Bone Marrow Oct3/4+ Cells Differentiate Into Cardiac Myocytes via Age-Dependent Paracrine Mechanisms

Benedetta A. Pallante, Inga Duignan, Daniel Okin, Andrew Chin, Michael C. Bressan, Takashi Mikawa, Jay M. Edelberg

Abstract—The mechanisms that govern the capacity of the bone marrow stem cells to generate cardiac myocytes are still unknown. Herein we demonstrate that the cardiomyogenic potential of bone marrow–derived Oct3/4+/cKit+/CXCR4+/CD34+/Sca1− cells is governed by age-dependent paracrine/juxtacrine platelet-derived growth factor (PDGF) pathways. Specifically, bone marrow cell cultures from both 3- and 18-month-old mice formed aggregates of Oct3/4+ cells circumscribed by PDGFRα+/Oct3/4+/Sca1− cells. In young (3-month) bone marrow cell cultures, induction of PDGF-AB preceded the induction of cardiac genes and was required for the generation of cardiomyogenesis. Indeed, in old (18-month) cultures, diminished PDGF-B induction was associated with impaired cardiomyogenic potential, despite having Oct3/4 levels similar to those in the young cells. Importantly, supplementation with PDGF-AB specifically restored the cardiac differentiation capacity of the old bone marrow cells. Together these results demonstrate that, regardless of age, the bone marrow niche contains Oct3/4 stem cells that are capable of differentiating into cardiac myocytes. Moreover, this differentiation is governed by age-dependent PDGF-AB–mediated paracrine/juxtacrine pathways that may be essential in the translation of bone marrow cell–mediated cardiomyogenesis. (Circ Res. 2007;100:e1-e11.)

Key Words: bone marrow • stem cells • myogenesis • Oct3/4 • PDGF

Previous studies have shown that bone marrow cells may have the ability to give rise to cells of the heart, including cardiac myocytes.1-3 Experimentally this process is notable, however, for the low survival rates4,5 and the limited differentiation efficiency2,3 of the bone marrow cells in the cardiac tissue. Moreover, evidence of the fusion of bone marrow cells to preexisting cardiac myocytes in the heart has called into question the potential of bone marrow cells to generate new cardiac myocytes.5,7 Thus, to address this controversy, we elected to use an in vitro approach to define the cell population(s), factors, and mechanisms by which adult bone marrow cells may give rise to cardiac myocytes. Studies demonstrating that the adult bone marrow contains highly plastic cells with multilineage differentiation potential8,9 have suggested that bone marrow may be capable of giving rise to myocyte-like cells.10-12 The differentiation of these bone marrow cells into cardiac myocytes may be highly dependent on microenvironmental and trophic signals. Specifically, recent reports have highlighted the importance of inductive extracellular cues in the cardiac differentiation of adult stem cells.13,14 In particular, endothelial progenitor cells (EPCs) and EPC-derived growth factors have been shown to enhance cardiac tissue regeneration mediated by resident cardiac stem cells.13,15 Moreover, endothelial cells have been shown to direct embryonic stem (ES) cell cardiac differentiation,16 suggesting that endothelial cell/EPC trophism may regulate bone marrow–mediated cardiomyogenesis.

Previous work performed in our group has focused on the role of platelet-derived growth factor (PDGF) as an important mediator of the cardiac myocyte-endothelial/EPC interactions that govern cardiac vascular homeostasis and ischemic response in the adult heart.17,18 Indeed, the age-related impairment in PDGF-B induction pathways underlies a senescent predisposition to increased cardiac injury but can be reversed through targeting the functions of PDGF in the heart.18 Specifically, PDGF-AB is cardioprotective, acting as both as a proangiogenic and antiapoptotic agent. Moreover, we have found that PDGF-AB promotes the formation of bone marrow–derived myocardial bundles in rat models of myocardial infarction.19 These data together with the ability of PDGF to increase cardiac differentiation of murine ES cells19 suggest that PDGF pathways may be important in the cardiac differentiation of the highly plastic cells of the adult bone marrow.

Herein we describe the use of an in vitro system to identify the source of cardiomyogenic potential in the bone marrow.
and dissect the mechanisms regulating bone marrow cell cardiac differentiation. Importantly, the ability of bone marrow cell cultures to give rise to cardiac myocytes was confirmed by their spontaneous chronotropic activity, the expression of a range of cardiac genes and their electrophysiological properties. We then demonstrated that bone marrow–derived cardiac myocytes originate from aggregates of Oct3/4+/cKit+/CXCR4+/Sca1+/CD34+ cells in culture. Furthermore, we found that the cardiac myocyte differentiation of these aggregates is related to the age-associated changes in PDGF pathways. Specifically, cultures of bone marrow cells from both young (3-month) and old (18-month) mice gave rise to Oct3/4+ aggregates surrounded by PDGF receptor (R)α+ cells. In the young cultures, PDGF-B expression preceded the induction of cardiac myocyte differentiation. In the old cell cultures, a marked reduction in the PDGF-B induction was associated with a significant decrease in cardiac myocyte gene expression. Importantly, addition of PDGF restored the cardiomyogenic induction of the aging cultures, demonstrating the importance of local microenvironmental cues in the differentiation of bone marrow derived cells into functional cardiac myocytes.

Materials and Methods

An expanded Materials and Methods section is included in the online data supplement.

Statistical Analysis

Results are presented as mean±SEM. Mean group values were compared by using unpaired and paired t tests. A repeated-measures ANOVA with post hoc testing was also performed (SPSS software) (Figure 4C). Probability values of P<0.05 were considered statistically significant. A detailed description is included in the online data supplement.

Results

Bone Marrow Cells Differentiate Into Functionally Active Clusters of Cardiac Myocytes

Culturing bone marrow cells under conditions previously reported to promote bone marrow derivation of chronotropic activity and expression of α (adult-) and β (fetal)-myosin heavy chain (MHC),10 confirmed the differentiation of the bone marrow cells into clusters of cardiac myocytes (CCMs) as demonstrated using a panel of molecular and protein makers. Specifically, immunocytological (Figure 1A) and RT-PCR analysis (Figure 1B) of both bone marrow cell cultures and isolated spontaneously contracting cells revealed the expression of the following cardiac markers: the sarcomeric proteins cardiac troponin-T (cTnT), α-sarcomeric actinin, α-cardiac actin, α-MHC, β-MHC, light chain ventricular myosin (MLC-2V); the transcription factor Nkx2.5/csx; the gap junction proteins connexin43 (Cx43) and connexin40 (Cx40). The cardiac myocyte differentiation of the bone marrow cells was associated with the expression of β1- and β2-adrenergic receptors, which are critical in the regulation of cardiac chronotropic function (Figure 1C and 1D).20 Indeed, this cardiac gene profile correlated with observational studies of spontaneous chronotropy (Videos 1 and 2 in the online data supplement) in the newly formed CCMs (60.1±12.2 contractions per minute).

