Cardiovascular tissue engineering and regeneration based on adipose tissue-derived stem/stromal cells
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CHAPTER 3

Differential Mechanisms of Myocardial Conduction Slowing by Adipose Tissue-Derived Stromal Cells Derived from Different Species

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Under revision
Abstract
Stem cell therapy is a promising therapeutic option to treat patients after myocardial infarction. However, the (intramyocardial) administration of large amounts of stem cells might generate a pro-arrhythmic substrate. Pro-arrhythmic effects can be explained by electrotonic and/or paracrine mechanisms. The narrow therapeutic time window for cell therapy and the presence of comorbidities limit the application of autologous cell therapy. The use of allogeneic or xenogeneic stem cells are a potential alternative to autologous cells, but differences in the pro-arrhythmic effects of adipose derived stromal cells (ADSC) across species are unknown. Using micro-electrode arrays and micro-electrode recordings we obtained local unipolar electrograms and action potentials from monolayers of neonatal rat ventricular myocytes (NRVM) that were co-cultured with rat, human or pig ADSC (rADSC, hADSC, pADSC). Monolayers of NRVM were cultured in the respective conditioned medium to investigate paracrine effects. We observed significant conduction slowing in all cardiomyocyte cultures containing ADSC, independent of species used (p<0.01). All co-cultures were depolarized compared to controls (p<0.01). Only conditioned medium taken from co-cultures with pADSC and applied to NRVM monolayers demonstrated similar electrophysiological changes as the corresponding co-cultures. We have shown that independent of species used, ADSC cause conduction slowing in monolayers of NRVM. In addition, pADSC exert conduction slowing mainly by a paracrine effect, were as the influence on conduction by hADSC and rADSC is preferentially by electrotonic interaction.
Differential Mechanisms of Myocardial Conduction Slowing by ADSC from Different Species

Introduction

Up to one third of the patients with myocardial infarction develop heart failure despite improvements in reperfusion therapy (1). Stem cell-based therapy has been suggested as a promising therapeutic modality to improve cardiac function in these patients (2-4). However, there are concerns for the potential pro-arrhythmic effects of stem cell therapy (5-7). One proposed mechanism for the pro-arrhythmic potential is the formation of electrotonic interaction between cardiomyocytes and stem cells, allowing interaction between the interior of the two cells (8). The membrane potential of mesenchymal stem cells is approximately \(-35\) mV (9,10). As a consequence, electrotonic coupling between a stem cell and a ventricular myocyte is expected to cause depolarization and a change in the action potential morphology of the myocytes. This may result in conduction slowing, conduction heterogeneity and unidirectional conduction block, together facilitating re-entrant arrhythmias (11,12). A second suggested pathway is the involvement of paracrine factors that can either directly or indirectly (paracrine cross talk) influence cardiomyocyte and/or stem cell function (7). Another drawback of cell based therapies concerns the availability of stem cells. Autologous stem cells such as mesenchymal stem cells from bone marrow are not only rare but also difficult to obtain and expand to the large number required for treatment. Multipotent cells, such as adipose tissue-derived stromal cells (ADSC) are however highly abundant in liposaspirates, which are easy to obtain from healthy individuals. ADSC are not only abundantly present, they are a source of multi-potent cells capable of differentiating along multiple lineage pathways with little immunological effects (13,14). In addition, ADSC secrete a wide variety of factors known to stimulate angiogenesis(15) and neovascularization(16), making them clinically relevant for possible cell based therapies and their use is favored to date. However, the function of autologous stem cells can deteriorate due to age and risk factors such as hyperglycemia and hyperlipidemia, which are present in the elderly population where myocardial infarctions are most prevalent (17,18). Current studies that describe the safety and efficacy of allogeneic stem cells indicate these can be used as a “off-the-shelf” alternative for autologous stem cells (19,20). Also, xenogeneic stem cells, are considered an alternative to autologous stem cell administration and is described frequently (21-24). The potential difference in the pro-arrhythmic effects of adipose derived stromal cells across species is unknown. In this \textit{in vitro} study we specifically address the potential adverse electrophysiological effects of different adipose tissue-derived stromal cells have on a confluent layer of neonatal rat ventricular (cardio) myocytes (NRVM). We specifically studied the different (allogeneic and xenogeneic) species sources of ADSC namely rat, human and pig.
Chapter 3

