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Conduction Analysis in Mixed Cardiomyocytes-Fibroblasts Cultures using Microelectrode Arrays

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Abstract— **Models for cardiac arrhythmia currently exist primarily in *in-vivo* and computer simulation form. Towards the development of such a model *in-vitro*, a better understanding of electrical conduction in heterogeneous cultures is required. Increasing ratios of cardiomyocytes and fibroblasts were cultured on 500 x 500 μm arrays of 36 microelectrodes to study the emergence and properties of action potential propagation in mixed cultures. A minimum ratio of 70% cardiomyocytes to 30% fibroblasts was found to be necessary for detection of electrical activity. However, the establishment of a continuous, homogeneous depolarization wave across the culture required a higher proportion of cardiomyocytes; even a 90:10 ratio was unable to consistently produce a unidirectional, uniform depolarization wave as is seen in controls. This model underlines the importance and sensitivity of tissue homogeneity in supporting electrical conduction, and is especially relevant to studies of arrhythmia (reentry) and stem cell grafts.**

I. Introduction

CARDIAC arrhythmias are a common heart disorder and can lead to severe complications, making it a significant area of both *in-vitro* and *in-vivo* research. The primary cause of arrhythmia lies in abnormalities in electrical conduction of the heart. This conduction normally begins with the generation of an action potential by a cardiac pacemaker cell, resulting in the depolarization of surrounding cells, and consequently, the initiation of a propagation wavefront throughout the tissue. This regenerative process provides the basis for the subsequent unimpeded propagation of depolarization in cardiac tissue [1]. Some cardiac conditions such as ischemia [2] or infarct [3] can lead to changes in tissue structures, notably formation of non-conductive fibrous tissue. These non-functional regions can vary in size from single cells interspersed to large blocks, leading to disruption of conduction, blockage, and subsequent arrhythmias [4]. Current treatments for such cardiac arrhythmias include radio-frequency catheter ablation, which while effective, is costly and often presents complications [5]. Other treatments aim at regenerating functional tissue within the damaged heart, using stem cell transplantation for instance [6]. However, such approaches are also not immune to conduction problems and associated arrhythmogenicity, as the properties of the conduction through the cell graft must match those of the host tissue in order to provide unimpeded conduction. With inhomogeneous grafts, either from impure stem cell-derived populations or through diffuse integration within the host, the risk of non-uniform, or even lack of, conduction arises. A minimum proportion of functional cardiac cells must be present to support a uniform depolarization wavefront, and it is important to understand the relationship between tissue homogeneity and conduction. While also useful for *in-vitro* modeling of arrhythmia, it is especially important for determining appropriate purity levels of stem cell-derived cardiomyocytes for effective cardiac stem cell therapy [7]. Without such an understanding of the requirements for proper conduction, using an impure population of differentiating embryonic stem cells may encourage arrhythmia more than contributing to improved regeneration and repair (see Fig 1(c)).

In order to investigate this purity-conduction relationship, cellular models can be developed that are more controlled than *in-vivo* studies [8], and more realistic than mathematical models [9]. Initial studies combining contractile and non-contractile tissues have been conducted by Camelliti, *et al.*, to observe patterned tissues and their properties and test the viability of such an *in-vitro* model [10]. These studies have indicated that *in-vitro* models of heterogeneous mixtures of cardiac tissues can adequately mimic *in-vivo* conditions, and thus are suitable for assessing the electrical properties necessary for conduction. Other work with optical mapping of mixed cultures of neonatal rat ventricular myocytes and mesenchymal stem cells (MSCs) has further demonstrated the merit of the *in vitro* model for studying conduction properties [11].

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We report here on the use of microelectrode arrays (MEA, see Fig 1a,b) to characterize conduction properties in pure and mixed co-cultures of HL-1 cardiomyocytes and IMR-90 fibroblasts, using ratios covering complete conduction block, observation of ectopic foci, and unimpeded conduction. IMR-90 fibroblasts were employed due to their non-conductive properties; alternatively, cardiac fibroblasts are highly conductive when merged with other cardiac pacemaker cells, and thus are ineffectual as blocks in conduction.

