Mechanisms controlling the acquisition of a cardiac phenotype by liver stem cells

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Communicated by Harry Rubin, University of California, Berkeley, CA, January 19, 2007 (received for review October 9, 2006)

The mechanisms underlying stem cell acquisition of a cardiac phenotype are unresolved. We studied early events during the acquisition of a cardiac phenotype by a cloned adult liver stem cell line (WB F344) in a cardiac microenvironment. WB F344 cells express *a priori* the transcription factors GATA4 and SRF, connexin 43 in the cell membrane, and myoinositol 1,4,5-triphosphate receptor in the perinuclear region. Functional cell–cell communication developed between WB F344 cells and adjacent cocultured cardiomyocytes in 24 h. *De novo* cytoplasmic $[Ca^{2+}]_c$ and nuclear $[Ca^{2+}]_{nu}$ oscillations appeared in WB F344 cells, synchronous with $[Ca^{2+}]_i$ transients in adjacent cardiomyocytes. The $[Ca^{2+}]$ oscillations in the WB F344 cells, but not those in the cardiomyocytes, were eliminated by a gap junction uncoupler and reappeared with its removal. By 24 h, WB F344 cells began expressing the cardiac transcription factors Nkx2.5, Tbx5, and cofactor myocardin; cardiac proteins 24 h later; and a sarcomeric pattern 4–6 days later. Myoinositol 1,4,5-triphosphate receptor inhibition suppressed WB F344 cell $[Ca^{2+}]_{nu}$ oscillations but not $[Ca^{2+}]_c$ oscillations, and L-type calcium channel inhibition eliminated $[Ca^{2+}]$ oscillations in cardiomyocytes and WB F344 cells. The use of these inhibitors was associated with a decrease in Nkx2.5, Tbx5, and myocardin expression in the WB F344 cells. Our findings suggest that signals from cardiomyocytes diffuse through shared channels, inducing $[Ca^{2+}]$ oscillations in the WB F344 cells. We hypothesize that the WB F344 cell $[Ca^{2+}]_{nu}$ oscillations activate the expression of a cardiac specifying gene program, ushering in a cardiac phenotype.

calcium signal transduction | cardiomyogenesis | gap junctions

Extracardiac stem cell use in early phase clinical trials and animal experiments has raised the possibility that stem cell therapy may be able to repair the damaged heart (for review see refs. 1 and 2). The mechanisms underlying the acquisition of a cardiac phenotype by stem cells have not been elucidated. In some cases, fusion between stem cells and cardiomyocytes has been proposed as the basis for the apparent "transdifferentiation" (3). We (4) and others (5, 6) have demonstrated that extracardiac stem cells can acquire a cardiac phenotype when placed in a cardiac microenvironment without the benefit of fusion with surrounding myocytes.

We have found that cells from a stem cell line (WB F344) derived from a cloned single nonparenchymal epithelial cell isolated from the liver of a young adult rat (for review see ref. 7) respond *in vivo* (8) and in culture (4) to signals from a cardiac microenvironment and acquire a cardiomyocyte phenotype. Understanding the mechanisms that regulate the acquisition of a cardiac phenotype by a clone-derived stem cell (for example, WB F344 cells) may prove valuable in selecting and manipulating stem cells from other sources, e.g., autologous stem cells, and so enhance their potential for successful use in cell therapy.

In the current study we examined the earliest events associated with the acquisition of a cardiomyocyte phenotype by WB F344 cells cocultured with rat neonatal heart cells, a cardiac environment model system (4). We find that cell–cell communication through shared and functional gap junction channels is established between WB F344 cells and adjacent neonatal myocytes as early as 24 h in coculture. This communication is associated with novel cytoplasmic ($[Ca^{2+}]_c$) and nuclear ($[Ca^{2+}]_{nu}$) calcium oscillations in the WB F344 cells that are synchronous with $[Ca^{2+}]_i$ transients in juxtaposed myocytes. The $[Ca^{2+}]_{nu}$ oscillations require myoinositol 1,4,5-triphosphate receptor (IP3R) stimulation and are associated with *de novo* expression of cardiac transcription factors in the WB F344 cells. We hypothesize that collectively these events usher in the acquisition of a cardiac phenotype in the WB F344 stem cell line.

Results

Early Intercellular Communication Between Neonatal Myocytes and WB F344 Cells. Because WB F344 cells express connexin 43 (Cx43), the predominant isoform expressed in ventricular myocytes, we examined whether myocytes and WB F344 cells develop shared Cx43-derived gap junctions. Twenty-four hours after dsRed fluorescent WB F344 cells were cocultured with heart cells, functional cell–cell communication between their cytoplasm and juxtaposed neonatal cardiomyocytes was demonstrated by fluorescence recovery after photobleaching (Fig. 1). When in older cocultures (4–6 days old) WB F344 cells had acquired a cardiac phenotype, Cx43 was preferentially localized at the interface between neonatal cardiomyocytes and differentiated WB F344-derived myocytes, as we previously reported (4) [supporting information (SI) Fig. 7].

