

# Acquired Cell-to-Cell Coupling and “Cardiac-Like” Calcium Oscillations in Adult Stem Cells in a Cardiomyocyte Microenvironment

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**Abstract**— Adult-derived stem cells have recently been found to respond *in vivo* to inductive signals from the microenvironment and to differentiate into a phenotype that is characteristic of cells in that microenvironment. We examined the differentiation potential of an adult liver stem cell line (WBF344) in a cardiac microenvironment *in vitro*. WBF344 cells were established from a single cloned non-parenchymal epithelial cell isolated from a normal male adult rat liver. Genetically modified, WBF344 cells that express  $\beta$ -galactosidase, green fluorescent protein (GFP) or mitochondrial red fluorescent protein (DsRed) were co-cultured with rat neonatal cardiac cells. After 4 -14 days, we identified WBF344-derived cardiomyocytes that were elongated, binucleated and expressed the cardiac specific proteins cardiac troponin T, cardiac troponin I and N cadherin. These WBF344-derived cardiomyocytes also exhibited myofibrils, sarcomeres, and a nascent sarcoplasmic reticulum. Furthermore, rhythmically beating WBF344-derived cardiomyocytes displayed “cardiac-like” calcium transients similar to the surrounding neonatal cardiomyocytes. Fluorescent recovery after photobleaching demonstrated that WBF344-derived cardiomyocytes were electrically coupled with adjacent neonatal cardiomyocytes through gap junctions (GJs). Collectively, these results support the conclusion that these adult-derived liver stem cells respond to signals generated in a cardiac microenvironment *in vitro* acquiring a cardiomyocyte phenotype and function. The identification of micro-environmental signals that appear to cross germ layer and species specificities should prove valuable in understanding the regulation of normal development and stem cell differentiation *in vivo*.

This work was supported by the National Institutes of Health grants RO1 HL42250 and RO1 HL67358)

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## I. INTRODUCTION

Transplantation of stem cells into diseased hearts is actively being investigated as a therapeutic measure to improve cardiac function [1-5]. Cell transplantation therapy delivers isolated cells to injured tissue where the transplanted cells differentiate and remodel in response to the surrounding microenvironment, leading to tissue regeneration and functional repair. Nevertheless, the promise of myocardial repair using stem cells has not been achieved because of low rates of engraftment and associated cardiac arrhythmia. The viability of stem cell transplantation as a therapeutic modality will require that the stem cell be fully integrated into the host tissue. Integration will depend on: 1.) the physical incorporation of the cell into the cardiac syncytium, and 2.) the differentiation into a phenotype supporting normal electrical propagation, electromechanical coupling and contraction. We found that cells from a stem cell line (WBF344) derived from a cloned single non-parenchymal epithelial cell, from an adult rat liver [6], respond to signals in a cardiac microenvironment, *in vivo* [7], and acquire a differentiated cardiomyocyte phenotype. Yet, the process of engraftment and ability of these stem cells to functionally integrate electrically and mechanically with host cardiac tissue is not understood and is technically challenging to study *in vivo*.

The focus of this research was to use an *in vitro* cardiac cell microenvironment to facilitate the study of stem cell engraftment and differentiation to a cardiac phenotype. Our preliminary studies suggest that a determining factor in successful donor cell engraftment and differentiation is the cardiac cell microenvironment, i.e. WBF344 stem cells do not differentiate in the absence of cardiomyocytes or in media conditioned with cardiomyocytes. A two-dimensional cardiac cell culture system provides control over host tissue composition, structure and the microenvironment. This modeled microenvironment is important for developing strategies to enhance the efficiency of stem cell engraftment as well as accelerating cellular coupling and the development of adult-like membrane characteristics.

## II. MATERIALS AND METHODS

### *Cardiac Microenvironment*

A cardiac microenvironment was generated by

establishing cardiomyocyte cultures. Neonatal cardiomyocytes were isolated from the hearts of 1 day-old Sprague-Dawley rats in accordance with accepted guidelines for the care and treatment of experimental animals at the University of North Carolina School of Medicine and the National Institutes of Health. Neonatal cardiomyocytes were isolated, plated on laminin-coated cover slides and grown in Richter's medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal calf serum. Cardiomyocyte cultures were maintained for 48 hours before WBF344 cells were added.

