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Histological Validation of Heart Slices as a Model in Cardiac Research

Masahiro Kaneko[1], Steven R. Coppen[1], Satsuki Fukushima[2], Magdi H. Yacoub[2] and Ken Suzuki[1,2]*

[1]*Translational Medicine and Therapeutics, William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, Charterhouse Square, London, EC1M 6BQ, UK*

[2]*Harefield Heart Science Centre, Imperial College London, Harefield, Middlesex, UB9 6JH, UK*

Abstract

Recently vibratome-cut heart slices has been proposed as a new model in cardiac research, particularly in studying functional integration of transplanted donor stem cells into the host myocardium. This emerging model has, over co-culture models, a theoretical advantage in maintaining the 3-dimensional cardiac structure and physiological cell-cell connections; however, further validation is needed for this to be an established model. Particularly, the ability of cardiomyocytes in heart slices to connect with administered donor cells remains uncertain.

Heart slices of 300 μm thickness were cut from adult and embryonic rat hearts using a vibratome and cultured. Adult heart slices maintained localized contraction for up to 3 days. Various histological studies demonstrated that the myocardial morphology was well preserved with necrotic cells being restricted to the edges of slices, but the number of apoptotic cells in the slices increased with culture periods (16.0 ± 3.2% at day 2 and 59.1 ± 8.3% at day 6). Importantly, immunohistolabeling and western blotting showed that connexin43 expression, which is a prerequisite for gap junction formation between donor-derived and host cardiomyocytes, was markedly diminished by 1 day in culture, suggesting rapid functional deterioration of adult heart slices. In contrast, embryonic heart slices remained contracting at least for 35 days with myocardial morphology and connexin43-involved gap junctions remaining intact, though in the embryonic, immature pattern.

Collectively, both adult and embryonic rodent heart slices, in the current style, have critical limitations to assess integration between donor-derived and host cardiomyocytes. Further study to improve the quality of heart slices is warranted.

Keywords: Heart slice; Stem cell therapy; Cell-cell communication; Gap junction; Connexin43

Introduction

Vibratome-cut heart slices is an emerging model in cardiac research, particularly in studying functional integration of transplanted donor stem cells with host cardiomyocytes [1,2]. This study aimed to validate this possible model by means of histological investigations.

Myocardial regeneration by stem cell transplantation is a new possible therapy to treat heart failure [3-5]. For the success of this strategy, it is vital for transplanted donor-derived cardiomyocytes to functionally integrate into the host myocardium [6]. Particularly, formation of appropriate intercellular communication *via* gap junctions, the structures responsible for the electrical coupling [7], with host cardiomyocytes is essential for donor-derived cardiomyocytes to contract in a synchronous manner without producing arrhythmias [6]. However, these aspects have not been fully investigated due to limited availability of suitable models. It was reported that the function of cell-cell connection could be assessed using viable whole hearts injected with donor cells *in vivo* or *ex vivo* [8,9], but these models are extremely demanding to establish, requiring highly-specific expertise and equipment. *In vitro* models currently available consist of co-culture of donor cells with isolated neonatal or adult cardiomyocytes. These 2-dimensional models are simple but do not reproduce a clinically-relevant situation. Therefore, development of a simple, widely-accessible, clinically-relevant model to study functional connections between donor-derived and host cardiomyocytes will be of great value for future progress of stem cell therapy for myocardial regeneration.

