Generation of Functional Cardiomyocytes From Adult Mouse Spermatogonial Stem Cells

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Abstract—Stem cell–based therapy is a promising approach for the treatment of heart failure. Adult stem cells with the pluripotency of embryonic stem cells (ESCs) would be an ideal cell source. Recently, we reported the successful establishment of multipotent adult germline stem cells (maGSCs) from mouse testis. These cultured maGSCs show phenotypic characteristics similar to ESCs and can spontaneously differentiate into cells from all 3 germ layers. In the present study, we used the hanging drop method to differentiate maGSCs into cardiomyocytes and analyzed their functional properties. Differentiation efficiency of beating cardiomyocytes from maGSCs was similar to that from ESCs. The maGSC-derived cardiomyocytes expressed cardiac-specific L-type Ca\(^{2+}\) channels and responded to Ca\(^{2+}\) channel–modulating drugs. Cx43 was expressed at cell-to-cell contacts in cardiac clusters, and fluorescence recovery after photobleaching assay showed the presence of functional gap junctions among cardiomyocytes. Action potential analyses demonstrated the presence of pacemaker-, ventricle-, atrial-, and Purkinje-like cardiomyocytes. Stimulation with isoproterenol resulted in a significant increase in beating frequency, whereas the addition of cadmium chloride abolished spontaneous electrical activity. Confocal microscopy analysis of intracellular Ca\(^{2+}\) with isoproterenol resulted in a significant increase in beating frequency, whereas the addition of cadmium chloride abolished spontaneous electrical activity. Confocal microscopy analysis of intracellular Ca\(^{2+}\) in maGSC-derived cardiomyocytes showed that calcium increased periodically throughout the cell in a homogenous fashion, pointing to a fine regulated Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores. By using line-scan mode, we found rhythmic Ca\(^{2+}\) transients. Furthermore, we transplanted maGSCs into normal hearts of mice and found that maGSCs were able to proliferate and differentiate. No tumor formation was found up to 1 month after cell transplantation. Taken together, we believe that maGSCs provide a new source of distinct types of cardiomyocytes for basic research and potential therapeutic application. (Circ Res. 2007;100:1615-1625.)

Key Words: spermatogonial stem cells ■ cardiac differentiation ■ gap junction ■ L-type Ca\(^{2+}\) channels ■ cell transplantation

When heart muscle is damaged with myocyte apoptosis or necrosis, functional contracting cardiomyocytes are replaced by nonfunctional scar tissue. Experimental evidence suggests that the heart harbors a resident population of stem cells able to differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells. A subset of native cardiac progenitors has been identified in hearts of newborn mice, rats, and humans. Despite the possible existence of these populations and their ability to contribute to cardiac repair, these intrinsic mechanisms are inadequate to restore the cardiac function of a failing heart. Therefore, a potential therapeutic approach for treatment of heart failure is to replace lost cardiomyocytes with new functional ones as an alternative to whole-heart transplantation.

Several groups have shown that functional cardiomyocytes can be differentiated from embryonic stem cells (ESCs), derived from the inner cell mass of preimplantation embryos. ESC-derived cardiomyocytes have been characterized by their developmentally controlled expression of cardiac-specific genes, proteins, and ion channels. Excitation–contraction coupling and electrophysiologic specialization also have been observed. Application of ESCs or ESC-derived cardiomyocytes for treatment of heart failure has already been tested in animal models and has demonstrated beneficial effects. However, to prevent the rejection of the implant, it is necessary to make the cells immunocompatible with the recipient. In addition, the use of human ESCs has encountered opposition that has led to considerations regarding limited availability.

To circumvent these problems, adult stem cells are under investigation. Transdifferentiation of adult stem cells in vivo into cardiomyocytes has been shown by some investigators but not by others. Moreover, only a few experiments have suggested that adult stem cells could generate...
cardiomyocytes under in vitro conditions. However, this has also been challenged by other studies.

We recently showed that stem cells from adult mouse testis, similar to ESCs, are pluripotent. Spermatogonial stem cells (SSCs), a unique population of germline stem cells in adult testis, have the capability to self-renew and to produce daughter cells destined to differentiate into spermatogonia throughout life. We have successfully isolated SSCs from adult testis and established in vitro culture conditions to convert SSCs into ESC-like cells, the so-called multipotent adult germline stem cells (maGSCs). These maGSCs show phenotypic characteristics similar to those derived from ESCs. They can spontaneously differentiate into derivatives of all three germ layers in vitro. These data suggest that SSCs could be a new and promising source of adult stem cells for myocardial regeneration.

The purpose of the present study was to analyze the complex functional properties of cardiomyocytes derived from maGSCs in vitro and to analyze the behavior of undifferentiated maGSCs in normal hearts of mice in vivo after transplantation. Using molecular, cellular, and physiological assays, we found that maGSC-derived cardiomyocytes had similar properties to those derived from ESCs. They exhibited characteristics typical of heart cells in early stages of cardiac development. After transplantation of undifferentiated maGSCs into normal mouse hearts, they were able to proliferate and differentiate into vascular endothelial and smooth muscle cells in vivo, and no tumor formation was found up to 1 month after cell delivery.