Materials and Methods

Methods

Isolation and culturing of neonatal rat ventricular myocytes

All animal experiments were approved by the local Animal Experiments Committee (AMC, University of Amsterdam and UMCG, University Medical Center Groningen) and carried out in accordance with national and institutional guidelines. Briefly, hearts were explanted from one to two days old Wistar rats. Ventricles were dissected into pieces and dissociated with trypsin (Becton Dickinson BV, Breda, The Netherlands) and collagenase (Worthington Vollenhove, The Netherlands, 230 units/mg). Cells were pre-plated to minimize fibroblasts contamination. The remaining myocardial cells were plated in fibronectin (BD Biosciences, Breda, The Netherlands) coated multi-electrode-arrays (MEAs; Multi Channel Systems MCS GmbH, Reutlingen, Germany) at a density of $1.4 \times 10^5$ cm$^{-2}$.

Isolation and culture of adipose tissue-derived stromal cells

ADSC were isolated and cultured as described previously (25). Inguinal rat fat (male, Wistar, 7-8 months), porcine subcutaneous abdominal fat (male, 3-4 months, kindly provided by the department of experimental surgery of the AMC) and human subcutaneous abdominal fat (donated by healthy patients with body mass index below 30; Bergman Clinics, The Netherlands) was used. Tissue was minced and washed extensively with PBS, before being subjected to dissociation steps with collagenase (Roche Diagnostics, Mannheim, Germany). The obtained stromal vascular fraction was then incubated with erythrocyte lysis buffer after which the cells were seeded at a density of $4 \times 10^4$ cells/cm$^2$, ADSC were propagated at a 1:2 ratio and used from passage 3 onwards. Cells were referred to either rat ADSC (rADSC) or human ADSC (hADSC) or pig ADSC (pADSC). The use of liposuction material as source of ADSC was approved by of the local Ethics Committee of University Medical Center Groningen, given the fact that it was considered anonymized waste material. Yet, for every one of these anonymous donations the clients gave their consent after information.

Experimental conditions

To investigate effects of ADSC, co-cultures of NRVM and ADSC were prepared. Four days after seeding NRVM, ADSC were added to the monolayers at a ratio of 1:1, and two days later electrophysiological measurements were performed. To assess paracrine effects, conditioned medium was collected from monolayers of NRVM (Cme NRVM), co-cultures (Cme NRVM: ADSC) and confluent cultures of ADSC (Cme ADSC). Medium was also collected from co-cultures with transwell inserts: this medium was referred to as 'Cme transwell ADSC'. In transwell experiments NRVM and ADSC are cultured together without making direct contact. Cme was filtered (0.22µm) before being added to monolayers of NRVM only on day four of culture and two days prior to measurements.

Electrical mapping and microelectrode measurements

Electrophysiological parameters were determined by mapping of the electrical activity of the
monolayers. MEAs harbored 60 electrodes terminals, aligned in an 8 by 8 matrix with terminals in the core portion of the MEA (Supplemental Fig.1). Cultures were stimulated using a bipolar extracellular stimulus electrode (twice diastolic stimulation threshold, 1 or 2 ms pulse width). Conduction velocity (CV), and conduction heterogeneity were determined from the unipolar electrograms recorded. For each experiment two monolayers of NRVM only served as control. Values obtained under different conditions were compared with the values of control monolayers of the same isolation. Resting membrane potential (RMP) and upstroke velocity of an action potential were determined from action potentials recorded during micro-electrode measurements.