Vibratome-cut heart slices have been proposed to serve as such a model [1,2,10-17]. In contrast to other *in vitro* models such as co-culture, the slice (usually 200-300 μm thick) can maintain the 3-dimensional cardiac structure with presence of all the different cardiac cell types. This property would provide donor cells with clinically-mimicking *in vivo* cardiac environment, enabling formation of connections with host cardiomyocytes as would occur in the whole heart. In addition, this model can be relatively-easily used for assessing functional cell-cell communication between donor-derived cells and host cardiomyocytes *in vitro*. Micro-injection of small fluorescent dyes into a single donor-derived cardiomyocyte within a cultured slice [1], loading slices with calcium/voltage sensitive dyes [9] or pre-labelling of donor cells with caged fluorescent dye transferable via gap junctions after photoactivation [18], will enable visualisation of the 3-dimensional cell-cell communication via gap junctions using confocal and multi photon microscopy [19]. It is also possible to evaluate the spread of excitation in a wide myocardial area, which contains donor-derived cells, by microelectrode arrays [11]. Furthermore, this model allows for investigating changes in electrophysiological properties of donor cells after cell transplantation by using whole-cell patch clamp technique [11]. Finally, this model enables to carry out multiple trials from one heart, reducing the number of animals used.

***Corresponding author:** Ken Suzuki, William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary, University of London, Charterhouse Square, London, EC1M 6BQ, UK, Tel: +44 (0)20 7882 8233; Fax: +44 (0)20 7882 8256; E-mail: ken.suzuki@qmul.ac.uk

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Slices from other organs including brain, liver, and placenta have been validated and successfully used as a model possessing the structural and functional features of *in vivo* tissues [20-22]. However, validation of this heart slice model has not been fully carried out. Although the previous reports briefly observed that the cardiac structure of the slices was preserved during culture, it remains uncertain whether cardiomyocytes within these cultured slices maintain appropriate viability and functions. In this study, we evaluated the suitability of the heart slices, either from adult or embryonic rodents, as a model for cell transplantation research. Particular attention was paid to the expression of connexin43 (Cx43), the major gap junction protein in ventricular cardiomyocytes and is a prerequisite for the formation of gap junctions between donor-derived and host cardiomyocytes [23].

Materials and Methods

Heart slice preparation and culture

All animal tissue was obtained in accordance with the UK Animals (Scientific Procedures) Act of 1986. The hearts were removed from adult (200g, male; n=4) and 19-day embryos (n=4) of Sprague-Dawley rats (Charles-River UK) and immediately placed into ice-cold Krebs solution (120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1 mM $MgSO_4$, 25 mM $NaHCO_3$, 11.5 mM glucose, pH 7.4). Left ventricular free walls of the hearts were collected and then embedded in low-melt agarose. Subsequently, the samples were covered with ice cold Krebs solution as quickly as possible, and slices (300 μm thick) were cut longitudinally from these hearts using a vibratome (Micro-Cut H1200, Energy Beam Sciences, Connecticut, USA). Slices were quickly transferred on nylon mesh (0.1 mm) to a 24 well plate and just enough ice cold Krebs solution added to keep the slices wet and cold. The plates were then incubated at 37°C for 20 minutes to allow attachment of the slices to the well surface with gradual warming. Warm culture medium was then added. Two types of medium were tested, RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics [24], or DMEM with 10% Medium199, 10% horse serum, 5% FBS and antibiotics [25]. Efficiency of these medium to culture isolated cardiomyocytes *in vitro* has been well validated and these were presumed to be most appropriate to culture heart slices as well. As a result, the latter medium was selected as spontaneous contraction of the slices appeared better (data not shown). The slices were cultured at 37°C in a humid atmosphere with 5% CO_2.

