Stem cell differentiation requires a paracrine pathway in the heart

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ABSTRACT **Members of the transforming growth factor β1 (TGF-β) superfamily—namely, TGF-β and BMP2—applied to undifferentiated murine embryonic stem cells up-regulated mRNA of mesodermal (Brachyury) and cardiac specific transcription factors (Nkx2.5, MEF2C). Embryoid bodies generated from stem cells primed with these growth factors demonstrated an increased potential for cardiac differentiation with a significant increase in beating areas and enhanced myofibrillogenesis. In an environment of postmitotic cardiomyocytes, stem cells engineered to express a fluorescent protein under the control of a cardiac promoter differentiated into fluorescent ventricular myocytes beating in synchrony with host cells, a process significantly enhanced by TGF-β or BMP2. In vitro, disruption of the TGF-β/BMP signaling pathways by latency-associated peptide and/or noggin prevented differentiation of stem cells. In fact, only host cells that secrete a TGF-β family member induced a cardiac phenotype in stem cells. In vivo, transplantation of stem cells into heart also resulted in cardiac differentiation provided that TGF-β/BMP2 signaling was intact. In infarcted myocardium, grafted stem cells differentiated into functional cardiomyocytes integrated with surrounding tissue, improving contractile performance. Thus, embryonic stem cells are directed to differentiate into cardiomyocytes by signaling mediated through TGF-β/BMP2, a cardiac paracrine pathway required for therapeutic benefit of stem cell transplantation in diseased heart.—Behfar, A., Zingman, L. V., Hodgson, D. M., Rauzier, J.-M., Kane, G. C., Terzic, A., Pucéat, M. Stem cell differentiation requires a paracrine pathway in the heart. *FASEB J.* 16, 1558–1566 (2002)**

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ISCHEMIC HEART DISEASE is a leading cause of heart failure precipitated by the death of highly vulnerable cardiomyocytes (1). The heart under stress primarily responds with cell hypertrophy rather than proliferation due to a limited mitotic capacity of differentiated cardiomyocytes (2). This restricts repair of the injured myocardium to replacement by fibrotic tissue disrupting proper contractile function despite the potential of some myocytes to participate in postinfarction regeneration (3). In this regard, a novel therapeutic approach has emerged based on repopulating injured tissue with cells of myogenic phenotype. Indeed, different cell types have been grafted into the heart, including myoblasts, cardioblasts, and fetal or neonatal cardiomyocytes (4–9). Although promising, use of already differentiated muscle cells suffers from several limitations. The cardiac area colonized by implanted cells is small due to a low dividing capacity of such terminally differentiated cells (3). Moreover, long-term survival and electrical coupling of these cells within the myocardium remains controversial (10, 11).

Recently, the plasticity of uncommitted stem cells has opened new perspectives in tissue regeneration (12–14). Adult bone marrow and hematopoietic stem cells have been successfully engrafted into ischemic hearts, differentiating into smooth muscle, endothelial, and cardiac phenotypes (15, 16). Evidence has been provided that such engrafted stem cells may improve the function of diseased heart (15). Although use of undifferentiated cells may resolve some of the limitations observed with differentiated myoblasts or myocytes, recent findings have called for caution regarding the use of adult stem cells that by fusion take the phenotype of recipient cells, negating a therapeutic benefit (17, 18). Indeed, much remains to be understood before stem cell-based therapies can be used effectively for cardiac repair.

One of the fundamental questions is how pluripotent stem cells respond to the host environment and differentiate toward a specific cell phenotype. More specifically, the molecular signals that induce commitment, proliferation, and differentiation of stem cells into cardiomyocytes within an infarcted myocardium are unknown (15).

Therefore, we focused our investigation on mecha-

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nisms underlying differentiation of stem cells grafted onto cardiomyocytes, normal, and postinfarcted myocardium. We report that members of the transforming growth factor β1 (TGF-β) superfamily commit undifferentiated stem cells into the cardiac lineage. The paracrine function of host cardiomyocytes in secreting TGF-β and/or BMP2 underlies cardiac differentiation of engrafted pluripotent stem cells, resulting in a gain-of-function of the infarcted myocardium. These findings favor the use of growth factor-committed stem cells for cell therapy in the diseased heart.

MATERIALS AND METHODS

Embryonic stem cell differentiation

The CGR8 murine embryonic stem cell line (19) was propagated in BHK21 medium supplemented with pyruvate, nonessential amino acids, mercaptoethanol, 7.5% fetal calf serum (FCS, Biomedia, Paris, France), and the leukemia inhibitory factor (LIF) obtained from LIF-D cells (20). Differentiation was carried out in hanging drops (21) of differentiation medium (BHK21 with 20% FCS without LIF) in which embryoid bodies were formed at 2 days (D0–2). Embryoid bodies were incubated for 3 days (D2–5) in suspension and for at least 7 days (D6–12) on gelatin-coated dishes or laminin-coated glass coverslips (20).

Generation of stem cell clones expressing a reporter gene

The CGR8 cell clone was engineered to express the enhanced cyan fluorescent protein (ECFP) under the control of the cardiac-specific α-actin promoter, subcloned upstream of ECFP using *Xho*I and *Hin*dIII restriction sites of the promoterless pECFP vector (Clontech, Palo Alto, CA). This α-actin promoter (actinECFP) construct was linearized using *Xho*I, electroporated into CGR8 stem cells, and colonies were screened for the construct by PCR after G418 treatment. Alternatively, the promoter of the ventricular myosin light chain 2 (MLC2v) was used to confirm ventricular differentiation (20). Some actinECFP stem cell clones were engineered to be resistant to TGF-β superfamily signaling through expression of noggin or a dominant negative mutant of the TGFβRII receptor (ΔKTGFβRII) (22). Noggin or ΔKTGFβRII cDNA were subcloned in the pcDNAhygromycin vector (Clontech) using *Xho*I and *Bam*HI or *Kpn*I and *Hin*dIII restriction sites, respectively. After electroporation of linearized plasmids into CGR8 stem cells, hygromycin-resistant colonies were screened by PCR for construct expression.