**Materials and Methods**

**In Vitro Differentiation of maGSCs**

Established maGSC cultures (SSC5, C57BL6 line 9, FVB line 5, and 129 line 2) from different mouse strains were maintained on a feeder layer of mouse embryonic fibroblasts in DMEM (Invitrogen) supplemented by 15% FCS (Invitrogen), l-glutamine (Invitrogen, 2 mMol/L), β-mercaptoethanol (Serva, 50 μm/L), nonessential amino acids (Invitrogen, stock solution diluted 1:100), and 106 units/mL recombinant human leukemia inhibitory factor (Chemicon) (ie, standard ESC culture conditions) as previously described. Standard ESC culture conditions were used for cultivation of mouse MPI-II ESCs.

For differentiation of maGSCs, the hanging drop method described for mouse ESC differentiation was applied for the formation of embryoid bodies (EBs). See the expanded Materials and Methods section in the online data supplement.

**Induction of Cardiac Differentiation**

Our initial studies show that maGSC lines (SSC5, SSC6, and SSC10) derived from double transgenic mice (Sra8-EGFP/Rosa26) can spontaneously differentiate into contracting cardiac clusters. Here we report that maGSCs derived from wild-type mice of 3 different strains (C57BL6, FVB, and 129/Ola) can also spontaneously differentiate into cardiomyocytes by inducing EB formation (Figure 1A through 1C). Spontaneously and rhythmically contracting cells appeared as clusters and were identified in approximately 40% of individual EBs (n=144; 3 independent experiments) derived from C57BL6 line 9 at day 5+2 (ie, 2 days after plating of 5-day-old EBs), increased to as many as 80% of the EBs by day 5+8, and declined to 50% by day 5+17 (Figure 1C). The size of beating cardiac clusters was =5% to 20% of EB outgrowths by day 5+8. The percentages of beating EBs derived from C57BL6 line 9 during differentiation was similar to those derived from line SSC5 and ESCs (Figure 1C). Similar results were seen in FVB line 5 (Figure 1C). However, lower differentiation efficiency was seen in 129/Ola line 2 in comparison with line SSC5 and ESCs (Figure 1C). The remainder of the experiments then focused on EBs or cardiomyocytes derived from C57BL6 line 9 and SSC5. RT-PCR assays showed that cardiomyocytes derived from C57BL6 line 9, similar to those derived from SSC5, expressed cardiac gene products in a developmentally controlled manner (data not shown).

**Functional L-type Calcium Channels of maGSC-Derived Cardiomyocytes**

In cardiac muscle, where Ca2+ influx across the sarcolemma is essential for contraction, the dihydropyridine-sensitive L-type calcium channel represents the major entry pathway of extracellular Ca2+. Using immunofluorescence staining with a specific antibody, we could show that the α1 subunit of L-type calcium channels was expressed in cardiomyocytes at day 5+6 in a striated pattern (Figure 1D through 1F). The function of L-type calcium channels was examined in beating cardiac clusters at day 5+15 by evaluating chronotropic effects of cardioactive drugs. The L-type calcium channel activator (S)-BayK 8644 (1,4 dihydropyridine-type) showed dose-dependent positive chronotropic effects on the beating frequency of maGSC-derived cardiomyocytes, whereas the L-type calcium channel blocker diltiazem (1,5-benzoazepine-type) showed dose-dependent negative chronotropic effects (Figure 1G). Treatment with 10-4 mol/L (S)-BayK 8644 doubled the beating frequency. Treatment with 10-5 mol/L diltiazem almost completely blocked the contractions. Contractions recovered to a normal frequency 24 hours after removal of the drug. These results indicate that functional L-type calcium channels exist in the maGSC-derived cardiomyocytes.

**Functional Cell-to-Cell Communication**

To analyze the cell-to-cell coupling in the synchronously contracting cardiac clusters, we performed double-staining information is available in the expanded Materials and Methods section in the online data supplement.
using antibodies against either pan-cadherin and α-actinin or connexin 43 (Cx43) and cardiac troponin T. We found that pan-cadherin localized at cell–cell junctions, indicating the presence of adhering junctions between cardiomyocytes (Figure 2A through 2C). In addition, Cx43 staining indicated the presence of gap junctions between cells in cardiac clusters (Figure 2D through 2F). Functional coupling between cells was confirmed by fluorescence recovery after photobleaching analysis. Fluorescence recovery of a gap junction–permeable dye (calcein–acetoxymethyl ester [AM]) was observed consistently for cardiomyocytes within 5 minutes after photobleaching (Figure 3A). When cells were exposed to carbeneoxolone (50 μmol/L), a gap junction uncoupler, the fluorescence recovery was disrupted (Figure 3B). Fluorescence in cardiomyocytes recovered to 25.6±5.6% (n=15) after 30 seconds and carbeneoxolone significantly blocked the percentage of refill to 4.3±2.4% (n=9; P<0.05) (Figure 3C). The magnitude of functional gap junctions (or the gap junction permeability) was assessed by the rate of fluorescence recovery (k), which reflected the diffusion of

Figure 1. Cardiac differentiation of mouse maGSCs. A and B, Undifferentiated maGSCs (A) differentiated into cardiomyocytes by inducing formation of EBs (B). C, Percentage of EBs containing beating cardiac clusters during differentiation. D and E, Double immunostaining of cardiac cells by antibodies against sarcomeric myosin heavy chain (D) and cardiac-specific L-type calcium channels (E). F, Overlay of D and E. Nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI). G, Effect of diltiazem and (S)-BayK 8644 on the beating frequency of cardiomyocytes derived from maGSCs. *P<0.05, **P<0.01, ***P<0.001. Scale bar: 100 μm (A and B); 25 μm (D through F).
calcine-AM from unbleached neighbors into a laser-bleached cell. The recovery rate in maGSC-derived cardiomyocytes was 0.47±0.06 min⁻¹ (n=15), and the application of carbeneoxolone resulted in a significantly lower rate of recovery (k=0.04±0.04 min⁻¹; n=7; P<0.05; Figure 3D). These observations suggest that gap junctions function as a conduit of intercellular communication between cardiomyocytes and play an important role in cell-to-cell communication, essential for the synchronization of myocardial contractile activity and intact electromechanical coupling.

