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A simplified protocol for the isolation and culture of cardiomyocytes and progenitor cells from neonatal mouse ventricles

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ABSTRACT

The neonatal heart is a very useful tool for the study of biochemical pathways and properties of cardiomyocytes and as it has the potential to regenerate for a brief period of time from birth; it is also useful to study cardiac regeneration. However, as the heart matures, this proficiency for regeneration is reduced. This regenerative potential may be influenced by the microenvironment of the heart in the early stages of postnatal development and therefore, cell cultures derived at this stage may contain functional cardiomyocytes and progenitor cells. The aim of this study was to identify key steps in the isolation and culture of such early stage—neonatal mouse hearts to allow maximum migration of cardiomyocytes from the explant and their maintenance as functional, long term cultures. Explant cultures of mouse ventricles preserved 3-dimensional structure and generated migrating layers of cardiomyocytes that expressed alpha sarcomeric actin which could be further sub-cultured by enzymatic dissociation. Western blotting demonstrated expression of c-KIT, GATA4, alpha sarcomeric actin and connexin43 proteins after 20 days of explant culture. ACTA1, GATA4, and CX43 continued to express in five weeks old explant cultures while the c-KIT protein was expressed up to two passages during sub-culture. Real time PCR and SQRT PCR also demonstrated gene expression of cardiomyocyte markers in long term cultures. Migrating cells from the explants assembled into contracting spheroids after subculture and expressed the c-KIT protein. Progenitor markers CD44, CD90, and extracellular proteins, periostin and vimentin demonstrated the preservation of cellular heterogeneity in such cultures. Supplementation with Hydrocortisone maintained a cardioprotective environment and reduced the non-myocyte population. This is an optimized and efficient method for the generation of neonatal heart cultures that is not labor intensive and does not require supplementation with cytokines.

1. Introduction

Isolation and culture of cardiac stem cells and progenitor cells is essential for *in vitro* studies of cardiac functions and cardiac regeneration. Neonatal cardiomyocytes have been shown to proliferate and regenerate after partial surgical resection in hearts of one day old mice for a brief period of time from birth (Porrello et al., 2011). However, such regenerative potential is limited (Mahmoud et al., 2014; Porrello et al., 2011) and the heart loses this potential after the first week of postnatal life as cells withdraw from the cell cycle (Ahuja et al., 2007). The regenerative potential of such early stage cardiomyocytes and endogenous cardiac stem and progenitor cells (CSC) may be determined by the unique microenvironment or stroma of the early stage-neonatal heart. These unique populations may lose their regenerative characteristics in primary cultures unless they are cultured in their original microenvironment. Most methods for isolation of cardiac cells are based on enzymatic digestion followed by differential plating based on time (Sreejit et al., 2008) or cell-adhesion to specific substrates (Louch et al., 2011). These methods are very useful for isolation and generation of homogenous cultures of specific cell types such as cardiomyocyte, however, whole organ culture or explant culture may be more suitable for culturing progenitor cell types such as CSC. While primary cultures of dissociated neonatal cardiomyocytes are used to study cardio-protection and intracellular signaling pathways, they are insufficient for studies on normal cardiovascular development, function and regeneration of the diseased myocardium. The enzymatic dissociation reduces gap junction formation between

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cardiomyocytes because of low seeding densities, making the generation of functioning primary cultures dependent on the initial yield of cells from tissue. Preservation of the actual microenvironment in culture will maintain the autocrine, paracrine, and intercellular interactions of the original tissue that will benefit progenitor cells.

The explant culture method preserves conditions where cells continue to differentiate and develop a tissue organization resembling the *in vivo* structure (Davis et al., 2009; Pampaloni et al., 2007). A perceived drawback of this method is the presence of a sizeable non-myocyte population of the heart that makes up 70% of the cell population. However, this population is essential for the heart's electrical and chemical equilibrium (Banerjee et al., 2007). Therefore, in this study, conditions were standardized for the explant to remain viable and shed all cell types in culture, including the progenitor populations. By identifying and improving key aspects of the extraction procedure such as excision, sectioning and plating of the heart, and culture conditions such as regulation of serum concentration, we were able to culture explants that shed cells which expressed stem cell, developmental and functional cardiac markers even after sub-culture. The modified protocol allows the migration and formation of synchronous contractile regions from the explant and these cultures could be maintained with no loss in contractile activity or supplementation with complex medium and cytokines that are generally required for culture of progenitor or cardiac stem cells (Louch et al., 2011). This protocol generates primary cultures that allow the use of a broad range of techniques including electrophysiology, calcium imaging, cell mechanics, immunohistochemistry, and protein biochemistry. Importantly, these cultures can be used for longer term studies which are not possible using acutely isolated cells of the heart. The basic culture conditions of this protocol do not allow the influence of cytokines and other stimuli that can be present *in vivo* but are not native to the heart.