#### *WBF344 Cell Labeling*

To identify WBF344 cells in the cardiac microenvironment, WBF344 cells intended for immunocytochemistry were genetically modified to express the *Escherichia coli* lac Z gene ( $\beta$ -galactosidase). WBF344 cells intended for live cell imaging and functional evaluation were transfected with either a plasmid containing a green fluorescent protein gene (GFP) or pDsRed-Mito (BD Biosciences Clontech Laboratories, Palo Alto, CA). Briefly, cells were transfected and grown in Richter's medium supplemented with 10% FCS for 48 hrs. Fluorescent cells were fluorescence activated cell sorted (FACS), amplified in culture, and cloned at limiting dilutions. Genetically modified or transfected WBF344 cells were harvested from subconfluent cultures, washed, and resuspended in cell culture medium. The WBF344 cells were counted and plated at 300 to 500 cells per 22-mm cover slide with the neonatal cardiac cells. The co-cultures were grown in a 5% CO<sub>2</sub>, 95% air environment at 37°C and interrupted at 4 to 14 days. In control experiments, WBF344 cells were grown in conditioned medium collected from 4- to 6-day-old neonatal cell cultures. Furthermore, neonatal cells were cultured on 0.02- $\mu$ m Anopore membrane inserts (Nunc, Naperville, IL) that physically separated them from WBF344 cells in the same culture.

#### *Immunocytochemistry*

Genetically modified WBF344 cells grown on cover slides were rinsed in a sodium phosphate buffer and fixed in a 2% formaldehyde, sodium phosphate buffer, pH 7.3. After three washes with phosphate buffer, the  $\beta$ -galactosidase reaction was performed by incubating the fixed cells in the X-gal substrate for 2 to 4 hours. Expression of cardiac-specific proteins was demonstrated using antibodies against cardiac myosin heavy chain (Chemicon International, Temecula, CA), cardiac troponin I (cTnI), cardiac troponin T (cTnT) and connexin 43 (Cx43, Zymed, San Francisco, CA). An anti-myogenin antibody (PharMingen, San Diego, CA) was used to rule out a skeletal muscle phenotype. Rhodamine or fluorescein isothiocyanate anti-rabbit antibodies were used as secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA).

#### *Fluorescence Recovery after Photobleaching (FRAP)*

Functional GJ mediated cell-to-cell communication was measured in WBF344-GFP/cardiomyocyte co-cultures using

a FRAP assay. The cell cultures were labeled by incubation in 1 $\mu$ mol/L SNARF-calcein AM (Molecular Probes, Eugene, OR) in modified Tyrode's for 30 minutes. SNARF-calcein has a molecular weight less than 1000 daltons, allowing it to pass through the connexons of neighboring cells. De-esterification inside the cell renders SNARF-calcein membrane impermeant. The cell cultures were washed several times with dye-free modified Tyrode's before measurements were recorded. Cell cultures were placed on the stage of a Zeiss LSM410 laser-scanning confocal microscope. High-resolution optics were focused on the co-cultures, identifying native cardiomyocytes and GFP transfected WBF344-derived cardiomyocytes. A 488-nm excitation wavelength and 540  $\pm$ 25-nm band pass emission filter were used to locate the WBF344-GFP cells. Using Zeiss software, a region of interest encompassing 90% of a WBF344-derived cardiomyocyte was selected. A high-intensity laser pulse bleached the SNARF-calcein molecules within the region of interest. This caused an immediate loss of SNARF-calcein fluorescence emission. SNARF-calcein diffusion into the bleached cell from adjacent unbleached cardiomyocytes was recorded in subsequent confocal images. Monitoring the fluorescence emission intensity of the SNARF-calcein in the region of interest as a function of time resulted in a single exponential recovery curve, yielding a rate constant for the fluoroprobe transport ( $k = \text{min}^{-1}$ ), a measure of GJ permeability.[8]. Inhibition of cell-to-cell GJ coupling was measured in the co-cultures with FRAP in the presence of 100  $\mu$ mol/L carbenoxolone (Sigma-Aldrich).

#### *Measurement of Intracellular Calcium*

A multisite ratiometric fiber optic recording and imaging system provided high temporal and spatial resolution recordings of intracellular calcium ([Ca<sup>2+</sup>]<sub>i</sub>) transients from whole cells in the co-cultures. WBF344-GFP/cardiomyocyte co-cultures were labeled with indo-1 AM, washed with fluoroprobe-free medium and placed on the stage of an inverted Olympus IMT-2 microscope. Co-cultures were electrically stimulated with a platinum bipolar electrode. [Ca<sup>2+</sup>]<sub>i</sub> transients were recorded with an Olympus 40X/1.4 NA UV oil immersion objective. An excitation wavelength of 350 nm was focused on the co-culture, and indo-1 emission fluorescence was collected for up to 2 seconds through a 406-nm BP and 460-nm LP filter to two identical, precisely aligned fiber optic arrays. The ratio of the emission fluorescence (406 nm/460 nm) provided a measure of [Ca<sup>2+</sup>]<sub>i</sub>. Recorded signals were normalized for individual fiber optics. Fluorescent signals were collected simultaneously from eight sites (16 channels) at a sampling rate of 70 kHz.