***De Novo* Calcium Oscillations in the WB F344 Cells.** dsRed fluorescent WB F344 cells were examined for $[Ca^{2+}]$ signals 18–24 h after their addition to primary neonatal rat heart cell cultures. $[Ca^{2+}]_c$ oscillations were detected in the cytoplasm of approximately 1 in 20 WB F344 cells that were adjacent to a neonatal cardiomyocyte (Figs. 2 and 3*A*). The WB F344 cell $[Ca^{2+}]_c$ oscillations had the same frequency but lower amplitude than $[Ca^{2+}]_i$ transients in adjacent spontaneously contracting or paced cardiomyocytes (Fig. 2). The calcium transients in the spontaneously beating neonatal myocyte, measured at different distances from the cell membrane, had the same kinetics and amplitude. This suggests that calcium-induced calcium release is the mechanism underlying the calcium transients in these myocytes. In contrast, WB

Author contributions: W.E.C., W.B.C., J.W.G., P.A.W.A., and N.N.M. designed research; B.J.M.-B., G.L.E., H.-S.K., and N.N.M. performed research; W.B.C. and J.W.G. contributed new reagents/analytic tools; B.J.M.-B., G.L.E., H.-S.K., and N.N.M. analyzed data; and N.N.M. wrote the paper.

The authors declare no conflict of interest.

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Abbreviations: Cx43, connexin 43; IP3R, myoinositol 1,4,5-triphosphate receptor; cVDCC, cardiac L-type voltage-dependent calcium channel; 2-APB, 2-aminoethoxydiphenyl borane.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0700416104/DC1.

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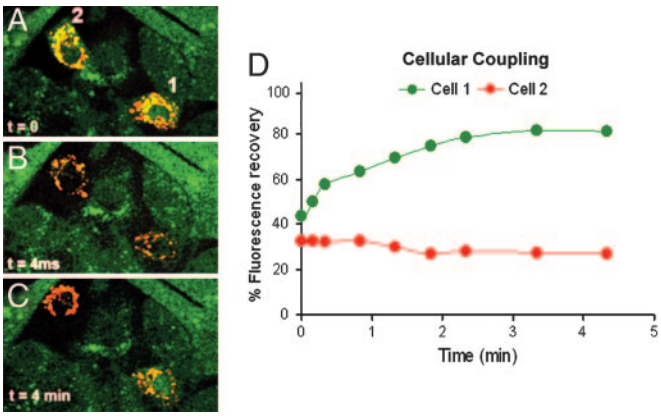

Fig. 1. Gap junction-mediated cell–cell communication recorded in early (24–48 h) WB F344/cardiomyocyte cocultures. (*A* and *B*) Calcein-labeled coculture with WB F344 cell 1 and cell 2 adjacent to cardiomyocytes before laser excitation (*A*) and immediately after exposure to high-intensity laser excitation (*B*). (*C*) Fluorescence recovery in WB F344 cell 1 through shared gap junction channels with adjacent cardiomyocytes. It is presumed that WB F344 cell 2 was not functionally coupled with adjacent myocytes because no fluorescence recovery was recorded. (*D*) Graph of fluorescence recovery vs. time in WB F344 cell 1 and cell 2.