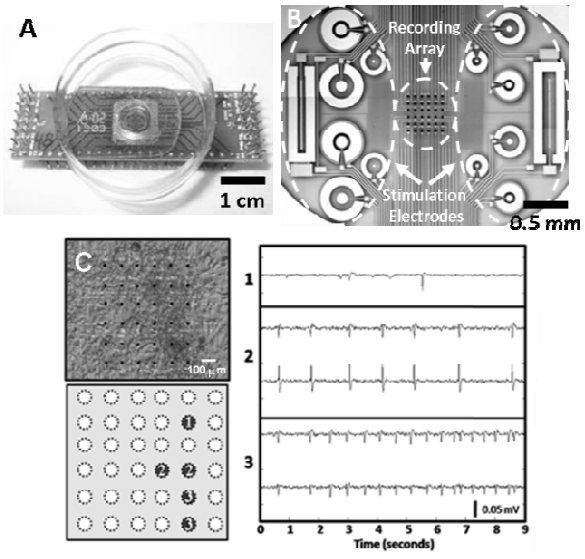

Figure 1: (A) Image of an assembled microelectrode array, which is glued and wire-bonded to a printed circuit board carrier. (B) Micrograph of the MEA. An array of 6x6 electrodes is located in the center used for electrical sensing, while larger auxiliary electrodes on the periphery may be used for stimulation [12]. (C) Differentiating human embryonic stem cells monitored over a MEA. Electrodes detecting signal are represented as darkened spot over a diagram of the array. Three different rhythms are observed within the same culture, implying a high degree of conduction block and motivating the need for greater understanding of the threshold for conduction.

II. Methods

A. Microelectrode Array Instrumentation

In determining the appropriate proportions of cardiomyocytes to support conduction, a microelectrode array was used consisting of 6 × 6 platinum electrodes with 22 µm diameters spaced on 100 µm centers, as discussed in [12]. Data was acquired through 32 channels, with the four corner electrodes excluded. Petri dishes with 35 mm-diameters and drilled through-hole 1 cm in diameter were fixed to the package using bio-compatible epoxy (EP42HT, Master Bond; Hackensack, NJ).

Signals from the MEA were processed by a custom recording system consisting of a 32-channel amplifier with two-stage gain of 60 dB, 7 Hz 1st-order high-pass cutoff, and 8th-order low-pass cutoff at 3 kHz, as previously reported in [13]. Data were digitized with 12-bit resolution at 10 kHz and acquired by a custom-designed visualization and analysis tool, written in Matlab™ (The MathWorks; Natick, MA) [12].

Conduction patterns were analyzed by coordinating the local activation time, as defined as the point of maximum negative slope of the extracellular action potential, with its spatial location on the MEA. Conduction magnitude and direction were extracted from adjacent triplets of active electrodes [14], and represented as a vector.

B. Cell Cultures

The heterogeneous population of cells consisted of murine atrial tumorgenic cell line HL-1 cardiomyocytes [15] transfected to express green fluorescent protein (GFP) through a LentiLox viral vector, and human diploid fibroblast-like IMR-90 cells [16]. HL-1 cells were cultured in Claycomb media (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 mM norepinephrine (Sigma-Aldrich), 100 units/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA), and 4 mM L-glutamine (Invitrogen). The IMR-90 cells were cultured in a solution containing 89% DMEM (Invitrogen), 10% FBS (Hyclone), and 1% penicillin–streptomycin (Invitrogen). The GFP-labeled HL-1 cardiomyocytes were imaged using 450 nm excitation and 515 nm emission wavelengths.

C. Mixed Populations Preparation

Heterogeneous populations of HL-1 cardiomyocytes and IMR-90 fibroblasts were prepared by mixing pure suspensions of known densities. Ratios of the two cell types were adjusted by volumetric ratio. Pre-mixed suspensions were then plated in Petri dishes or on the MEA using HL-1 cell culture medium. For experiments on MEA, a total of 40k cells/cm^2 were plated on each MEA to provide a confluent layer within a day. Recordings were performed one day after plating.

III. Results

In order to create a functional population of integrated cells, the threshold ratio of contractile HL-1 cardiomyocytes to non-contractile IMR-90 fibroblasts must be determined. Using the MEAs, different heterogeneous cell populations were analyzed for the presence of electrical signal. Preliminary data was taken over larger intervals of 25:75, 50:50, 75:25, and 100:0 proportions of cardiomyocytes to fibroblasts (N=5 for each ratio) to draw a coarse estimate of the minimum ratio for electrical activity to be detected. No signal was detected under the 75:25 ratio category; thus, smaller intervals between 50:50 and 100:0 (N=5 for each ratio) were taken to determine a more accurate floor for conduction within the heterogeneous population (see Fig. 2). A 70:30 ratio was consistently observed as the threshold necessary to ensure any signal detection. In four out of the five MEAs plated with a 70:30 ratio, action potentials were sensed in only two to three channels, implying that the population of IMR-90 fibroblasts inhibited electrical propagation through the HL-1 cardiomyocytes. Indeed, the observed rhythms were often asynchronous between channels, demonstrating that the 70:30 ratio, while sufficient for islets of activity, was not enough to support continuous conduction throughout the whole array.

