFs, TGF- β s, BMPs, CSF, IGFs, EGF, NGF, and ILs. Pathway analysis of identified proteins visualized the interactome triggered by cytokine stimulation and demonstrated secreted candidate cardirotrophic factors downstream of TNF- α integrated through p38-driven networks with individual proteins depicted in their corresponding cellular compartments. Blue nodes reflect detection by proteomic analysis, and yellow nodes reflect ELISA confirmation. (F) Example of LTQ-FT shotgun tandem mass spectrum showing IGF-2 peptide (amino acids 74–89). This approach was used to provide a more sensitive means of detecting the presence of secreted proteins. *, $P < 0.05$.

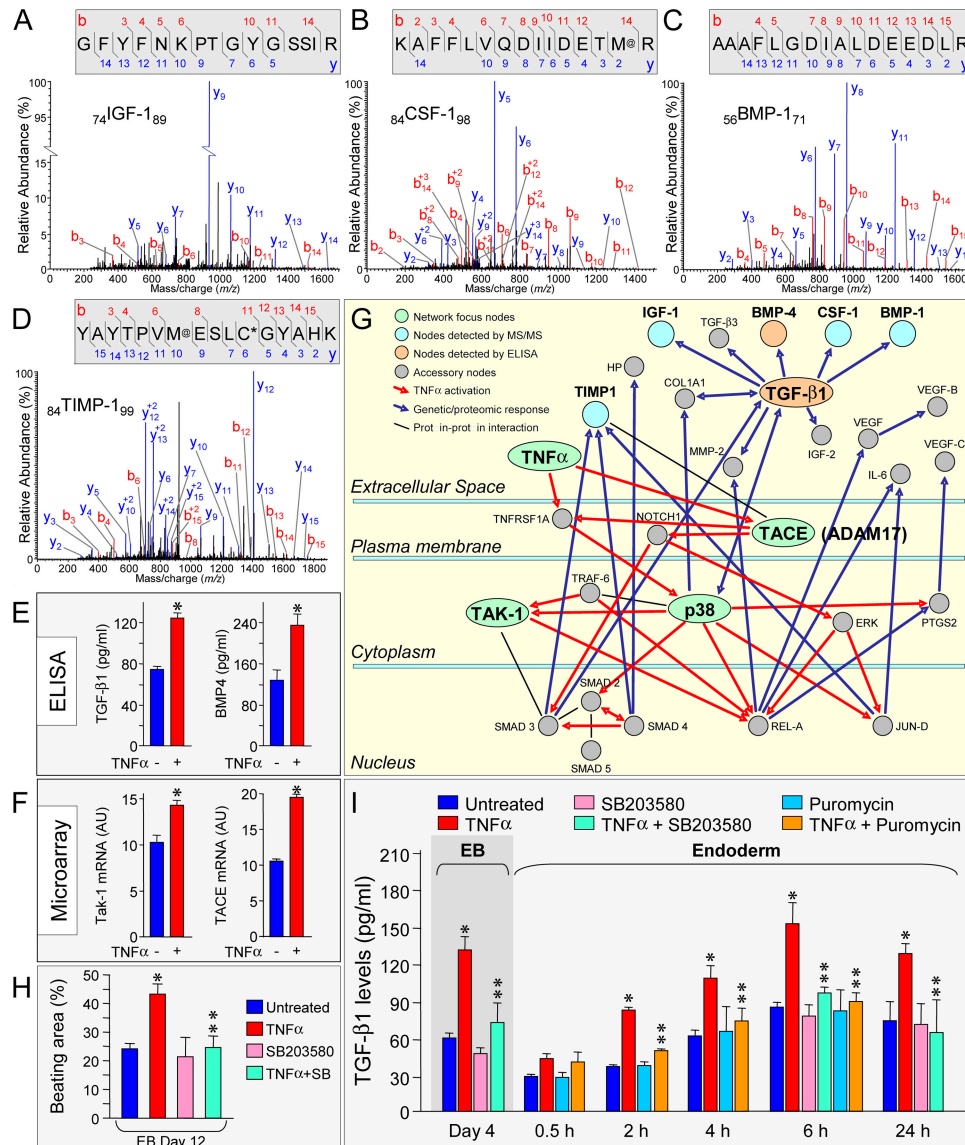


Figure 5. Cardiac priming of the endoderm by TNF- α requires p38 signaling. (A–D) LTQ-FT shotgun tandem mass spectrum of selected growth factors including IGF-1, CSF-1, BMP-1, and tissue inhibitor of metalloprotease-1. (E) TGF- β 1 and BMP4 were up-regulated by twofold as determined by ELISA-based quantification. (F) Expression profiling of endodermal mRNA before and after TNF- α treatment revealed up-regulation of TAK-1 and TACE (Adam17) by 1.5- and 2-fold, respectively. (G) Factors identified by proteomic and genomic analysis integrate to highlight p38 as a central component in the signaling network established after TNF- α priming of the endoderm. Expression of each node presented in this

interactome was confirmed by gene chip and tandem MS-MS dissection. (H) Pharmacological inhibition of p38 MAPK using SB203580 (5 μ M) blunted the procardiogenic effect of TNF- α on embryoid bodies. (I) TGF- β secretion promoted by TNF- α was seen in both the embryoid body and the isolated ventral endoderm. Antagonism of p38 with SB203580 or inhibition of protein synthesis using the ribosomal inhibitor, puromycin, abrogated TNF- α enhancement of TGF- β production. * and ** indicate $P < 0.05$ with respect to untreated and TNF- α -treated embryoid bodies or endoderm, respectively.

shotgun proteomics, and expression was confirmed by gene array analysis (Fig. 5, A–D). TGF- β 1 and BMP-4 were found up-regulated by twofold as demonstrated on ELISA (Fig. 5 E). Expression of BMP-2, VEGF-A, -B, and -C, IL-6, EGF, and haploglobin was probed by gene array analysis and verified by ELISA (Fig. 5 G). Expression profiling of endodermal mRNA after TNF- α treatment revealed up-regulation of TGF- β -activated kinase (TAK)-1 and TNF- α converting

enzyme (TACE) (Adam17) by 1.5- and 2-fold, respectively (Fig. 5 F). Increase in TACE expression indicates NOTCH1-dependent activation of the SMAD system (43) in addition to p38 (44), whereas increased TAK-1 expression mediates SMAD3 and p38 activation (45, 46). Pathways analysis demonstrated a nonstochastic assembly based on cellular compartments of TNF- α -up-regulated elements identified and confirmed by genomic and proteomic evaluation,

highlighting p38 as an integral node of intracellular signaling induced by cytokine stimulation of the endoderm (Fig. 5 G). The central role of the mitogen-activated protein kinase (MAPK) p38 was confirmed as application of the p38 antagonist SB203580 to embryoid bodies blunted TNF- α action (Fig. 5 H). As pathway analysis revealed TGF- β as a node directly downstream of p38, ELISA evaluation of TGF- β secretion was made after p38 inhibition with SB203580 and protein synthesis inhibition with the ribosomal antagonist puromycin. Direct application of SB203580 to day 4 embryoid bodies or isolated visceral endoderm-like cells eliminated TNF- α up-regulation of TGF- β secretion (Fig. 5 I). Furthermore, puromycin treatment of isolated endoderm inhibited the TNF- α -dependent boost in TGF- β secretion, indicating induction of protein synthesis. Thus, subtractive genomic and proteomic profiling of the endoderm before and after TNF- α stimulation identifies secreted endoderm-derived cardiotropic factors and reveals p38 as an intracellular mediator of TNF- α cardiogenic priming.