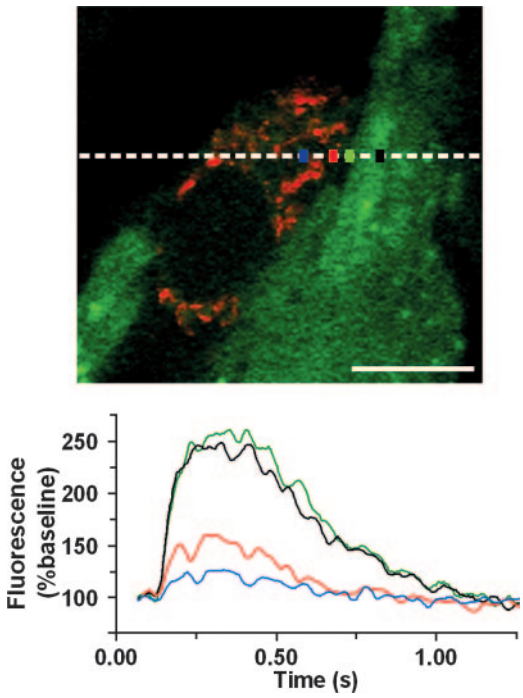

Fig. 2. Calcium signals acquired from undifferentiated WB F344 cells and adjacent cardiomyocytes. (*Upper*) Image of dsRed WB F344 cell adjacent to cardiomyocyte in coculture labeled with fluo-4 AM. Confocal line scan microscopy was used to record intracellular calcium oscillations along the dotted line. (*Lower*) Tracings correspond to the areas marked by the color-coded dots in the line scan. Shown are signal-averaged calcium signals at the immediate cardiomyocyte (green)/WB F344 cell (red) interface, 4 μm away from the cell interface in the WB F344 cell (blue), and 4 μm away from the interface in the cardiomyocyte (black). Decreased amplitude of the $[Ca^{2+}]$ signal is demonstrated in the WB F344 cell with distance from the cell interface (red and blue lines) suggesting a diffusion-mediated signal but not in the cardiomyocyte (green and black lines), where a uniform $[Ca^{2+}]_i$ within the cardiomyocyte is believed to be mediated by a calcium-induced calcium release process. The calcium oscillations in the WB F344 cells depend on direct contact between the WB F344 cells and adjacent myocytes. WB F344 cells cultured alone or distant from cardiomyocytes did not demonstrate oscillations even when paced, suggesting that a humoral factor secreted into the medium did not induce these oscillations. (Scale bar: 10 μm.)

F344 cell $[Ca^{2+}]_c$ oscillation amplitude and rate of rise were highest in the region where the WB F344 cell membrane was closest to that of the cardiomyocyte and decreased with distance away from the cardiomyocyte, suggesting a diffusion-mediated calcium signal rather than calcium-induced calcium release (Fig. 2). WB F344 cells cultured alone or in coculture but distant from cardiomyocytes did not demonstrate any $[Ca^{2+}]$ oscillations or change in phenotype, suggesting that a humoral factor released into the culture medium did not induce these transformations. After addition of the gap junction blocker carbenoxolone, the WB F344 cell $[Ca^{2+}]_c$ signals were eliminated, whereas the cardiomyocyte $[Ca^{2+}]_i$ transients were maintained. After carbenoxolone removal, the WB F344 cell $[Ca^{2+}]_c$ oscillations reappeared (Fig. 3*B*). The WB F344 cell nucleus makes up a large portion of the cell volume. Nuclear $[Ca^{2+}]_{nu}$ oscillations were detected in ≈1 in 40 WB F344 cells, usually when the nucleus was no more than 2–3 μm away from an adjacent cardiomyocyte (Fig. 3*A*, line 2). The WB F344 cell $[Ca^{2+}]_{nu}$ oscillations were most prominent in the perinuclear region and had the same frequency as those in the cytoplasm (Fig. 3*C*).

In agreement with other investigators (9), we found using immunocytochemistry that WB F344 cells express IP3R that is preferentially localized in the perinuclear region and endoplasmic reticulum (Fig. 3*D*). The IP3R inhibitors, 2-aminoethoxydiphenyl borane (2-APB), used at 4 μmol/liter, and xestospongin C, used at 5 μmol/liter (not shown), suppressed the nuclear $[Ca^{2+}]_n$ oscillations in the WB F344 cells but not the cytoplasmic $[Ca^{2+}]_c$ oscillations in either the WB F344 cells or the cardiomyocytes (Fig. 3*C*). Nifedipine, an L-type calcium channel antagonist, used at 1 μmol/liter eliminated the cardiomyocyte $[Ca^{2+}]_i$ transients and the WB F344 cell $[Ca^{2+}]_c$ and $[Ca^{2+}]_{nu}$ oscillations (SI Fig. 8).

Acquisition of a Cardiac Phenotype. We examined the expression of transcription factors that combine to specify a cardiac phenotype (Nkx2.5, Tbx5, GATA4, SRF, myocardin, and Mef2c) (for review see ref. 10) in dsRed fluorescent WB F344 cells that were cultured alone and in coculture with heart cells. WB F344 cells cultured with neonatal heart cells and harvested by FACS demonstrated *de novo* expression of Nkx2.5, Tbx5, and myocardin, and up-regulation of Mef2c as early as 24 h after coculture (Fig. 4). The expression of these transcription factors increased further by 72 h (Fig. 4). The expression of GATA4 and SRF was not changed (data not shown).

Expression at the RNA level of the cardiac specific proteins cardiac troponin T (data not shown) and cardiac troponin I (data not shown), and the cardiac L-type voltage-dependent calcium channel (cVDCC) (Fig. 4), was detected in the WB F344 cells 48–72 h after the initiation of coculture. Four to 7 days later some WB F344 cells adjacent to cardiomyocytes acquired a cardiac phenotype, as previously described (4). They exhibited a striated sarcomeric pattern that contained cardiac troponin T (SI Fig. 9). In seeking further evidence of gene reprogramming in WB F344 cells cocultured with neonatal cardiac cells, we examined the expression of a stem cell marker (c-Kit, a stem cell factor receptor) in WB F344 cells cultured alone or with neonatal heart cells. This was compared with the expression of cardiac myocyte proteins, the cVDCC, and the sarcomeric proteins cardiac troponin T and cardiac troponin I. We found in WB F344 cells cocultured with heart cells that c-Kit expression decreased with time in culture as the expression of cVDCC increased (Fig. 4).