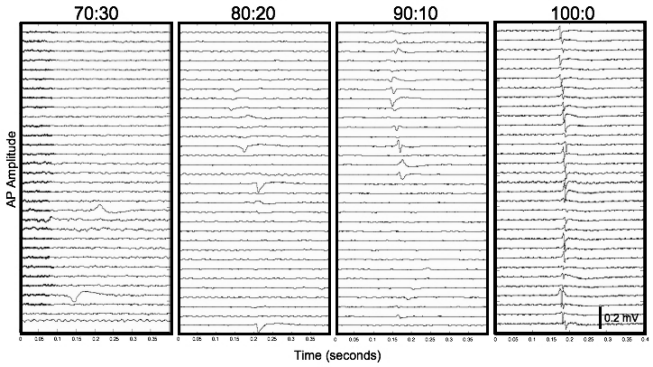

Figure 2: Plot of all 32 traces of a MEA with different proportions of HL-1 cardiomyocytes to IMR-90 fibroblasts. (A) Signals detected in 70:30 ratio culture, (B) 80:20, (C) 90:10, and (D) 100:0 control culture. Signals are only seen in specific channels in the 70:30 culture, with no sign of homogenous conduction, as is seen in the 100:0 control. The 80:20 and 90:10 cultures demonstrate intermediary behavior depicting groups of beating and non-beating cells.

In the samples of the 70:30 cardiomyocyte to fibroblast plating ratio, asynchronous electrical activity was observed in an average of two channels with a standard deviation of approximately one, indicating the presence of patches of independent beating, each with an area covering about one electrode. The 80:20 ratio samples delineate a similar pattern of beating, averaging 12 channels with a standard deviation of two, grouped into two beating patches covering about six electrodes each. The 90:10 plot appropriately demonstrates a small depolarization wavefront throughout the heterogeneous population (conduction pattern observed in Fig. 3(a)), with one large patch of beating that covers about 20 active electrodes, with a standard deviation of three electrodes. The 90:10 samples, while displaying pathways of action potential propagation, were still unable to support a uniform depolarization wave across the culture. Lastly, the 100:0 controls demonstrated the expected behavior of homogenous cultures, with unidirectional propagations (all 32 electrodes active; see Fig. 3(b)).

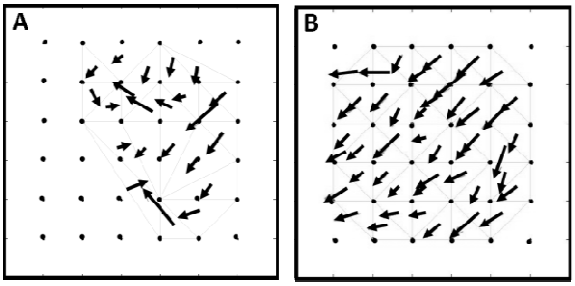

Figure 3: Representation of conduction across a MEA. Microelectrodes are displayed as solid circles. (A) Propagation exhibited by a heterogeneous population of ratio 90:10 cardiomyocytes to fibroblasts where the conduction pattern is impeded by non-conductive fibroblasts. Action potentials were not observed on all electrodes. Note the non-uniform propagation wavefront resulting from tissue heterogeneity. (B) 100:0 (pure) cultures of cardiomyocytes support continuous unidirectional propagation throughout the homogenous population.

As shown in Fig. 4, a monotonically increasing number of active electrodes was observed between the 70:30 and 100:0 ratios. Increasing the threshold ratio past 70:30, the density and overall area of beating foci increased, as less non-conductive tissue is impeding the conduction path.

GFP-expressing cardiomyocytes were used to visually confirm through fluorescent microscopy the ratio of cardiomyocytes to fibroblasts, as well as the homogeneity of the mixture. Although the pure population of cardiomyocytes was confluent, GFP expression was not ubiquitous throughout the entire culture of transfected cells. Nonetheless, the fluorescence observations confirmed the relative differences between the successive proportions of cardiomyocytes as seen in Fig. 5.