Synergy of endoderm-derived cardiotropins achieves cardiopoietic programming

The cardiogenic aptitude of individual candidates identified within the TNF- α -primed endodermal secretome was tested on embryonic stem cells in monolayer and found to increase the expression of early cardiac transcription factors Nkx2.5 and MEF-2C localized to the cytosol (Fig. 6 A). Induction with single recombinant factors, e.g., BMP-2 or -4, TGF- β 1, IGF-1 or -2, and FGF-2 or -4, was, however, not sufficient to mediate nuclear import of cardiac transcription factors in differentiating stem cells, a critical step for definitive engagement into the cardiac program (Fig. 6 A; reference 47). Rather, the synergy of factors (TGF- β 1, BMP-2 and -4, activin-A, VEGF-A, IL-6, FGF-2 and -4, IGF-1 and -2, and EGF) identified in the secretome, used as a recombinant cocktail regimen, induced nuclear translocation of Nkx2.5 by day 2, and of later cardiac transcription factors MEF-2C and GATA4 by day 4, indicative of definitive commitment to cardiac differentiation (Fig. 6 B). In this way, the cardiogenic cocktail demonstrated a capacity to recruit from embryonic stem cells a monolayer of cells en route to cardiac maturation. This intermediate cell phenotype—termed cardiopoietic progenitor cells—completed the cardiac differentiation program by day 7, demonstrating definitive expression of myofibrillar proteins (α -actinin) and sarcomeric organization (Fig. 6 B). Specifically, when day 4 cardiopoietic stem cells at 10,000 cells/ml were continuously cultured in the presence of the cardiogenic cocktail, sarcomeric differentiation was achieved by day 7 in $\geq 10\%$ of cells, day 9 in $\geq 30\%$ of cells, and day 12 in $\geq 65\%$ of cells in culture (Fig. 7, A–C). Removal of the cardiogenic cocktail after 4 d of recombinant stimulation resulted in continued engagement of cardiopoietic cells in the cell cycle dividing every 36 h without differentiation into cardiomyocytes by day 9 ($\leq 5\%$ of cells), and withdrawal from cell cycle upon confluence (Fig. 7 D). Electron microscopy further established the transitional state of cardiopoietic cells

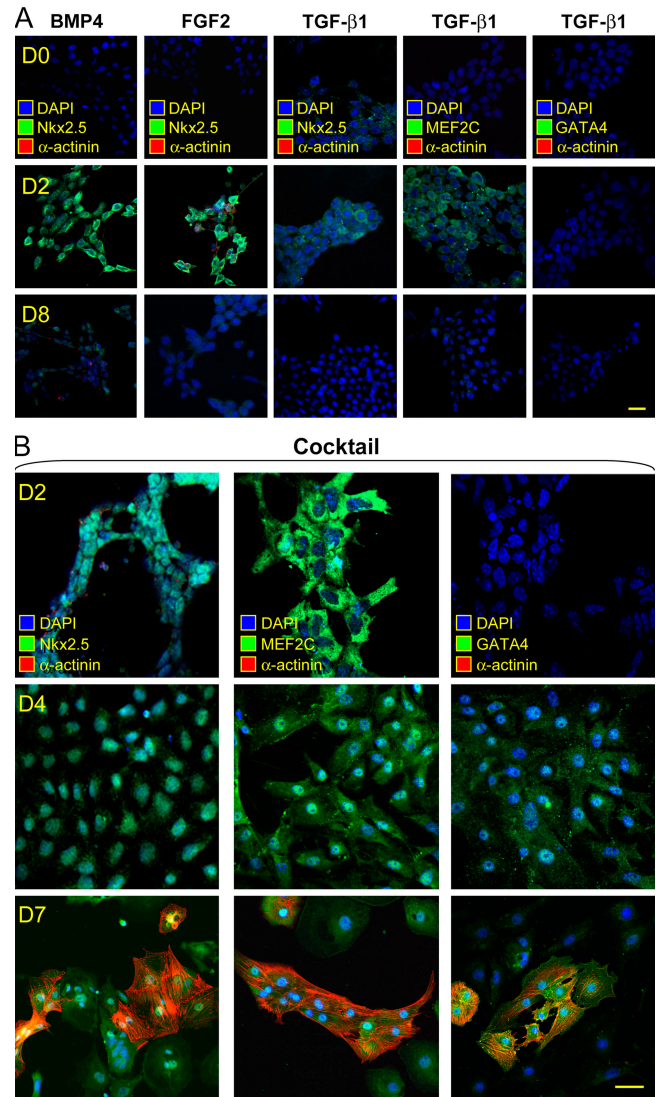


Figure 6. Synergy of recombinant secretome components drives cardiogenesis of embryonic stem cells in monolayer, revealing a cardiopoietic population. (A) Untreated embryonic stem cells (D0, row 1) or cells treated for 2 d (D2, row 2) or 8 d (D8, row 3) with individual components (i.e., BMP-4, FGF-2, and TGF- β 1) did not demonstrate cardiogenic specification as expression of cardiac transcription factors (Nkx2.5, columns 1–3; MEF-2C, column 4; GATA-4, column 5) was limited to the cytosol at D2 and diminished in expression by D8. (B) Combinatorial stimulation with identified secretome components resulted by day 2 in nuclear translocation of Nkx2.5 with strong cytosolic expression of MEF-2C (D2, row 1), followed by recruitment of the cardiopoietic phenotype with nuclear translocation of Nkx2.5, MEF-2C, and GATA-4 (D4, row 2) to yield cardiomyocytes with completed sarcomerogenesis by day 7 demonstrated by α -actinin (D7, row 3). Bars: (A) 20 μ m; (B) 15 μ m.

relinquishing a phenotype of high nucleus-to-cytosol ratio typical of embryonic stem cells (48), while acquiring a progressively mature cardiac structure (Fig. 7 E). Thus, the identified cardiopoietic cell population demonstrated maintained mitotic activity, a remnant property of the embryonic source, and reproducibly acquired contact inhibition with execution