Expression of Nkx2.5, Tbx5, and myocardin was significantly decreased in the presence of the IP3R inhibitor 2-APB, used

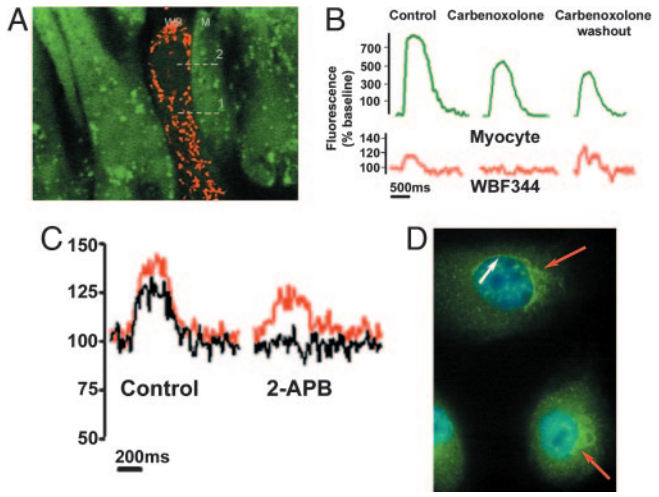

Fig. 3. Inhibition of calcium signals acquired from undifferentiated WB F344 cells cocultured with cardiomyocytes. (*A*) Representative image of undifferentiated WB F344 cell adjacent to cardiomyocyte in coculture. Cardiomyocyte $[Ca^{2+}]_i$ transients and adjacent WB F344 cell $[Ca^{2+}]_c$ oscillations or cardiomyocyte $[Ca^{2+}]_i$ transients and adjacent WB F344 cell $[Ca^{2+}]_n$ oscillations were acquired by line scan confocal microscopy in the region along dotted line 1 or line 2, respectively. (*B*) WB F344 cell $[Ca^{2+}]_c$ oscillations (red) were synchronous with the cardiomyocyte $[Ca^{2+}]_i$ transients (green) during control conditions. The line scan was recorded from an area that corresponds to the dotted line 1 in *A*. WB F344 cell $[Ca^{2+}]_c$ oscillations were abolished in the presence of the gap junction uncoupler carbenoxolone, whereas the cardiomyocyte $[Ca^{2+}]_i$ transients were maintained. Approximately 30 min after the carbenoxolone was washed out of the culture medium, the WB F344 cell $[Ca^{2+}]_c$ oscillations were restored. (*C*) Inhibition of WB F344 cell $[Ca^{2+}]_{nu}$ oscillations. Cytoplasmic $[Ca^{2+}]_c$ (red) and nuclear $[Ca^{2+}]_{nu}$ (black) signals were recorded in a WB F344 cell in coculture before and after the addition of the IP3R inhibitor 2-APB. The tracings represent calcium signals recorded from a WB F344 cell nuclear region that corresponds to dotted line 2 in *A*. The WB F344 cell $[Ca^{2+}]_{nu}$ signals (black) were significantly suppressed in the presence of the IP3R inhibitor. (*D*) IP3R was localized by immunocytochemistry to the perinuclear region (white arrow and green fluorescence in *D*) and the endoplasmic reticulum (red arrows) of WB F344 cells. *D* is a merged image of FITC fluorescence and DAPI nuclear counterstain (×63 oil immersion objective lens).