**Action Potential Characteristics**

In mature cardiac cells, depolarization of the cell membrane during the AP activates L-type Ca²⁺ channels, leading to Ca²⁺ influx and subsequent release of Ca²⁺ from intracellular calcium stores. To characterize whether mouse maGSC-derived cardiomyocytes could enter a fully differentiated cardiac phenotype, the dissociated cardiomyocytes were analyzed by patch-clamp. Mouse maGSC-derived cardiomyocytes showed spontaneous APs (Figure 4A). We examined the shape and properties of APs from 68 single beating cardiomyocytes. Four major types of APs characteristic for pacemaker- (n=8), ventricle- (n=23), atrial- (n=9), and Purkinje-like (n=9) cells were found (Figure 4A) with distinct morphologies at day 5+9. This classification was based on the shapes (Figure 4A) and the properties of the AP as measured by upstroke velocity (dV/dtₘₐₓ), AP amplitude (APA), AP duration at 90% and 80% of repolarization, and maximum diastolic potential as summarized in the supplemental Table I. Pacemaker-like APs are characterized by prominence of phase 4 depolarization, slow dV/dtₘₐₓ, and a smaller APA. The ventricle-like APs can be distinguished by the presence of a significant plateau phase of the AP, resulting in a long duration, and high dV/dtₘₐₓ and APA. The atrial-like APs show a triangular shape with a short duration and high dV/dtₘₐₓ and APA. The Purkinje-like APs are characterized by the presence of a notch and plateau-phase and high dV/dtₘₐₓ. In addition, a subset of cells (n=19) showed an intermediate AP phenotype (Figure 4B), which exhibited characteristics of both ventricle- and pacemaker-like morphology (slow dV/dtₘₐₓ, long duration; supplemental Table I).

To determine the functional expression of β-adrenergic receptors in cardiomyocytes, we studied the effects of β-adrenergic agonist isoproterenol (1 μmol/L) on APs. The isoproterenol stimulation resulted in the significant increase of AP frequency from 0.80±0.15 to 1.62±0.21 Hz (n=5; P<0.05; Figure 4C), demonstrating that β-adrenergic receptors are present in maGSC-derived cardiomyocytes and stimulation of these receptors produces a positive chronotropic response. Furthermore, cadmium (0.5 mmol/L), a nonspecific blocker of voltage-gated Na⁺ and L-type Ca²⁺ channels, completely abolished spontaneous APs (Figure 4D), proving that Na⁺ and Ca²⁺ channels critically contribute to the observed APs. A further hint for the involvement of fast depolarizing Na⁺ channels is AP upstroke velocities in the magnitude of 30 to 50 V/s⁻¹ for ventricle-, Purkinje-, and atrial-like APs (supplemental Table I). In particular, ventricle- and Purkinje-like APs showed plateau phases obviously caused by Ca²⁺ channel currents.

**Ca²⁺ Transients and Ca²⁺ Sparks**

We assessed the spontaneous intracellular Ca²⁺ fluctuations in maGSC-derived cardiomyocytes using confocal microscopy. A typical triangle-shaped cardiomyocyte during low-diastolic [Ca²⁺], and high-systolic [Ca²⁺], is presented in Figure 5A. Calcium increased homogenously throughout the cell, pointing to a fine-regulated Ca²⁺ release from intracellular Ca²⁺ stores, most likely the sarcoplasmic reticulum (SR) (supplemental Figure I; supplemental Video 1). The amplitudes of Ca²⁺ transient measured in maGSC-derived cardiomyocytes were 464±77 nmol/L (filled bar; n=15) versus 287±69 nmol/L measured in ESC-derived cardiomyocytes (open bar; n=5; statistically not significant; Figure 5B). Both are in the range of amplitudes measured in adult cardiomyocytes. Using line-scan mode, rhythmic Ca²⁺ transients were found (Figure 5C) and even small elementary Ca²⁺ release events (Ca²⁺ sparks; Figure 5D), which are mainly attributable to SR Ca²⁺ release through a cluster of ryanodine receptors (RyRs), appeared. Ca²⁺ sparks were previously
described in increasing numbers at later stages of cardiomyocytes derived from ESCs.29

The cardiac RyR2 serves as the major SR calcium-release channel to mediate the rapid rise of cytosolic free calcium. We found that the gene encoding RyR2 was expressed during EB differentiation (Figure 6A). In addition, genes encoding phospholamban, SERCA2a, and Na\(^+\)/Ca\(^{2+}\) exchanger (NCX)1, as well as calsequestrin, a high-capacity Ca\(^{2+}\)-binding protein in the SR, were expressed (Figure 6A). This expression pattern was similar to those described for cardiac differentiation of ESCs.5 To study the distribution of RyR2 and the Ca\(^{2+}\)-handling proteins SERCA2a and NCX1 throughout cardiomyocytes, we performed double staining in maGSC-derived cardiomyocytes at day 5. We found mainly a diffuse distribution of RyR2 in maGSC-derived cardiomyocytes (Figure 6B). Of note, we observed partial organization of RyR2 in striated-like structures (Figure 6B, arrow), which are not found in cardiomyocytes until postnatal day 6 when SR begins to organize.30 Immunochemical staining revealed that SERCA2a and NCX1 were mainly expressed in a fine granular, network-like pattern throughout cardiomyocytes (Figure 6C and 6D) comparable to those of neonatal cardiomyocytes.30,31 These results suggest that the organization of the SR is not fully developed in maGSC-derived cardiomyocytes.