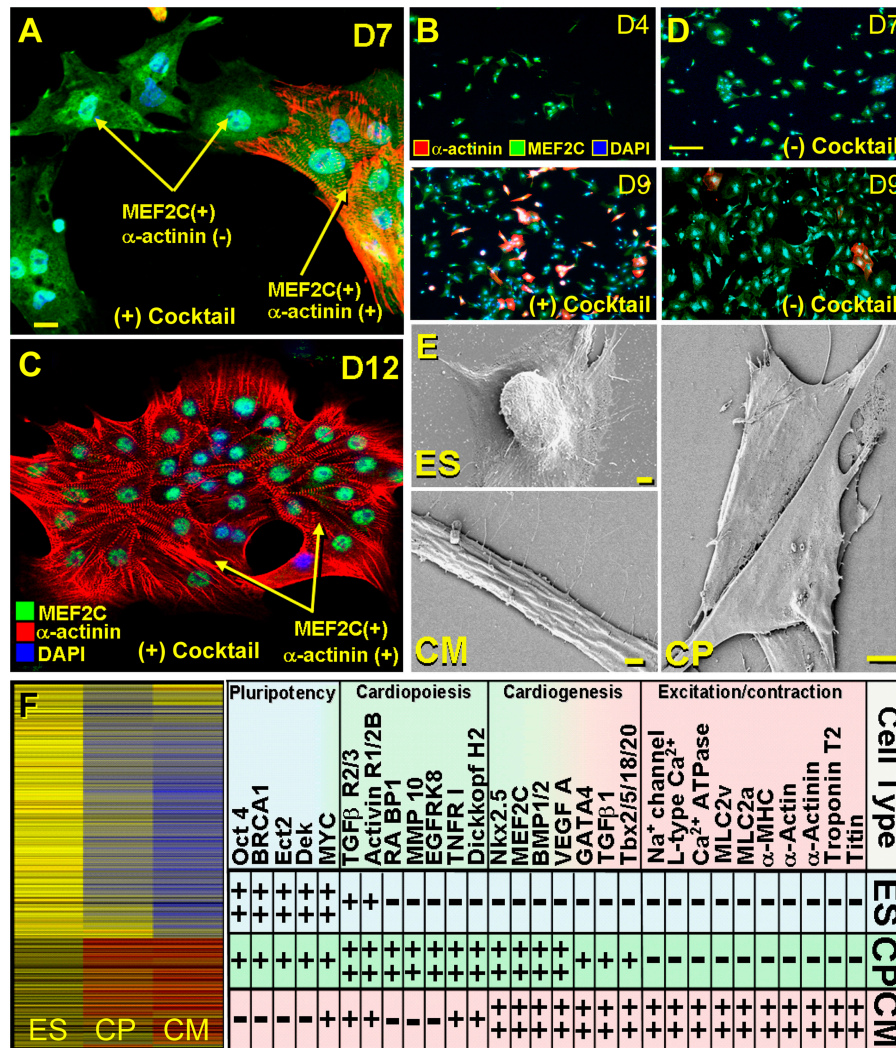


Figure 7. Cardiopoietic cells demonstrate loss of oncogenic and pluripotent markers. (A–D) MEF-2C (green), α -actinin (red), and DAPI (blue) staining revealed cardiopoietic cells with nuclear translocation of MEF-2C without sarcomeric expression, spontaneously differentiating into cardiomyocytes by day 7 (D7) of cocktail treatment (A). Cardiopoietic cells plated at 10,000 cells/ml (B, top), when placed in medium containing cocktail components ((+) Cocktail), differentiated into cardiomyocytes with $\geq 10\%$ of cells α -actinin positive by day 7 (A), $\geq 30\%$ of cells positive by day 9 (B, bottom), and $\geq 65\%$ of cells positive by day 12 (C) of stimulation. Without cocktail stimulation, cardiopoietic cells continued to proliferate without differentiation when placed in a low serum medium devoid of cocktail components ((-) Cocktail, D). In this condition, spontaneous differentiation into cardiomyocytes was observed in $\leq 1\%$ of cells by day 7 (D7, D top)

and $\leq 5\%$ of cells by day 9 (D9, D bottom). (E) On scanning electron microscopy, the intermediate phenotype of cardiopoietic stem cells (CP) contrasts from that of embryonic stem cells (ES) with high nucleus-to-cytosol ratio, and from that of rod-shaped mature cardiomyocytes (CM). (F) Transcriptome profiling of embryonic stem cells (ES), cardiopoietic stem cells (CP), and cardiomyocytes (CM); left) reveals that combinatorial priming induces a switch from a gene expression profile of pluripotency (ES) to cardiopoiesis (CP) and cardiogenesis (CP and CM) before cardiomyocyte maturation and excitation-contraction coupling (CM). –, background expression; +, definitive expression used as baseline; ++, ≥ 2 -fold increase in expression. Bars: (A; also applies to C) 5 μm ; (D; also applies to B) 50 μm ; (E, CP) 5 μm ; (E, ES) 10 μm ; and (E, CM) 2 μm .

of the cardiac program under the combinatorial guidance of the cardiogenic cocktail composed of factors identified in the TNF- α -primed endodermal secretome.

Cardiopoietic cells discard markers of oncogenesis with loss of pluripotency

Genomic expression profiling demonstrated progressive loss of pluripotent traits associated with cardiac specification of

embryonic stem cells (Fig. 7 F). Dissection of the cardiopoietic transcriptome revealed down-regulation in markers of pluripotency (Oct4; reference 27) and oncogenicity (MYC and DEK; reference 49) along with activation of cardiogenic pathways (MEF-2C, Nkx2.5, GATA4, and Tbx2), preceding expression of the excitation-contraction machinery (L-type Ca²⁺ channel, MLC2v) typical of the cardiac lineage (Fig. 7 F). Differentiation of embryonic stem cells untreated

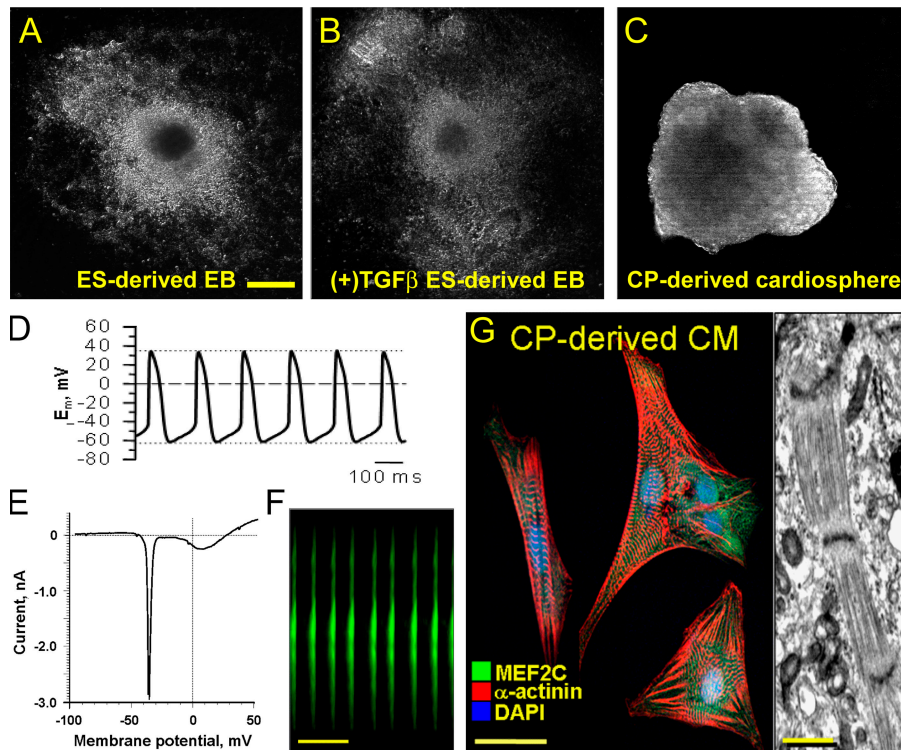


Figure 8. Cardiopoietic stem cells are confined to the cardiac program. (A) Hanging drop differentiation of embryonic stem cells resulted in differentiation of cells into all three germinal layers. (B) Treatment of embryonic stem cells with a single component (e.g., TGF-β1) of the cardiogenic cocktail increases stem cell aptitude for cardiogenesis—demonstrated by increased beating area—but still results in trigeminal embryoid body formation indicating maintained pluripotency. (C) Differ-

entiation of cardiopoietic cells generated cardiospheres demonstrating definitive propensity toward cardiogenesis. (D–G) Isolation of cells from cardiopoietic cell-generated cardiospheres yielded functional cardiomyocytes demonstrating action potential (E), ion current activity (F), and calcium transients (G) along with organized sarcomerogenesis recapitulating myocyte structure on confocal and transmission electron microscopy (G, inset). Bars: (A–C) 500 μm; (F) 3 s; (G) 20 μm; (G, inset) 1 μm.

or treated with single factors from the cardiogenic cocktail yielded three germinal layer embryoid bodies (Fig. 8, A and B), reflecting a maintained pluripotency. In contrast, day 4–recruited cardiopoietic cells no longer produced trigeminal layer embryoid bodies and instead formed beating cardiospheres, demonstrating commitment to the cardiac program (Fig. 8 C). Dissociation of cardiospheres released cells with hallmark features of cardiomyocytes, including action potential activity (Fig. 8 D), ion current profiles (Fig. 8 E), rhythmic calcium transients (Fig. 8 F), and sarcomeric organization (Fig. 8 G). Thus, derived cardiopoietic cells exchange the pluripotent and oncogenic molecular profile of embryonic stem cells for definitive commitment to cardiac lineage.