RT-PCR and real-time quantitative PCR

Total RNA was prepared from CGR8 stem cells as described (20, 23). After reverse transcription, 10 ng cDNA was used for real-time quantitative PCR, performed with a Lightcycler and the SYBR green fast start kit (Roche, Germany). Primers used in real-time PCR were as follows: Brachyury forward 5′-GACTTCGTGACGGCTGACAA-3′ and reverse 5′-CGAGTCTGGGTGGATGTAG-3′; MEF2C forward 5′-AGATACCCACAACACACCACGCGCC-3′ and reverse 5′-ATCCTTCAGAGAGTCGCATGCGCTT-3′; Nkx2.5 forward 5′-CATTTTACCCGGGAGCCTACGGTG-3′ and reverse 5′-GCTTTCCGTCGCCGCCGTG-3′; β-tubulin forward 5′-CCGGACAGTGTGGCAACCAGATCGG-3′ and reverse 5′-TGGCCAAAAGGACCTGAGCGAACGG-3′; Myf 5 forward 5′-GGAGATCCTCAGGAATGCCAT-3′ and reverse 5′-TGCTGTTCTTTCGGGACCAGA-3′; ICAM forward 5′-GACTTCACCATTGAGTGCACGGTGT-3′ and reverse 5′-TATACCCACCATGGGGCCGTAGATC-3′. The reaction contained 1 μL of Master SYBR green I mix (*Taq* DNA polymerase, buffer, deoxynucleoside trisphosphate mix, and SYBR green I dye), 3 mM $MgCl_2$, and 0.5 μM of each primer to which 2 μL of diluted cDNA was added. A standard concentration curve was established by serial dilution of gel-purified gene specific PCR fragments. Data were normalized using β-tubulin as an index of cDNA content after reverse transcription. Amplification included initial denaturation at 95°C for 8 min, 45 cycles of denaturation at 95°C for 3 s, annealing at 60–65°C for 8–10 s, and extension at 72°C for 7–10 s performed at a temperature transition rate of 20°C/s. Fluorescence was measured at the end of each extension step. After amplification, a melting curve acquired by heating the product to 95°C, cooling to and maintaining at 70°C for 20 s, then slowly (0.3°C/s) heating to 95°C was used to determine the specificity of PCR products, confirmed by gel electrophoresis.

Cell imaging

Fluorescent images of EBs or isolated cells were acquired on a LEICA microscope with objectives mounted on a piezoelectric device, digitized on-line with a Micromax 1300YHS CCD camera (Princeton, NJ), and stored as volume files ('stack' of z-section images) using the Metamorph software (Universal Imaging, Downington, PA). ECFP was detected in embryoid bodies or isolated cells illuminated with a Hg lamp at 400 ± 20 nm with CFP fluorescence recorded by a X114–2 CFP LEICA filter cube that consists of a DM 455 dichroic mirror and a 480 ± 30 nm emission filter. Beating areas in embryoid bodies were identified by videomicroscopy using the stream acquisition mode and their size quantified with the region measurement option of Metamorph. The sum of individual beating areas was normalized to the total size of the embryoid body. Embryoid bodies (12-day-old) were fixed in 3% paraformaldehyde for 30 min, permeabilized for 30 min with 1% Triton X-100, and immunostained as described (20). In situ immunostained sarcomere-specific proteins, actinin, or MLC2v were visualized in 0.1 μm optically z-sectioned embryoid bodies. Similarly, immunohistochemistry of heart sections was performed on paraffin sections. To improve resolution and signal-to-noise ratio, images were restored using Huygens software (Huygens 2.2.1, Scientific Volume Imaging, Hilversum, The Netherlands) and visualized using Imaris (Bitplane, Switzerland). Calculations were performed on an Octane workstation (Silicon Graphics, Los Angeles, CA).

Coincubation of stem cells and cardiomyocytes

Cardiomyocytes isolated from 2- to 3-day-old neonatal rat ventricles and purified on a Percoll (Pharmacia, Uppsala, Sweden) gradient (24) were plated at a density of 2×10^5 cell per 35 mm dishes and cultured for 3 days. At this stage, cells are postmitotic and do not dedifferentiate in culture (25). Coincubation was then performed with 10^4 CGR8 stem cells expressing ECFP under the control of either the cardiac-specific α-actin or ventricular specific MLC2v promoter, untreated or treated with the 2.5 ng/mL TGF-β or 5 ng/mL bone morphogenetic protein (BMP2) (R&D System, Abingdon, UK), resuspended in differentiation medium, and added to the cardiomyocyte-containing dish. Cardiac differentiation of stem cells was monitored by appearance of ECFP fluorescence using videomicroscopy (20).

Coculture of stem cells with BMP-secreting or nonsecreting C3H10T1/2 cells

Native C3H10T1/2 cells were cultured in DMEM supplemented with 10% FCS. The C9 cell clone, secreting BMP2, was engineered from the C3H10T1/2 cell line using an inducible BMP2 expression vector (ptTATop-BMP2). C9 cells were routinely cultured in the presence of 1 μg/mL doxycycline to prevent premature BMP2 expression (26). Next, 10^4 CGR8 stem cells expressing ECFP under the control of the cardiac α-actin promoter were cocultured with confluent native C3H10T1/2 or BMP-secreting C9 cells in the absence of doxycycline.