In addition, WBF344-DsRed/cardiomyocyte co-cultures were labeled with the calcium sensitive fluoroprobe, fluo-4 AM (Molecular Probes, Eugene, OR). Line scanning confocal recordings were acquired at the interface of WBF344-DsRed cells and cardiomyocytes with the Zeiss LSM510 inverted confocal microscope. An excitation wavelength of 488 nm and emission filter wavelength

515±15 nm was used to record  $[Ca^{2+}]_i$  signals. The line scan rate was 1.92 ms per 1024 pixel line. The cardiomyocytes beat spontaneously or were electrically stimulated with a platinum bipolar electrode. Rectangular impulses, 1 ms in duration, 2x threshold intensity, were delivered continuously to the co-cultures at a cycle length of 1 Hz (Grass Instruments). The resulting line scan images were analyzed with ImageJ software. Fluorescence measurements from line scans are expressed as a ratio (R) of fluorescence intensity (F) divided by the basal cell fluorescence ( $F_0$ ),  $R = F/F_0$ .

### III. RESULTS

#### *WBF344-Derived Cardiomyocytes*

WBF344 cells that attached to the coverslips away from the neonatal cardiac cells behaved as normal WBF344 cells when plated in the absence of neonatal cardiac cells. The WBF344 cells multiplied to form nests of confluent, small polygonal cells, and were coupled via Cx43. At the periphery of WBF344 nests, WBF344 cells bordered by neonatal cardiac cells acquired a phenotype characteristic of developing cardiomyocytes in culture, exhibiting double nuclei and occasionally demonstrating cross-striations by light microscopy. The number of WBF344-derived cardiomyocytes examined at 7 days in the co-culture was  $39.8 \pm 8.1$  ( $n = 6$ , mean  $\pm$  SEM) per coverslip. The WBF344-derived cardiomyocytes expressed the cardiac proteins, myosin, cTnI, cTnT, and Cx43. They did not express skeletal muscle myogenin. The striated pattern obtained with the immunolocalization of cardiac myosin, cTnI, and cTnT was consistent with localization of these proteins in the cardiac sarcomeres. By confocal microscopy, neonatal cardiomyocytes were not found to underlie or overlap the WBF344-derived cardiomyocytes. The diffuse distribution of Cx43 on the WBF344-derived cardiomyocyte cell membrane reflects the relative immaturity of these cardiomyocytes. Contact between the WBF344 cells and neonatal cells in the cardiac microenvironment appeared to be necessary for WBF344 cells to acquire the cardiomyocyte phenotype. WBF344 cells did not acquire the cardiomyocyte phenotype when grown in conditioned media from neonatal cardiac cell cultures, or when grown on Anopore membrane inserts that prevented their direct contact with the neonatal cardiac microenvironment.

#### *Functional Cell-to-cell Communication*

FRAP demonstrated functional cell-cell communication between the cytoplasm of WBF344 cells and adjacent cardiomyocytes. When the WBF344 cells were seeded adjacent to neonatal cardiomyocytes, Cx43 was preferentially located at the cell membrane between the WBF344 cells and the neonatal cardiomyocytes. Four types of cell-to-cell interactions were identified: **(A)** adjacent neonatal rat cardiomyocytes; **(B)** WBF344-derived cardiomyocyte adjacent to rat neonatal cardiomyocyte; **(C)** undifferentiated WBF344 cell adjacent to rat neonatal cardiomyocyte; and **(D)** adjacent undifferentiated WBF344 cells. The highest rate of fluorescence recovery was

measured in **(A)** (adjacent neonatal cardiomyocytes)  $k = 0.082 \pm 0.009$ , the lowest rate of fluorescence recovery was measured in **(C)** (neonatal cardiomyocytes adjacent to undifferentiated WBF344 cells)  $k = 0.029 \pm 0.006$ . The rate of fluorescence recovery measured in **(B)** (neonatal cardiomyocyte adjacent to WBF344-derived cardiomyocyte) was ~60% of that measured in **(A)** (adjacent neonatal cardiomyocytes)  $k = 0.049 \pm 0.006$  vs.  $k = 0.082 \pm 0.009$ ,  $P < 0.01$ . In addition the rate of fluorescence recovery in **(B)** (neonatal cardiomyocyte adjacent to WBF344-derived cardiomyocyte)  $k = 0.049 \pm 0.006$ , was approximately equal to that measured in **(D)** (adjacent undifferentiated WBF344 cells)  $k = 0.057 \pm 0.010$ . When 100  $\mu$ mol/L carbenoxolone was added to the co-cultures, fluorescence recovery in all cells was inhibited, demonstrating decreased GJ permeability in the presence of this GJ uncoupler.