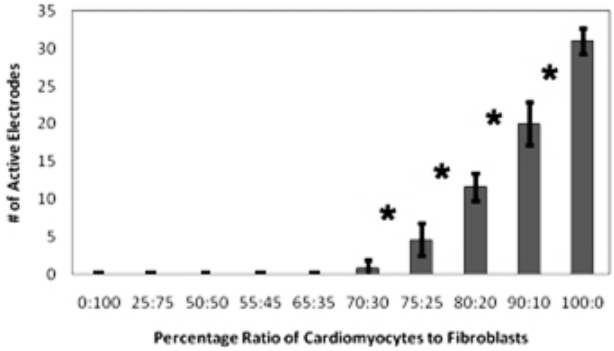

Figure 4: Relationship between the proportion of cardiomyocytes to fibroblasts and the average number of electrodes detecting electrical activity (N=5 for each ratio; *P<0.05). Action potentials began to be consistently observed on multiple electrodes at a 70:30 ratio, although activity was not consistently synchronous. With increasingly higher ratios of cardiomyocytes, larger regions of synchronous activity were observed. However, uniform propagation was only found in pure populations of cardiomyocytes.

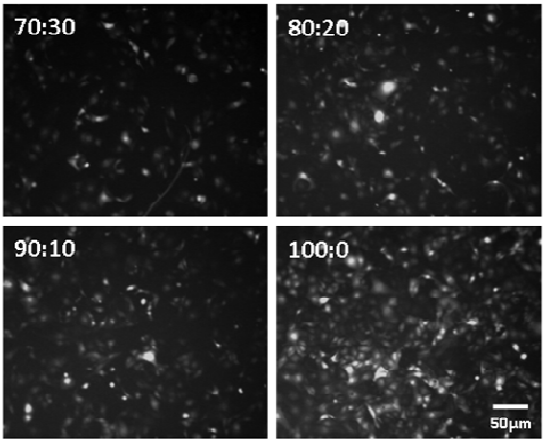

Figure 5: Visual detection of GFP-expressing cardiomyocytes in mixed co-cultures with fibroblasts, at several mixing ratios. The monotonic increase in expression of GFP follows the increasing ratio of cardiomyocytes.

IV. Discussion

A heterogeneous population of cardiomyocytes and non-conductive tissue is proposed as a model for understanding conduction properties.

When culturing such mixtures of cells, a 70:30 proportion proved to be the minimum ratio necessary to support electrical activity in the culture. The lack of signal detection in ratios below 70:30 was likely due to insufficient cardiomyocyte connectivity and/or uniformity to support

limited conduction or even beating. As the proportion of HL-1 cardiomyocytes increased in the heterogeneous population, greater electrical activity was observed. However, even a 90:10 ratio of HL-1s to fibroblasts did not exhibit homogeneous conduction throughout the culture. Instead, patches of non-conductive tissue impeded the unidirectional propagation of the depolarization wavefront. It was expected that when cardiomyocytes heavily dominated the heterogeneous population, a more reliable conduction uninhibited by the simulated "scar tissue" would be observed. However, it is now fitting to infer from the data collected that a very high ratio (close to 100:0 cardiomyocytes:fibroblasts) is necessary to sustain a conduction that is homogeneous enough to prevent arrhythmic behavior. Thus, it can be concluded that though electrical activity is observed at relatively low ratios of HL-1 cardiomyocytes to IMR-90 fibroblasts, conduction is especially sensitive to any presence of IMR-90 fibroblast tissue. This finding may appear to contrast with earlier studies of fibroblast-mediated conduction in the heart tissue [17], but it should be noted that IMR-90s are not cardiac fibroblasts, and the presented data strongly suggests that they do not participate in conduction, possibly because of lack of gap junction expression. Also, similar studies of conduction in mixed cultures of ventricular myocytes and MSCs have shown a lower susceptibility to presence of non-cardiac cells, although it is known that MSCs will form gap junctions with cardiac tissue [18].

The model presented in this study has its limitations – it only considers a two dimensional environment, and simulates a homogenous, anisotropic mixture of myocytes and fibroblasts. However, it clearly points towards a high sensitivity of the conduction properties of cardiac tissue to the presence of dispersed, non-conductive cells.

V. Conclusion

This study demonstrated the requirements necessary for proper conduction through a heterogeneous population. It was seen that though signal was detected at ratios of 70:30 cardiomyocytes:fibroblasts, continuous conduction was not observed until ratios close to 100:0 (pure cardiomyocytes population) were implemented. This result has potential implication in stem cell transplantation studies, addressing concerns regarding the proportion of cardiomyocytes necessary for improved regeneration and repair. Another important application of this result is the development of an *in-vitro* model for arrhythmia, where conduction blocks may be artificially created to initiate reentry patterns.

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