In vivo stem cell injection

CGR8 stem cells (5×10^5) expressing ECFP under the control of the actinECFP with or without noggin or the ΔKTGFβRII mutant (22) were trypsinized, resuspended in serum free medium, and injected in isoflurane-anesthetized (3% induction; 1.5% maintenance) mice. Injections were made through the left chest wall as well as via an abdominal approach through the diaphragm into the left ventricle using a 26 gauge needle. Three to 4 wk later, hearts were excised, cryofixed, and sliced in 4 μm-thick sections at 0.5 mm increments through the tissue. ECFP expression was visualized using a Zeiss Axioplan epifluorescence wide field microscope.

Myocardial infarction model

Myocardial infarction was induced in both Sprague-Dawley and Wistar rats by in situ ligature of the left coronary artery (27). Four weeks after surgery, rats were anesthetized with ketamine (70 mg/kg) and xylazine (15 mg/kg) or isoflurane (3% induction; 1.5% maintenance) and the heart was exposed after thoracotomy. Concomitantly, trypsinized $3\cdot10^5$ cells (suspended in 20 μL medium) or medium without cells (sham) injected along the border zone of the infarcted area at three different locations (below the left atrium, in the middle portion of the left ventricle, and at the apex) using a 27 gauge needle. Five weeks later, animals were either killed with phenobarbital or assessed for function with echocardiography. Hearts from killed animals were rapidly removed; ventricles were sliced in two transverse sections fixed with 4% paraformaldehyde. The infarcted area with surrounding tissue was embedded in paraffin and successive 5 μm sections were cut for immunohistology. Sections were stained with an anti-MLC2v antiserum (20), anti-connexin 43 polyclonal antibody (Sigma France) or the Verhaeff-Van Gieson stain for collagen and elastin (IMEB Inc., San Marcos, CA). Echocardiography was performed on isoflurane-anesthetized rats using a 5 MHz transducer on an ultrasonographic scanner (Vingmed System FiVe, GE Medical Systems, Milwaukee, WI). Parasternal short axis views with M-mode were acquired at the ventricular base immediately distal to the mitral valve. Ejection fraction was calculated as follows: $EF = (S^2 - D^2)/D^2 \cdot 100$, where S is the systolic and D the diastolic dimension (expressed in cm).

RESULTS

TGF-β and BMP2 up-regulate cardiac transcription factors in embryonic stem cells

To determine whether undifferentiated stem cells could be specifically committed to a cardiac cell lineage by growth factors, embryonic stem cells were treated (24 h) with TGF-β or BMP2 in 3.5% or 7.5% FCS-containing medium in the presence of LIF. Stem cells deprived of LIF, a suppressor of differentiation, lose their compact appearance and with it the potential for mesodermal differentiation (**Fig. 1**). Real-time quantitative PCR revealed that TGF-β and BMP2 both significantly up-regulated mRNA levels of Brachyury, a mesodermal transcription factor (Fig. 1*a*). TGF-β and BMP2 also significantly increased mRNA encoding Nkx2.5 (Fig. 1*b*) and MEF2C (Fig. 1*c*), early and late markers in cardiac differentiation (28), while maintaining colony morphology (Fig. 1*d*). In preliminary experiments, concentration response curve revealed that induction of transcription factors was most effective at 2.5 and 5 ng/mL of TGF-β and BMP2, respectively. In contrast, expression of Myf5, a skeletal muscle marker, or ICAM, an endothelial cell marker, was not significantly up-regulated (data not shown). Thus, TGF-β growth factor family members promote in stem cells induction of the cardiac gene program.

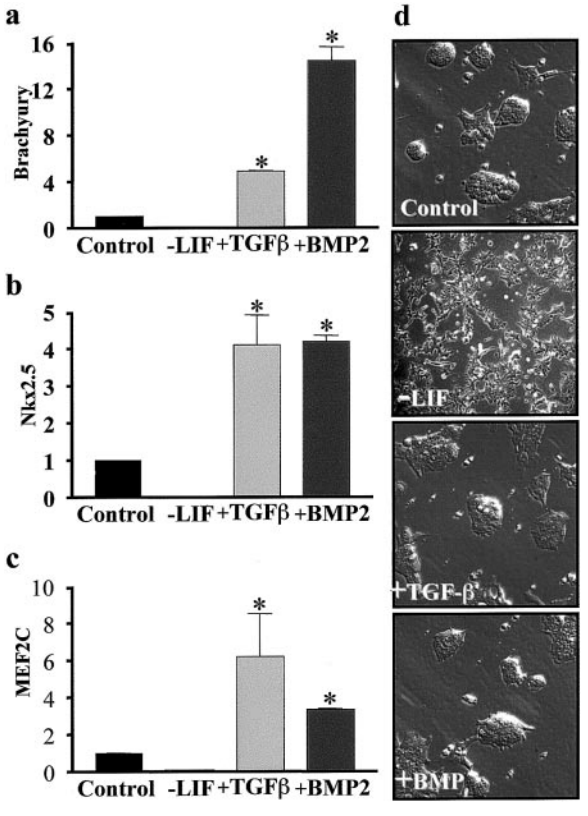

Figure 1. TGF-β- and BMP2-induced cardiac commitment of embryonic stem cells. CGR8 stem cells were left untreated (control) or were treated for 24 h with TGF-β (2.5 ng/mL) or BMP2 (5 ng/mL) in a low-serum and LIF-containing medium. Alternatively, cells were grown in media without LIF, TGF-β, and BMP2 (−LIF). Expression of mRNA for Brachyury (*a*), Nkx2.5 (*b*), and MEF2C (*c*) was measured using real-time quantitative PCR. Data are means (±SE; $n=3$ each) of the ratio between expression of gene of interest vs. tubulin presented as multiples of control. *Significant difference from control ($P<0.01$). *d)* Morphology of stem cell colonies under different experimental conditions.