Effect of Cardiac Environment on Development of maGSCs In Vivo
We further tested the effect of cardiac environment on the development of maGSCs in vivo. CM-DiI-labeled maGSCs (0.5 to 1\texttimes;10^6) were injected into the left ventricular free wall of female C57BL/6 mice. Given that the transplanted cells were Dil-labeled, whereas the myocardium of the recipient mice was not, the fate of donor cells was readily monitored by fluorescence microscopic examination. Two days after cell application, DiI-labeled cells could be found again in mice (n=5; Figure 7A). Hematoxylin/eosin (H&E) staining showed that these cells in small clusters were stained in blue (Figure 7D). One week after cell application, Dil-labeled cells could be found again in the recipients (n=4; Figure 7B). This data were confirmed by H&E staining of heart sections revealing that one week post-injection of cells, left ventricular regions of mice frequently contained larger clusters of cells with large nuclei which were stained dark blue (Figure 7E). One month after cell injection, Dil-labeled cells could still be found in heart sections of mice (n=6; Figure 7C). However, they no longer contained large nuclei and were not stained dark blue (Figure 7F). Furthermore, none of the transplanted hearts showed evidence of tumor or teratoma formation on histological evaluation.

In addition, we tested whether cells could migrate into hearts after intravenous cell delivery. We found that only very
a few single cells could be detected at 2 days after application (data not shown).

The fate of donor cells following intramyocardial injection was determined using immunohistochemical assays. In transplanted hearts at 1 week after cell injection, immunolabeling with an antibody specific for Oct4, a germline-specific transcription factor often used to characterize pluripotent stem cells, revealed large aggregates of labeled cells in the myocardium (Figure 8A). In a parallel section, the Oct4-positive cell aggregates were also stained dark blue by H&E staining (data not shown), suggesting the presence of undifferentiated and nonmature cells in the left ventricles of recipients. As shown in Figure 7E, there were cells with elongated nuclei adjacent to the dark blue–stained cells. They were Oct4 negative (Figure 8A), suggesting that these cells lost their pluripotency. However, when the sections were stained with an antibody against the cell proliferation marker Ki-67, we found that some cells with elongated nuclei were positive for Ki-67 (Figure 8C), indicating that they still have the proliferation potential. One month after cell transplantation, no Oct4- or Ki-67–positive cells were found (Figure 8B and 8D). Immunolabeling for cardiac troponin T showed that none of the transplanted cells expressed cardiac troponin T at either 1 week (supplemental Figure IIA through IIC) or 1 month (supplemental Figure IID through IIIF). When the sections at 1 month were stained with antibodies against von Willebrand factor (vWF) and smooth muscle-α-actin (SM-α-actin), we found that some of labeled cells were positive for vWF (Figure 8E through 8G) and SM-α-actin (Figure 8H and 8I), indicating that the transplanted maGSCs could differentiate into vascular endothelial and smooth muscle cells. In addition, Masson–Goldner trichrome staining showed that fibrosis (light blue staining) developed in the heart at 1 week (Figure 7H) and 1 month (Figure 7I) postinjection of cells, whereas almost no fibrosis was visible in the transplants at 2 days (Figure 7G).

Taken together, these findings demonstrate that transplanted maGSCs are able to proliferate and differentiate in the normal heart. Some cells lost their pluripotency but still were proliferating 1 week after cell delivery. After 1 month, no proliferating cells were observed, and until this time, no tumor or teratomas were formed. Although transplanted

Figure 4. AP characteristics of spontaneously contracting cardiomyocytes at day 5+9. A, Exemplary original traces of APs. Distinct AP morphologies representing pacemaker-, ventricle-, atrial-, and Purkinje-like cardiomyocytes could be discriminated. B, In a subset of cardiomyocytes, an intermediate phenotype between pacemaker- and ventricle-like APs was found. C, Application of isoproterenol (Iso) (1 μmol/L) resulted in an increase in AP frequency and irregularity. D, Nonselective blockade of L-type Ca2+ and Na+ channels with cadmium (0.5 mmol/L) completely abolished cardiac APs.
maGSCs can differentiate into vascular endothelial and smooth muscle cells, no mature cardiomyocytes developed.

**Discussion**

We report here that cardiomyocytes can be generated from multiple mouse maGSC lines with an efficiency comparable to cardiac differentiation of mouse ESCs. The cardiomyocytes derived from maGSCs express L-type calcium channels and respond to cardioactive drugs. Functional gap junctions are present between cardiac cells. AP analysis demonstrates the presence of pacemaker-, ventricle-, atrial-, and Purkinje-like cardiomyocytes. The cardiomyocytes also exhibit the complex functional properties of native cardiomyocytes, including a positive or negative response to β-adrenergic stimulation or Ca\(^{2+}\) blockers and an intact calcium cycling. After intramyocardial injection, maGSCs are able to proliferate and differentiate without tumor formation.