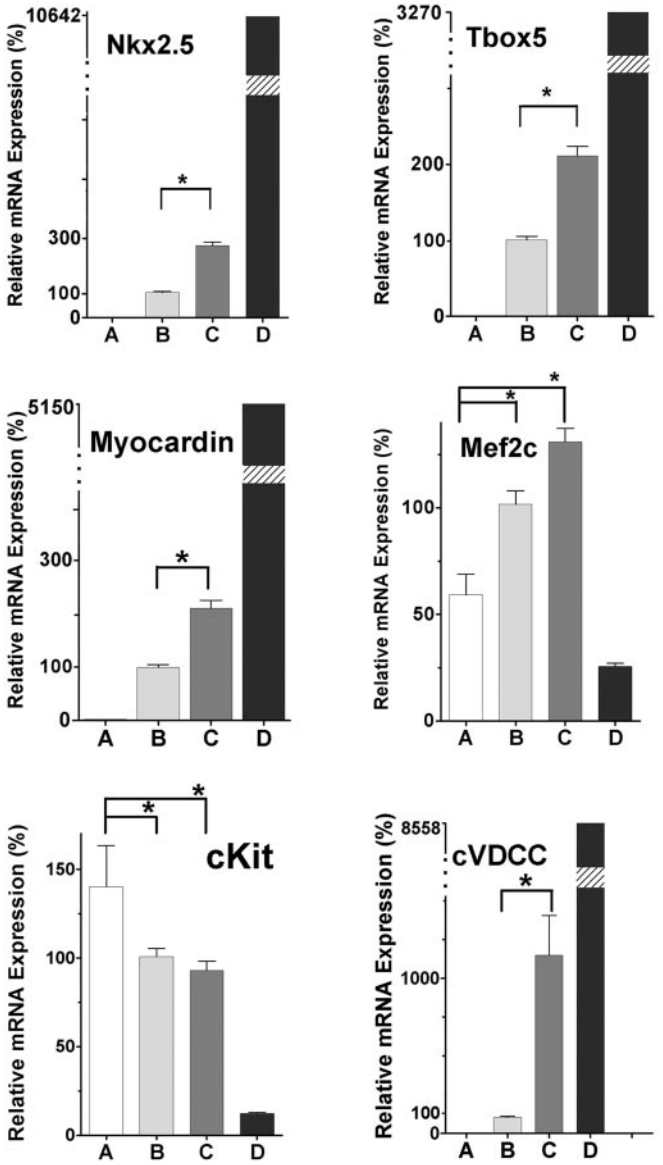

Fig. 4. RNA levels (expressed as fold change) of cardiac transcription factors, Nkx2.5, Tbx5, myocardin, and Mef2c, WB F344 cells cultured alone (column A), WB F344 cells cocultured with heart cells for 18–48 h (column B), WB F344 cells cocultured with heart cells for 48–72 h (column C), and cardiac cells cultured alone (column D). Gene reprogramming is noted by the decrease in expression of c-Kit and the increase in expression of the cVDCC with time in culture in the same conditions. The bars show mean ± SEM. *, $P < 0.001$. (See primer sequences in SI Table 1.)

overnight in the culture medium at 4 μmol/liter (Fig. 5). This inhibitor suppressed the WB F344 cell $[Ca^{2+}]_{nu}$ oscillations (Fig. 3*C*). The expression of Nkx2.5, Tbx5, and myocardin was significantly decreased in the presence of the L-type calcium channel blocker nifedipine, used overnight in the culture medium (Fig. 5). Nifedipine inhibition did not persist as some myocytes were still contracting after exposure to 4 μmol/liter nifedipine overnight.

The FACS-harvested dsRed fluorescent WB F344 cells contained a mixed population of WB F344 cells; a small population of WB F344 cells that had formed couplings with myocytes and a majority of WB F344 cells that were seeded either next to nonmyocyte heart cells or away from cardiomyocytes. This majority of WB F344 cells proliferated and formed nests of cells with a typical WB F344 cell phenotype. With time in culture, these proliferating WB F344 cells became an increasingly larger proportion of the WB F344 cell population, decreasing the relative number of FACS-harvested WB F344 cells that were responding to signals from adjacent myocytes.

We examined in a sorted FACS dsRed fluorescent WB F344 cell population the extent of contamination with neonatal heart cells using postsort assessments and found that contamination was <1%. This is consistent with the PCR analysis of microsatellite DNA where we exploited the strain-specific allelic differences in DNA microsatellite markers between Sprague–Dawley neonatal rat heart cells and WB F344 cells cloned from an American Fischer 344 (F344) rat. No Sprague–Dawley microsatellite DNA was detected in PCR amplicons from the dsRed fluorescent WB F344 cell population harvested from cocultures with neonatal heart cells (Fig. 6). In control experiments, we were able to detect to <10% contamination by Sprague–Dawley cells in a Fischer cell population. These results also suggest that, if fusion occurred between the Fischer WB F344-derived cells and the surrounding Sprague–Dawley heart cells, this event was infrequent.

Discussion

Overview. We demonstrate in WB F344 cell/neonatal heart cell cocultures that a signal is transmitted from the cardiomyocytes into juxtaposed WB F344 cells through shared functioning,

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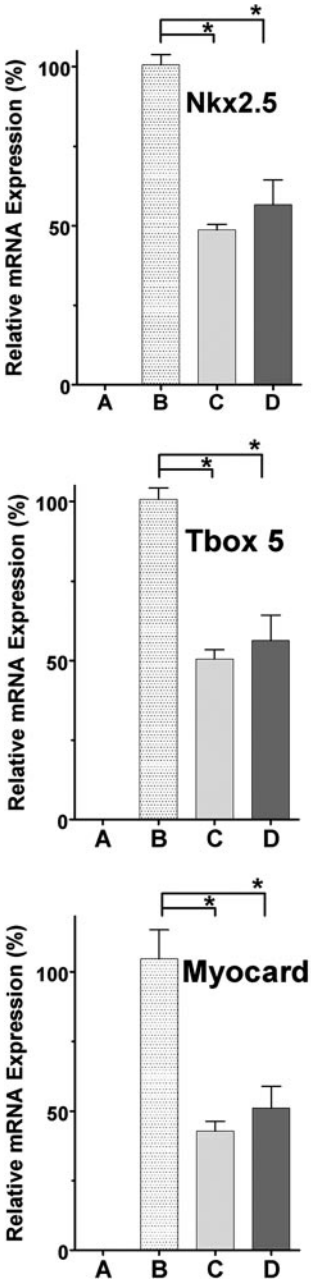

Fig. 5. Suppression of cardiac transcription factor expression with inhibitors. Expression (noted in fold change) of the cardiac transcription factors Nkx2.5, Tbx5, and myocardin in WB F344 cells cultured alone (column A), WB F344 cells cocultured with heart cells for 18–24 h (column B), WB F344 cells cocultured with heart cells in the presence of the IP3R inhibitor 2-APB for 18–24 h (column C), and WB F344 cells cocultured with heart cells in the presence of the cVDCC inhibitor nifedipine for 18–24 h (column D). The bars show mean ± SEM. *, $P < 0.001$.