**Immunostainings**
Cells plated in 12-wells plate used for immunofluorescence were cultured under the same conditions as cells on MEAs. Briefly, after fixation in 4% PFA cells were permeabilized and blocked before staining with primary and secondary antibodies. Examination was performed by Leica SPE confocal laser scanning and Leica Application Suite Advanced Fluorescence software.

**Statistical analysis**
Continuous and normally distributed variables are presented as mean ± standard deviation, and compared using an independent T-test and in case of more than two groups a one-way ANOVA was performed with the Bonferroni correction as post-hoc analysis. In case of a skewed distribution data are presented as median with the interquartile range (IQR) and tested with the Mann Whitney test, in case of more than two groups a Kruskal-Wallis analysis was performed with post-hoc analysis using the Dunn’s test. A p-value of <0.05 was considered statistically significant. All graphs were made using GraphPad Prism software version 5 (Graphpad Software, La Jolla, Ca, USA).

**Results**

**Effects of co-culturing ADSC with NRVM**
Monolayers of NRVM co-cultured with rADSC demonstrated conduction slowing compared to monolayers of NRVM only (Fig. 1A). On average conduction velocity was 14.4±3.2 cm/s in monolayers of NRVM co-cultured with rADSC compared to 20.0±1.6 cm/s in control monolayers (p<0.001, Fig. 1B). Similar to rADSC, monolayers that were co-cultured with either hADSC (13.0±2.8 cm/s) or pADSC (8.0±3.9 cm/s) also demonstrated significant conduction slowing compared to their respective controls (19.3±2.4 and 20.2±2.8 cm/s respectively, p<0.001, Fig 1. A and B). Conduction heterogeneity in monolayers of NRVM co-cultured with rADSC demonstrated a trend to be higher compared to control monolayers (9.11 (3.8) vs. 6.2 (1.95) ms, p=0.056, Fig. 1C). Heterogeneity in co-cultures with hADSC was on average higher than in control monolayers (10.3 (5.9) vs 7.2 (5.1) ms p< 0.01; Fig 1C). Monolayers co-cultured with pADSC (13.3 (17.7) ms) also demonstrated a significant increase in conduction heterogeneity compared to monolayers of NRVM only (6.4 (2.9) ms, p<0.001, Fig. 1C).
Figure 1. Effect of ADSC on conduction velocity and heterogeneity in monolayers of NRVM. A: Activation map of a monolayer of NRVM, a monolayer cultured with rADSC, a monolayer with hADSC and a monolayer with pADSC respectively. Conduction velocity is determined along white arrows perpendicular to isochronal lines. B: conduction velocity of controls and different co-cultures and C: conduction heterogeneity. * indicates p< 0.001 compared to the monolayers of NRVM.

Effects of conditioned medium of NRVM: ADSC

In order to determine the mechanisms behind the conduction slowing, we cultured monolayers of NRVM in conditioned medium (Cme) obtained from the various co-cultures. Conduction velocity in monolayers of NRVM cultured in Cme of the NRVM: rADSC co-cultures was not different from conduction velocity (19.2±2.0 cm/s) or conduction heterogeneity (7.0 (5.4) ms) in control monolayers (21.8±1.8 cm/s and 5.9 (1.9) ms; p=ns, Fig. 2A and B). Conduction velocity in monolayers of NRVM cultured with Cme of NRVM: hADSC co-cultures was also not affected compared to controls (18.5±2.2 vs. 19.0±1.2 cm/s, p=ns; Fig. 2A). Conduction heterogeneity was not affected either when monolayers of NRVM were cultured in Cme NRVM: hADSC (4.9 (2.0) vs. 5.3 (1.9), p=ns; Fig. 2B). In contrast, Cme NRVM: pADSC slowed conduction velocity significantly compared to control monolayers (7.0±2.9 vs. 19.6±2.4 cm/s, p<0.001, Fig. 2A). Conduction heterogeneity was also significantly increased by Cme NRVM: pADSC compared to control monolayers (16.3 (13.2) vs. 5.5 (1.5) ms, p<0.001; Fig. 2B). Cme NRVM served as control for the conditioned medium conditions and did not differ from control monolayers in any of the groups (Fig. 2A and B). The conduction velocity or the heterogeneity in monolayers co-cultured with pADSC was not significantly different from the CV or the heterogeneity in monolayers of NRVM cultured in Cme NRVM: pADSC (compare...
Conditioned medium of the co-cultures NRVM: pADSC affected conduction properties of monolayers of NRVM. To distinguish whether this effect is attributed to soluble factors of pADSC or whether there is an interaction (cross talk and/or electrotonic connections) between pADSC and NRVM we further explored the effects of Cme pADSC and Cme transwell pADSC. Monolayers of NRVM cultured in Cme transwell pADSC and Cme pADSC both demonstrated significant lower conduction velocities compared to controls (16.3±2.4, 14.6±1.6 vs. 19.6±1.8 cm/s, respectively, p< 0.05, Fig. 3A). Conduction heterogeneity was only affected by Cme pADSC (10.4 (5.0) vs. 5.7 (3.8) ms, p<0.05; Fig. 3B). Conditioned medium obtained from only hADSC and rADSC did not elicit an effect on conduction velocity or the heterogeneity of NRVM monolayers (Supplemental Fig. 2). Direct application of Cme NRVM: pADSC did not have an effect (results not shown).