The use of a cell therapy approach to replace lost cardiomyocytes with new cardiomyocytes that could be grafted would represent an invaluable technique for the treatment of heart failure. Such an application has already been demonstrated in animal models using ESCs as well as other adult stem cells. However, some studies have indicated that the capacity of transdifferentiation of other adult stem cells into cardiomyocytes may be limited or that fusion of stem cells with cardiomyocytes may occur. In contrast, ESCs have been proven to be pluripotent. However, the use of human ESCs has encountered opposition that has led to considerations regarding limited availability. Therefore, adult stem cells with the pluripotency of ESCs would be ideal for cell-based regeneration strategies.

Our results show that mouse maGSCs, derived from SSCs in culture, are similar to ESCs. When maGSCs were cultured as EBs in hanging drops, as described for mouse ESCs, they spontaneously differentiated into functional cardiomyocytes with an efficiency similar to that seen with ESCs. Spontaneously beating cardiomyocytes could be derived from approximately 80% of EBs. Beating frequency could be modulated by isoproterenol, indicating functional β-adrenergic receptors. AP measurements showed 4 major types of APs that were characteristic of specialized cardiomyocytes from sinus node, ventricle, atrium, and Purkinje fibers, and similar to those found in ESC-derived cardiomyocytes.

Excitation–contraction coupling systems of maGSC-derived cardiomyocytes showed all major components and resembled those of neonatal cardiomyocytes regarding the degree of maturation. This included L-type calcium channels...
sensitive to the calcium channel activator (S)-BayK8644 and the calcium channel blocker diltiazem, as well as calcium binding and cycling proteins, such as RyR, calsequestrin, sarcoplasmic reticulum calcium pump, phospholamban, and sarcolemmal sodium calcium exchanger.

We also observed organization of RyRs in a striated-like structure in maGSC-derived cardiomyocytes, similar to those in postnatal cardiomyocytes. Development of SR and functional excitation–contraction coupling is obvious from the presence of Ca\(^{2+}\) sparks and Ca\(^{2+}\) transients in maGSC-derived cardiomyocytes. Critically important for cardiac function, we show that maGSCs derived cardiomyocytes express the gap junction protein Cx43 and develop cell-to-cell coupling. Again, cardiomyocytes derived from maGSCs were structurally and functionally comparable to those obtained from ESCs.

The finding that transplanted maGSCs are able to proliferate in the heart is consistent with the known self-renewal property of the cell source. We observed that maGSCs transplanted into normal hearts lost their proliferating potential at 1 month and differentiated into vascular endothelial and smooth muscle cells but did not differentiate into cardiomyocytes. When maGSCs were cultured as EBs, which resemble early postimplantation embryos, they were able to spontaneously differentiate into functional cardiomyocytes in vitro at day 5+8. However, they could not spontaneously differentiate into cardiomyocytes in the normal heart at 1 month postinjection of cells. It is possible that cardiac differentiation may occur at later time points that have not been investigated yet. Alternatively, local microenvironment might be a critical determinant of the fate of the transplanted maGSCs. This is also in line with previous studies showing that in the absence of myocardial infarction, only rare transplanted ESCs remained in the heart at 2 weeks. However, undifferentiated mouse ESCs transplanted directly into the infarcted heart could differentiate into cardiomyocytes and vascular smooth muscle and endothelial cells. It will be interesting to investigate whether maGSCs or predifferentiated maGSCs can contribute to the cardiac and vascular lineages in the absence of fusion after cellular transplantation into the infarcted heart.

Although maGSCs proliferated after transplantation, under the present conditions, no teratoma/tumor formation was observed, despite the known ability of these cells to form teratomas under certain conditions. This seems consistent with earlier studies transplanting mouse ESCs into rat or mouse hearts. However, this is in contrast to a recent study showing that undifferentiated ESCs formed teratomas in both normal and infarcted hearts of nude or immunocompetent syngeneic mice. Even allogenic ESCs caused teratomas, but these were immunologically rejected after several weeks. Of course, we cannot exclude that maGSCs may
form teratomas with experimental conditions different from those used in the present study. However, absence of teratoma formation may also suggest that our maGSCs are different from mouse ESCs in this regard.

The present findings open new possibilities for basic research on cardiac development as well as cardiac regeneration. SSCs can be easily derived from transgenic animals to study the effects of genetic manipulation on myocyte development and maturation. Moreover, these functional cardiomyocytes may be able to engraft into the damaged host myocardium and function as cardiomyocytes after transplantation. Most importantly, if the present technique could be transferred to human tissue, this would open new options for human cardiac regeneration without the ethical problem associated with ESCs. SSCs and maGSCs could be obtained from testicular biopsies without the use of human embryonic tissue. Moreover, the availability of immunocompatible tissue for autotransplantation would circumvent immunological problems associated with ESC-based therapy. Finally, we postulate that regeneration strategies using maGSCs may be based on techniques that have been developed previously for ESCs.

In conclusion, we have demonstrated that functional cardiomyocytes can be derived from adult stem cells. These maGSC-derived cardiomyocytes can now be tested for their ability to restore the function of damaged hearts in animal models. A major challenge will be to produce functional cardiomyocytes derived from human SSCs and to use them in cell-based therapies for heart disease.