TGF-β and BMP2 induced-commitment of ES cells favors cardiac differentiation within embryoid bodies

Embryonic stem cells unprimed or primed with TGF-β and BMP2 (24 h) were allowed to differentiate within embryoid bodies (20, 21). Within 7 days, beating clusters appeared within the mesodermal layer (**Fig. 2*a***). On day 9, the time required for maximal beating activity (20), contracting areas were threefold larger in embryoid bodies formed from growth factor-primed stem cells compared to untreated controls (Fig. 2*b*). Immunostaining of actinin, a protein distributed in z-discs of sarcomeres, revealed extensive myofibrillar regions in embryoid bodies from stem cells treated with TGF-β or BMP2, in contrast to smaller areas in controls (Fig. 2*c–e,* left panels). Enhanced myofibrillogenesis induced by TGF-β or BMP2 was not detrimental to sarcomeric architecture, which remained highly organized (Fig. 2*c–e,* right panels). Accordingly, similar beating rates (beats/s) of 1.2 ± 0.3 (n=5), 1.1 ± 0.3 (n=5), and 1.0 ± 0.2 (n=10) and sarcomeric unit sizes (in μm) of 2.0 ± 0.2 (n=10), 1.9 ± 0.3 (n=5), and 1.8 ± 0.3 (n=5) were observed in embryoid bodies from controls, TGF-β- and BMP2-primed stem cells, respectively. Thus, pretreatment of embryonic stem cells with TGF-β growth factor members results in embryoid bodies with greater areas of cardiac differentiation and normal sarcomeric organization.

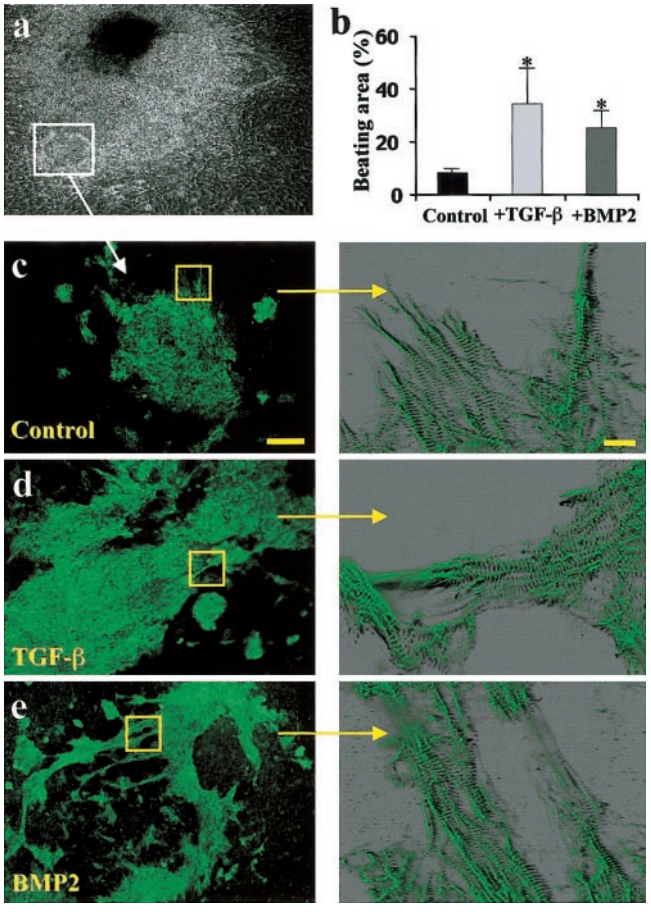

Figure 2. TGF-β and BMP2 enhance the potential of stem cells for cardiac differentiation in embryoid bodies. Embryoid bodies were formed in a hanging drop from untreated CGR8 stem cells (*a*) or stem cells primed with TGF-β and BMP2. On day 7 after initiation of differentiation, beating clusters were observed within the mesodermal layer. *b)* Total beating area measured on day 9 and expressed as percentage of total embryoid body area. Data were obtained from at least 3 experiments in each group. Embryoid bodies generated from untreated stem cells (*c*) or cells treated for 24 h with TGF-β (*d*) or BMP2 (*e*) were fixed and immunostained with an anti-actinin antibody and a secondary FITC-conjugated antibody. *c–e)* Left panels: 10× magnification; yellow bar indicates 100 μm. Right panels: 63× magnification; yellow bar indicates 15 μm.

In vitro cardiac differentiation of stem cells grafted onto cardiomyocytes enhanced by TGF-β and BMP2

Undifferentiated stem cells carrying the fluorescent ECFP probe under the control of the cardiac α-actin promoter (actinECFP cells) failed to emit fluorescence in the absence of a host cellular milieu (not illustrated). However, when grafted onto postmitotic isolated ventricular cardiomyocytes, actinECFP stem cells expressed ECFP vigorously after 5 days of coculture (**Fig. 3*a***), indicating that the cardiac α-actin promoter was turned on. In fact, cells expressing ECFP fluorescence demonstrated immunostaining of the ventricular marker myosin light chain 2 (MLC2v; Fig. 3*a,* inset), indicating ventricular phenotype. Similarly, stem cells expressing ECFP under the control of the MLC2v promoter (20) instead of the α-actin promoter, also fluoresced within 7 days in the presence of host cardiomyocytes (data not illustrated). Cardiac differentiation of stem cells was augmented by pretreatment with TGF-β (5 ng/mL) or BMP2 (2.5 ng/mL) before engraftment onto host cardiomyocytes (Fig. 3*b–d*). All differentiated stem cells displayed positive staining for connexin 43 (Fig. 3*e*), a gap junction protein required for intercellular communication expressed in ventricular myocytes (29) and ES cell-derived cardiomyocytes (30). Indeed, the beating pattern of stem cell-derived cardiomyocytes and host cardiac cells was synchronous on time-lapse videomicroscopy (data not shown). Thus, cardiac cells provide an environment in which stem cells can differentiate into cardiac lineage, an effect enhanced by stem cell priming with TGF-β growth factor members.