#### *Intracellular Calcium*

In established co-cultures (7-14 day), using the multisite ratiometric fiber optic recording and imaging system,  $[Ca^{2+}]_i$  oscillations were recorded in WBF344-derived cardiomyocytes adjacent to cardiomyocytes. The  $[Ca^{2+}]_i$  oscillations were of similar amplitude and duration to the cardiomyocyte. In contrast, in the same co-culture,  $[Ca^{2+}]_i$  signals were not recorded from WBF344 cell nests that had not acquired a cardiac phenotype. Increasing the frequency of the pacing stimulus to 2 Hz resulted in a corresponding increase in the frequency of  $[Ca^{2+}]_i$  transients in the neonatal cardiomyocytes. However,  $[Ca^{2+}]_i$  oscillations in the WBF344-derived cardiomyocytes evidenced 2:1 or higher degrees of conduction block, reflecting the relative immaturity of the WBF344-derived cardiomyocytes.

High resolution line scanning confocal microscopy used to confirm the  $[Ca^{2+}]_i$  transients at the cellular level detected  $[Ca^{2+}]_i$  oscillations in WBF344-derived myocytes adjacent to neonatal cardiomyocytes. Measured  $[Ca^{2+}]_i$  transients in the WBF344-derived myocytes at the cellular interface showed a slower rate of rise, shorter amplitude and longer duration than  $[Ca^{2+}]_i$  transients measured in adjacent cardiac cells. These results suggest the relative immaturity of the WBF344-derived cardiomyocytes. In control experiments, we found that homogeneous WBF344 cultures did not demonstrate oscillatory  $[Ca^{2+}]_i$  signals even when electrically stimulated.

### IV. DISCUSSION

An *in vitro* cardiac cell microenvironment was used to facilitate the study of stem cell engraftment and differentiation to a cardiac phenotype. We demonstrated that 1.) WBF344 stem cells acquire a cardiac phenotype in an *in vitro* cardiac microenvironment, 2.) GJ mediated cell-to-cell coupling as measured with FRAP is present in the WBF344-derived cardiomyocytes, although less robust than in the cardiac neonatal myocytes, 3.) Rate-dependent modulation of conduction was observed in WBF344-derived cardiomyocytes relative to neonatal cardiomyocytes

indicating immaturity of these WBF344-derived cardiomyocytes one to two weeks in culture, 4.) WBF344-derived cardiomyocytes contacting neonatal cardiac myocytes acquire an attenuated calcium signal, synchronous with the  $[Ca^{2+}]_i$  transients in the adjacent cardiomyocyte and 5.) WBF344-derived cardiomyocytes demonstrate structural and functional integration into the cardiac cell culture as determined by phenotype, cell-to-cell coupling and signal propagation.

In contrast, when WBF344 cells engrafted away from neonatal cardiac cells, were separated from the neonatal cardiac cells through a barrier membrane, or were grown in a neonatal cardiac cell preconditioned medium, the WBF344 cells multiplied and formed confluent nests of undifferentiated WBF344 cells. These results suggest that inductive signals present in the neonatal cardiac microenvironment acted on the WBF344 cells through either cell-cell or cell-extracellular matrix contact, rather than as soluble factors alone. We speculate that Cx43, being the predominant isoform expressed in ventricular cardiomyocytes, might facilitate the transfer of cardiogenic signals from the neonatal cardiomyocytes to adjacent undifferentiated WBF344 cells through shared connexons. Indeed, it has previously been demonstrated that the WBF344 cells in culture are joined by GJs and express liver and cardiac connexin isoforms, namely Cx43 and Cx26 [9-12].

The results of this study confirm that WBF344-derived cardiomyocytes that acquire a cardiomyocyte phenotype also function as cardiomyocytes. Functional integration was verified by observing the presence of  $[Ca^{2+}]_i$  oscillations in response to propagated electrical wavefronts and the diffusion of a fluorescent probe between WBF344-derived cardiomyocytes and adjacent neonatal cardiomyocytes. These studies show that WBF344-derived cardiomyocytes cycle intracellular  $[Ca^{2+}]_i$  during contraction, a characteristic of cardiomyocytes. Two observations, however, indicate that the electrical properties of the WBF344-derived cardiomyocytes are not as well developed as the neonatal cardiomyocytes. First, cell-to-cell coupling, as measured by FRAP, was decreased between WBF344-derived cardiomyocytes and neonatal cardiomyocytes. Secondly, electrical coupling or excitability of the WBF344-derived cardiomyocytes, as measured by the effect of increasing the pacing rate, appeared to be decreased compared to neonatal cardiomyocytes.

Finally, the clonal nature of the WBF344 cell eliminates the contribution from other cell types, providing an ideal experimental system to understand the plasticity of adult stem cells and to investigate, *in vitro*, the signals that induce lineage commitment and phenotypic differentiation.

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