**Figure 2.** Effect of Cme ADSC: NRVM on conduction velocity and heterogeneity in monolayers of NRVM. Effects on A: conduction velocity and B: conduction heterogeneity in monolayers of NRVM cultured in the condition medium obtained from the different co-cultures.* indicates p< 0.01 compared to control monolayers and monolayers of NRVM cultured in Cme NRVM.
Figure 3. Effect of Cme pADSC transwell and Cme pADSC on conduction velocity and heterogeneity in monolayers of NRVM. Effects on A: conduction velocity and B: conduction heterogeneity in monolayers of NRVM cultured in condition medium obtained from the transwell co-cultures and pADSC culture, * indicates p<0.05.

Micro-electrode measurements
Micro-electrode measurements were performed to study whether the observed conduction slowing could be explained by depolarization. As expected, monolayers of NRVM co-cultured with rADSC, hADSC and pADSC were depolarized compared to control monolayers (resting membrane potential (RMP) -50.95±9.45 vs. -65.06±5.98 mV, -52.6±15.2 vs. -71.2±13.1 mV and -44.7±16.2 vs. -66.0±7.9 mV respectively, p<0.01, Fig. 4). Although monolayers cultured in Cme NRVM: rADSC and Cme NRVM: hADSC demonstrated no effect on conduction velocity, these monolayers were depolarized compared to controls (-55.4±6.2 vs. -65.1±6.0 mV and -52.1±12.8 vs. -71.2±13.1 mV, respectively, p<0.01). Cme NRVM: pADSC elicited heterogeneous conducting slowing and these cultures were also depolarized compared to controls (-44.0±9.0 vs. -66.0±7.9 mV, p<0.01, Fig. 4). Depolarization in monolayers of NRVM induced by Cme NRVM: pADSC was significantly more compared to depolarization induced by Cme NRVM: rADSC and Cme NRVM: hADSC.

Figure 4. Effects of ADSC and conditioned medium on membrane potential. The effects of co-culturing A: rADSC, B: hADSC and C: pADSC together with NRVM and the effects of conditioned medium on resting membrane potential. * indicates p< 0.001 compared to control monolayers (N = impalements).
**Relationship between RMP and conduction velocity**

A theoretical sigmoid relation exists between RMP and CV (26,27). We studied whether the relation between local RMP and conduction velocity was maintained in co-cultures and after culturing in the presence of Cme. Figure 5A shows the relation between RMP and CV in the three different co-cultures as well as in the pooled control monolayers (NRVM). In a similar fashion figure 5B shows the combined data of monolayers subjected to Cme of the various species and their corresponding pooled controls. In both panels a sigmoid function is fitted through the combined data points (black lines). Because the average data do not appear to deviate from the theoretical sigmoid function the figure show that the degree of depolarization of each monolayers is the main determinant of the conduction velocity and that the degree of depolarization is different in the various conditions (see also Fig. 4).