Differentiation of stem cells induced by TGF-β/BMP2 paracrine signals from host cells

The heart harbors an endocrine function (31). Cardiomyocytes and cardiac fibroblasts release members of the TGF-β family, including TGF-β (32, 33) and BMP2 (34), involved in embryonic cardiomyogenesis (35). Cardiac differentiation of actinECFP stem cells in coculture with postmitotic ventricular cardiomyocytes (**Fig. 4*a***) was disrupted by the blockers of TGF-β or BMP2 receptor-mediated signaling (36, 37). Latency-associated peptide (LAP; Fig. 4*b*) and noggin (Fig. 4*c*) used individually or in combination (Fig. 4*d*) prevented

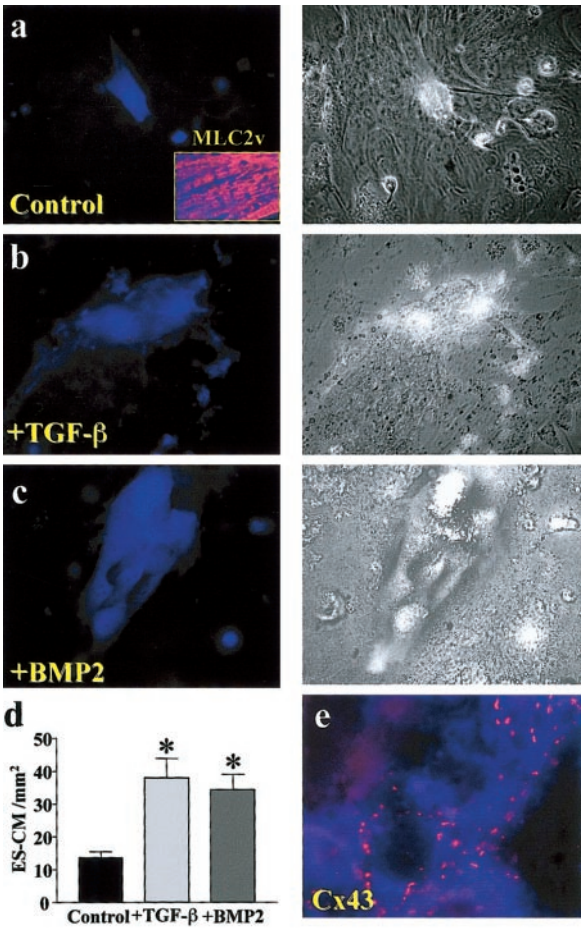

Figure 3. TGF-β and BMP2 commit stem cells grafted onto cardiomyocytes in vitro. Stem cells expressing ECFP under the control of the α-actin promoter were cocultured with ventricular cardiomyocytes. *a)* On day 5 after plating stem cells, cyan fluorescence was observed as was red MLC2v immunostaining (inset). *b–d)* Stem cells pretreated for 24 h with TGF-β (*b*) and BMP2 (*c*) displayed enhanced cyan fluorescence corresponding to a greater number of fluorescing embryonic stem cell-derived cardiomyocytes (ES-CM; *d*). *Significant difference from control ($P<0.01$, $n=3$ in each group). *a–c)* Transmitted light microscopy images are shown to the right of corresponding fluorescent images. *e)* Connexin 43 (Cx43) antibody was used to detect integration of differentiated stem cells with host cardiomyocytes. The panel is typical of multiple images obtained from 3 separate cocultures.

cardiac differentiation of stem cells as shown by the absence of ECFP expression. In the presence of LAP and noggin, stem cells formed clusters of undifferentiated cells (Fig. 4*b–d*), and the number of stem cell-derived cardiomyocytes was essentially negligible compared to control (Fig. 4*e*). Removal of LAP and noggin while in coculture with cardiomyocytes allowed stem cells to regain the ability to differentiate and express ECFP (Fig. 4*f*).

To directly assess the role of a host cell and of cell–cell interactions in stem cell differentiation, actin-ECFP cells were cocultured with embryonic C3H10T1/2 fibroblasts, wild-type, or genetically engineered as the C9 cell clone to secrete BMP2. Cardiac differentiation of actinECFP stem cells manifested through expression of fluorescence, was detected within 5–7 days in cells cocultured with the BMP2-secreting C9 cell clone, but not with C3H10T1/2