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Disclosures

None.
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**Materials and Methods**

**Cell Culture and Differentiation of Embryoid Bodies**

Established maGSC cultures (SSC5, C57BL6 line 9, FVB line 5 and 129 line 2) from different mouse strains were cultivated on a feeder layer of primary mouse embryonic fibroblasts on gelatin (0.1%)-coated petri dishes (Nunc) in Dulbecco’s modified Eagle’s minimal essential medium (DMEM, 4.5 g/L glucose; Invitrogen) supplemented by 15% heat-inactivated fetal calf serum (FCS, selected batches, Invitrogen), L-glutamine (Invitrogen, 2 mmol/L), β-mercaptoethanol (β-ME, Serva, final concentration 50 µmol/L), non-essential amino acids (NEAA, Invitrogen, stock solution diluted 1:100) and 10³ Units/ml recombinant human leukemia inhibitory factor (LIF; ESGRO, Chemicon) (= standard ESC culture conditions) as previously described.¹ Standard ESC culture conditions were used for cultivation of mouse MPI-II ESCs.

For differentiation of maGSCs, mouse maGSCs were cultivated as embryoid bodies (EBs) in hanging drops in Iscove’s modified Dulbecco’s medium (IMDM, Invitrogen) supplemented with 20% FCS, L-glutamine, NEAA and α-monothioglycerol 3-mercapto-1,2-propandiol (MTG, final concentration 450 µmol/L, Sigma) as described for standard mouse ESC differentiation.² Briefly, cells (n = 400) in 20 µL differentiation medium were placed on the lids of petri dishes filled with phosphate buffered saline (PBS) and cultivated in hanging drops for 2 days and in bacteriological petri dishes for 3 days. EBs were plated separately onto gelatin-coated 24-microwell or 6-cm culture plates at day 5. For the determination of cardiac differentiation efficiency, spontaneously beating cardiomyocytes were investigated from the outgrowths of EBs cultured in 24-microwell plates at various stages during differentiation. The percentage of EBs containing beating cardiomyocytes was used as a measure of the efficiency of cardiomyocyte differentiation. At least three independent experiments (n = 48 EBs per experiment) were performed.
In vitro Response of Beating EBs to Pharmacological Agents

A day before the experiment, cells were fed with 1 mL/well differentiation medium and beating areas were photographed and marked for later identification. The next day, the beating frequency for each area was measured by visual inspection before adding the drugs. To examine pharmacological responses, drugs (all from Sigma) were added to the culture at the lowest dose. The beating frequency was monitored after cultures were incubated with drugs at 37°C for 5 min. The procedure was repeated several times by sequentially adding additional doses of the drug followed by monitoring the beating frequency. The results are presented as the mean beating frequency ± standard error of the mean measured for at least 15 beating areas.

Isolation of Cardiomyocytes

Cardiomyocytes were isolated as single cells from the beating areas of EBs mechanically using a micro-scalpel under an inverted microscope. Tissues were collected in Tyrode’s solution with (in mmol/L) 137 NaCl, 5,4 KCl, 1,2 MgSO4, 1,2 Na2HPO4, 10 glucose, 20 HEPES (pH 7.4 with NaOH). The isolated clusters were digested in collagenase type 2 (310 U/mL, Worthington) supplemented Tyrode’s solution with 30 µmol/L CaCl2 at 37°C for 30 minutes. For the isolation of small cardiac clusters, the incubation time was shortened to 15 minutes. The cells were then resuspended and dissociated in 100 µL of KB medium² at 37°C for 30 minutes. The cell suspensions were transferred into tissue culture plates containing gelatin-coated cover slips and incubated in differentiation medium at 37°C overnight. Cardiomyocytes began rhythmical contractions after 24 hours and were used for electrophysiological assays and immunostaining 2-3 days later.

Immunocytochemistry

For immunostaining, isolated cardiomyocytes were first incubated with the antibodies against sarcomeric α-actinin (rabbit polyclonal antibody, clone 653, kindly from Prof. D.O. Fürst, University of Potsdam, Germany), cardiac troponin T (Ab-1, mouse monoclonal antibody,
clone 13-11, Lab Vision), L-type Ca$^{2+}$ channel $\alpha$ (Ca,1.2a, rabbit polyclonal antibody, Alomone labs), connexin 43 (rabbit polyclonal antibody, Lab Vision), pan-cadherin (mouse monoclonal antibody, clone CH-19, Sigma), sarcoplasmic/endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA2a; mouse monoclonal antibody, clone 2A7-A1, Affinity Bioreagents), sodium/calcium exchanger 1 (NCX1; mouse monoclonal antibody, clone 6H2, Chemicon), and ryanodine receptor (RyR2; mouse monoclonal antibody, clone 34C, Affinity Bioreagents). Afterwards, specimens were incubated with the fluorescent antibodies: FITC-conjugated goat anti-rabbit (Dianova) for $\alpha$-actinin; Cy3-conjugated goat anti-mouse IgG + IgM (Dianova) for L-type Ca$^{2+}$ channel $\alpha$, SERCA2a, NCX1, and RyR2; FITC-conjugated goat anti-mouse IgG (Dianova) for troponin T; Cy3-conjugated goat anti-rabbit (Dianova) for connexin 43. Specimens were count stained with 4,6-diamino-2-phenylindole (DAPI; Sigma) and analyzed using a fluorescence microscope.