Based on the development of the bone marrow cells into phenotypic cardiac myocytes, day-7 CCMs were selected for functional analysis. Data were acquired using a microelectrode array (Figure 1E, a and b), before (Figure 1E, c) and after (Figure 1E, d) stimulation with 1 μmol/L isoproterenol, showing an increase in the depolarization rate, depolarization amplitude, and maximum electropotential amplitude (Table 1). Together, these findings confirmed the reactivity of CCMs to β-adrenergic stimulation and thus the phenotypic function of the bone marrow–derived cardiac myocytes.

Cardiac Myocytes Derive From Oct3/4+ Cells, Forming Stem Cell Aggregates or Bone Marrow–Derived Spheres

Observational studies revealed that clusters of cTnT+ cells (28.6±4.5% of total CCMs cells) form at day 7 at the periphery of spherical cellular aggregates, termed bone marrow spheres (BMSs) (Figure 2A, c and d). The number of these BMSs reached a peak at day 7 (6.0±1.9 BMS/animal, day 5; 10.7±1.9, day 7; 5.8±2.3, day 14), when spontaneous chronotropic activity was also observed in ~1/3 of the BMSs (32.9±10.6%). The size of the CCMs budding from the periphery of the BMSs gradually increased between days 10 and 14, when they detached and were observed in suspension (Figure 2A, e and f).

Notably, the formation of BMSs and subsequent CCMs was highly dependent the concentration of cells at the time of initial plating. Specifically, serial dilution studies revealed that cell density is critical in the formation of BMSs and CCMs chronotropy (Table 2). The highest number of BMSs was observed in the wells with 7.0×105 cells/well, whereas at lower concentrations (3.5×105 to 1.7×106 cells/well) few BMSs were formed and none developed chronotropic activity.

Experiments were then conducted to determine the mechanisms governing the generation of cardiac myocytes from the BMSs. To isolate the BMS cells from the non-BMS cells, spheres were selected by microdissection at day 5, before the onset of chronotropic activity and cultured on fibronectin. Onset of chronotropic activity was observed at day 14 (Figure 2B, a and b) in ~1/3 of the BMSs (N=7/24). In addition, approximately one-third of the cell clusters in suspension at day 21 were found to be cTnT+ (N=67/214). These findings represent a 1-week delay in the onset of chronotropic activity and CCMs formation, compared with control cultures, suggesting that an interaction with cells and/or extracellular signals outside of the BMS may be important for cardiac myocyte differentiation. Alternatively, we considered that the mechanical disruption of the BMS might account for the delay in cardiac myocytes generation. To test this, genetically labeled LacZ BMSs were isolated at day-5 whole bone marrow cell cultures prepared from ROSA-26 mice, dissociated into single-cell suspensions and mixed with intact wild-type whole bone marrow cell cultures forming BMSs. Notably, the presence of cTnT+/β-galactose+ (β-Gal+) cells in day-14 CCMs, which were composed predominantly of β-Gal- cells, and the lack of cTnT+/β-Gal+ cells alone (Figure 2B, c and d), supported the importance of microenvironmental interactions in the generation of bone marrow–derived cardiac myocytes.
The temporal patterns of cardiac differentiation and the morphology of the BMSs were similar to those observed in studies of ES cell–derived embryoid bodies and cardiospheres derived from resident cardiac stem cells. Based on these parallels, molecular and immunocytochemical studies were conducted to assess the stem cell nature of the BMSs capable of giving rise to the cardiac myocytes. RT-PCR analysis of the bone marrow–derived BMSs demonstrated the expression of Oct3/4 as well as the stem cell markers nanog, Dppa3, and prominin-1/AC133 and the progenitor cell marker nestin (Figure 2C). Quantitative RT-PCR revealed that the levels of Oct3/4, nanog, and Dppa3 in the day-7 BMSs were 10% of those observed in ES cells. Between day 7 and 14, there was a significant down regulation in the expression of these genes (unpaired t test; Oct3/4, P<0.04; nanog, P<0.019; Dppa3, P<0.005) as well as prominin-1 (P<0.04). Notably, however, immunostaining revealed the continued presence of Oct3/4 in the day-7 and -14 BMSs (Figure 2D, e and f). Conversely, the levels of expression of nestin increased in day-14 BMSs compared with day 7 (P<0.003), indicating a possible transition from a more undifferentiated state to a progenitor state committed to the cardiac lineage (Figure 2D, g).

Derivation of cardiac myocytes from Oct3/4+ cells localized in the BMSs was then confirmed by confocal imaging of day-14 BMSs double stained for Oct3/4 and cTnT (Figure 2E, a through c; supplemental Video 3). Z-Stack imaging of day-14 BMSs revealed that Oct3/4+ cells concentrate in the core of the BMSs (Figure 2E, a), whereas cTnT+ cells predominately localized to the periphery of the BMSs (Figure 2E, b and c). Detailed analysis revealed the presence of cTnT+ Oct3/4+ cells in the

