STAT3-Dependent Mouse Embryonic Stem Cell Differentiation Into Cardiomyocytes
Analysis of Molecular Signaling and Therapeutic Efficacy of Cardiomyocyte Precommitted mES Transplantation in a Mouse Model of Myocardial Infarction

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Abstract—Pluripotent embryonic stem (ES) cell therapy may be an attractive source for postinfarction myocardial repair and regeneration. However, the specific stimuli and signal pathways that may control ES cell–mediated cardiomyogenesis remains to be completely defined. The aim of the present study was to investigate (1) the effect and underlying signal transduction pathways of leukemia inhibitory factor (LIF) and bone-morphogenic protein-2 (BMP-2)-induced mouse ES cell (mES-D3 line) differentiation into cardiomyocytes (CMC) and (2) the efficacy of CMC precommitted mES cells for functional and anatomical cardiac repair in surgically-induced mouse acute myocardial infarction (AMI) model. Various doses of LIF and BMP-2 and their inhibitors or blocking antibodies were tested for mES differentiation to CMC in vitro. CMC differentiation was assessed by mRNA and protein expression of CMC-specific markers, Connexin-43, CTI, CTT, Mef2c, Tbx5, Nkx2.5, GATA-4, and αMHC. LIF and BMP-2 synergistically induced the expression of CMC markers as early as 2 to 4 days in culture. Signaling studies identified STAT3 and MAP kinase (ERK1/2) as specific signaling components of LIF+BMP-2–mediated CMC differentiation. Inhibition of either STAT3 or MAPK activation by specific inhibitors drastically suppressed LIF+BMP-2–mediated CMC differentiation. Moreover, in mouse AMI, transplantation of lentivirus-GFP–transduced, LIF+BMP-2 precommitted mES cells, improved post-MI left ventricular functions, and enhanced capillary density. Transplanted cells engrafted in myocardium and differentiated into CMC and endothelial cells. Our data suggest that LIF and BMP-2 may synergistically enhance CMC differentiation of transplanted stem cells. Thus augmentation of LIF/BMP-2 downstream signaling components or cell type specific precommitment may facilitate the effects of ES cell–based therapies for post-MI myocardial repair and regeneration. (Circ Res. 2007;101:0-0.)

Key Words: ES cells  ■  LIF  ■  STAT3  ■  BMP-2  ■  cardiomyocytes

Myocardial ischemia is the leading cause of mortality in the US. Tissue engineering by stem cell therapy for the replacement of lost or damaged tissue with biologically compatible substitutes is a promising therapy for cardiac repair. Recent evidence in animal models has shown that both embryonic and adult-derived stem cells have the capacity to differentiate into cardiovascular cell types including cardiomyocytes (CMC) and to improve cardiac function when transplanted after cardiac injury. These findings lead to several ongoing human clinical trials. Although these findings are promising, this form of treatment is still in a very premature stage. One of the experimentally promising strategies is that transplantation of fetal or neonatal CMC results in successful integration into the recipient heart and also improves heart disorders. However, the clinical application of such cell transplantation therapy is hampered by the lack of an available source of human CMC. In this regard, embryonic stem (ES) cell–derived CMCs are a potential candidate for the donor cells. On the other hand, it is still difficult to control and induce the specific differentiation of ES cells solely toward CMCs.

Much research is ongoing to perfect the specific types of stem cells used, the microenvironment in which they are exposed to, as well as a continuum of other considerations that are vital for successful differentiation. The mouse ES (mES) cells were shown to differentiate into variety of cell types, including CMCs. Although much emphasis, and rightly so, has been placed on the functional capacity of both
embryonic and adult stem cells to repair ischemic heart, molecular and signaling events that initiate the differentiation of stem cells to CMCs lineage are still in their infancy. Signaling studies attempting to elucidate the differentiation mechanisms are further complicated by the use of complex mixture of cytokine/growth factor “cocktails” used to induce differentiation toward a particular lineage in vitro. Some evidence suggests a possible role of single soluble factor in promoting CMC differentiation thereby making molecular and signaling studies of ES cell–derived CMC differentiation feasible. These include members of the transforming growth factor β (TGFβ) family, retinoic acid, and leukemia inhibitory factor (LIF) when provided at an appropriate timing and dosage.\(^9\) However, exact signaling pathways and molecular events as well as the extent to which single factor or 2 agents acting synergistically induced mES cell–derived CMCs in vitro and may promote cardiac repair in vivo, is still unknown. Our studies in mES cells indicate that LIF and bone morphogenic protein 2 (BMP-2), a member of TGFβ super family, can efficiently differentiate mES cells into CMCs via STAT3-dependent pathway. Accordingly, in this study we investigated that (1) LIF and BMP-2 synergistically induces mES cell differentiation into CMCs mediated via STAT3 signal transduction pathway and (2) the efficacy of mES cell transplantation of precommitted cells with LIF and BMP-2 for functional and histological cardiac repair in surgically-induced mouse acute myocardial infarction (AMI).