Fluorescence Recovery after Photobleaching (FRAP) Assay

Functional analysis of gap junctions in isolated cardiac clusters was performed by measuring the cell-to-cell diffusion of a fluorescent dye using a FRAP assay at room temperature. Briefly, cardiac clusters were loaded with the membrane-permeant fluorescent dye calcein AM (5 µmol/L; Invitrogen) in Ca$^{2+}$-free Tyrode solution for 20 min at 37°C. After washing away the excess extracellular fluorescent dye to prevent further loading, the cultures were bathed in Ca$^{2+}$-free Tyrode solution and placed on the stage of a Zeiss LSM 5 PASCAL laser-scanning confocal microscope. Using Zeiss software, a rectangular region encompassing 30-50% of a single cell within a cell cluster was selected and its fluorescence was bleached by a high-intensity laser pulse (488 nm, 3-5 s duration). This caused immediate loss of calcein fluorescence emission recorded through a 505 nm long-pass emission filter. Calcein redistribution from adjacent unbleached cells through connexin pores into the bleached region of interest (ROI) was recorded in subsequent confocal images acquired at 30 s intervals for up to 10 min using a low-intensity laser pulse. Fluorescence recovery within the ROI was plotted as a function of time and fit to a single exponential function: $I_{ROI}(t) = A \left(1 - e^{-kt}\right)$. 

$I_{ROI}(t) = A \left(1 - e^{-kt}\right)$
exp (\(-kt\)), where \(I_{ROI}(t)\) is the ROI fluorescence intensity at time \(t\), \(A\) is the amplitude of fluorescence recovery, and \(k\) is the rate of recovery. The latter is considered a measure of gap junction permeability.\(^3\) For the inhibitor studies, the cells were incubated in Ca\(^{2+}\)-free Tyrode solution containing the gap junction uncoupler 3β-hydroxy-11-oxoolean-12-en-30-oic acid 3-hemisuccinate (carbenoxolone, 50 µmol/L; Sigma).

**Action Potential Measurements**

The membrane potential \((E_m)\) of single cardiomyocytes was measured at room temperature with ruptured-patch whole cell current clamp as described previously.\(^1\) Fire-polished glass microelectrodes of >10 M\(\Omega\) resistance when filled with pipette solution were used. Pipette solution contained (in mmol/L) 120 potassium aspartate, 8 KCl, 7 NaCl, 1 MgCl\(_2\), 10 HEPES, 5 Mg-ATP, 0.3 Li-GTP (pH 7.2 with KOH). A normal Tyrode’s solution with 2 mmol/L CaCl\(_2\) served as bath solution. Liquid junction potentials were corrected before G\(\Omega\) seal establishment. Access resistance was typically ~20 M\(\Omega\) after patch rupture. Spontaneous action potentials (APs) were recorded immediately after patch rupture and followed for about 2 min. Signals were filtered with 2.9 and 10 kHz Bessel filters, and recorded with an EPC10 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) using the Patchmaster software. Action potentials were averaged (~10 APs per cell) and analysis was done using MS Excel\()^\text{®}\) to determine the maximum rate of rise of the AP upstroke (\(dV/dt_{\text{max}}\)), AP amplitude (APA), AP duration at 90/80% of repolarisation (APD 90/80) and the maximum diastolic potential (MDP).

In some experiments isoproterenol (1 µmol/L) or cadmium chloride (0.5 mmol/L) were added to the bath solution during recording. Signals were averaged and results visualized using Graphpad Prism\(\text{™}\) and SPSS SigmaPlot\(\text{®}\).

**Intracellular Calcium Measurements using Confocal Laser Microscopy**

Intracellular calcium ([Ca\(^{2+}\)]) signals were recorded after incubating cells with 10 µmol/L fluo-4 acetoxymethylester (Mobitec, Göttingen, Germany) for 15 min on a laser scanning confocal microscope (Zeiss LSM 5 PASCAL, Göttingen, Germany). Cells were washed with Tyrode’s
solution. Fluo-4 was excited via an argon laser (488 nm; 30 mW) and emitted fluorescence (F) was collected through a 505 nm long-pass emission filter. Changes in fluo-4 fluorescence (indicating fluctuation in cytosolic Ca\textsuperscript{2+}) were recorded in frame and line scan mode while the cells were beating spontaneously. The images were acquired and analyzed using Zeiss software and fluorescence signals were normalized to basal cell fluorescence after fluo-4 loading (F\textsubscript{0}). Intracellular Ca\textsuperscript{2+} was assessed using line scan modus and calibrated by the following pseudo-ratio equation\textsuperscript{2} \[ [\text{Ca}]_i = \frac{K_d(F/F_0)}{(K_d/([\text{Ca}]_i\text{rest}+1) - F/F_0)} \] with $K_d=1100$ nmol/L and $[\text{Ca}]_i\text{rest}=100$ nmol/L.

RT-PCR Analysis
Differentiating EBs at various stages were collected for total RNA isolation (Promega). 150 ng of DNase-treated RNA was used for first-strand cDNA synthesis. One-tenth of the resulting cDNA was taken as PCR template and amplified for 25-35 cycles. GAPDH was used as an internal control. Sequences of oligonucleotide primers used for RT-PCR analyses are available from the authors on request.

Transplantation of maGSCs
The present study used female C57BL/6 mice obtained from the animal facility of the University of Goettingen. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the local government authority. Immunosuppression with cyclosporine A (Sandimmun, Novartis) was applied to all animals used. Cyclosporine A was diluted 1:50 in 5% glucose and stored at -20°C. The dilution was injected once daily intraperitoneally so that the resulting dose was 10 mg/kg body weight.