![Figure 1. Bone marrow cells differentiate into functionally active cardiac myocytes. A, Immunostaining of day-14 spontaneously beating clusters of cardiac myocytes (CCMs) with anti-cTnT (Texas red), anti-Cx40 (fluorescein isothiocyanate, green), and anti-α-sarcomeric actinin (Texas red). Fluorescent images also show the DNA dye 4′,6-diamidino-2-pheylindole (DAPI) (blue). Bars=20 μm. B, RT-PCR analysis of day-0 to -14 whole bone marrow cells (BM) cultured with FGF-b and VEGF and of day-14 CCMs showing expression of the cardiac genes Nkx2.5/csx, α-cardiac actin, and fetal/β- and adult/α-MHC, light chain ventricular myosin (MLC-2V), Cx43, and Cx40. Controls: heart (positive); no cDNA (negative). C and D, Immunostaining of day-14 CCMs (C) and RT-PCR analysis of day-0 to -14 whole bone marrow cell cultures (BM) showing the expression of the α1- and the α2-adrenergic receptors (AR) (D). Fluorescent images also show the DNA dye DAPI (blue). Bar=20 μm. E, Reactivity to adrenergic stimulation was assessed by placing day-7 CCMs (arrow) into a microelectrode array chamber with 60 electrodes (arrowhead) (a) and recording the electric tracing (b) before (c) and after (d) stimulation with 1 μmol/L isoproterenol-HCl.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Depolarization Rate, Hz/sec</th>
<th>Depolarization Interval, ms</th>
<th>Depolarization Amplitude, μV</th>
<th>Maximum Peak, μV</th>
</tr>
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<tbody>
<tr>
<td>Baseline</td>
<td>5.5±0.3*</td>
<td>245±51*</td>
<td>378±49*</td>
<td>230±28*</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>10.1±1.4†</td>
<td>149±18†</td>
<td>562±64†</td>
<td>344±42†</td>
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</table>

Concentration of isoproterenol was 1 μmol/L. Paired t test. Groups with different symbols (* and †) are significantly different (P<0.05). Data were collected in 3 replicate experiments and are expressed as mean±SEM.
transition from the center to the periphery of the BMSs (Figure 2E, d through f), representing approximately one-fourth of the cardiac myocytes found in the BMSs (cTnT+ cells: Oct3/4+/H11001 cells: Oct3/4+/H11002, 2.9+/H11006 1.9%; Oct3/4+/H11002, 10.8+/H11006 1.9%, of total CCMs cells). To further confirm the key role played by Oct3/4+ cells in cardiac differentiation, targeted Oct3/4 small interfering (siRNA) was performed on day-1 bone marrow cell cultures (Figure 2F).

Reduction of Oct3/4 expression at day 3 significantly reduced day-7 expression of the cardiac marker β-MHC.

BMSs Are Formed by a Heterogeneous Population of Oct3/4+ Stem Cells
To investigate the in vivo origin of the Oct3/4+ population, we examined the bone marrow in situ. Immunostaining
confirmed the presence of the Oct3/4 cells in the bone marrow (Figure 3A, a). The cells were present as isolated cells, mostly localized close to the osteoblastic niche. Consistent with this, immunostaining of freshly isolated bone marrow cells confirmed Oct3/4 expression in rare, isolated cells (0.05±0.03% of cells) (Figure 3A, b).

In vitro analysis of the bone marrow cells revealed significant phenotypic heterogeneity within the Oct3/4 cell population that only partially overlapped with that of stem cell populations previously described (Figure 3B; supplemental Figure I). Notably, only a fraction of the Oct3/4+ cells contained for the stem cell marker cKit (17.1±1.7%; Figure 3B, a, and Figure 3C), which is also expressed by resident cardiac stem cells21,22 and CXCR4 (58.3±16.7%; Figure 3B, c, and Figure 3C), which has been found in association with Oct3/4 in a population of multipotent bone marrow stem cells previously described.23

Furthermore, the Oct3/4+ cells were negative for the hematopoietic cell markers CD34 and CD45, as well as the mesenchymal stem cell marker Sca1/CD31 (Figure 3B, a and g), which has been previously used to isolate cells for cardiac regeneration.12,23,24 Additional staining showed that a proportion of Oct3/4+ cells also expressed the vascular endothelial growth factor receptor, Flk1/VEGFR2 (21.5±5%) and the fibroblast growth factor receptor 1 (FGFR1) (16.4±12.1%), with only a small proportion of dual positive cells (6.6±4.6%; Figure 3B, e; 3C; supplemental Figure I). Indeed,

### TABLE 2. Effect of Cell Density on Day-7 BMS Numbers and Chronotropy

<table>
<thead>
<tr>
<th>Treatment</th>
<th>10^7/well</th>
<th>7.0x10^6/well</th>
<th>3.5x10^6/well</th>
<th>1.7x10^6/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMS, n/animal</td>
<td>7.0±1.2*</td>
<td>12.5±0.9†</td>
<td>0.5±0.3‡</td>
<td>0±0‡</td>
</tr>
<tr>
<td>Beating CCMs, %</td>
<td>2.5±0.3*‡ (36)</td>
<td>3.5±0.9*‡ (28)</td>
<td>0±0‡</td>
<td>0±0‡</td>
</tr>
</tbody>
</table>

Unpaired t test. Groups with different symbols (*, †, ‡) are significantly different (P<0.05). Data were collected in 3 replicate experiments and are expressed as mean±SEM.
cells positive for cKit, CXCR4, Flk1, or FGFR1 primarily composed the BMSs (Figure 3B, b, d, and f) highlighted the nonuniform composition of the Oct3/4+ cell aggregates that give rise to cardiac myocytes. Finally, although Oct3/4+ cells did not initially express CD45, CD45+ cells were observed at day 14 at the periphery of developing BMSs, possibly as a result of the differentiation process (Figure 3B, h).

Cardiac Differentiation of Bone Marrow Stem Cells Is Mediated by PDGFRα/Ligand Signaling Pathways

Immunostaining whole revealed that day-14 BMSs are surrounded by a distinct population of PDGFRα+ cells (Figure 4A, d) that are Oct3/4+, CD34+, and Sca1+ (Figure 4A, a through c and g). Analysis of phosphorylation patterns confirmed PDGFRα activation (Figure 4A, e and f). Notably, the predominant phosphorylation on PDGFRα Tyr720 (Figure 4A, e and g) as compared with Tyr754 (Figure 4A, f) is suggestive of signaling through an α receptor homodimeric complex rather than a heterodimerization with β receptor.25,26 Indeed, immunostaining demonstrated a lack of PDGFRβ (native or phosphorylated) within or adjacent the BMSs (Figure 4A, h, and Figure 4A, h, inset) and molecular analysis revealed expression of PDGFRα but not PDGFRβ in the bone marrow cell cultures or the BMSs themselves (Figure 4B).