Materials and Methods

Culture and Maintenance of D3-ES Cells
The D3-mES cells were obtained from ATCC and cultured in complete DMEM medium as described earlier.\(^10\)

Reagents
The murine recombinant LIF and anti-LIF were purchased from Chemicon International (Temecula, Calif), human BMP-2 was purchased from PeproTech (Rocky Hill, NJ), Cucurbitacin I (JAK-STAT inhibitor) and U0126 (MAPK inhibitor) were purchased from Calbiochem (La Jolla, Calif). Anti-JAK2 Ab and antiphospho-tyrosine mAb, 4G10, were purchased from Upstate Biotechnology, Inc (Lake placid, NY) and anti-phosphoserine mAb, 4A9 was purchased from Anaspec Inc. (San Jose, Calif). Antibodies specific to STAT3 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, Calif). The HRP conjugated secondary Abs and other chemi-

Quantitative RT-PCR
The quantitative RT-PCR for CMC markers in mES cells was performed as described earlier.\(^11\)

Immunofluorescence Staining
Cells were cultured in a gelatin coated 4-well culture slide in complete DMEM medium in the absence and presence various doses of LIF alone, BMP-2 alone, anti-LIF alone, cucurbitacin I alone, and with the combination all these agents for 4 days. Immunofluo-

Immunoprecipitation
The immunoprecipitation and Western blot analyses of STAT3 proteins was performed as described earlier.\(^10\)

SDS-PAGE and Western Blot Analysis
SDS-PAGE and Western Blot analyses were done as described previously.\(^10,11\)

Electrophoretic Mobility Shift Assay
Electrophoretic mobility shift assay experiments were performed using STAT3 synthetic oligonucleotides and nuclear proteins isolated from variously treated cells as described earlier.\(^1\) Specificity of STAT3 binding activity was determined by using anti-STAT3 antibody and excess unlabeled oligos in supershift and competition assays.

eGFP-Transduction for Cell Transplantation
For tracking of transplanted cells, mES cells were transduced with a lentivirus-eGFP construct (more than 60% transduction efficiency). After GFP transduction a subset of cells was cultured in the presence of LIF and BMP-2 for 36 hours before the cells were used for transplantation.

Mice and Establishment of AMI
All procedures were performed in accordance with the guidelines of Institutional Animal Care and Use Committee. The study involved 8-week-old male C57BL/6J mice (n=60; Jackson Laboratories). Mice underwent surgery to induce AMI by ligation of the left anterior descending coronary artery as described before.\(^12,13\) Animals were grouped into 3 and received intramyocardial injection of 5×10⁵ lentiviral-GFP–labeled mES cells pretreated with LIF and BMP-2, untreated mES cells, and saline, respectively, in total volume of 10 μL at 5 sites (basal anterior, mid anterior, mid lateral, apical anterior, and apical lateral) in the periinfarct area.

Physiological Assessments of LV Function Using Echocardiography
Physiological assessment of ventricular function was assessed by echocardiography just before AMI (base level) and 2 and 4 weeks after AMI as described before.\(^12,13\)

Histology and Immunochemical Staining of Tissues
Mice were euthanized and the aortas were perfused with saline. The hearts were sliced into 4 transverse sections from apex to base and fixed. Immunofluorescence staining was performed to determine CMC and endothelial cell differentiation of transplanted cells essentially as described earlier.\(^13\)

Fibrosis Area
For the measurement of fibrosis, tissues sections were embedded in paraffin and sectioned for elastic tissue/trichrome to measure the average ratio of the external circumference of fibrosis area to LV area.

Statistical Analyses
All experiments were performed at least 3 times with similar results. Results are presented as mean±SEM. Comparisons were done by ANOVA (GB-STAT; Dynamic Microsystems Inc.) or χ² test for percentages. All tests were 2-sided, and a probability value of less than 0.05 was considered statistically significant.