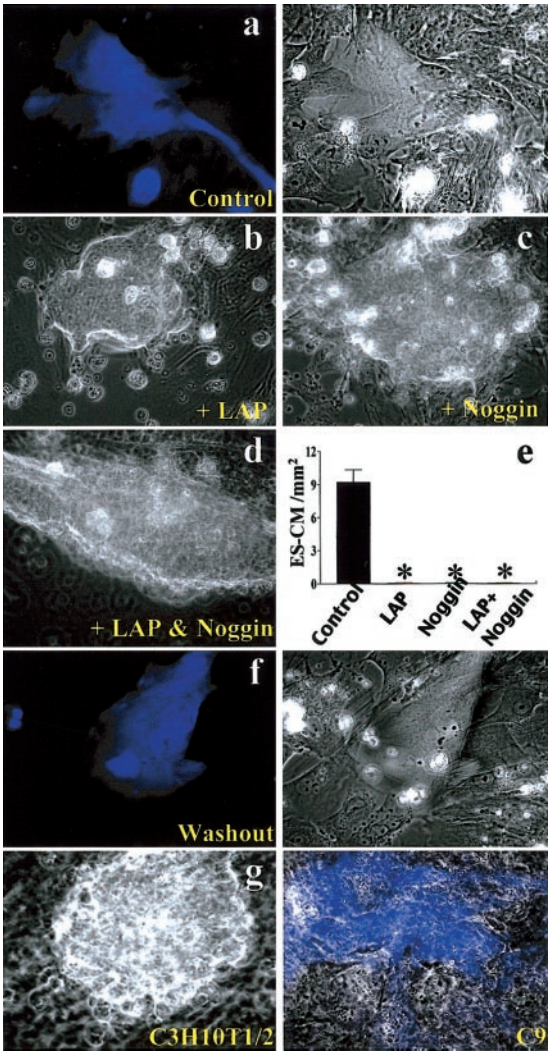

Figure 4. TGF-β/BMP2 paracrine signals from host cells differentiate stem cells. *a–f)* Stem cells expressing ECFP under the control of the α-actin promoter were cocultured with cardiomyocytes for 5 days. *a)* ECFP fluorescence (left) and phase (right) microscopy reveal cardiac differentiation of stem cells. Five day treatment of cocultures with latency-associated peptide (LAP; *b*), noggin (*c*), or both (*d*) prevented expression of cyan fluorescence indicating negligible cardiac differentiation. The concentration of LAP (5 nM) and the volume of the conditioned medium from CHO-expressing noggin were chosen for their efficacy to inhibit cardiac differentiation of stem cells within embryoid bodies. *e)* Data from 3 cocultures in each group expressed as mean (±SE) of the number of ECFP-expressing cells (ES-CM)/mm^2 of the microscope field. *f)* Within 2 days of removal of LAP and noggin, stem cells differentiated into ECFP-expressing cardiomyocytes. *g)* Whereas wild-type C3H10T1/2 embryonic fibroblasts not secreting BMP2 did not promote stem cell differentiation to fluorescing cardiomyocytes in coculture (left), the C9 cell clone secreting BMP2 promoted cyan fluorescence expression within 7 days, indicating differentiation of stem cells into cardiomyocytes (right). The experiment was performed on 3 cocultures with similar results.

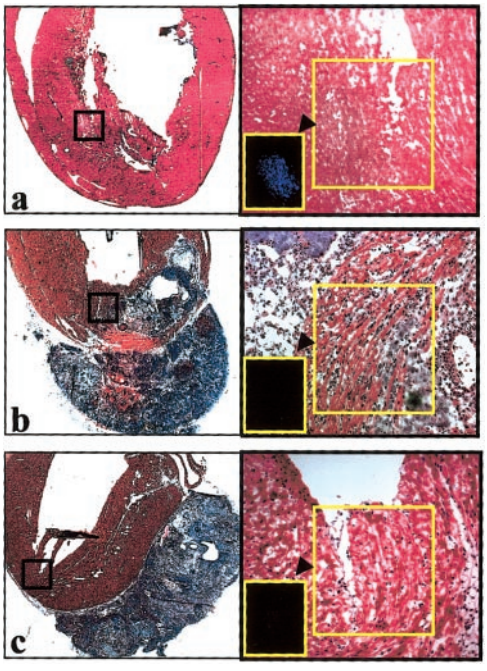

Figure 5. Paracrine stimulation by TGF-β family members required for in vivo stem cell differentiation. Hematoxylin-eosin stained cryosections of mouse hearts 4 wk after transplantation with stem cells. *a)* Heart injected with stem cells expressing ECFP under the control of the α-actin promoter (left) reveal fluorescent cardiomyocytes (right, inset) indicative of differentiated stem cells integrated within the host myocardium (right). *b, c)* Hearts injected with ECFP stem cells engineered to express noggin (*b*) or ΔKTGFβRII (*c*) reveal undifferentiated tumor formation with invasion of the myocardial wall (left) and absence of fluorescence (right, inset), indicating stem cells with compromised cardiac differentiation disseminated through the host myocardium (right). Right images are a magnification of the black inset on left images. Fluorescence was visualized in the area delimited by the yellow frame on the right images as indicated by arrows.

fibroblasts that do not secrete BMP2 (Fig. 4*g*). Thus, host cells through paracrine TGF-β/BMP2 signaling induce cardiac differentiation of stem cells.

In vivo stem cell differentiation requires paracrine stimulation by TGF-β family members

To assess stem cell differentiation in vivo, actinECFP cells were grafted into hearts of mice (n=3). After 4 wk, fluorescent myocytes expressing ECFP, indicative of stem cell differentiation, were integrated throughout the host myocardium (**Fig. 5*a***). In contrast, actinECFP cells engineered to express noggin or ΔKTGFβRII, disrupters of TGF-β family receptor-mediated signaling, failed to express ECFP fluorescence 4 wk post-transplantation, indicating the absence of cardiac differentiation (n=6; Fig. 5*b, c*). In fact, such stem cells incapable of responding to TGF-β receptor signaling remained undifferentiated and developed into invasive tumors in the hearts in one-third of the injected mice (Fig. 5*b, c*), an effect never seen from TGF-β-responsive stem cells (n=20, Fig. 5*a*). Thus, the paracrine effect of TGF-β family signaling is mandatory for proper cardiac differentiation of stem cells in vivo.