These data are further supported by our previous observation that PDGFRα+ cells localize to sites of bone marrow cell–mediated cardiac regeneration10 and further supports the hypothesis that the generation of bone marrow–derived cardiac myocytes from Oct3/4+ stem cells may be regulated by PDGF/PDGFRα signaling pathway. To test this hypothesis, we assessed the effects of neutralizing antibodies, selectively blocking PDGFRα or PDGFRβ, on the differentiation of bone marrow stem cells cultured for 7 days under cardiomyogenic conditions. Significantly, our results showed that blocking of PDGFRα, but not PDGFRβ, prevented the expression of β-MHC, used as a marker of cardiac differentiation (Figure 4B).

Gene profiling of Oct3/4, α- and β-MHC, PDGF-A, -B, -C, and -D in differentiating bone marrow cells cultures also suggested a correlation between cardiac differentiation of

Figure 4. Cardiac differentiation of bone marrow cells is regulated by PDGFRα/PDG signaling pathways. A, BMSs formed by Oct3/4+ cells are surrounded by a population of PDGFRα+/Sca1+ cells that do not express Oct3/4. Immunostaining of day-0 bone marrow cell cytopsins showed that PDGFRα+ cells (a, Texas red [TR]; b and c, fluorescein isothiocyanate [FITC]; arrows) do not express Oct3/4 (FITC; arrowhead) (a) and CD34 (TR) (b) but express Sca1 (TR) (c). Cells expressing PDGFRα (FITC; arrows) (d) were found at the periphery of day-14 BMSs. Activation of PDGFRα was confirmed by its phosphorylation on Tyr720 (p720-PDGFRα; FITC) (e), predominantly observed in the αα homodimer. Phosphorylation on Tyr754 (p754-PDGFRα; FITC) (f), usually associated with the αβ heterodimer, was only occasionally observed. Colocalization of pTyr720-PDGFRα (FITC) and Sca1 (Cy3) was also observed at the periphery of day-14 BMSs (arrows) (g). PDGFRα, in its native or phosphorylated form (TR) (h and h, inset) was not expressed in the BMSs. Bar=20 μm. B, RT-PCR results confirming expression of PDGFRα, -β-MHC, and of PDGF-A, -B, and -C with affinity for PDGFRα were observed in the BMSs. Controls: heart, positive; no cDNA, negative. Results also showed that incubation at day-0 with PDGFRα-neutralizing antibody prevented the expression of β-MHC in day-7 bone marrow cell cultures. No effect was observed in cells incubated with PDGFRβ-neutralizing antibody and controls, incubated with no antibodies or control goat serum. C, RT-PCR and quantitative RT-PCR (1 arbitrary unit [AU]=maximal relative value [RV] for each gene) of bone marrow cells cultured under cardiomyogenic conditions showed a direct temporal correlation between the downregulation of Oct3/4 and the upregulation of cardiac-specific genes, α- and β-MHC, used as a marker of cardiac differentiation.
bone marrow stem cells and PDGFRα/PDGF signaling pathways. RT-PCR and quantitative RT-PCR results (Figure 4C) confirmed the kinetics of the reduction in Oct3/4 expression and the induction of cardiac myocyte genes, similar to what has been previously described for ES cell cardiac myocyte derivation.16 The levels of expression of β-MHC became detectable at day 5 and dramatically increased at day 7, whereas α-MHC was not detectable until day 7 and peaked at day 14. Notably, induction of PDGF-A, -B, and -C, which can all form dimers (PDGF-AA, -BB, -AB, -CC) that can signal through PDGFRα,27 was inversely correlated with the decrease in Oct3/4 and preceded the expression of β-MHC and the development of chronotropic activity (Figure 4C). On the contrary, PDGF-D, which interacts only with PDGFRβ, remained at basal levels during differentiation. Together with the results of the receptor neutralization studies, these findings suggested that PDGFRα ligand(s) were central in the differentiation of cardiac myocytes from bone marrow–derived Oct3/4+ cells.

Cardiac Differentiation of Bone Marrow Stem Cells Is Mediated by PDGF-AB/PDGFRα Signaling Pathways

To better evaluate the significance of the PDGF pathways on cardiomyogenesis, we elected to compare cultures of bone marrow cells isolated from 3 month-old mice with cells isolated from 18-month-old mice, which have an impairment of PDGFRα/H9251 cardiomyogenic potential facilitated our assessment of this subpopulation of bone marrow cells can differentiate into cardiac myocytes in vitro. Indeed, we have shown that an autoaggregatory heterogeneous population of bone marrow–derived Oct3/4+ stem cells can differentiate into cardiac myocytes in culture. Together, these experiments enabled us to confirm the in vitro differentiation of bone marrow cells into cardiac myocytes.

Our identification of the BMSs as the source of cardiomyogenic potential facilitated our assessment of this subpopulation of cells. BMSs were morphologically similar to other stem cell aggregates with cardiomyogenic potential, such as ES cell–derived embryoid bodies16 and cardiospheres obtained by culturing resident cardiac stem cells.21 Indeed, the cardiac gene profiling and induction of spontaneous chronotropic function in the bone marrow cultures was also comparable to that observed during murine ES cell cardiac differentiation.16,30

Discussion

Previous reports had shown the ability of purified bone marrow cells to differentiate into a variety of cell types including cardiomyocyte-like cells.10–12,23 Here we focused on the mechanism by which a subpopulation of bone marrow cells can differentiate into cardiac myocytes in vitro. Indeed, we have shown that an autoaggregatory heterogeneous population of bone marrow–derived Oct3/4+ stem cells can differentiate into cardiac myocytes in culture. Moreover, we demonstrated the essential role of PDGF-AB–mediated pathways for this differentiation, revealing that a reversible impairment in trophic support and not the Oct3/4+ stem cell compartment itself underlies the age-related decline in cardiomyogenic capacity.

It has been previously described, by us and others, that bone marrow–derived cells have the ability to differentiate into cells with cardiac myocyte phenotypes.10–12 Extending on those observations, we have chronologically and mechanistically assessed the cardiomyogenic capacity of the adult murine bone marrow. Cardiac differentiation was confirmed by the onset of spontaneous chronotropic activity associated with the expression of a range of cardiac genes. Notably, the relative proportion of β-MHC and the rounded shape of the cells, including those with chronotropic activity is consistent with the development of fetal/neonatal cardiac myocytes and is similar to the cells generated by ES-derived embryoid bodies.28 Indeed, functionality was confirmed as has been previously assessed for differentiating ES cells.29 testing the responsiveness of the bone marrow–derived cardiac myocytes to β-adrenergic stimulation on multifield electrodes. Together, these experiments enabled us to confirm the in vitro differentiation of bone marrow cells into cardiac myocytes.