Results
LIF and BMP2 Synergistically Upregulate the mRNA and Protein Expression of Cardiomyocyte Markers in mES Cells
We tested various doses of LIF and BMP-2 for CMC differentiation of mES cells (supplemental Figure I, available
A combined treatment with 2 cytokines at specific doses of 5 ng/mL (LIF) and 3 ng/mL (BMP-2), respectively, resulted in marked mRNA upregulation of CMC-specific genes whereas neither of the 2 cytokines by themselves were sufficient for CMC differentiation (Figure 1A). Synergistic effect of LIF+BMP-2 on the expression of CMC-specific mRNAs was further corroborated by enhanced protein expression of selected CMC-specific markers (Figure 1B; connexin 43 [green] and α-sarcomeric actinin [red], double positive cells [yellow] in merged figure). The percentage of cells expressing these 2 markers was >90% on day 5, posttreatment (data not shown). Interestingly, the maintenance dose of LIF (10 ng) required to keep mES cells undifferentiated did not induce CMC differentiation either by itself or in combination with 3 ng/mL of BMP-2. Thus the synergy of BMP-2 with LIF for CMC differentiation appears to be LIF dose-dependent. LIF+BMP-2 treatment neither induced vascular smooth muscle cell or endothelial cell differentiation in nor had an impact of cell viability (supplemental Figures II and III).

LIF Induces Tyrosine and Serine Phosphorylation of STAT3 Proteins in mES Cells

Earlier studies have shown that LIF (10 ng/mL) induces the activation of STAT3 pathway which is required for self renewal of mES.10 We first examined the tyrosine phosphorylation of STAT3 by various doses of LIF and BMP-2. As shown in Figure 2A, within 30 minutes posttreatment, LIF induced STAT3 tyrosine phosphorylation at dose as low as 2 ng/mL, which was further enhanced at 5 ng and 10 ng/mL of LIF. However, no significant difference was observed between doses of 10 ng/mL or 5 ng/mL. BMP-2 alone also induced slight tyrosine phosphorylation of STAT3 which was comparable at various doses. Because LIF (5 ng) + BMP-2 (3 ng) specifically induced CMC differentiation of ES cells, we chose this dose combination for further signaling studies. We also evaluated time course of tyrosine and serine phosphorylation of STAT3 proteins in response to low dose of LIF (5 ng/mL), with maximal phosphorylation achieved at 30 minutes (supplemental Figure IVA and IVB). We used the 30-minute time point to further analyze the synergistic effect of BMP-2 and LIF on STAT3 phosphorylation in next series of experiments.

BMP-2 Synergistically Enhances LIF-Induced STAT3 Activation in mES Cells

We next examined the effect of BMP-2 on LIF-induced tyrosine and serine phosphorylation of STAT3 in mES cells. As shown in Figure 2B and 2C, compared with untreated cells, stimulation with 5 ng/mL LIF alone and 3 ng/mL BMP-2 alone resulted in moderate increase in tyrosine and serine phosphorylation of STAT3; however, combined treatment of both LIF and BMP-2 showed significant increase in both tyrosine and serine phosphorylation of STAT3 (P<0.01).

To further confirm synergistic upregulation of STAT3 activation by LIF+BMP-2, we determined STAT3-DNA binding activity as an independent measure of STAT3 activation. As shown in Figure 2D, nuclear proteins from either LIF- or BMP-2–treated mES cells resulted in the induction of STAT3 DNA binding activity; combination of LIF and BMP-2 increased STAT3 DNA binding activity (lane 4, P<0.01). The specificity of the STAT3 DNA binding was evident from the “supershift” of the band when anti-STAT3 antibodies were included in the electrophoretic mobility shift assay reactions (lane 6) and by the competition of binding activity by excess molar concentration of unlabeled STAT3 oligo.