In vivo differentiation of embryonic stem cells in diseased myocardium improves contractile function

To test whether stem cells respond to TGF-β/BMP in vivo in a diseased condition, we used an established model of heart failure after myocardial infarction (27) in which cardiomyocytes are known to maintain secretion of TGFβ (38, 39). Pluripotent actinECFP cells were injected into the area surrounding the infarcted tissue (**Fig. 6*a, b***). After 5 wk, cells expressed ECFP fluorescence at the site of injection (Fig. 6*c*) as well as within the neovascularized scar tissue (Fig. 6*d–e*). Fluorescent cells displayed a typical cardiac phenotype, including sarcomeric striations (Fig. 6*f*), and immunoreactivity for MLC2v, a ventricle-specific myosin light chain isoform (Fig. 6*g*). Immunostaining for the gap junction

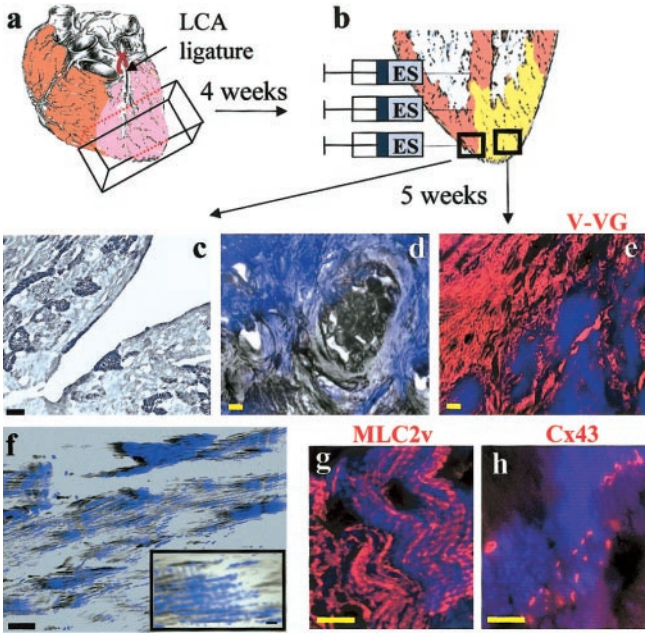

Figure 6. In vivo differentiation of stem cells in infarcted myocardium. *a)* Ligature of the left coronary artery (LCA) in rat causes myocardial infarction with development of cardiac failure within 4 wk. *b)* Stem cells expressing ECFP under the control of the cardiac α-actin promoter were injected into three peri-infarct locations 4 wk after infarction. *c–e)* Paraffin-embedded heart sections (5 μm) were imaged 5 wk after stem cell transplantation. Digital restoration of a z-stack of images acquired from the apical site of injection (*c*) or within the scar tissue (*d, e*) demonstrates numerous ECFP-expressing cells. *d)* Note the presence of neovascularization. *d, e)* Sections were stained with Verhaeff-Van Gieson (V-VG) to highlight collagen (in red) and elastin (black fibers around an erythrocyte-containing vessel). *f)* A digitally restored z-stack of images acquired at high magnification further reveals that stem cell-derived ECFP-expressing cardiomyocytes generated a network of myofibrils. Inset: magnified sarcomeres. *g, h)* Immunostaining of MLC2v (*g*) and connexin 43 (Cx43; *h*) in ECFP-expressing stem cell-derived cardiomyocytes. Scale bars indicate (in μm) 20 (*c*), 50 (*d, e*), 10 (*f*), 5 (*g, h*), and 2 (*f*, inset). The series of experiments was performed on 6 rats.

protein connexin 43 further revealed that these stem cell-derived cardiomyocytes were integrated with the surrounding heart tissue (Fig. 6*h*). In fact, compared with sham-injected hearts (n=3), echocardiography of stem cell transplanted hearts (n=4) revealed a significantly greater left ventricular ejection fraction (**Fig. 7*a***) as derived from M-mode images (Fig. 7*b*). Whereas in sham-injected hearts the infarcted anteroseptal wall was akinetic, the corresponding area in stem cell-injected hearts displayed contractility indicative of viable myocardium (Fig. 7*c*). A positive inotropic β-adrenergic response was observed in stem cell engrafted hearts but was absent in sham-injected failing hearts (unpublished results). Thus, stem cells differentiate into functional cardiomyocytes within infarcted hearts, resulting in improved function.

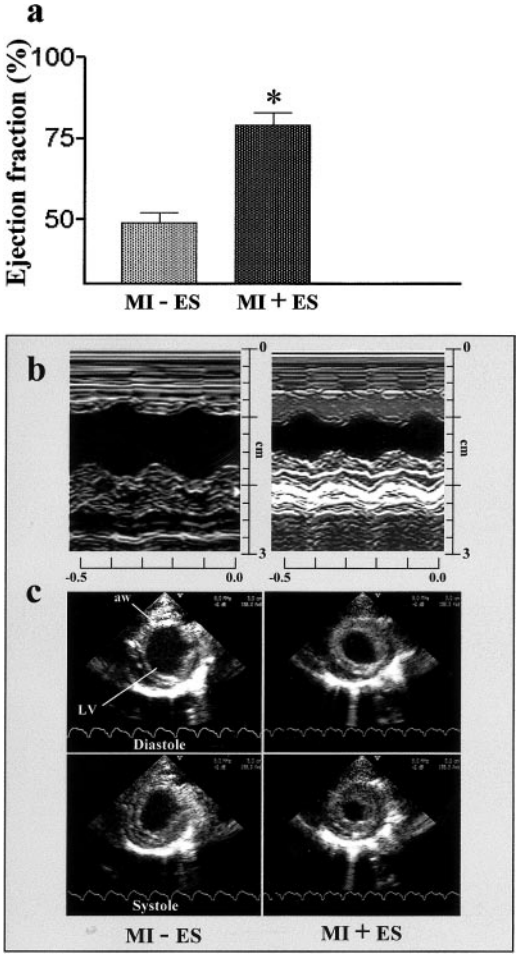

Figure 7. Gain of function of infarcted hearts after stem cell therapy. *a)* Average left ventricular ejection fraction 4 wk after sham-injection (MI-ES, n=3) or stem cell transplantation (MI+ES n=4) in rats with myocardial infarction. *Significant difference between the two groups (P<0.01). *b)* Representative M-mode scans obtained as 1-dimensional images at the greatest dimension of the left ventricular base as a function of time. Note improved mobility of the anterior wall (top of image) in the stem cell-transplanted (right) vs. the sham-injected (left) postinfarcted hearts. *c)* Two-dimension echocardiographic images through the base of the left ventricle (LV) in diastole (top) and systole (bottom). Note less dilatation and greater anterior wall (aw) contractility in the stem cell-transplanted (right) compared to the sham-injected (left) heart.