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Molecular and immunocytochemical analysis of the BMSs confirmed the expression of the stem cell markers Oct3/4, nanog, and Dppa3 which are also expressed by undifferentiated ES cells. The BMSs also expressed prominin-1 which was originally considered a progenitor cell marker but has been found, more recently, in adult stem cells in association with the pluripotency markers Oct3/4, Rex-1, Sox-1, and Sox-2 suggesting it might be expressed in more primitive cell types. Furthermore, downregulation of these genes, and in particular Oct3/4, in the BMSs during cardiac differentiation is consistent with what has been observed in mouse embryoid bodies. Moreover, the coexpression of Oct3/4 and cTnT cells in BMSs and the inverse temporal correlation between Oct3/4 expression and the induction of MHC offers further proof that the cardiac myocytes differentiate from a primitive subpopulation of Oct3/4 stem cells. Notably, the decrease in Oct3/4 coincided with the upregulation in nestin, a gene that has been shown to be...
coexpressed with early cardiac markers in ES cells committed to the cardiac lineage. Indeed, together these findings suggest that bone marrow-cell cardiac differentiation or bone marrow cell differentiation into cardiac myocytes might progress from a more plastic stem cell stage (Oct3/4<sub>high</sub>/nestin<sub>low</sub>) to an intermediate, lineage restricted, cardiac progenitor cell stage (Oct3/4<sub>low</sub>/nestin<sub>high</sub>).

Like recent studies showing that the cardiomyogenic potential of cardiac stem cells correlates negatively with CD31/Scal<sup>40</sup> and positively with cKit<sup>31,22</sup> the present findings suggest that cells of the BMSs may be similar to resident cardiac stem cells. Indeed, the phenotypic similarities we found between bone marrow-derived stem cells and cardiac stem cell populations support the possibility that the bone marrow and the heart may be part of the same stem cell compartment, potentially connected by the systemic circulation. Moreover, the characterization of the Oct3/4<sup>−</sup> cells with cardiomyogenic suggested they represent a heterogeneous population of cells that only partially overlapped with stem cell populations previously described. The lack of expression of CD34 and CD45 suggests that they do not belong to the hematopoietic compartment. The Sca1<sup>−</sup>/cKit<sup>−</sup>/CXCR4<sup>−</sup> phenotype of the cells, in association with their higher levels of Oct3/4 expression, suggests that they are distinct from the Sca1<sup>−</sup>/cKit<sup>−</sup>/Oct3/4<sup>−</sup> mesenchymal stem cell population<sup>41</sup> and the Sca1<sup>−</sup>/CXCR4<sup>−</sup>/Oct3/4<sup>−</sup> multipotent bone marrow stem cell subpopulation.<sup>23</sup>

In addition to identifying a source of the cardiomyogenic potential in the adult bone marrow, our data offer important mechanistic insights into the differentiation of bone marrow cells into functional cardiac tissue. Expression of Flk1 and FGFR1 in the Oct3/4<sup>−</sup> cells, albeit in a heterogeneous pattern, are consistent with the importance of VEGF and basic fibroblast growth factor (FGF-b) in the generation of bone marrow–derived cardiac myocytes.<sup>10</sup> In particular, the expression of Flk1 at sites of cardiac differentiation is consistent with the central support role of VEGF and FGF-b pathways in heart tissue development and regeneration.<sup>16,42</sup> Moreover, the expression of FGFR1 in Oct3/4<sup>−</sup> cells and the requirement for FGF-b for bone marrow cardiomyocyte differentiation parallels the cardiac differentiation of ES cells, where FGF-b/FGFR1 is necessary for the maturation of AFP<sup>+</sup> cardiomyogenic endoderm.<sup>43</sup>

Our molecular and phenotypic analysis also proved that PDGF-AB/PDGFRα signaling pathway is necessary for cardiac myocyte differentiation. Immunostaining and experiments with neutralizing antibodies confirmed the exclusive involvement of PDGF-α in cardiac differentiation. Gene profiling results also confirmed the involvement of PDGF-α-ligand interaction showing that PDGF chains with specific affinity for PDGFRα (PDGF-A, PDGF-B, PDGF-C) preceded induction of cardiac genes. Moreover, experiments with aging bone marrow cells linked the dysregulation of PDGF-B to the loss of cardiomyogenic potential and showed that this can be reversed only by supplementation of PDGF-AB. The reduction in the expression of cardiac genes observed after PDGF-A and -B siRNA in young bone marrow cell cultures also confirmed the specific involvement of PDGF-AB/PDGFRα pathway in cardiac differentiation. Our observations are consistent with previous reports showing that PDGF-AB high-affinity binding to PDGFRα is not only possible, but necessary for significant activation of target cells.<sup>44</sup> Furthermore, specific requirement for PDGF-AB/PDGFRα interaction for cardiac differentiation of bone marrow cells is also consistent with previous data showing that, although PDGFRα can bind different PDGF isoforms (PDGF-AA, -AB, BB, and CC), each receptor/ligand interaction activates distinct signaling pathways regulating different physiological processes.<sup>45</sup> Indeed, such interactions activate distinct PDGFRα phosphorylation patterns that are then transduced into different downstream signaling pathways. Specifically, we have previously demonstrated that PDGF-AB, but not PDGF-AA, mediates the PDGFRα-dependent activity of cardiac microvascular endothelial cells in their communication with cardiac myocytes.<sup>46</sup> Indeed, such ligand-specific regulation may involve conformational changes in PDGFRα that can govern signaling cascades, including the recruitment and/or binding of specific cofactors, to promote a microenvironment supportive of the generation of additional cardiac myocytes from generated from bone marrow–derived Oct3/4<sup>−</sup> cells. Functional advantage of PDGF-AB over PDGF-AA on stem cell differentiation might also be attributable to its stronger mitogenic properties<sup>47</sup> conferred by the presence of the B chain, homolog to the simian sarcoma virus (v-sis) transforming protein.<sup>48</sup>