UO126 and Cucurbitacin I Attenuates the Synergistic Effect of LIF and BMP-2 Induced Serine Phosphorylation of STAT3

Because cotreatment of mES cells with LIF and BMP-2 increased STAT3 activation, we next attempted to analyze the cross-link between LIF and BMP-2 signaling components
leading to synergistic activation of STAT3. Some evidence suggests the involvement of MAP kinase as a connecting link for BMP-LIF mediated STAT3 activation. To investigate whether MAPK might be mediating LIF/BMP-2 synergy, mES cells were cultured for 30 minutes with a combination of LIF and BMP-2 either in the absence and presence of specific MAPK inhibitor (UO126, 1 μmol/L) and serine/tyrosine phosphorylation of STAT3 was determined. As shown in Figure 3A and 3B, cotreatment with UO126 attenuated the synergistic effect of LIF and BMP-2 on tyrosine and serine phosphorylation of STAT3. Similarly, cotreatment of LIF+BMP-2–treated cells with STAT3 inhibitor, Cucurbitacin I (1 μmol/L), diminished LIF+BMP-2–mediated increase in STAT3 phosphorylation (Figure 3C and 3D). These data suggest that STAT3 cross-talk with MAPK may potentially be involved in the signaling pathway of LIF-BMP-2–mediated differentiation of mES cells into CMCs. Blocking of LIF receptor with anti-LIF also prevented the LIF- and BMP-2–induced increase in STAT3 phosphorylation (Figure 3C and 3D) suggesting that upstream signaling also plays an important role in CMC differentiation.
We next examined the requirement of both STAT3 and MAPK activation on LIF- and BMP-2–mediated CMC differentiation in mESCs. We found that inhibition of either STAT3 or MAPK activation depressed in mice receiving saline. However, treatment with uncommitted and precommitted mES cells significantly improved FS at day 28 (Figure 5B, at 4 weeks postsurgery in precommitted mES cell group $P<0.01$ versus control group). However, the difference in FS, although showing a better trend with precommitted ES cells, did not reach statistical significance when precommitted and noncommitted cell transplantation groups were compared ($P>0.05$). Additionally, we measured the % fibrosis area in all 3 groups of mice hearts, and it was significantly larger in the transplanted uncommitted mES cell group ($P<0.01$ versus saline group and in precommitted mES cell group $P<0.01$ versus control group). The fractional shortening (FS) was evaluated on day 7, 14, and 28 in all groups. Compared with presurgery (basal) level, FS was consistently depressed in mice receiving saline. However, treatment with uncommitted and precommitted mES cells significantly improved FS at day 28 (Figure 5B, at 4 weeks postsurgery in transplanted uncommitted mES cell group $P<0.01$ versus saline group and in precommitted mES cell group $P<0.01$ versus control group). The remarkable reduction in fibrosis indicates inhibition of scar tissue formation attributable to enhanced myogenesis and ischemic tissue repair. Although not specifically documented, these data may also imply a reduction in fibroblast activation.

**Intracardially Injected mES Cells Express CMC and Endothelial Markers In Vivo, Increase Capillary Density, and Decrease Myocardial Fibrosis**

Gains in post-AMI physiological heart function in mice transplanted with uncommitted and precommitted mES cells was further corroborated by histological evaluation of hearts from each groups of mice on day 28. Immunofluorescence staining was performed to determine CMC differentiation of the transplanted (GFP+) cells in the myocardium, tissue sections were costained with 2 specific CMC markers, connexin43 (green) and cardiac troponin I (red). The cells cultured in the presence of LIF+BMP-2 (b) showed significant increased number of CMCs specific marker tested whereas U0126 (c) and cucurbitacin I (d) significantly inhibit the protein expression of CMC-specific markers induced by LIF and BMP-2 in mES cells. *P<0.001. All experiments were repeated at least 3 times.

**Inhibition of STAT3 or MAPK Activation Represses Synergistic Effect of LIF and BMP-2 on CMC Differentiation in mESCs**

We next examined the requirement of both STAT3 and MAPK activation on LIF+BMP-2–mediated CMC differentiation of mES cells. Cells were cultured with LIF+BMP-2 or U0126 or cucurbitacin I for 2 days. Total cellular RNA was harvested and used to analyze quantitative mRNA expression of CMC specific markers, cardiac troponin I and T, GATA4, and Nkx2.5. As shown in Figure 4A, LIF- and BMP-2–induced expression of CMC transcripts was significantly downregulated by MAPK or STAT3 inhibitor. Similarly, protein expression of CMC markers was also attenuated in the presence of either STAT3 or MAPK inhibitor (Figure 4B), suggesting the cross-talk between LIF and BMP-2 pathways leads to enhanced STAT3 activation and consequent CMCs differentiation.