![Figure 5. Relationship between RMP and conduction velocity.](image)

**Cell characterization and gap junctions**

Confluent monolayers of NRVM and co-cultures were visualized with light microscopy and immunostaining at day six. Immunofluorescence staining was performed using the cardiomyocyte and ADSC marker, alpha actinin and CD44 respectively (Supplemental Fig. 3). Fluorescent microscopy results revealed that ADSC were scattered heterogeneously throughout the NRVM monolayer (Supplemental Fig. 3). Immunofluorescence was performed to visualize connexin 43 (Cx43) and connexin 45 (Cx45) on cardiomyocytes or ADSC. Co-cultures were stained for CD44 and the connexins Cx43 (Fig. 6.1A-D) and Cx45 (Fig. 6.2A-D). In monolayers of NRVM Cx43 and Cx45 are abundantly present (Fig. 6.1A and 6.2A). In monolayers of NRVM co-cultured together with either rADSC or hADSC Cx43 and Cx45 are also seen (indicated with white arrowheads). However, in the monolayers of NRVM co-cultured with pADSC Cx43 and Cx45 are rarely seen (Fig. 6.1D and Fig. 6.2D).
Figure 6. Immunofluorescence micrographs of the various cultures stained with CD44 and Cx43 and Cx45. Monolayers of NRVM are stained with CD44 and Cx43 (A1) and with CD44 and Cx45 (A2). Monolayers of NRVM co-cultured with rADSC (B1+B2), NRVM monolayers co-cultured with hADSC (C1+C2) or pADSC (D1+D2) are stained for the same markers.
**Figure 7.** Schematic illustration of the various interactions between NRVM and ADSC. The figure summarizes the study. First co-cultures of cardiomyocytes and ADSC are studied. In this scenario all situations are possible: electrotonic interactions and the various paracrine interactions, we can therefore not exclude or identify which of the situations explains the heterogeneous conduction slowing. The next step is to distinguish between electrotonic interactions and the paracrine interactions. Experiments with Cme transwell conditions can allow simple to complicated cross talk situations; paracrine only, paracrine + autocrine or paracrine + paracrine; where soluble factors of one cells leads to the secretion of soluble factors by the other cells, which in turn stimulates the first cell to secrete different soluble factors. Experiments done with Cme ADSC can only be explained by paracrine (1) effects of ADSC on NRVM, or paracrine factors from ADSC initiate NRVM to secrete soluble factors that have an autocrine effect (paracrine + autocrine (1)). The situations that are crossed out can also occur, however the focus of the study is on the effects ADSC have on NRVM conduction properties and not what effects NRVM have on ADSC, and therefore these situations are omitted. If we follow the logic of the scheme we can conclude that the primary mechanism for hADSC and rADSC is electrotonic as heterogeneous conduction slowing is not observed when Cme ADSC is used. When Cme ADSC and Cme transwell of pADSC is used we still observe heterogeneous conduction slowing suggesting the primary effect is paracrine based.