Cardiopoietic cell therapy offers safe myocardial repair

The therapeutic usefulness of derived cardiopoietic cells was demonstrated *in vivo* after echocardiography-guided myocardial transplantation. As seen with embryonic stem cell transplantation (Fig. 1, A–C), cardiopoietic cells proliferated and generated cardiomyocytes in the host myocardium (Fig. 9, A and B). However, the load yielding tumor-free outcome exceeded that of the embryonic source (Fig. 9 C). Delivery

of cardiopoietic cells at $\geq 3 \times 10^6$ per heart produced no incidence of tumors, in contrast to a $\sim 70\%$ teratoma outcome that resulted from implantation of an equivalent embryonic stem cell load (Fig. 9 C). Cardiopoietic cells tripled the area of engraftment maximally attainable from embryonic stem cell transplantation without resultant electrical ectopy or disruption of myocardial architecture and function (Fig. 9, D and E). In a chronic model of myocardial infarction with individual animals serving as their own control, delivery of cardiopoietic cells 8 wk after ligation of the left anterior descending artery resulted in improved anterior wall motion, as detected on short axis (Fig. 9 F) and M-mode (Fig. 9 G) echocardiographic evaluation. Echocardiography of the intraventricular area before and after cardiopoietic cell transplantation revealed on average a 30-mm² decrease in the circumferential area during systole 2 mo after transplantation (Fig. 9 H), improving fractional heart output by 35% (Fig. 9 I). On histopathological examination, infarcted hearts treated with cardiopoietic cells (10^5 mg/heart tissue) revealed that engrafted cells had advanced into the cardiac program expressing CFP under control of the cardiac-specific actin promoter (Fig. 10 A). Cardiopoietic cell-derived cardiomyocytes exhibited distinct sarcomeric striation determined by

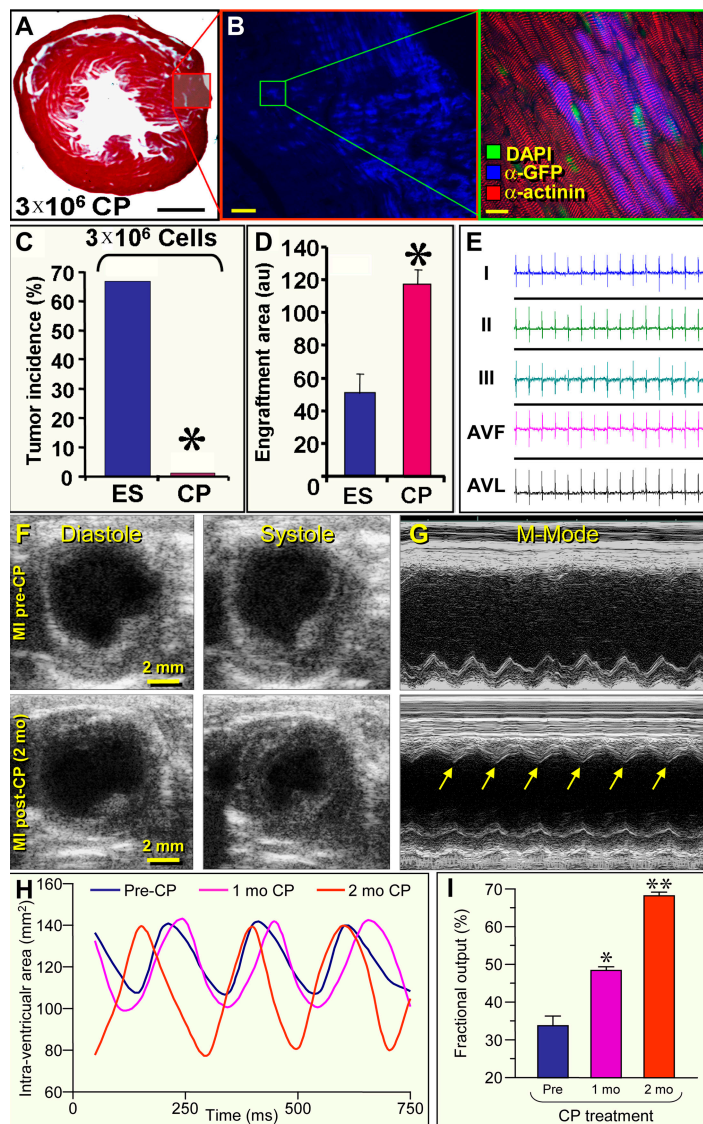


Figure 9. Cardiopoietic programming of embryonic stem cells eliminates tumorigenic risk in therapy. (A and B) Delivery of derived cardiopoietic cells (CP) at 3×10^6 /heart resulted in proper implantation and cardiac differentiation. (C and D) In contrast to embryonic stem cells (ES), treatment with cardiopoietic cells (CP) was not associated with tumor formation and led to a threefold increase in engraftment area. (E) Synchronous cardiac function monitored by multilead electrocardiography demonstrated no evidence for ectopy after delivery of cardiopoietic cells. I, II, III, AVF, and AVL indicate lead placement on electrocardiogram.

(F and G) Echocardiographic evaluation of intraventricular area before and after cardiopoietic cell transplantation in myocardial infarction shows reanimation of the akinetic anterior LV wall on short axis and improved fractional shortening demonstrated by two-dimensional M-mode echocardiography during the 2-mo follow-up period. (H) In comparison to hearts before treatment, a 10-mm² and 30-mm² decrease in circumferential area during systole was observed on 1- and 2-mo follow-up echocardiography, respectively, resulting in a 35% increase in fractional output 2 mo after treatment (I). Bars: (A) 2 mm; (B) 50 μ m; (B, inset) 10 μ m.

α -actinin staining and populated periinfarct regions (Fig. 10, A and B). Furthermore, Ki67, a nuclear protein expressed by proliferating cells in all phases of cell cycle (50), colocalized with DAPI-stained nuclei of CFP-expressing cardiopoietic cell-derived cardiomyocytes, indicating the continued ability of engrafted cells to divide (Fig. 10, A and B). Thus, programming of embryonic stem cells pretransplantation to generate the cardiac predetermined cardiopoietic cell type translated into safe tumor-free infarct repair.

DISCUSSION

Embryonic stem cells, although recognized for their potential in tissue repair, harbor the threat for neoplastic transformation because of an inherent risk for unguided differentiation, preventing translation of embryonic stem cell therapy into practice. A strategy to overcome this limitation was here developed with the discovery that the reprogramming cytokine TNF- α promotes cardiogenic specification of pluripotent embryonic stem cells. This previously unrecognized

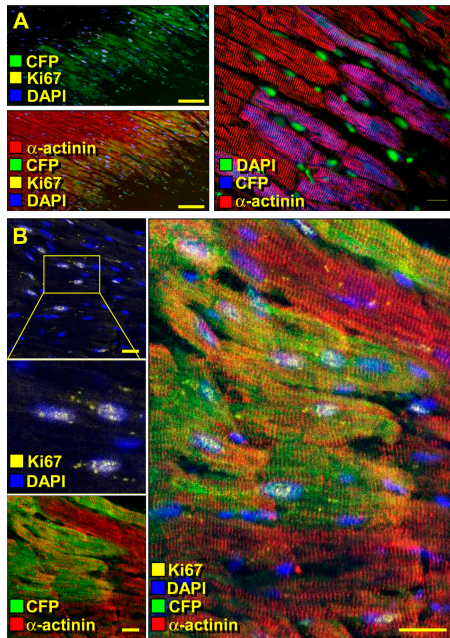


Figure 10. Cardiopoietic progenitor cells maintain cell cycle activity after engraftment into the infarcted heart. (A) Histopathological examination of treated infarcted hearts revealed cardiopoietic cell-derived cardiomyocytes incorporated into host myocardium around the infarct region. Low magnification confocal microscopy revealed large numbers of Ki67-expressing cardiopoietic cell-derived myocytes surrounding the infarcted region. While high magnification confocal evaluation demonstrates CFP positive cells with organized sarcomeres aligned with host myocardium (A, right). (B) Ki67-expressing cells on high magnification confocal microscopy were CFP positive, indicating cardiopoietic cells as their source. Bar: (A, left) 50 μ m; (A, right) 10 μ m; (B, left) 15 μ m; (B, right) 20 μ m.

function of the stress cytokine was demonstrated in vivo through cardiac-restricted transgenic overexpression of TNF- α , resulting in augmented cardiogenic competence of the host heart and safe incorporation of transplanted stem cells. Molecular dissection of the procardiogenic action of TNF- α in vitro resolved the identity of cardioinductive signals, which when applied to embryonic stem cells recruited cardiac predetermined progenitors, termed cardiopoietic progenitor cells. Use of cardiopoietic stem cells eliminated reliance on host signaling for differentiation, averting the malignant risk of pluripotency and achieving a critical step in the translation of stem cells into therapy.