**Intracardiac Injection of CMC Precommitted ES Cells Improves Postinfarct Left Ventricular Functions in a Mouse AMI**

The differentiation of mES cells treated with LIF and BMP-2 into CMCs observed in vitro, prompted us to determine whether ischemic myocardium could be protected by transplantation of these cells in a mouse AMI model. Mice underwent surgery to induce AMI by ligation of the left anterior descending coronary artery, as described before. We performed physiological assessment of the LV functions after AMI in all mice at basal level before surgery and on days 7, 14, and 28, post-AMI. The echocardiography revealed significant improvement in LVdV at day 28 after AMI in group of mice transplanted with uncommitted mES cells (Figure 5A, $P<0.05$ versus control) and precommitted mES cells (Figure 5A, $P<0.01$ versus control) as compared with saline group. Interestingly, transplantation of precommitted cells showed a significant improvement over noncommitted ES cells (Figure 5A, $P<0.05$). The fractional shortening (FS) was evaluated on day 7, 14, and 28 in all groups. Compared with presurgery (basal) level, FS was consistently depressed in mice receiving saline. However, treatment with uncommitted and precommitted mES cells significantly improved FS at day 28 (Figure 5B, at 4 weeks postsurgery in transplanted uncommitted mES cell group $P<0.01$ versus saline group and in precommitted mES cell group $P<0.01$ versus control group). However, the difference in FS, although showing a better trend with precommitted ES cells, did not reach statistical significance when precommitted and noncommitted cell transplantation groups were compared ($P>0.05$). Additionally, we measured the % fibrosis area in all 3 groups of mice hearts, and it was significantly larger in control group mice than the mice that received uncommitted mES cells (Figure 5C; $P<0.05$) or precommitted ES cells ($P<0.01$). The remarkable reduction in fibrosis indicates inhibition of scar tissue formation attributable to enhanced myogenesis and ischemic tissue repair. Although not specifically documented, these data may also imply a reduction in fibroblast activation.

**Intracardially Injected mES Cells Express CMC and Endothelial Markers In Vivo, Increase Capillary Density, and Decrease Myocardial Fibrosis**

Gains in post-AMI physiological heart function in mice transplanted with uncommitted and precommitted mES cells was further corroborated by histological evaluation of hearts from each groups of mice on day 28. Immunofluorescence staining was performed to determine CMC differentiation of the transplanted (GFP+) cells in the myocardium, tissue sections were costained with 2 specific CMC markers, cardiac troponin I (Figure 6A) and α-sarcomeric actinin (Figure 6B), and the GFP+ cells (green) expressing these 2 CMC proteins (red) were visualized as double positive (yellow) cells in the merged images. As shown in Figure 6A and 6B and quantified in Figure 6C, both uncommitted and precom-
mitted mES showed evidence of CMC differentiation in vivo, however, compared with uncommitted mES, precommitted mES showed a significantly higher number of cells coexpressing CMC markers ($P < 0.01$). Interestingly, precommitted cells showed typical CMC morphology and striation pattern, which was not evident in case of noncommitted mES (Figure 6A and 6B, insets). Contrary observations were revealed when endothelial cell differentiation of transplanted (GFP) cells was investigated. As shown in Figure 7A, GFP+CD31 double positive cells were observed in myocardial sections obtained from mice transplanted with uncommitted mES cells (b-GFP transplanted cells, e- Phase contrast image merged with CD31 cells, h- double positive cells merge with dapi) and precommitted ES cells (c-GFP+transplanted cells, f- Phase contrast image merged with CD31+ cells, i- double positive cells merge with dapi). The endothelial differentiation in myocardial tissues was also quantified by counting the double positive cells of CD31+GFP cells in high visual field and was shown in Figure 7B. Higher number of GFP+CD31 double positive cells was observed in uncommitted ES cells (180±7) than in the precommitted ES cells (98±4, $P < 0.05$). These data suggest that the precommitted mES cells tend to differentiate more into CMCs than to endothelial cells. Tissue sections were stained with BS1 lectin to determine the capillary density at the border zone of the infracted myocardium. As shown in Figure 7C, significantly higher capillaries/visual field were observed in the mice receiving uncommitted mES cells (180±7) than in control mice and a higher number of capillaries in uncommitted cell transplantation group compared with precommitted cell transplantation group ($P < 0.05$). This data may suggest formation of resistance arterioles by undifferentiated ESCs and the reduction of this response when committed cells are used.