Together these findings highlight the importance of the local microenvironment in regulating the differentiation potential of the bone marrow–derived Oct3/4<sup>−</sup> stem cells and are consistent with data showing the ability of PDGF-AB<sup>18</sup> to increase the number of cardiac myocyte bundles in rat models of myocardial infarction and presence of high concentrations of PDGFRα<sup>+</sup> cells in areas of cardiac regeneration.<sup>10</sup> Our data are also consistent with previous reports showing involvement of PDGF-B and PDGFRα in ES cells cardiac differentiation<sup>19</sup> in vitro and during development.<sup>49</sup>

Our dilution studies served to further support the importance of cell–cell interactions in the differentiation of bone marrow–derived cardiac myocytes. Specifically, we showed that cell density is critical to the aggregation and subsequent differentiation of the BMSs from the bone marrow cells in culture. To this end, we speculate that the cardioplasticity of the bone marrow cells requires a heterogeneous population of Oct3/4<sup>−</sup> cells that can interact with PDGFRα cells to promote the generation of cardiac myocytes. Thus, it is likely that the isolation of individual Oct3/4<sup>−</sup> cells in the bone marrow may be essential in preserving the plasticity and/or quiescence of this subpopulation of stem cells. Indeed, we speculate that paracrine/juxtacrine pathways in the bone marrow may govern the integrity of the Oct3/4<sup>−</sup> cells, potentially preventing their aggregation and possible differentiation in the bone marrow itself. Outside of the bone marrow, such as in an in vitro culture system, our results have shown that the Oct3/4<sup>−</sup> cells can spontaneously aggregate and differentiate. Moreover, we demonstrated that both Oct3/4<sup>−</sup> expression and self-aggregation are preserved in the aging bone marrow and that age-associated impairment in cardiac regenerative potential<sup>50</sup> is determined by a reversible dysregulation of the PDGF-AB/PDGFRα signaling pathways. This result is in
contrast with previous reports that linked the age-related loss of cardiomyogenic potential to a reduction of a specific bone marrow stem cell population (CXCR4+/Oct3/4+/Sca1+). This discrepancy can be explained by the different experimental approaches. Previous studies focused on the purification and characterization of multipotent stem cells, whereas the present study focused on the mechanisms of cardiac differentiation. For this purpose, unfractonated bone marrow cells were used to preserve and study the complex interactions of heterogeneous populations of cells involved in cardiac differentiation, which may not have been previously appreciated.

Notably, the potential of bone marrow–derived Oct3/4+ cells to differentiate into cells other than cardiac myocytes was not the focus of this present work and the mechanisms governing their differentiation into noncardiac cells remains to be defined. Previous studies have demonstrated the significance of Oct3/4 in the extended nuclear plasticity of stem cells.51,52 Notably, although the BMS Oct3/4 level was significantly higher compared with the expression levels reported in multipotent adult stem cells (MAPCs),9,41 suggesting that the murine BMSs may have lineage plasticity similar to that observed in the MAPCs. To this end, we speculate that the noncardiac differentiation of the BMS Oct3/4+ cells may be dependent paracrine/juxtacrine communications that are analogous the PDGF pathways that drive cardiac myocyte generation. Indeed, we hypothesize that the identification of such cell–cell interactions in other tissue microenvironments may enable the development of approaches to directly the plasticity of the BMS Oct3/4+ cells. In conclusion, the present data identify a cardiac stem cell population in the bone marrow and the paracrine/juxtacrine mechanisms that regulate its differentiation. Moreover, these data suggest that strategies aimed at targeting the bone marrow–derived Oct3/4+ cells and that their interactions of PDGF-AB with PDGFRα+ cells may be used to enhance cardiac regeneration, particularly in older individuals.

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Disclosures
None.

References


(a) Supplemental Materials and Methods

Animals. Experiments with 3- and 18-month-old C57Bl/6, wildtype (WT) or LacZ labeled ROSA-26 mice were performed in compliance with the Institutional Animal Care and Use Committee of the Weill Medical College of Cornell University.

Bone marrow cell culture. Whole bone marrow cells (BMCs) were isolated and cultured in 5% CO₂ in air (7.5±0.5 x 10⁶ cells/well/4cm²), on fibronectin coated (0.1µg/ml) 12 well plates, for 7-14 days, in “complete medium” consisting of Iscove’s Modified Dulbecco’s Medium (Gibco) supplemented with 15% FBS (ATCC), 50µg/ml heparin-sodium salt (Sigma), 1% (v/v) Penicillin/Streptomycin (Cellgro), 10ng/ml recombinant human (rh) VEGF (#293-VE), 5ng/ml rhFGF-basic (FGF-b; #233-FB). In some experiments the culture media was also supplemented with 10ng/ml rhPDGF-AB (#222-AB), rhPDGF-AA (#221-AA), rhPDGF-BB (#220-BB), recombinant mouse PDGF-CC (#1447-PC) or rhPDGF-DD (#1159-SB). All growth factors were purchased from R&D Systems. Cultures were monitored daily for the development of bone marrow spheres (BMS) and onset of spontaneous chronotropy (3 replicates; 3 animals/replicate). BMS numbers were calculated per animal, therefore do not reflect the slight, animal-dependent variability in cell numbers (30.7±2.1x10⁶ BMCs/animal).

Derivation of cardiac myocytes from BMS. Day-5 WT BMS, purified by microdissection, were cultured in complete medium, on fibronectin coated 24 well plates (2 replicates; 10-15 BMS/3 animals/replicate) at a concentration of 1 BMS/well and
immunostained at day-21 for cTnT. Samples containing 1, 5, 10 β-Gal\(^{-}\)-BMS, microdissected at day-5 from ROSA-26 BMCs cultures, were trypsinized into single cell suspensions and added, at the three different concentrations, to wells containing developing BMS of WT cells plated 5d before at a density of 5x10^6 cells/well. The day-14 cardiomyocyte clusters (CCMs) were subsequently fixed and double stained for X-Gal\(^{1}\) and cTnT to confirm their chimeric derivation from the ROSA-26 and WT cells.