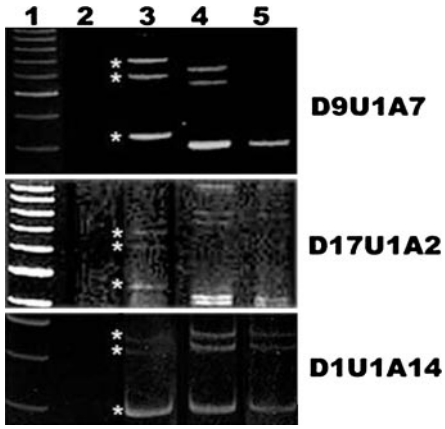

Fig. 6. Polymorphic microsatellite markers. By using the D9U1A7 set of microsatellite markers, the amplicons from the control or FACS-harvested Fischer WB F344 cells DNA (lanes 4 and 5) are shifted down compared with the PCR product from control Sprague–Dawley DNA (lane 3, white asterisks in *Top*). Lane 1, DNA ladder; lane 2, no DNA control. By using a second set of markers, D17U1A2, three amplicons (white asterisks in *Middle*) are present only in the Sprague–Dawley control DNA (lane 3) but not in the harvested WB F344 cells. By using a third set of markers, D1U1A14, the amplicons from the Fischer WB F344 DNA (lanes 4 and 5) are shifted up compared with the PCR product from the Sprague–Dawley DNA (white asterisks in *Bottom*). (See primer sequences in SI Table 2.)

presumably Cx43-derived gap junction channels. This signal induces in the WB F344 cell "cardiac-like" calcium oscillations that have the same frequency as the calcium transients in adjacent cardiomyocytes. The WB F344 cell calcium oscillations are detected in the cytoplasm ($[Ca^{2+}]_c$) and the perinuclear region ($[Ca^{2+}]_{nu}$). The $[Ca^{2+}]_{nu}$ signals appeared to be the product of IP3R stimulation and to be associated with the expression of cardiac transcription factors Nkx2.5, Tbx5, and the cofactor myocardin and up-regulation of Mef2c because both the $[Ca^{2+}]_{nu}$ oscillations and the *de novo* expression of the cardiac transcription factors were suppressed with use of IP3R inhibitors. We hypothesize that these mechanisms contribute, together with prior expression of SRF and GATA4 in the WB F344 cells, to their acquisition of a cardiac phenotype.

Our finding of calcium oscillations in the WB F344 cells early after coculture is consistent with previous investigations in early ES cell-derived cardiomyocytes (11). $[Ca^{2+}]_i$ oscillations were found to be present in ES cells independent of their expression of a cVDCC or ryanodine-sensitive Ca^{2+} release channel.

WB F344 cells in monoculture do not express the cVDCC but begin to express an increasing amount of cVDCC, as some acquire a cardiac phenotype in coculture (Fig. 4). That the calcium oscillations in the WB F344 cells are lost when carbenoxolone, a gap junction uncoupler, is introduced into the medium and are re-acquired with its removal, whereas nifedipine acutely eliminated the calcium transient in the cardiomyocyte and in the WB F344 cell, lead us to conclude that the early calcium oscillations in the WB F344 cells before they acquire a cardiac phenotype are dependent on cardiomyocyte excitation and cell–cell communication between the cardiomyocytes and the WB F344 cells.

Calcium Signaling. Evidence that calcium signals trigger transcriptional responses is mounting (12–26). Calcium-driven transcription of cell-specific genes has been described in lymphocytes (15, 16), neuronal cells (17), smooth muscle cells (18), skeletal muscle (19), and cardiomyocytes (14, 20, 21). In the heart, transcription under physiological conditions is believed to be "under a constant Ca^{2+}-dependent surveillance" (14). Calcium sensing cascades through calmodulin and its downstream effectors, CaMK (22) and calcineurin (15), PKC (23), and Ras (24) have been reported to transduce calcium signals into transcriptional responses specific for each cell type. Furthermore, it has been suggested that the diversity in gene transcription responses depends on the amplitude, frequency, and duration of the calcium signal and its spatiotemporal properties (12, 22, 25, 26). Oscillating nuclear calcium signals have been reported to increase the efficiency and specificity of gene transcription (25, 26) and are believed to regulate the expression of transcription