Cell viability/apoptosis detection and immunofluorescent labeling

Cultured slices were removed at set time points, washed with phosphate buffered saline (PBS) and placed in PBS containing 1 μg/ml DAPI for 1 minute to stain the nuclei of dead or damaged cells. The slices were then embedded in OCT mounting medium and frozen in liquid nitrogen. Cryosections (10 μm thick), cut at 90 degrees to the plane of the slices, were mounted on poly-lysine coated slides and DAPI positive nuclei observed immediately to determine the degree of cell damage or death. For Cx43 labeling, sections were fixed with methanol at -20°C, washed with PBS, blocked with 1% bovine serum albumin in PBS, incubated with monoclonal anti-Cx43 (Chemicon, MAB3068, 1 in 250 dilution) for 2 hours. These were again washed, incubated with anti-mouse secondary antibody conjugated with Alexa596 (Amersham, 1 in 500 dilution) for 1 hour, washed, counterstained with 1 μg/ml DAPI and mounted [26]. For apoptosis detection, cryosections were air-dried overnight, rehydrated, and fixed with 4% formaldehyde freshly prepared from paraformaldehyde. DNA fragmentation was detected by incorporation of biotinylated nucleotides using the CardioTACS *in situ* apoptosis detection kit (R&D Systems) according to the manufactures instructions. Sections were counterstained with Nuclear Fast Red (Sigma-Aldrich).

Western blot analysis

Cultured slices were removed at set time points, washed with PBS and snap frozen in liquid nitrogen. Western blot analysis was carried out as described previously [26]. Ten μg total protein was loaded per well on 12.5 % SDS polyacrylamide gels. Proteins were transferred to PVDF membrane and probed for Cx43 (MAB3068 monoclonal antibody, Chemicon, 1 in 500 for 2 hours) or sarcomeric actin (Dako Cytomation, 1 in 1000 for 2 hours). Bands of interest were detected using alkaline phosphatase-conjugated secondary antibodies (Chemicon, 1 in 1000 for 1 hour) and enzyme activity revealed by reaction with BCIP/NBT colour substrate (Perbio Science UK Ltd, Cramlington, UK). Blots were scanned and analyzed using ImageJ software. Cx43 levels were normalized to the sarcomeric actin.

Results

Morphology and viability of cultured adult heart slices

The adult heart slices stably attached to the bottom of the culture wells with the support of the nylon mesh for at least 1 week. Regional spontaneous contractions were observed at several sites in the heart slices until day 3 but not after that. Growing colonies of migrated fibroblasts were observed around the slices. Microscopic observation showed that myocardial structure was not disrupted for 6 days and

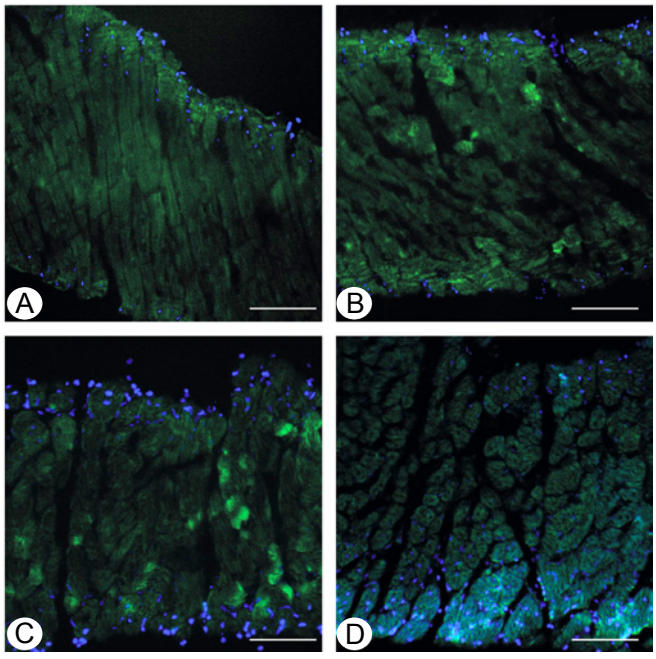

Figure 1: Morphology and viability of cultured adult heart slices. (A) Freshly cut vibratome slices of adult rat heart (non-fixed) were stained with DAPI to reveal dead/damaged cells at the periphery of the slice (blue). Cardiomyocytes were seen with their autofluorescence (green). DAPI-nuclear staining was still restricted to the outer layers of cells in slices which had been cultured for 2 (B) and 3 days (C). After 6 days in culture (D), DAPI-nuclear staining was still most pronounced at the periphery of the slice with some extracellular staining (released DNA from dead cells) but some nuclei were also stained towards the middle of the slice. Please note that the myocardial structure was maintained throughout the period (A-D). One representative picture from 4 slices (from 4 different hearts) at each time point is presented. Bar markers = 100 μm.