**Embryonic stem cell culture.** The ES-cell line ES-D3 (ATCC) was cultured following the supplier guidelines.

**Reagents.** Samples were stained with the following primary antibodies: rabbit anti-Oct3/4 (#se9081; sc5279), rabbit anti-Flk1 (#sc0315), rabbit anti-cKit (#sc168), goat anti-Scal/Ly6 (#AF1226; R&D Systems), mouse anti-CD34 (#sc7324), mouse anti-FGFR1 (#133100; Zymed; 500\(\mu\)g/ml), rabbit anti-PDGFR\(\alpha\) (#sc338), goat anti-PDGFR\(\beta\) (#sc1627), mouse anti-CXCR4 (#MAB171; R&D Systems), rabbit anti-pTyr720-PDGFR\(\alpha\) (#sc12910R), preferentially phosphorylated in the \(\alpha/\alpha\) homodimer,\(^2\) rabbit anti-pTyr754-PDGFR\(\alpha\) (#sc12910R), preferentially phosphorylated in the \(\alpha/\beta\) heterodimer,\(^3\) goat anti-pTyr770-PDGFR\(\beta\) (#sc12910R), goat anti-CD45 (#sc1121), rabbit anti-β1 (#sc568) or −β2-AR (#sc570), rabbit anti-Nestin (#PRB315C; Covance; 1mg/ml), goat anti-Cx40 (#sc20466), anti-αSA (#A7811; Sigma) and goat anti-cTnT (#sc8121). Mouse antibodies were biotinylated before use (#HC301801; InnoGenex). The secondary antibodies included: FITC-donkey anti-rabbit (#sc2090), FITC-avidin (#A2011; Vector Laboratories; 1mg/ml), TR-avidin (#A2016; Vector Laboratories; 1mg/ml), TR-donkey anti-goat (#sc2783), FITC-donkey anti-goat (#sc2724), Alexa Fluor
350 anti-goat (#A21081; Molecular Probes; 2mg/ml). For confocal microscopy, Cy3-mouse anti-goat (#205165108; Jackson ImmunoResearch; 1mg/ml) was also used. All antibodies were purchased from Santa Cruz Biotechnology (primary antibodies: 200µg/ml; secondary antibodies 400µg/ml), unless otherwise noted, and were used at a final concentration of 2-5µg/ml. Samples were finally mounted with the nuclear dyes DAPI (#; InnoGenex) or 5µM To-pro-3 iodide (A21081; Molecular Probes; 1mM) and mounted with Vectashield (H1000; Vector Laboratories).

**Immunostaining.** Cells were prepared on cytospin slides (2x10^5 cells/slide; 2 replicates; 8 fields/2 cytospin slides), while microdissected CCMs and BMS (diameter=40-200µM, cell number=148±85 cells/BMS) were processed in 40µl microdrops (3 replicates; 10 BMS or CCMs/3 animals/replicate). Samples were fixed for 10min, at room temperature (RT), in 4% paraphormaldehyde (PFA) followed by 12min at RT in 0.25% Triton X-100/Tris Buffer Solution (TBS: 50mM Tris-HCl-pH 7.4, 200mM NaCl) or 20min at -20°C in 95% ethanol/5% acetic acid (nestin). For surface antigen detection, specimen were fixed for 20min at 4°C in 2% PFA. After incubating for 20min at RT in 1% BSA/0.03% Tween-20 in PBS or TBS (blocking buffer), specimen were incubated overnight at 4°C with the primary antibodies, followed by 45min incubation at RT with the correspondent fluorochrome-conjugated secondary antibodies. Incubation with labeled avidin was followed by 10min block with unlabeled avidin and 1h incubation, at RT with another secondary antibody. All antibodies were diluted in blocking buffer.

Paraffin sections of decalcified femurs and tibiae were stained with anti-Oct3/4 and anti-rabbit-horseradishperoxidase, followed by DAB chromogenic reaction (DAB; #sc2018; Santa Cruz Biotechnology).
**Fluorescent microscopy.** Fluorescent images were acquired using a Nikon Eclipse E600 upright microscope equipped with: a Mercury Lamp; 40x/0.75 and 20x/0.50 Plan Fluor objectives; triple filter combination for FITC (Abs/Em=488-492/520), TR (Abs/Em=596/620) and DAPI (Abs/Em=350/450); a Spot camera and Spot Advanced program (Diagnostic Instruments Inc.). Confocal images were generated with a Zeiss Laser Scanning System (LSM 510) equipped with a Pan-Neofluar 25x/0.8 oil objective and the following laser/filter combinations: Argon-488nm, for FITC (Abs/Em=488-492/520); HeNe1-543nm, for Cy3 (Abs/Em=550/570nm); HeNe2-633nm for To-pro 3 iodide (Abs/Em=642/661nm). For each specimen Z-stacks (1µm/slice, 35-50 slices/stack) still images and Z-stack animations were acquired and edited using the Zeiss LSM 510 software.

**Movies.** Movies of contracting cardiac myocytes were acquired using a Nikon Coolpix x5000 digital camera connected to a Nikon Eclipse TE 2000-U inverted microscope.

**Microelectrode Array (MEA).** Day-7 CCMs (3 replicates; 10 CCMs/3 animals/replicate) were placed in a MEA (Multi Channels Systems) equipped with a 200/10 MEA-60 culture chamber, a MEA amplifier (MEA1060-1BC). Data were acquired for 120sec, at baseline and 2min after addition of 1µM isoproterenol-HCl, and were analyzed using the MC-Rack software (Multi Channels Systems).
**PDGF-receptor blocking experiments.** BMCs from 3 month-old mice were seeded in 12w plates at a concentration of $10^7$ cells/well and were cultured for 17h in 700µl serum-free culture medium supplemented with: 100µg control goat IgGs (#sc-2018; Santa Cruz) or 100µg neutralizing goat IgGs selectively blocking either PDGFRα (AF-307-NA; R&D Systems) or PDGFRβ (AF-385; R&D Systems). After 17h medium cells were resuspended in 1x complete medium. The medium was changed at day-5 and cells were harvested at day-7 for molecular analysis.

**Gene silencing by siRNA.** Day-1 (Oct3/4 siRNA) or day-3 (PDGF-A, -B and –C siRNAs) BMCs cultured in complete medium on fibronectin coated 24w plates (5x10^6 cells/well; 4 wells/animal; 4-10 animals/experiment) were transfected for 7-8h in transfection medium (#sc36868) supplemented with 8µl transfection reagent (#sc29528) and 200nM siRNA duplexes targeting Oct3/4 (#sc29419), PDGF-A (#sc39704), PDGF-B (#sc3706) or PDGF-C (#sc39708). Cells were cultured for additional 17h in complete medium with 100nM siRNA duplexes and 4µl transfection reagent. Control samples were transfected with scrambled siRNA sequences (#sc37007). Gene silencing was assessed 24h (Oct3/4 siRNA; day-3) or 72h (PDGF-A, PDGF-B, PDGF-C siRNAs; day-7) after transfection by RT-PCR and Quantitative RT-PCR of the silenced gene. All reagents were purchased from Santa Cruz Biotechnology.