DISCUSSION

A promising approach for cardiac repair is cell-based repopulation of diseased myocardium. However, the originally used differentiated cardioblasts possess a limited mitotic aptitude, reducing their potential to replace extensive necrotic areas after ischemic injury. In this regard undifferentiated stem cells have a higher proliferative capability, and adult stem cells have recently been shown to differentiate into cardiomyocytes when engrafted in hearts before or shortly after infarction, improving cardiac function (15, 16). No data, however, are available on the potential for differentiation of stem cells engrafted into a diseased heart weeks after infarction, a more clinically relevant situation. Here we provide the first evidence that 4 wk after infarction, embryonic stem cells can extensively repopulate scar tissue within neovascularized (40) scar tissue. Ventricular specification of differentiated stem cells was proved by positive staining for MLC2v, a specific ventricular sarcomeric protein. Such cells expressed the gap junction protein connexin 43, suggesting integration and synchronization with the host tissue. This resulted in enhancement of wall motion in the infarct zone and significant improvement of ventricular contractile performance, including recovery of the β-adrenergic response. This together with the recent observation that heart injection of differentiated cardiomyocytes, but not fibroblasts, improved cardiac contractile function (41) argues in favor of a gain in function provided by contractile cardiomyocytes differentiated from ES cells. Altogether, our findings provide the first example of the potential therapeutic benefit of undifferentiated embryonic stem cells for the diseased heart.

Rejection of the stem cell graft by the host was not noted. This could be due to the absence of MHC antigen expression by stem cells (42). Stem cells can induce down-regulation of the host immune response; in fact, engraftment may be successful without immunosuppression due to induction of mixed immune chimerism favoring long-term graft acceptance (42). Here, stem cells were injected into the myocardium without coadministration of immunogenic molecules, which could explain the absence of mobilization of T cells, whose pool is likely to be modest (43).

Our findings are in line with previous work showing the propensity of embryoid bodies formed from embryonic stem cells to regenerate spinal cord when engrafted into the appropriate host environment (44). In fact, the present study provides a direct demonstration that the cardiac host environment is sufficient to commit undifferentiated embryonic stem cells with a high repertoire of potential fates toward a very specific cell lineage, namely, the cardiac ventricular myocyte. Growth and differentiation factors determine the fate of stem cells (45). TGF-β and BMP2 released by cardiac

myocytes or fibroblasts (34, 46, 47) are mandatory in early cardiogenesis in avian or *Xenopus* embryos (35, 48, 49). These factors are secreted chronically after myocardial infarction (38). Here, members of the TGF-β family induced strong expression of mesodermal as well as early and late cardiac markers, indicating their ability to mediate cardiac commitment of mammalian embryonic stem cells. TGF-β- and BMP2-treated stem cells generated embryoid bodies with extensive myofibrillar networks and, in the presence of host cells secreting a TGF-β family member, readily differentiated into cardiomyocytes with sarcomeric units similar to those of mature cardiac cells. The requirement for TGF-β and BMP2 is underscored by use of disrupters of TGF-β/BMP2 receptor-mediated signaling, LAP (37), noggin (36), and ΔKTGFβRII (22), which prevented differentiation of stem cells in vitro or in vivo. Thus, stem cells unable to respond to TGF-β/BMP2 signals remained undifferentiated and could proliferate into invasive tumors. This provides direct evidence that TGF-β superfamily members secreted by the heart (39, 50) are essential for stem cell differentiation into cardiomyocytes, thereby demonstrating a paracrine role for the heart in this process.

In principle, the molecular mechanism underlying TGF-β/BMP2 induced cardiac differentiation could be due to an 'instructive' or 'selective' modality (51). The first refers to TGF-β- and BMP2-directed cardiac differentiation and prevention of other fates, such as myogenesis (52). The second refers to cardiac differentiation induced by other conditions, such as cell–cell interaction, in which TGF-β and BMP2 favor selection through a proliferative or survival advantage (34). TGF-β and BMP2 actively up-regulated expression of cardiac transcription factors in stem cells, and the absence of TGF-β/BMP2 signaling kept stem cells undifferentiated. Only coculture of ES cells with C9 cells secreting BMP2 (but not with wild-type C3H10) cells induced their cardiac differentiation, suggesting that cell–cell interaction alone was insufficient to secure cardiac differentiation. Thus, our data rather suggest an instructive mechanism underlying a true differentiation action for TGF-β and BMP2 on stem cells.

In summary, we found that TGF-β superfamily members trigger in embryonic stem cells the expression of otherwise silent genes (Nkx2.5, MEF2C), a hallmark of cardiac phenotype, and activate cardiac promoters after stem cell engraftment. Driven by these differentiation factors, stem cell-derived cardiomyocytes integrate with the surrounding myocardium, express ventricular specific proteins, and contract in synchrony with the host. These three criteria, recently described as necessary for establishing the occurrence of a cell fate change (45), are demonstrated here. Finally, our demonstration that stem cell-derived cardiomyocytes improve contractile performance of infarcted hearts, presents the opportunity for exploitation of the paracrine TGF-β/BMP2 function of the heart in optimizing stem cell-based therapeutic strategies for cardiac repair. FJ

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