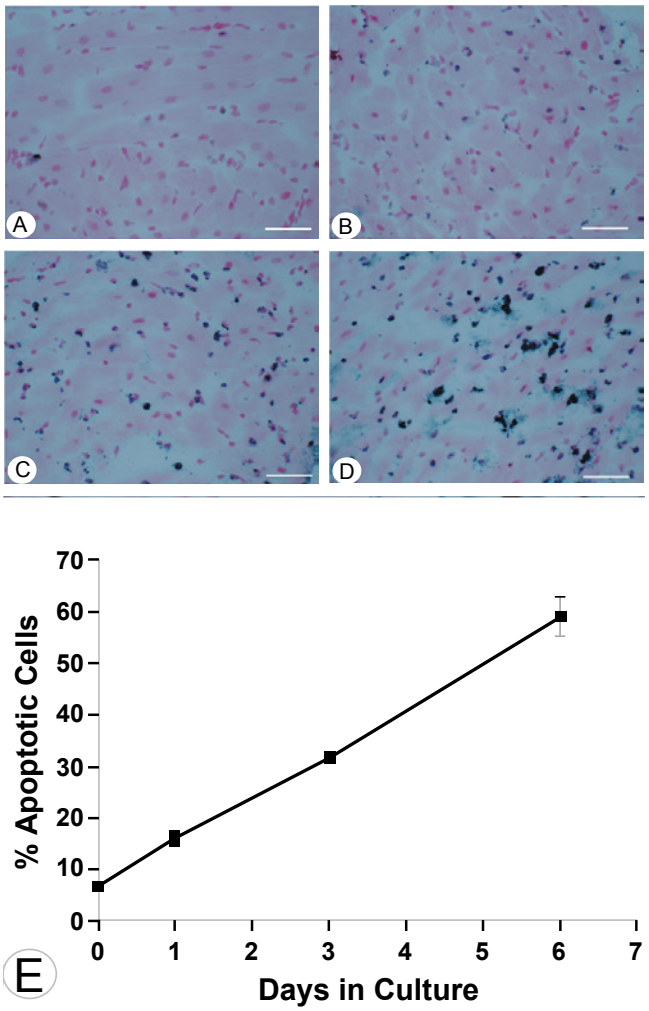

Figure 2: Apoptosis of cultured adult heart slices. Apoptosis labeling revealed low numbers of apoptotic cells (blue nuclei; 6.7 ± 1.7%), including cardiomyocytes, in freshly cut slices of adult rat hearts (A). The number of apoptotic cells increased proportionally with the number of days of culture (E), 16.0 ± 3.2% after 1 day (B), 31.8 ± 1.0% after 3 days (C) and 59.1 ± 8.3% after 6 days (D). Sections counterstained with Nuclear Fast red. Bar markers = 50 µm. n = 4 (one slice per heart) at each time point.

that morphology of the majority of cardiomyocytes appeared to be well maintained (Figure 1A-D). DAPI staining of freshly cut slices showed that only a small number of cells at the periphery of the slices were dead or damaged (Figure 1A). After 2 days in culture it was still only the cells towards the surface of the slices which took up the DAPI (Figure 1B). Even after 3 and 6 days in culture, most of cardiomyocytes inside of the slices continued to be negative for DAPI uptake with maintained morphology, whereas there was an increase of the number of DAPI-positive nuclei at marginal sites (Figure 1C & 1D). These findings were identical among all slices generated from 4 different hearts (one slice per heart). While on the other hand, apoptosis detection demonstrated that a considerable, increasing number of cardiomyocytes and other cell types were apoptotic during the course of culture. Only a low frequency (6.7 ± 1.7 %) of apoptosis was detected in the freshly cut adult rat heart slices (n=4; one slice from each of 4 animals), but this steadily rose with time with 16.0 ± 3.2 % and 59.1 ± 8.3 % of cells being apoptotic by day 1 and 6 in culture, respectively (Figure 2).