Transplantation of embryonic stem cells outside of the embryonic environment is disruptive to the normal differentiation program (51, 52). Although the host heart, through paracrine release of cardiogenic factors, can guide stem cell toward cardiogenic differentiation (34, 53, 54), the capacity of the foster milieu to secure stem cell specification and mimic embryonic conditions is finite (24, 26), with uncontrolled growth resulting from increased stem cell loads. This indicates that noncardiogenic signaling within a pluripotent stem cell population (55) can overcome the limited cardio-

instructive signaling of the host heart (34, 56). The resulting teratoma emanating from an otherwise nontumor-prone myocardium contained a multiplicity of tissue types representative of all three germinal layers, confirming their stem cell origin (12) and revealing that environmental cues play a critical role in affecting stem cell behavior (57, 58). Tumorigenic outcome was precipitated by disrupting the capacity of stem cells to recognize paracrine cardiogenic signals, highlighting the importance of intact TGF- β superfamily signaling in cardiac specification (59). Conversely, enriching the cardiogenic capacity of the host microenvironment through cardiac-specific TNF- α expression and up-regulation of TGF- β family members prevented aberrant growth patterns. This indicates successful competition with noncardiogenic signals that are innate to embryonic stem cells, providing the necessary cues for cardiogenic specification. This finding is in line with works that implicate up-regulation of TNF- α and TGF- β as components of the myocardial stress response (36, 60, 61), contributing to the observation that transplantation of stem cells into diseased myocardium results in incorporation without tumor formation (18, 20, 62). Collectively, these findings underscore the importance of cross-talk between stem cells and the host environment in determining cell fate after transplantation and provide evidence that manipulation of this interaction can achieve the desired outcome.

Although cardiac-restricted overexpression of TNF- α was efficacious in driving stem cell differentiation in vivo, the feasibility of translating such transgenic approach into clinical practice remains uncertain. Moreover, the unpredictable nature of the heart microenvironment in the setting of disease (63, 64), including variable up-regulation of cytokine and growth factors after myocardial injury (65, 66), may not be reliable in counteracting noncardiogenic signaling in differentiating embryonic stem cells to ensure safety. Recapitulation of the procardiogenic action of TNF- α in vitro using the embryoid body model allowed dissection of the mechanism of cytokine action providing a paradigm by which to relinquish the noncardiogenic potential of embryonic stem cells and bypass the reliance on the cardiogenic competence of host myocardium. By targeting the endoderm, TNF- α activated the TAK1/p38 pathway as in the injured heart (61), promoting cardiogenesis via increased secretion of endodermal cardiogenic factors. Although coculture of embryonic stem cells with the endoderm can guide cardiogenesis (67), the present study reveals the identity of secreted proteins through subtractive proteomic analysis of the endodermal secretome. Identified proteins within the primed secretome clustered into networks downstream of TNF- α and included potent cardiogenic factors, such as members of the TGF- β and FGF families (15, 68) whose combined application secured commitment to the cardiac program, recapitulating the influence of the natural embryonic milieu. As individual components of the derived cardiogenic cocktail enhance cardiac specification in vitro (34, 69) or in vivo (20), their combinatorial use achieved guided

cardiogenesis of embryonic stem cells in a monolayer allowing profiling of cardiopoiesis. This approach enabled capture of cardiopoietic progenitor cells with phenotypic and genotypic features that demonstrate diminished tendency for unguided differentiation and loss of pluripotent and oncogenic markers characteristic of undifferentiated stem cells (55), with translocation of cardiac transcription factors into the nucleus securing cardiac specification. Untreated embryonic stem cells demonstrate their pluripotency by forming three-layer embryoid bodies (48, 70). Treatment of embryonic stem cells with single cardiogenic agents, although promoting cardiomyocyte content, did not eliminate trigeminal differentiation. In contrast, recruitment of cardiopoietic cells through application of the cardiogenic cocktail on a monolayer of embryonic stem cells eliminated pluripotent propensity, generating cardiospheres indicative of definitive commitment to the cardiac program.

Having secured cardiogenic specification, cardiopoietic cells provided a safe source for cell therapy. With demonstrated survivorship and completion of the cardiac program upon transplantation, along with sarcomeric alignment and electrical coupling within the host myocardium, this cell type fulfilled established criteria for implantation (24, 71). Cardiopoietic cells averted tumor formation on engraftment into healthy hearts at doses that carry high risk for tumorigenesis with embryonic stem cells. Further, cardiopoietic cells maintained a capacity to proliferate within scar tissue, leading to functional recovery after engraftment into infarcted hearts. This is the first example where delivery of embryonic-derived cardiac progenitors achieved proper myocardial integration without risk for unguided growth. This work thus demonstrates that use of cardiopoietic cells eliminates reliance on host heart signaling for differentiation, a limitation that has precluded pluripotent embryonic stem cells as a safe therapeutic option. Honing cellular plasticity to nullify malignant risk, therefore, achieves a critical step in translation of stem cells into therapy.

MATERIALS AND METHODS

Protocols were approved by Mayo Clinic Institutional Animal Care and Use Committee.

Embryonic stem cell-based cardiopoiesis. Murine embryonic stem cells were differentiated into embryoid bodies using the hanging-drop method, with cardiac differentiation monitored by epifluorescence using α -actinin antibody (1:1,000) and live microscopy (34, 48). RNA was processed by quantitative RT-PCR using the Light Cycler (Roche) and QuantiTect SYBR Green kit (QIAGEN) to quantify Nkx2.5 (forward, reverse primers: 5'-TGCAGAAGGCAGTGGAGCTGGACAAGCC-3' and 5'-TTGCACTTGTAGCGACGGTCTGGAACCA-3'), MEF-2C (5'-AGATACCCACAACACACCACGCGCC-3' and 5'-ATCCTTCAGAGACTCGCATGCGCTT-3'), GATA-4 (5'-GGAATTCAAGATGAACGGCATCAAC-3' and 5'-TGAATTCTCAACCTGCTGGCGTCTTAGA-3'), and β -MHC (5'-GCCAAAACACCAACCTGTCCAAGTTC-3' and 5'-CTGCTGGAGAGGTTATTCCTCG-3') mRNA expression normalized to β -tubulin. From day 7 embryoid bodies, cardiopoietic progenitor cells were isolated by Percoll purification and visualized through laser confocal examination using MEF-2C (1:400; Cell Signaling Technologies), Nkx2.5 (1:300; Santa Cruz Biotechnology, Inc.), GATA-4 (1:300, Santa Cruz Biotechnology; Inc.),

and α -actinin (1:1,000; Sigma-Aldrich). In a subset of experiments, the endodermal layer was eliminated from embryoid bodies through generation of hanging drops in the presence of recombinant leukemia inhibitory factor (10 U/ μ l) once differentiation has been initiated (38). Isolated visceral endoderm-like cells were derived from an F9 cell population (American Type Culture Collection) with retinoic acid (1 μ M), dibutyl cAMP (0.5 mM), and theophylline (0.5 mM), with phenotype confirmed through comparison with END-2 cells (67). Conditioned medium obtained after 24 h of culture was used for dissection of procardiogenic signaling by proteomic analysis. To stimulate cardiopoiesis of embryonic stem cells cultured in monolayer at 100 cells/cm², cells were stimulated with recombinant TGF- β 1 (2.5 ng/ml), BMP-2 and -4 (5 ng/ml), activin-A (5 ng/ml), FGF-2 and -4 (10 ng/ml), IL-6 (100 ng/ml), IGF-1 and -2 (50 ng/ml), VEGF-A (10 ng/ml), and EGF (2.5 ng/ml) either in singular or combinatorial fashion with cellular response monitored by confocal microscopy. When recruited from a monolayer of embryonic stem cells, the cardiopoietic population was enriched using a dual interface Percoll gradient to separate sarcomere-rich high density cardiomyocytes (34) from the lower density sarcomere-poor cardiopoietic phenotype. Cardiopoietic cell proliferation and purity was assessed by ArrayScan multi-channel fluorescence automated microscopy (Cellomics) using MEF-2C and α -actinin antibodies, along with DAPI nuclear staining. Cell morphology was resolved by field emission scanning or transmission electron microscopy. Action potentials and voltage-current relationships were acquired by patch-clamp electrophysiology. Calcium dynamics were tracked in Fluo 4-AM-loaded cells using laser confocal line scanning (48).