**Discussion**

The development of robust culture condition is essential to delimit the ES cell culture environment and thereby define and control signaling inputs that direct CMC differentiation. Using this approach, we have shown that inclusion of BMP-2 regulates LIF-mediated maintenance of undifferentiated status of mES cells and induces their differentiation into CMCs. Although combined effect of LIF and BMP-2 therapeutic function is known from sheep AMI, the exact signaling mechanism of CMC differentiation in response to these 2 cytokines is not well elucidated by this study. Our study thus not only substantiates LIF/BMP-2–mediated CMC differentiation but also provides mechanistic insights into this process.

Murine ES cells can be cultured for prolonged periods in an undifferentiated state in the presence of LIF or on the feeder layer of mouse embryonic fibroblasts. In contrast to human ES cells, mouse ES cells require LIF for self-renewal. LIF, a member of IL-6 family of cytokines, initially identified as a cytokine capable of inducing differentiation of M1 myeloid leukemia cells, was later shown to have differentiation inhibitory activity on ES cells. These opposite effects of LIF may reflect the differential functions of 2 LIF isoforms. LIF exists in 2 isoforms, as diffusible molecule (D-LIF) and as an extra cellular matrix-bound isoform (M-LIF), with distinguishable functions. Interestingly, subpicomolar concentrations of paracrine D-LIF are necessary and sufficient for the onset of differentiation in CMCs, whereas increasing concentrations of D-LIF attenuate ongoing differentiation of CMCs. Thus, LIF seemingly acts both as a differentiation-inducing as well as differentiation-inhibiting factor depending on dose, bioavailability of 2 isoforms, and the timing and dose of LIF treatment.

The effect of LIF is mediated through a cell surface receptor complex composed of a low affinity LIF receptor...
and gp130, a common receptor subunit of the IL-6 family. On binding to its receptor, LIF induces dimerization of LIFR/gp130 complex, leading to the activation of JAK-STAT, Ras-MEK-ERK, and other signaling pathways. Mutation of STAT3-interacting tyrosines on gp130, targeted deletion of STAT3, or overexpression of a dominant negative STAT3 all abrogate the ability of LIF to maintain self-renewal of ES cells, and constitutive expression of activated STAT3 prevents differentiation of ES cells after the withdrawal of LIF. The beneficial effect of STAT3 in heart is already established. Downregulation of STAT3 has been associated with end-stage heart failure in patients. More-
over, multiple studies showed that the activation of STAT3 promotes CMC survival and hypertrophy as well as cardiac angiogenesis in response to various pathophysiologic stimuli, strongly suggesting that STAT3 is beneficial for the heart.23,24,26 STAT3-KO mice are embryonically lethal, and conditional CMC-restricted STAT3-KO mice showed enhanced susceptibility to cardiac injury caused by myocardial ischemia, systemic inflammation, or drug toxicity.23,24

BMPs belong to the TGF-β super family and are expressed in lateral endoderm and ectoderm. Expression of cardiac-specific proteins by BMP-2 appears to be mediated by the transcription factors GATA-4 and Nkx2.5. Administration of soluble BMP-2 or BMP-4 to explants culture induces full cardiac differentiation in stage 5 to 7 anterior medial mesoderm, a tissue that is normally not cardiogenic.27 It has been shown that TGF-β and BMP-2 induced upregulated cardiac specific transcription factors Nkx2.5 and Mef2c.27,28 Taken together, these observations suggest that BMP alone may not be sufficient to promote CMC differentiation of mES cells.29 Our studies in mES cell line indicated that LIF and BMP-2 could efficiently differentiate mES cells into CMCs via their synergistic activation of STAT3. However, the exact modulations by BMP-2 in LIF-STAT3 pathway required for the maintenance of mES cell pluripotency or self-renewal on one hand and leading to their differentiation into CMCs on the other are understudied and not well defined. The requirement of MAPK as an intermediate signal for enhanced STAT3 activation in response to LIF+BMP-2 observed in our study thus provides evidence of LIF+BMP-2 signaling cross-talk in mES cell CMCs differentiation.