Expression of Cx43 in cultured adult heart slices

Immunofluorescent labeling demonstrated that freshly cut heart slices of adult rat expressed abundant Cx43 (Figure 3A) which was in the typical pattern for gap junction labeling in the adult rodent ventricle [27]. However, noticeably, after 1 day in culture, the Cx43 labeling was largely diminished in the adult rat heart slices with only patches of the slices having easily defined Cx43-gap junctions (Figure 3B). The Cx43 expression further diminished to such a degree that it was difficult to detect at 3 days (Figure 3C) and 6 days (Figure 3D) in culture. These findings were consistent among all the slices generated from 4 different hearts. Such a quick reduction of Cx43 in rat adult heart slices (n=4 at each point; one slice from each of 4 animals) was confirmed by Western blotting (Figure 4A & B). Only the freshly cut slices contained a similar amount of Cx43 to the intact normal heart and in all three phosphorylation states. The total amount of Cx43 in adult slices of rat dramatically decreased to approximately 30% of fresh samples by day 1, followed by further reduction to 13% at 6 days in rat slices in culture. Phosphorylated Cx43 rapidly disappeared and only the dephosphorylated form present by 1day in culture. Even the dephosphorylated form was barely detectable after 3 days in culture.

Characterisation of Embryonic Heart Slices

Heart slices of 19-day rat embryo also stably attached to the bottom of the culture dish with a support of nylon mesh for as long as 1 month. Freshly cut rat embryonic heart slices showed a typical pattern of myocardial structure and morphology of the embryonic heart [28]. Even though fibroblasts migrated from the slices during culture period, the overall structure of the slices was almost maintained. This was associated with a large number and area of persistent spontaneous regional contractions. Regional spontaneous contractions at multiple sites were observed even after 35 days in culture. In addition, in contrast to the adult slices, embryonic heart slices maintained their Cx43-containing gap junctions for a longer period. Immunolabeling

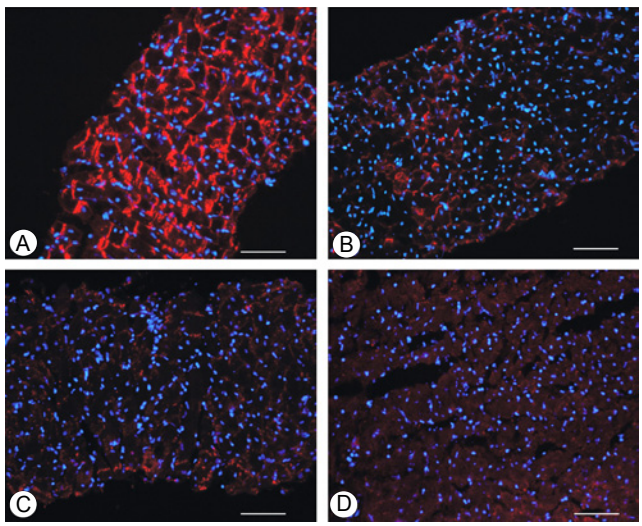

Figure 3: Immunolabeling for Cx43 in adult heart slices. Immunolabeling showed robust expression of Cx43 (red label) at the intercalated discs in sections of freshly cut adult rat heart slices (A). Nuclei were counter-stained in blue with DAPI in the fixated sections. After 1 day in culture (B) the intensity of Cx43 labeling was much reduced. The Cx43 labeling was further reduced after 3 days in culture (C) and was non-existent after 6 days (D). One representative picture from 4 slices (from 4 different hearts) at each time point is presented. Bar markers = 100 µm.