2. Materials and methods

2.1. Extraction of the heart and explant culture

Neonatal Swiss Albino mice were procured from King's Institute, Chennai, Tamil Nadu, India. All procedures were approved by the Institutional Animal Ethics Committee (IIT Madras, India) and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Pups aged between 12 and 18 h were used. The neonatal mouse was euthanized by cervical dislocation. Sternotomy was done in the midline using fine scissors (iris or Castro-Viejo Scissors). Vascular forceps were used to retract the rib cage and expose the heart and lungs. The forceps were passed under the base of the heart avoiding the lungs and the whole heart was removed gently without damaging the myocardial tissue in one swift pull. The excised heart was placed in pre-warmed (37 °C) Dulbecco's modified Eagle's medium-F12 (DMEM/F12) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM Non-essential amino acids (Life technologies, USA). Fresh vascular forceps (with non-serrated edges) were used to gently squeeze out any residual blood from the explanted heart. The forceps were used to pinch and hold the explanted heart between the base and the apex gently and using a surgical scalpel (BP handle 4, Size 20), the ventricles were sliced to produce sections that were approximately1 mm^3 in volume (Fig. 1A1). A single ventricle was sliced perpendicular to the long axis followed by cuts parallel to the long axis. Progressive transverse slices were made in the same manner on all subsequent sections generating 18–20 explants. This mode of sectioning allowed minimal handling and slicing. While sectioning, deft, uniform and single slices are used, reducing damage to the tissue. These explants were transferred to a 60 mm dish with 2 mL of fresh DMEM/F12 using a wide mouth micropipette tip (Fig. 1A2). The explants were cultured in DMEM F/12, with a serum supplement of 5% FBS (Low Serum—LS), a serum supplement of 10% FBS (High Serum—HS) and a serum supplement of 10% FBS plus 5% Horse serum (High Serum+—HS+). The sections were placed in the dish spaced 8–10 mm diameter between individual explants (Fig. 1A6). The explants are observed for contractile activity and cultures were incubated at 37 °C, 5% CO_2 for 48 h for explants to adhere to the surface of the plate. Medium was changed every 48 h for the duration of the culture. All procedures were carried out in medium pre-warmed to 37 °C. Ideally the protocol generates a culture from a single ventricle under 15 min. The protocol is comparatively efficient to methods based on enzymatic dissociation (Supplementary Table 3).

2.2. Enzymatic dissociation of cells shed by explant

The migratory and contractile phase bright regions of cells were sub-cultured by partial enzymatic dissociation. The phase bright dense cellular regions dissociate from the rest of the explant cultures in 0.25%Trypsin-EDTA (1 mL, Life Technologies, USA) at 37 °C incubated for 2 min. 3 mL of DMEM/F12 10% FBS was used to neutralize the digestion process and the entire volume was collected gently by pipetting or swirling the plate without dislodging adherent cells. After centrifugation at 80 g for 5 min and re-suspension in fresh medium, the dislodged explants sediment is replated in a fresh dish in DMEM/F12 supplemented with 5% or 10% FBS. We observed the phase bright regions to self assemble into spherical aggregates that are contractile. Differential enzymatic dissociation is selective for the contractile cells because fibroblasts have stronger adhesions to the culture plate and their dissociation takes longer, while the cardiomyocytes and stromal cells detach from the TCP earlier.

2.3. PCR amplification using semi-quantitative and real time PCR

Total RNA was isolated using Trizol reagent (Sigma-Aldrich, USA) and converted to cDNA using MMLV-RT (Thermo scientific, USA) with oligo-dT primers (NEB, USA) according to manufacturer's protocol. Real time PCR was carried out as previously reported (Shyamsunder et al., 2013a,b) using SYBR green Real time PCR kit from Qiagen on an Eppendorf mastercycler, eprealplex (Eppendorf, Germany). Relative mRNA expression was determined by normalization to the expression of a housekeeping gene, beta-actin. (Primer list is provided in Supplementary Table 1).

2.4. Western blotting

Western blotting was carried out as previously reported (Shyamsunder et al., 2013a) and total protein was isolated using radio immunoprecipitation assay (RIPA) lysis buffer containing protease inhibitors. Protein was estimated for concentration, subjected to 10% SDS-PAGE and electroblotted onto BioRad, 0.22 μM nitrocellulose membrane (BioRad Laboratories, USA). Primary antibodies for c-KIT (1:100; Cell signaling Technologies, USA), PPAR-γ (1:500; Cell signaling Technologies, USA), GATA4 (1:150; Santa Cruz Biotechnology, USA), Connexin43 (CX43; 1:500; Abcam, USA), Alpha sarcomeric actin (ACTA1; 1:500;Sigma-Aldrich, USA) and Beta Actin (ACTB; 1:1000; Sigma-Aldrich, USA) were used along with goat anti-rabbit and anti-mouse horseradish peroxidase conjugated secondary antibodies (1:10,000; Sigma-Aldrich, USA). Antibody-reactive proteins were detected by means of enhanced chemiluminescence, Pierce ECL Plus western blotting detection