factors that are different from factors regulated by cytoplasmic calcium signals (27–29). Nuclear calcium signals are suspected to be triggered by the release of calcium from the nuclear envelope calcium stores through nuclear membrane IP3R stimulation (21, 30). As we demonstrate here and as previously reported by other investigators (9), WB F344 cells express IP3R localized to the perinuclear region. The IP3R inhibitor suppressed the $[Ca^{2+}]_{nu}$ signals in the WB F344 cells and were associated with a significant decrease in expression of the cardiac transcription factors, Nkx2.5, Tbx5, and myocardin, suggesting that at this early stage the process may be reversible. These findings lead us to hypothesize that cardiac-like $[Ca^{2+}]_{nu}$ oscillations in the WB F344 cell have a role in triggering the expression of a cardiac specifying gene program that results in their acquisition of a cardiac phenotype.

Cell Coupling. We (4) and others (6, 31, 32) have found that stem cell expression of Cx43 and their close proximity with cardiomyocytes may be important in the stem cell acquiring a cardiac phenotype in culture. Cx43-derived gap junctions have been reported to be permeable to small molecules such as IP3 and calcium ions (for review see ref. 33). Because the IP3R inhibitors, xestospongin C and 2-APB, did not eliminate $[Ca^{2+}]_c$ oscillations in the WB F344 cells, we suggest that these oscillations were not the result of IP3 triggering release of calcium from the WB F344 cell cytoplasmic stores. Rather another mechanism such as calcium ions diffusing from the myocyte through shared gap junction channels may have taken place. Our finding that the WB F344 cell $[Ca^{2+}]_c$ oscillations decrease in amplitude with distance from the juxtaposed cardiomyocyte, potentially the product of a calcium-diffusion coefficient, is consistent with this interpretation. In contrast, we find that the nuclear $[Ca^{2+}]_{nu}$ oscillations in the WB F344 cells are dependent on IP3R activity in the perinuclear region.

Functional Cx43-derived gap junctions between stem cells and adjacent myocytes appear to provide a conduit through which the stem cell receives signals from the myocyte. We suggest that Cx43 expression is one of the important criteria to be used in the selection of stem cells for potential therapy in the heart. In agreement with our results, stem cells known to develop anatomical couplings with myocytes and to express Cx43 (31) such as mesenchymal stem cells (6, 32) have been demonstrated to acquire a well differentiated cardiac phenotype. Failure to express Cx43 may underlie the potential life-threatening dysrhythmias observed when other types of stem cells, such as skeletal myoblasts, have been used in stem cell therapy in the heart (2). Cell–cell communication that allows electrical coupling between the donor stem cell-derived cardiomyocyte and the recipient cardiomyocyte appear to be an important criterion for successful stem cell treatment of the patient with a failing heart.

Materials and Methods

Cell Cultures and Genetic Modification of WB F344. WB F344 cells and 1-day-old neonatal Sprague–Dawley rat ventricular cells were cocultured as previously described (4). WB F344 cells were transfected with a plasmid that encodes a dsRed fluorescent protein targeted to the mitochondria following the vendor's specifications (BD Biosciences Clontech Laboratories, Palo Alto, CA). They were seeded with neonatal heart cells at a ratio of 1/10 to 1/100 and maintained in culture up to 6 days.

Fluorescence Recovery After Photobleaching. Fluorescence recovery after photobleaching measured cell–cell communication via functional gap junction channels between dsRed fluorescent WB F344 cells, cultured alone or with heart cells. All cells were intracellularly labeled with the fluoroprobe calcein AM, as previously described (4). Clean diffusion through functional gap junctions into the cytoplasm of a bleached WB F344 cell from an adjacent unbleached cardiomyocyte was recorded for 10 min at 30- to 120-sec intervals.

Confocal Imaging of Calcium Signals. dsRed fluorescent WB F344 cells, cultured alone or in coculture with heart cells, were incubated in the calcium sensitive fluoroprobe, fluo-4 AM (5 μmol/liter; Molecular Probes, Eugene, OR) for 30 min at 20°C and subsequently washed several times with dye-free, phenol-red free Richter's medium plus 2% horse serum before measurements were recorded.

Confocal fluorescence images were acquired to record cell position, alignment, and geometry with a LSM510 inverted laser-scanning confocal microscope with high-resolution optics ($\times$63/1.4 N.A. oil immersion objective and environmental chamber; Carl Zeiss Microimaging, Thornwood, NY). The line scan mode with an excitation wavelength of 488 nm and an emission filter wavelength of 515 $\pm$ 15 nm were used for quantitative analysis of $[Ca^{2+}]$ signals. A line spanning a neonatal cardiomyocyte and an adjacent WB F344 cell was defined, and fluorescence emission was collected at a rate of 1.92 ms per 1,024-pixel line. In separate experiments, a line was selected that also spanned cardiomyocyte and the adjacent WB F344 cell and its nucleus (Fig. 3*A*, line 2). The cardiomyocytes beat spontaneously or were electrically stimulated with a platinum bipolar electrode, using rectangular pulses, 1 ms in duration, 2$\times$ threshold voltage, 1 Hz (Grass Instruments, Quincy, MA). The resulting time series images were analyzed with Image J software. Fluorescence signals were normalized by dividing the cell fluorescence (F) by cell fluorescence recorded before stimulation/contraction (F_o).