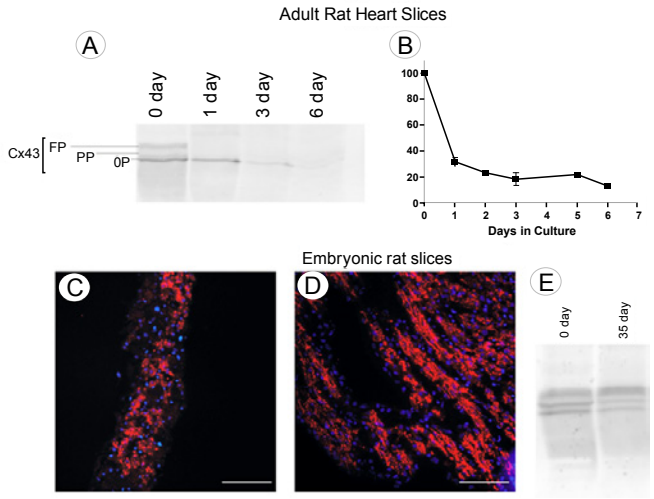

Figure 4: Different Cx43 expression between adult and embryonic heart slices. Western blots showed that the total Cx43 content of rat heart slices decreased with the increasing number of days in culture (A). Only freshly cut slices (0 day) contained all three phosphorylated forms of Cx43. Quantification (B) showed an approximately 70% decrease in total Cx43 expression after 1 day, decreasing 13% in rat slices after further days in culture. FP/PP, fully/partly phosphorylated forms of Cx43; 0P, dephosphorylated form of Cx43. n=4 at each time point.

Rat embryonic heart slices remained viable for longer in culture. Cx43 labeling of embryonic heart slices after 6 days in culture (C) showed positive gap junctional labeling similar in appearance to that observed in whole embryonic rat heart sections (D). One representative picture from 4 slices (from 4 different hearts) at each time point is presented. Bar markers = 100 µm. Western blot analysis (E) demonstrated that even after 35 days in culture levels of Cx43 remained similar to those observed in freshly-cut (day 0) slices and that all three phosphorylated forms were present.

showed that Cx43 expression was not reduced up to 6 days in culture (Figure 4C) and the expression pattern (intracellular localization) was similar to that observed in the intact embryonic heart (Figure 4D). Furthermore, Western blot analysis quantitatively confirmed that the expression level of Cx43 in embryonic heart slices was preserved for a long time (87.5 % of fresh sample at day 35) in all three phosphorylation states (Figure 4E).

Discussion

Our histological investigations demonstrated that while the majority of the adult heart slices remained intact in structure and morphology for up to 6 days, there were increasing amounts of apoptosis during the course of culture. Furthermore, expression of Cx43, a key player in the formation of cell-cell communication between donor-derived and host cardiomyocytes, was rapidly and substantially down regulated in the cardiomyocytes of the adult heart slices. On the other hand, embryonic heart slices well maintained the morphology as well as Cx43 expression for more than one month, though in the embryonic pattern. These results would provide cautions to the future use of this experimental model for stem cell transplantation research.