Transgenic mice. Enrichment of the myocardium with TNF- α was induced by cardiac-restricted overexpression of this cytokine using the α -myosin heavy chain promoter linked to the TNF- α transgene as described (72). Wild-type females were bred with transgenic males, and resultant heterozygous transgenic offspring (TNF- α -TG) identified by tail-cut PCR were compared with wild-type littermates (73).

Genomics. Total RNA was isolated using the Micro-to-Midi isolation kit (Invitrogen) and subjected to comparative gene expression profiling by labeled cRNA hybridization to the mouse genome 430 2.0 microarray (Affymetrix). Data acquired using the GeneChip Scanner 3000 was analyzed with the Genespring GX 7.3 microarray data software bioinformatics suite (Agilent Technologies), restricting the derived gene list to identify differentially expressed genes defined by a >1.5-fold difference and $P < 0.05$. Data population sets were normalized to the undifferentiated phenotype and quality filtered to eliminate background noise before hierarchical clustering (74). The dataset is available as series GSE6689 at National Center for Biotechnology Information (National Center for Biotechnology Information) on Gene Expression Omnibus (GEO) website.

Proteomics. Endodermal cells were cultured with serum-free Glasgow modified Eagle's medium. Derived conditioned medium was centrifuged and filtered with protein quantified, concentrated (Amicon Ultra 5 kD cut-off), and requantified for volumetric normalization. The protein equivalent of 5 ml conditioned medium was resuspended in isoelectric focusing (IEF) buffer containing urea (7 M), thiourea (2 M), CHAPS (2% wt/vol), and DeStreak (15 mg/ml; GE Healthcare). Proteins were resolved in the first dimension using immobilized pH gradient IEF strips (Bio-Rad Laboratories) at pH 3–10, 4–7, and 6–11, and in the second dimension by 7.5% and 15% SDS-PAGE. Proteins visualized by silver staining were isolated, destained, and trypsin digested (75) with extracted peptides subjected to high performance liquid chromatography–electrospray ionization tandem mass spectrometry using a ThermoFinnigan LTQ. Alternatively, concentrated protein was prepared for multidimensional liquid chromatography protein analysis (76) by reduction, alkylation, and sequential digestion with endoprotease Lys-C (Sigma-Aldrich; enzyme to substrate ratio 1:125) and trypsin (Promega; enzyme to substrate ratio 1:100). Peptides were then subjected to strong cation exchange chromatography, and each strong cation exchange fraction was analyzed by liquid chromatography–electrospray ionization

tandem mass spectrometry using a ThermoFinnigan LTQ-FT. Proteins were identified from tandem mass spectrometry (MS-MS) data using SEQUEST and Mascot search algorithms in silico mining of the SwissProt database. Identified proteins were quantified with enzyme-linked immunosorbent assay, and their cardioprotective potency was evaluated. Molecules identified by proteomic screening were introduced to a bioinformatics model generating a network based on interrelationships between orthologs mined by the Ingenuity Pathways Analysis. A scale-free network was generated to reflect levels of subcellular compartmentalization.

Stem cell transplantation. Under isoflurane anesthesia, echocardiography with a 15-MHz probe (Acuson) was used to guide myocardial delivery of embryonic or cardiopoietic cells engineered for in situ tracking (70). Cardiac performance was monitored by echocardiography in the short axis with a two-dimensional M-mode probing in the long axis, Doppler pulse wave analysis, and 12-lead electrocardiography (77, 78). Harvested heart tissue was fixed for 1 h in 3% paraformaldehyde, paraffin sectioned, and subjected to antigen retrieval using CFP antibody for cell tracking (1:500; Molecular Probes) in combination with α -actinin for sarcomere visualization and DAPI nuclear stain. Myocardial infarction was induced by coronary ligation of the left anterior descending artery (18).

Statistical analysis. Comparison between groups was performed using a standard Student's *t* test of variables with 95% confidence intervals.

We thank D.C. Muddiman for guidance in protein identification, J. Nesbitt and L. Rowe for technical assistance, and D.L. Mann, M.D. Schneider, R.M. Harland, and C. Mummery for generous gifts of TNF- α transgenic mice, Δ TGF β R11 cDNA, noggin cDNA, and END-2 cells, respectively.

This work was supported by National Institutes of Health, American Heart Association, Marriott Heart Disease Research Program, Marriott Foundation, Ted Nash Long Life Foundation, Ralph Wilson Medical Research Foundation, Heart and Stroke Foundation of Canada, and Asper Foundation. M. Puceat is supported through Institut National de la Santé et de la Recherche Médicale U421. A. Behfar is supported by the Clinician-Investigator Program at the Mayo Clinic.

The authors have no conflicting financial interests.