The effect of inhibiting cell–cell gap junction coupling on the $[Ca^{2+}]$ signals was examined by using the reversible gap junction uncoupler, carbenoxolone (3β-hydroxy-11-oxoolean-12-en-30-oic acid 3-hemisuccinate; Sigma–Aldrich, St. Louis, MO). Intracellular calcium signals were recorded at 10 and 30 min after 100 μmol/liter carbenoxolone was added to culture medium and 30 min after the carbenoxolone was washed out. In separate conditions, the cocultures were exposed to the IP3R inhibitors, 2-APB (Tocris, Ellisville, MO) at 4 μmol/liter or xestospongin C (Sigma–Aldrich) used at 5 μmol/liter and to the L-type calcium channel antagonist nifedipine (Sigma–Aldrich) used at 1 μmol/liter. The effect of these inhibitors on WB F344 cell $[Ca^{2+}]_c$ and $[Ca^{2+}]_{nu}$ oscillations was examined before and after the addition of these inhibitors.

PCR Amplification. dsRed fluorescent WB F344 cells were harvested from cocultures on days 1–3 by using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with a 70- or 100-μm nozzle, a 488-nm argon laser for excitation of the dsRed protein, and a 530 $\pm$ 15-nm bandpass filter for monitoring fluorescent emission. Postsort assessments examined the extent of contamination of the sorted fluorescent WB F344 cell population by nonfluorescent neonatal cardiac cells.

Real-time RT-PCR was carried out, as previously described (34) (see SI Table 1 for primer and probe sequences), in triplicate on RNA isolated from WB F344 cells cultured alone and from WB F344 cells cocultured with neonatal heart cells in the absence or presence of 2-APB (4 μmol/liter) or nifedipine (4 μmol/liter) for 18–24 h. The expression of the cardiac specifying transcription factors Nkx2.5, Tbx5, GATA4, SRF, the cofactor myocardin and Mef2c, the cardiac proteins cardiac troponin T and cardiac troponin I, and the cVDCC was examined over time in culture. The expression of the stem cell marker c-Kit was examined in WB F344 cells before and after coculture with neonatal heart cells.

Fischer Versus Sprague–Dawley Genomic Microsatellite Amplification. The WB F344 cell line was isolated and cloned from an American Fischer 344 (F344) rat, and the neonatal cardiomyocytes were harvested from Sprague–Dawley rats. Genomic DNA microsatellite markers (primer sequences in SI Table 2) were amplified by PCR, and the purity of the dsRed fluorescent WB F344 cell population harvested by FACS from cocultures with neonatal cardiomyocytes was examined. PCRs were run in easy-start PCR tubes (Molecular BioProducts, San Diego, CA) by using the manufacturer's protocol and 70 ng of genomic DNA template, an annealing temperature of 55°C, and 40 cycles of amplification in a total volume of 20 μl. The product was analyzed on 8% polyacrylamide (30% acrylamide: 0.8% Bis-acrylamide, Ultra Pure Protogel; National Diagnostics, Atlanta, GA) at 100 V for 4 h. Ethidium bromide staining and visualization on a UV Transilluminator revealed whether or not samples contained chimeric amplicon patterns (Fig. 6). We tested our ability to detect contamination in WB F344 cell populations with Sprague–Dawley cells by mixing different proportions of the two cell populations and by using the microsatellite markers to distinguish between the two cell types through a distinct PCR amplicon pattern (data not shown). This analysis was furthermore used to evaluate the presence and extent of cell and/or nuclear fusion between the Fischer-derived WB F344 cells and Sprague–Dawley neonatal heart cells.

Immunocytochemistry. dsRed fluorescent WB F344 cells were cocultured with neonatal heart cells on laminin-coated coverslips. Immunocytochemistry was done as previously described (4). We investigated the expression and localization of IP3R in the WB F344 cells using an IP3R antibody that recognizes all three IP3R isoforms (Chemicon, Temecula, CA). FITC-labeled anti-rabbit antibody was used as the secondary antibody (Rockland Immunochemicals, Gilbertville, PA). All immunoreactions were run in parallel without primary antibody as controls for background staining. DAPI was used as the nuclear counterstain. Immunofluorescent signals were observed by using a Zeiss LSM5 Pascal Confocal Laser Scanning Microscope equipped with argon laser, HeNe lasers, and a $\times$63 oil immersion objective.

This work was supported in part by National Institutes of Health Grant HL67385.

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