Although a subpopulation of stem cells from bone marrow has been reported to give rise to CMCs, the extent of the differentiation process was poor.30 This finding suggests that these cells might be used to improve neoangiogenesis rather than cardiomyogenesis. Alternate, potentially nonallogeneic sources of cells are therefore needed to regenerate damaged myocardium in patients with severe heart failure. To achieve regeneration of scarred postinfarction myocardium, the high plasticity and the likely tolerance to hypoxia of pluripotent ES cells are attractive features. The pluripotency of mES differentiation, however, adds the burden of unrestricted proliferation and teratoma formation when transplanted in vivo. Recently, it has been shown that transplantation of undifferentiated murine ES cells cause teratomas and immune response in heart.31 We did not observe any visibly obvious tumor in hearts of mice transplanted with mES likely because of lower number of transplanted cells (50 000). The recent study by Nussbaum et al31 also did not find any teratoma formation when fewer than 100 000 undifferentiated mES cells were transplanted. The fate of mES cell differentiation can be directed by ex vivo addition of appropriate growth factors to precommit them toward specific cell lineages,32 including the cardiac phenotype.17 Furthermore, the commitment process is translated into a differentiation program when cells are engrafted into diseased organs of small animals provided that they find a favorable humoral environment.31,32 In a recent preclinical study, transplantation of cardiac-committed mES cells in sheep AMI showed not only a significant improvement in scarred area but also the tolerance of mES cells by sheep.16 However, the exact mechanism of CMC differentiation is not well elucidated by this study. The LIF+BMP-2–induced enhanced CMC specific gene expression and the elucidation of downstream signaling components that lead to CMC differentiation, observed in our study, may in future be potentially modifiable therapeutic targets to precommit ES cells to a cardiac lineage and may make these cells more efficacious for regenerating injured myocardium.

Figure 7. Engraftment and endothelial differentiation of transplanted mES cells in infarcted myocardium. A, Representative figures showing colocalization of transplanted GFP+ (green) and endothelial-specific marker CD31 (red). Upper panel (a–c) shows injected cells, middle panel (d–f) shows merged image of phase contrast and fluorescence CD31 marker, and lower panels (g–i) show merge image of GFP+ and CD31 endothelial marker, demonstrate that injected cells colocalized with neovascularization. B, Capillary density was measured by CD31 staining showing significantly higher in uncommitted mES cells than the precommitted mES cells with LIF and BMP-2 *P<0.05.

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References
32. Rajasingh et al Cardiomyocyte Precommitted mES in Cardiac Repair 9
33. Rajasingh J, Bright JJ. 15-Deoxy-Delta(12,14)-Prostaglandin J(2) reg-
STAT3-Dependent Mouse Embryonic Stem Cell Differentiation Into Cardiomyocytes. Analysis of Molecular Signaling and Therapeutic Efficacy of Cardiomyocyte Precommitted mES Transplantation in a Mouse Model of Myocardial Infarction

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Supplementary Data

**Figure S1**: Dose-dependence induction of Cardiomyocyte differentiation of mES cells. mES cells (D3) were treated with indicated doses of LIF and BMP2 alone or in combination. RNA was harvested and was subjected to quantitative mRNA analysis of two CMC-specific transcripts, Cardiotroponin I and connexin 43. Neither LIF nor BMP2 alone induced CMC specific mRNA induction at any dose tested. However, LIF (5ng/ml) and BMP2 (3ng/ml) specifically induced significant induction of these two transcripts (p<0.001). A modest induction of connexin 43 was also observed with 5ng/ml dose of LIF and BMP2.

![Figure S1](image-url)
**Figure S2:** LIF+BMP2 treatment does not induce VSMC/skeletal muscle cell or EC differentiation. Cells were treated with indicated doses of LIF and BMP2. RNA was extracted and analyzed for quantitative mRNA expression of indicated VSMC and EC genes. No significant difference from basal expression (LIF 10ng/ml) for any of these genes was observed.
**Figure S3: LIF or BMP2 do not affect mES cell survival.** Cells were treated as indicated for days 2 and 5. A subset of cells, treated in triplicates, was stained with Trypan Blue and cell death was documented by dye-uptake under microscope. No differences in cell viability were observed between various treatment conditions (A). (B) A subset of variously treated cells was assayed for TUNEL staining to document apoptotic cells. Number of apoptotic cells remained same on days 2 and 5 under different treatment conditions (<2%).

**Figure S3**

![Graph A](image1.png)

![Graph B](image2.png)
**Figure S4:** Immunoprecipitation and Western blot analyses showing time kinetics of LIF induced tyrosine and serine phosphorylation of STAT3. The optimal (A) tyrosine phosphorylation of STAT3 and (B) serine phosphorylation of STAT3 in mES cells following stimulation with LIF in 30 min.