Submitted: 6 September 2006

Accepted: 16 January 2007

REFERENCES

- Anversa, P., and B. Nadal-Ginard. 2002. Myocyte renewal and ventricular remodeling. *Nature*. 415:240–243.
- Orlic, D., J. Hill, and A. Arai. 2002. Stem cells for myocardial regeneration. *Circ. Res.* 91:1092–1102.
- Dimmeler, S., A. Zeiher, and M. Schneider. 2005. Unchain my heart: scientific foundations of cardiac repair. *J. Clin. Invest.* 115:572–583.
- Murry, C., L. Field, and P. Menasche. 2005. Cell-based cardiac repair. *Circulation*. 112:3174–3183.
- Van Laake, L., D. Van Hoof, and C. Mummery. 2005. Cardiomyocytes derived from stem cells. *Ann. Med.* 37:499–512.
- Wollert, K., and H. Drexler. 2005. Clinical applications of stem cells for the heart. *Circ. Res.* 96:151–163.
- Bartunek, J., S. Dimmeler, H. Drexler, F. Fernandez-Aviles, M. Galinanes, S. Janssens, J. Martin, A. Mathur, P. Menasche, S. Priori, et al. 2006. The consensus of the task force of the European Society of Cardiology concerning the clinical investigation of the use of autologous adult stem cells for repair of the heart. *Eur. Heart J.* 27:1338–1340.
- Sanchez, P., J. San Roman, A. Villa, M. Fernandez, and F. Fernandez-Aviles. 2006. Contemplating the bright future of stem cell therapy for cardiovascular disease. *Nat. Clin. Pract. Cardiovasc. Med.* 3:S138–S151.
- Schachinger, V., S. Erbs, A. Elsasser, W. Haberbosch, R. Hambrecht, H. Holschermann, J. Yu, R. Corti, D.G. Mathey, C.W. Hamm, et al. 2006. Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. *N. Engl. J. Med.* 355:1210–1221.
- Mangi, A., N. Noiseux, D. Kong, H. He, M. Rezvani, J. Ingwall, and V.J. Dzau. 2003. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nat. Med.* 9:1195–1201.
- Behfar, A., and A. Terzic. 2006. Derivation of a cardiopoietic population from human mesenchymal stem cells yields cardiac progeny. *Nat. Clin. Pract. Cardiovasc. Med.* 3:S78–S82.
- Thomson, J., J. Itskovitz-Eldor, S. Shapiro, M. Waknitz, J. Swiergiel, V. Marshall, and J. Jones. 1998. Embryonic stem cell lines derived from human blastocysts. *Science*. 282:1145–1147.
- Donovan, P., and J. Gearhart. 2001. The end of the beginning for pluripotent stem cells. *Nature*. 414:92–97.
- Sachinidis, A., B. Fleischmann, E. Kolossov, M. Wartenberg, H. Sauer, and J. Hescheler. 2003. Cardiac specific differentiation of mouse embryonic stem cells. *Cardiovasc. Res.* 58:278–291.
- Lev, S., I. Kehat, and L. Gepstein. 2005. Differentiation pathways in human embryonic stem cell-derived cardiomyocytes. *Ann. N.Y. Acad. Sci.* 1047:50–65.
- Menasche, P. 2005. The potential of embryonic stem cells to treat heart disease. *Curr. Opin. Mol. Ther.* 7:293–299.
- Smith, A. 2001. Embryo-derived stem cells: of mice and men. *Annu. Rev. Cell Dev. Biol.* 17:435–462.
- Hodgson, D.M., A. Behfar, L. Zingman, G.C. Kane, C. Perez-Terzic, A.E. Alekseev, M. Puceat, and A. Terzic. 2004. Stable benefit of embryonic stem cell therapy in myocardial infarction. *Am. J. Physiol.* 287:H471–H479.
- Kehat, I., L. Khimovich, O. Caspi, A. Gepstein, R. Shofti, G. Arbel, I. Huber, J. Satin, J. Itskovitz-Eldor, and L. Gepstein. 2004. Electromechanical integration of cardiomyocytes derived from human embryonic stem cells. *Nat. Biotechnol.* 22:1282–1289.
- Menard, C., A.A. Hagege, O. Agbulut, M. Barro, M. Morichetti, C. Brasselet, A. Bel, E. Messas, A. Bissery, P. Bruneval, et al. 2005. Transplantation of cardiac-committed mouse embryonic stem cells to infarcted sheep myocardium. *Lancet*. 366:1005–1012.
- Kolossov, E., T. Bostani, W. Roell, M. Breitbach, F. Pillekamp, J.M. Nygren, P. Sasse, O. Rubenchik, J.W. Fries, D. Wenzel, et al. 2006. Engraftment of engineered ES cell-derived cardiomyocytes but not BM cells restores contractile function to the infarcted myocardium. *J. Exp. Med.* 203:2315–2327.
- Singla, D., T. Hacker, L. Ma, P. Douglas, R. Sullivan, G. Lyons, and T. Kamp. 2006. Transplantation of embryonic stem cells into the infarcted mouse heart. *J. Mol. Cell. Cardiol.* 40:195–200.
- Behfar, A., D.M. Hodgson, L.V. Zingman, C. Perez-Terzic, S. Yamada, G.C. Kane, A.E. Alekseev, M. Puceat, and A. Terzic. 2005. Administration of allogenic stem cells dosed to secure cardiogenesis and sustained infarct repair. *Ann. N.Y. Acad. Sci.* 1049:189–198.
- Lafamme, M., and C. Murry. 2005. Regenerating the heart. *Nat. Biotechnol.* 23:845–856.
- Cao, F., S. Lin, X. Xie, P. Ray, M. Patel, X. Zhang, M. Drukker, S. Dylla, A. Connolly, X. Chen, et al. 2006. *In vivo* visualization of embryonic stem cell survival, proliferation, and migration after cardiac delivery. *Circulation*. 113:1005–1014.
- Foley, A., and M. Mercola. 2004. Heart induction: embryology to cardiomyocyte regeneration. *Trends Cardiovasc. Med.* 14:121–125.
- Nichols, J., B. Zevnik, K. Anastasiadis, H. Niwa, D. Klewe-Nebenius, I. Chambers, H. Scholer, and A. Smith. 1998. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell*. 95:379–391.
- Schultheiss, T., S. Xydias, and A. Lassar. 1995. Induction of avian cardiac myogenesis by anterior endoderm. *Development*. 121:4203–4214.
- Olson, E., and D. Srivastava. 1996. Molecular pathways controlling heart development. *Science*. 272:671–676.
- Harvey, R. 2002. Patterning the vertebrate heart. *Nat. Rev. Genet.* 3:544–556.
- Harvey, R. 1996. NK-2 homeobox genes and heart development. *Dev. Biol.* 178:203–216.
- Lin, Q., J. Schwarz, C. Bucana, and E. Olson. 1997. Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. *Science*. 276:1404–1407.