Adequate expression of Cx43 in host cardiomyocytes is a vital requirement for a model to be suitable for studying intercellular communication between donor-derived and host cardiomyocytes after stem cell transplantation. In addition, Cx43 plays a role in the electrophysiology of cardiomyocytes [29]. Thus, we considered Cx43 expression as the barometer to validate the availability of the cultured heart slices for cell transplantation research. However, results of the present study, to our disappointment, described a limitation of adult rat heart slices for this purpose. Despite an intact appearance, adult heart slices showed a marked reduction of Cx43 expression, rapidly to 30% of fresh samples at day 1 and further to 5-13% at day 6. In addition, phosphorylated Cx43 quickly disappeared by day 1 in culture and the Cx43 remaining was all in the dephosphorylated form. This was associated with a significant amount of apoptosis increasing in a time-dependent manner. Approximately 15% of cells were apoptotic in adult rat heart slices at day 1 and 60% at day 6. This is consistent with the previous report [17]. They demonstrated that, in particular, the contractility of heart slices at day 1 after generation had reduced to only one-tenth of that of fresh heart slices. Also, they could measure action potential parameters of surviving cardiomyocytes in the heart slices at day 28, but most of these parameters were significantly deteriorated compared to freshly-cut heart slices. However, these data are not totally negative for the use of adult heart slices; it could be acceptable to use this model in a short-term experiment, where freshly cut slices are immediately used. In fact, previous reports [10,13-16] successfully utilized this option. Similarly, immediate evaluation of freshly cut slices from adult hearts which had been injected with stem cells may also be feasible to investigate integration of the donor cells. However, it is clear that the adult heart slice requires significant improvement to be used as a recipient of cell transplantation (addition or injection of cells into the slices). For this purpose, at least 1-2 days culture is needed after cell transplantation so that intercellular connections between donor-derived and host cardiomyocytes can develop; however, adult heart slices would, based on our results here, largely lose the ability to form gap junctions during this time in culture.

A major cause of such dysfunction and apoptosis of cultured adult heart slices was presumed to be environmental stress including mechanical damage, hypoxia, free radical-mediated insult, inappropriate culture conditions and so on. We have tested several strategies to minimize these insult, including myocardial preservation during the cutting process by keeping the samples cool using cold-Krebs solution with selection of suitable culture medium and the use of nylon mesh to enhance the attachment of the slices to the surface of the culture dish. We made an effort to complete these processes for heart slice generation as quickly as possible. Despite these efforts, we were not able to sufficiently protect cultured adult heart slices from apoptosis or down regulation of Cx43 expression. Although this study focused on the validation of heart slices generated using common protocols, further investigations to refine a protocol for improving and better maintaining the quality of adult heart slices are needed. Continuous or repeated electrical stimulation, which could attenuate the Cx43 down regulation, may be an option. In addition, the cut thickness could affect the viability and quality of slices particularly in adult cardiomyocytes that are larger than neonatal/embryonic cardiomyocytes. However, given that the length of adult rat cardiomyocytes is 130-135 µm [30], it is considered that 300 µm thickness in our study would be generous enough to maintain a significant proportion of undamaged cells with intact intercalated discs and gap junctions in the slices.

Pillekamp and co-workers [11] have reported that embryonic rat heart slices exhibit normal histology and electrophysiological function with only marginal apoptosis for at least 24 hours. Our results in the present study are consistent with their observations. We demonstrated that, in contrast to adult heart slices, embryonic heart slices remained viable with active contractions for as long as 1 month in culture. Importantly, Cx43-containing gap junctions were much better preserved in the embryonic heart slices (87.5% of fresh samples at day 35 in culture) compared to adult ones. Furthermore, all three different

phosphorylation forms were maintained. Therefore, embryonic slices may provide a better model for the study of donor cell integration into the host myocardium than the adult heart slices. However, one needs to bear in mind the disadvantages in using embryonic tissues. It is known that the structure of the embryonic/neonatal rodent heart is not fully matured and the cardiomyocytes do not have the adult morphology and function [31]. The expression pattern of the gap junction protein is also different between adult and embryonic/neonatal hearts [23,32]. Thus, relevance of this model may be limited as a model of the adult heart. Having said this, culture of neonatal cardiomyocytes is, nowadays, a commonly-used model in cardiac research and is widely accepted as an established model even in cell therapy-related research [33,34]. Similarly, heart slices derived from 19-day embryos, just before birth, could be accepted as a useful model which will provide principal proof of concept in basic science.

In conclusion, we have defined the viability and quality of the adult and embryonic rodent heart slices and found that both kinds of slice have limitations as a model to study cell-cell communication between donor-derived and host cardiomyocytes after stem cell transplantation. Further study to improve the quality of this promising model is warranted.

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