**RT-PCR and Quantitative RT-PCR.** RNA was isolated from microdissected BMS or CCMs (20-30 BMS or CCMs/3-5x10^3 cells/6 animals/replicate), freshly isolated cell samples (10^7 cells/animal/replicate) and tissue samples (ovaries and liver; 100mg). RNA
was treated with RNAse-free DNAse, (1U/1µg RNA; Qiagen) and 1-2µg total RNA was reverse transcribed with Omniscript Reverse Transcriptase (Qiagen). PCR reaction mix contained 0.5µg/2.0µl template cDNA/reaction, HotStarTaq polymerase (Qiagen), 0.8-1µM of the required primer combination (Supplemental Table 1). PCR products were visualized by 2% agarose gel electrophoresis. Quantitative RT-PCR assays were carried out using a Sybr Green Master Mix (Applied Biosystems) and the primer sets and PCR profiles previously described. Data are expressed as relative values (RV)=2^{ΔCt} x10^3 and ΔCt= (specific gene cycle n° - β-actin cycle n°); arbitrary units, AU, where data were normalized to the relative value (RV) of a control group of choice (=1 AU). Reverse transcription step or template cDNA were omitted in controls. All experiments were carried out in triplicate.

References


(b) Supplemental Statistical Analysis

An unpaired *t*-Test (Fig. 2C) was used to compare the mean levels of expression (3 samples/group), for each gene (Oct3/4; Dppa3; Nestin; Nanog; Prominin-1), in different tissue or cell samples (BMS-d7; BMS-d14; ES-cells; ovary; liver). Samples in different groups were collected from unrelated animals.

A Repeated Measures-ANOVA with Post Hoc testing and Bonferroni’s correction for multiple comparisons (Fig. 4C), was used to compare the changes in the mean levels of expression for each gene (Oct3/4; \(\alpha\)-MHC; \(\beta\)-MHC; PDGF-A; PDGF-B; PDGF-C), occurring over 4 different time points (day-0; day-5; day-7; day-14), in bone marrow cell cultures (N=3).

An unpaired *t*-Test (Fig. 5B) was used to compare mean levels of expression for each gene (Oct3/4; PDGFR\(\alpha\); \(\beta\)-MHC; PDGF-A; PDGF-B; PDGF-C), at each time point (day-0; day-7; day-14), in cultures set-up from different age group animals, young or old (N=3, young; N=3, old).
Supplemental Figure 1. Single colour images supplemental to Figures 3B (a, c, e, g) and 4A (a-c). Freshly isolated bone marrow cells were prepared on cytopsin slides and stained for: (A) cKit (TR) and Oct3/4 (FITC); CD34 (TR) and Oct3/4 (FITC); Sca1 (TR) and Oct3/4 (FITC); (B) CXCR4 (FITC) and Oct3/4 (Cy3); CD45 (Cy3) and Oct3/4 (FITC); (C) Flk1 (TR) and Oct3/4 (FITC); FGFR1 (TR) and Oct3/4 (FITC); PDGFRα (TR) and Oct3/4 (FITC); (D) PDGFRα (FITC) and CD34 (TR); Sca1 (TR) and PDGFRα (FITC); Texas Red or TR, red; Cy3, red; Fluoresceinisothyocynate or FITC, green; Alexa Fluor 350, blue.
(d) Supplemental Videos

**Supplemental Video 1.** Video of day-7 bone marrow spheres (BMS) generating, at their periphery, clusters of cardiac myocytes (CCMs) with spontaneous chronotropic activity (QuickTime movie, 17.3 MB).

**Supplemental Video 2.** Video of a day-14 cluster of cardiac myocytes (CCMs) that has detached from a bone marrow sphere (BMS) and is present in suspension (QuickTime movie, 17.2 MB).

**Supplemental Video 3, 4.** Video, supplemental to Figure 2E, confirming the derivation and localization of cardiac myocytes in the BMS. Video 3 was generated from Z-stack confocal images (1µm/slice, 44µm) of day-14 BMS stained for cardiac Troponin-T (Cy3), Oct3/4 (FITC) and the nuclear dye To-pro-3 (blue). cTnT+ cells are localized at the periphery of the BMS and radiate from Oct3/4+ cells, that are localized in the centre of the nucleus (QuickTime movie, 33.0 MB). Video 4 (QuickTime movie, 33.0 MB) shows a X-axis three dimensional projection of the same specimen. cTnt+ cells can be observed at the periphery of the BMS, while they are radiating outwards and forming clusters of cardiac myocytes (CCMs).

**Supplemental Videos 5, 6.** Videos, supplemental to Figure 5A, confirming expression of Oct3/4 and PDGFRα in day-14 BMS derived from bone marrow cell cultures of both 3-month old (young; Supplemental Video 5; QuickTime movie 30.7 MB) and 18-month old (old; Supplemental Video 5; QuickTime movie 19.5 MB) and mice. The videos were
generated from Z-stack confocal images (1µm/slice; young, 41µm; old, 26µm) of day-14
BMS stained for Oct3/4 (FITC), PDGFRα (Cy3) and the nuclear dye To-pro-3 (blue).
cTnT+ cells are localized at the periphery of the BMS and radiate from Oct3/4+ cells, that
are localized in the centre of the nucleus (QuickTime movie, 33.0 MB).
### (d) Supplemental Table 1. Primer pairs used for RT-PCR and Quantitative RT-PCR analysis

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AT: annealing temperature; β-, -α-MHC: β-, α-myosin heavy chain; ; Connexin40, 43: Cx40, 43; Dppa3: Developmental pluripotency-associated 3; MLC-2V: Light Chain Ventricular Myosin; PDGF−A, -B, -C, -D: Platelet-derived growth factor−A, −B, −C, −D; PDGFRα or PDGFRβ: PDGF receptor α or β; β1-, β2-AR: β1-, β2-adrenergic receptor.