33. Durocher, D., F. Charron, R. Warren, R.J. Schwartz, and M. Nemer. 1997. The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. *EMBO J.* 16:5687–5696.
34. Behfar, A., L.V. Zingman, D.M. Hodgson, J. Rauzier, G.C. Kane, A. Terzic, and M. Puceat. 2002. Stem cell differentiation requires a paracrine pathway in the heart. *FASEB J.* 16:1558–1566.
35. Locksley, R., N. Killeen, and M. Lenardo. 2001. The TNF and TNF receptor superfamilies. *Cell.* 104:487–501.
36. Mann, D. 2003. Stress-activated cytokines and the heart. *Annu. Rev. Physiol.* 65:81–101.
37. Maltsev, V., A. Wobus, J. Rohwedel, M. Bader, and J. Hescheler. 1994. Cardiomyocytes differentiated *in vitro* from embryonic stem cells developmentally express cardiac-specific genes and ionic currents. *Circ. Res.* 75:233–244.
38. Bader, A., A. Gruss, A. Hollrigel, H. Al-Dubai, Y. Capetanaki, and G. Weitzer. 2001. Paracrine promotion of cardiomyogenesis in embryoid bodies by LIF modulated endoderm. *Differentiation.* 68:31–43.
39. Edgeworth, J., P. Freemont, and N. Hogg. 1989. Ionomycin-regulated phosphorylation of the myeloid calcium-binding protein p14. *Nature.* 342:189–192.
40. Xin, H., T. Senbonmatsu, D. Cheng, Y. Wang, J. Copello, G. Ji, M. Collier, K. Deng, L. Jeyakumar, M. Magnuson, et al. 2002. Oestrogen protects FKBP12.6 null mice from cardiac hypertrophy. *Nature.* 416:334–338.
41. Smart, N., A. Hill, J. Cross, and P. Riley. 2002. A differential screen for putative targets of the bHLH transcription factor Hand1 in cardiac morphogenesis. *Gene Expr. Patterns.* 2:61–67.
42. Kwon, Y., A. Kashina, I. Davydov, R. Hu, J. An, J. Seo, F. Du, and A. Varshavsky. 2002. An essential role of N-terminal arginylation in cardiovascular development. *Science.* 297:96–99.
43. Blokzijl, A., C. Dahlqvist, E. Reissmann, A. Falk, A. Moliner, U. Lendahl, and C.F. Ibanez. 2003. Cross-talk between the Notch and TGF-beta signaling pathways mediated by interaction of the Notch intracellular domain with Smad3. *J. Cell Biol.* 163:723–728.
44. McDermott, E.P., and L.A.J. O'Neill. 2002. Ras participates in the activation of p38 MAPK by interleukin-1 by associating with IRAK, IRAK2, TRAF6, and TAK-1. *J. Biol. Chem.* 277:7808–7815.
45. Benus, G.F., A.T. Wierenga, D.J. de Gorter, J.J. Schuringa, A.M. van Bennekum, L. Drenth-Diephuis, E. Vellenga, and B.J. Eggen. 2005. Inhibition of the transforming growth factor beta (TGFβ) pathway by interleukin-1β is mediated through TGFβ-activated kinase 1 phosphorylation of SMAD3. *Mol. Biol. Cell.* 16:3501–3510.
46. Hanafusa, H., J. Ninomiya-Tsuji, N. Masuyama, M. Nishita, J. Fujisawa, H. Shibuya, K. Matsumoto, and E. Nishida. 1999. Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-beta-induced gene expression. *J. Biol. Chem.* 274:27161–27167.
47. Perez-Terzic, C., R.S. Faustino, B.J. Boorsma, D.K. Arrell, N.J. Niederlander, A. Behfar, and A. Terzic. 2007. Stem cells transform into a cardiac phenotype with remodeling of the nuclear transport machinery. *Nat. Clin. Pract. Cardiovasc. Med.* 4:S68–S76.
48. Perez-Terzic, C., A. Behfar, A. Mery, J.M. van Deursen, A. Terzic, and M. Puceat. 2003. Structural adaptation of the nuclear pore complex in stem cell-derived cardiomyocytes. *Circ. Res.* 92:444–452.
49. Shachaf, C., A. Kopelman, C. Arvanitis, A. Karlsson, S. Beer, S. Mandl, M. Bachmann, A. Borowsky, B. Ruebner, R. Cardiff, et al. 2004. MYC inactivation uncovers pluripotent differentiation and tumour dormancy in hepatocellular cancer. *Nature.* 431:1112–1117.
50. Laflamme, M., J. Gold, C. Xu, M. Hassanipour, E. Rosler, S. Police, V. Muskheli, and C. Murry. 2005. Formation of human myocardium in the rat heart from human embryonic stem cells. *Am. J. Pathol.* 167:663–671.
51. Rohwedel, J., U. Sehmeyer, J. Shan, A. Meister, and A. Wobus. 1996. Primordial germ cell-derived mouse embryonic germ cells *in vitro* resemble undifferentiated stem cells with respect to differentiation capacity and cell cycle distribution. *Cell Biol. Int.* 20:579–587.
52. Wobus, A., G. Kaomei, J. Shan, M. Wellner, J. Rohwedel, G. Ji, B. Fleischmann, H. Katus, J. Hescheler, and W. Franz. 1997. Retinoic acid accelerates embryonic stem cell-derived cardiac differentiation and enhances development of ventricular cardiomyocytes. *J. Mol. Cell. Cardiol.* 29:1525–1539.
53. Olson, E., and M. Schneider. 2003. Sizing up the heart: development redux in disease. *Genes Dev.* 17:1937–1956.
54. Rosenblatt-Velin, N., M. Lepore, C. Cartoni, F. Beermann, and T. Pedrazzini. 2005. FGF-2 controls the differentiation of resident cardiac precursors into functional cardiomyocytes. *J. Clin. Invest.* 115:1724–1733.
55. Stewart, R., M. Stojkovic, and M. Lako. 2006. Mechanisms of self-renewal in human embryonic stem cells. *Eur. J. Cancer.* 42:1257–1272.
56. Heng, B., T. Cao, H. Haider, D. Wang, E.K.-W. Sim, and S. Ng. 2004. An overview and synopsis of techniques for directing stem cell differentiation *in vitro*. *Cell Tissue Res.* 315:291–303.
57. Schuldiner, M., O. Yanuka, J. Itskovitz-Eldor, D.A. Melton, and N. Benvenisty. 2000. Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc. Natl. Acad. Sci. USA.* 97:11307–11312.
58. Cooke, M., M. Stojkovic, and S. Przyborski. 2006. Growth of teratomas derived from human pluripotent stem cells is influenced by the graft site. *Stem Cells Dev.* 15:254–259.
59. Mery, A., E. Papadimou, D. Zeineddine, C. Menard, A. Behfar, L.V. Zingman, D.M. Hodgson, J. Rauzier, G.C. Kane, C. Perez-Terzic, et al. 2003. Commitment of embryonic stem cells toward a cardiac lineage. *J. Muscle Res. Cell Motil.* 24:269–274.
60. Irwin, M., S. Mak, D. Mann, R. Qu, J. Penninger, A. Yan, F. Dawood, W.-H. Wen, Z. Shou, and P. Liu. 1999. Tissue expression and immunolocalization of tumor necrosis factor-α in postinfarction dysfunctional myocardium. *Circulation.* 99:1492–1498.
61. Matsumoto-Ida, M., Y. Takimoto, T. Aoyama, M. Akao, T. Takeda, and T. Kita. 2006. Activation of TGFβ-TAK1-p38 MAPK pathway in spared cardiomyocytes is involved in left ventricular remodeling after myocardial infarction in rats. *Am. J. Physiol.* 290:H709–H715.
62. Nelson, T.J., Z.D. Ge, J. Van Orman, M. Barron, D. Rudy-Reil, T.A. Hacker, R. Misra, S.A. Duncan, J.A. Auchampach, and J.W. Lough. 2006. Improved cardiac function in infarcted mice after treatment with pluripotent embryonic stem cells. *Anat. Rec. A Discov. Mol. Cell. Evol. Biol.* 288:1216–1224.
63. Stanton, L., L. Garrard, D. Damm, B. Garrick, A. Lam, A. Kapoun, Q. Zheng, A. Protter, G. Schreiner, and R. White. 2000. Altered patterns of gene expression in response to myocardial infarction. *Circ. Res.* 86:939–945.
64. Kittleson, M., K. Minhas, R. Irizarry, S. Ye, G. Edness, E. Breton, J. Conte, G. Tomaselli, J. Garcia, and J. Hare. 2005. Gene expression analysis of ischemic and nonischemic cardiomyopathy. *Physiol. Genomics.* 21:299–307.
65. Porto, I., A. Leone, F. Crea, and F. Andreotti. 2005. Inflammation, genetics, and ischemic heart disease. *Cytokine.* 29:187–196.
66. Kempf, K., G. Haltern, R. Futh, C. Herder, S. Muller-Scholze, H. Gulker, and S. Martin. 2006. Increased TNF-alpha and decreased TGF-beta expression in peripheral blood leukocytes after acute myocardial infarction. *Horm. Metab. Res.* 38:346–351.
67. Mummery, C., D. Ward-van Oostwaard, P. Doevendans, R. Spijker, S. van den Brink, R. Hassink, M. van der Heyden, T. Opthof, M. Pera, A. de la Riviere, et al. 2003. Differentiation of human embryonic stem cells to cardiomyocytes. *Circulation.* 107:2733–2740.
68. Lough, J., M. Barron, M. Brogley, Y. Sugi, D. Bolender, and X. Zhu. 1996. Combined BMP-2 and FGF-4, but neither factor alone, induces cardiogenesis in non-precordial embryonic mesoderm. *Dev. Biol.* 178:198–202.
69. Kawai, T., T. Takahashi, M. Esaki, H. Ushikoshi, S. Nagano, H. Fujiwara, and K. Kosai. 2004. Efficient cardiomyogenic differentiation of embryonic stem cell by fibroblast growth factor 2 and bone morphogenetic protein 2. *Circ. J.* 68:691–702.
70. Meyer, N., M. Jaconi, A. Landopoulou, P. Fort, and M. Puceat. 2000. A fluorescent reporter gene as a marker for ventricular specification in ES-derived cardiac cells. *FEBS Lett.* 478:151–158.
71. Menasche, P. 2004. Embryonic stem cells pace the heart. *Nat. Biotechnol.* 22:1237–1238.
72. Sivasubramanian, N., M. Coker, K. Kurrelmeyer, W. MacLellan, F. DeMayo, F. Spinale, and D. Mann. 2001. Left ventricular remodeling in

- transgenic mice with cardiac restricted overexpression of tumor necrosis factor. *Circulation*. 104:826–831.
73. Hodgson, D.M., L.V. Zingman, G.C. Kane, C. Perez-Terzic, M. Bienengraeber, C. Ozcan, R.J. Gumina, D. Pucar, F. O’Coilain, D. Mann, et al. 2003. Cellular remodeling in heart failure disrupts K_{ATP} channel-dependent stress tolerance. *EMBO J*. 22:1732–1742.
74. Chung, S., P.P. Dzeja, R.S. Faustino, C. Perez-Terzic, A. Behfar, and A. Terzic. 2007. Mitochondrial oxidative metabolism is required for the cardiac differentiation of stem cells. *Nat. Clin. Pract. Cardiovasc. Med*. 4:S60–S67.
75. Gharahdaghi, F., C. Weinberg, D. Meagher, B. Imai, and S. Mische. 1999. Mass spectrometric identification of proteins from silver-stained polyacrylamide gel. *Electrophoresis*. 20:601–605.
76. Link, A., J. Eng, D. Schieltz, E. Carmack, G. Mize, D. Morris, B. Garvik, and J. Yates. 1999. Direct analysis of protein complexes using mass spectrometry. *Nat. Biotechnol*. 17:676–682.
77. Kane, G.C., A. Behfar, R.B. Dyer, D. O’Coilain, X.K. Liu, D.M. Hodgson, S. Reyes, T. Miki, S. Seino, and A. Terzic. 2006. *KCNJ11* gene knockout of the Kir6.2 K_{ATP} channel causes maladaptive remodeling and heart failure in hypertension. *Hum. Mol. Genet*. 15:2285–2297.
78. Yamada, S., G.C. Kane, A. Behfar, X.K. Liu, R.B. Dyer, R.S. Faustino, T. Miki, S. Seino, and A. Terzic. 2006. Protection conferred by myocardial ATP-sensitive K^+ channels in pressure overload-induced congestive heart failure revealed in *KCNJ11* Kir6.2-null mutant. *J. Physiol*. 577:1053–1065.