Intramyocardial Injection of maGSCs
Mice were anesthetized with isoflurane, intubated with a 22 gauge (G) plastic cannula and ventilated with a mixture of 2% isoflurane in ambient air (150 breaths/min, tidal volume 150
µl) by use of a MiniVent (Type 845, Harvard Apparatus). The heart was exposed by a left lateral thoracotomy via the 4th intercostal space and the pericardium was opened. Five hundred thousand to one million cells (SSC5) labelled with a fluorescent carbocyanine dye CM-Dil (Invitrogen) were injected in a volume of 50 µl by use of a 30G steel cannula connected to a 50 µl Hamilton syringe via a PE10 tube. The total volume was applied in 5-7 injections of 7-10 µl each covering the area of the anterolateral wall of the heart that was accessible via the thoracotomy. Afterwards, the thorax was closed with single sutures before the skin was adapted with polypropylene 6-0 sutures. The mice were placed on a heating pad to recover until fully awake.

Intravenous Application of maGSCs

Mice were anesthetized and ventilated as described above. The jugular vein was exposed via a median cervical incision. Cannulation of the vein was performed with a 30G steel cannula connected to an insulin syringe with a 29G needle via a PE10 tube. One million maGSCs labelled with CM-Dil were injected in a volume of 100 µl. After the injection the skin was closed and the mice were allowed to recover as described above.

Histochemical and Immunohistological Analysis of Hearts

Hearts were harvested, fixed in 4% buffered-formalin, embedded in paraffin and sectioned at 6 µm on a microtome. Haematoxylin-Eosin (H&E) staining and Masson-Goldner trichrome staining of the sections was performed on an automated immunostainer (Ventana Medical Systems, Tucson, AZ) according to the manufacturer's protocol. For immunohistology, non-specific immunoreactivity was blocked by incubation of sections in 4% BSA. The antibody panel used included monoclonal mouse anti-Ki-67 (Dako) and anti-Oct3/4 (Santa Cruz Biotechnology) at dilutions of 1 : 50; monoclonal mouse anti-cardiac troponin T (cTnT; LabVision, Fremont, CA) at a dilution of 1 : 1000; monoclonal mouse anti-smooth muscle-α-actin (SM-α-actin; Sigma) at a dilution of 1:8000; polyclonal rabbit anti-von Willebrand factor (vWF; Dako) at a dilution of 1:100. The antibodies Ki-67 and Oct3/4 were detected with
Sections incubated with cTnT, vWF and SM-α-actin antibodies were reacted with a fluorescein-conjugated secondary antibody.

Data Analysis and Statistics

All data was expressed as mean value ± standard error of the mean (SEM). Student’s unpaired t-test was used to test for significant difference. Double sided P-values of P<0.05 were considered significant.


**Supplementary Table 1** Characteristics of APs in maGSC-derived single cardiomyocytes

<table>
<thead>
<tr>
<th></th>
<th>Pacemaker-like</th>
<th>Ventricle-like</th>
<th>Atrial-like</th>
<th>Purkinje-like</th>
<th>Intermediate</th>
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<tr>
<td>AP Amplitude (mV)</td>
<td>62.8±6.9</td>
<td>93.1±3.0</td>
<td>88.3±5.6</td>
<td>96.4±5.3</td>
<td>73.6±3.2</td>
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<td>APD 90 (ms)</td>
<td>227.7±12.4</td>
<td>314.8±21.3</td>
<td>162.8±19.1</td>
<td>287.6±23.9</td>
<td>338.8±15.8</td>
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<tr>
<td>APD 80 (ms)</td>
<td>203.3±12.3</td>
<td>281.7±20.8</td>
<td>120.2±14.9</td>
<td>185.8±17.3</td>
<td>310.4±15.1</td>
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<tr>
<td>Max. dV/dt (Vs⁻¹)</td>
<td>2.5±0.6</td>
<td>31.6±3.8</td>
<td>43.5±5.4</td>
<td>49.4±6.2</td>
<td>2.9±0.6</td>
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<tr>
<td>MDP (mV)</td>
<td>-38.1±3.4</td>
<td>-54.5±2.0</td>
<td>-56.9±2.0</td>
<td>-57.4±2.4</td>
<td>-42.6±2.3</td>
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</table>

Data are mean±SEM. *n* indicates the cell number; APA, AP amplitude; APD90/APD80, AP duration measured at 90% or 80% repolarization; Max. dV/dt, maximum rate of rise of AP; and MDP, maximum diastolic potential.
Supplementary Figure Legends

Supplementary Figure 1
Real time [Ca$^{2+}$]$_i$ transients. Frames (recorded every ~149 ms) of one [Ca$^{2+}$]$_i$ transient cycle of the cardiomyocyte shown in Figure 5A are presented.

Supplementary Figure 2
Absence of cardiac differentiation of maGSCs after transplantation into the normal heart. Immunostaining of the transplants using an antibody against cardiac Troponin T (cTnT) showed that DiI-labeled transplants (A, D) were negative for cTnT at 1 week (B) and 1 month (E). (C) Overlay of A and B. (F) overlay of D and E. Scale bar, 25 µm. Nuclear staining with DAPI.

Supplementary Video Legends

Supplementary Video 1
Short video sequence of real time [Ca$^{2+}$]$_i$ transients as detected by fluo-4 fluorescent dye and recorded every 149 ms.