**Discussion**

In this study we have shown that application of ADSC, regardless of the species origin, causes heterogeneous conduction slowing in monolayers of NRVM. The conduction effect could be attributed to electrotonic interaction and/or paracrine mechanisms. To distinguish between these mechanisms we first investigated the effects of conditioned medium obtained from the various co-cultures. Only conditioned medium from co-cultures of NRVM and pADSC replicated the effects observed in the co-cultures. This indicates the involvement of soluble factors and possible paracrine cross talk between the two cell types, in a deleterious way. In man and rat the paracrine effects could not be replicated, suggesting that electrotonic coupling plays a more prominent role in these species. The existence of paracrine cross talk between cardiomyocytes and non-cardiomyocytes has been suggested by Pedrotty et al. (28) and others (7,29). Pedrotty et al. demonstrated that conditioned medium from a culture of cardiac fibroblasts altered electrophysiological properties of NRVM. However, when the same fibroblasts were grown in the presence of NRVM and the resulting conditioned medium was used, all arrhythmogenic effects disappeared, suggesting that cardiomyocytes were ‘activated’ to produce protective factors that protects them from damaging soluble factors secreted by the fibroblasts (28). In order to discriminate whether the observed heterogeneous conduction slowing could be attributed to paracrine cross talk between NRVM and pADSC or solely to the soluble factors of pADSC, we used transwell inserts. In these cultures pADSC and NRVM are unable to physically connect, eliminating electrotonic interactions, but allowing the exchange of soluble factors. Conditioned medium from transwell conditions were used to culture monolayers of NRVM and the results were compared to those obtained in conditioned medium from pADSC only. Our results show that ADSC produce adverse soluble factors that slow the conduction velocity of NRVM monolayers. Because the conduction induced by Cme pADSC (16.3±2.4 cm/s) and Cme transwell pADSC (14.6±1.6 cm/s) is larger than in the corresponding co-cultures (8.0±3.9 cm/s, Fig. 1) we deduce that the reduction of conduction velocity by pig ADSC does at least in part depend on direct interaction between...
the ADSC and NRVM. Because the conditioned medium obtained from the NRVM: pADSC co-culture fully replicated the effect of the corresponding co-culture (compare Fig. 1 and 2) the intercellular interaction is likely fully juxtacrine/paracrine. In the interaction between NRVM and ADSC derived from man and rat, electrotonic coupling likely plays a role. First, condition medium obtained from the co-culture of NRVM and rat or human ADSC did not replicate the results from the corresponding co-culture and the physical presence of the ADSC is therefore required for the production of conduction slowing. However, conditioned medium of NRVM: rADSC and NRVM: hADSC co-cultures did induce depolarization in NRVM monolayers that was not different from the depolarization in the co-cultures. This suggests that soluble factors are responsible for the depolarization, but that this is not sufficient for conduction slowing. The relation between RMP and conduction velocity is non-linear (26,27) and it is well possible that the depolarization induced by conditioned medium of rADSC and hADSC co-cultures was slightly less than the conditioned medium of pADSC co-cultures and therefore insufficient to lead to a conduction slowing. The degree of depolarization, however, was the same in all subsets. Therefore, it is more probable that the depolarization induced by the soluble factors alone is not enough to induce the heterogeneous conduction slowing in rat and human ADSC co-cultures, and additional intercellular coupling is required. The coupling between the ADSC and the myocytes would then provide additional depolarization (9), may lead to additional capacitative loading of the NRVM (30), or cause interference with sodium channel function. We have also shown that the relationship between the RMP and the conduction velocity does not deviate from the theoretical sigmoid relation and that it is the same in co-cultures and in monolayers subjected to Cme alone (Fig. 5). This suggests that the degree of depolarization determines conduction velocity in each condition. Whether the RMP is determined by paracrine factors or by electrotonic coupling depends on species and conditions. From the immunofluorescence data we deduce that connexins (Cx43 and Cx45) are present at the interface between NRVM and ADSC in the co-cultures (with rADSC and hADSC). Although we are not sure that the connexins are electrically connecting the cell interiors of either cells (this would require double voltage clamp, which is not feasible in a co-culture), this may be the basis of heterogeneous conduction slowing in these co-cultures. In contrast, connexins are barely present in co-cultures with pADSC. Taken together these data support the idea that electrotonic interaction is the main contributor of the significant heterogeneous conduction slowing in co-cultures with rADSC and hADSC. All cells have a wide secretome of soluble factors that are secreted and that can influence the behavior and the secretome of other cells. However, these soluble factors in turn can be influenced by environmental factors as well as other soluble factors secreted either by other cells or indirectly by the cell itself (autocrine). The exact nature of the soluble factor(s) responsible for inducing the observed heterogeneous conduction slowing is unlikely to be identified and outside the scope of this paper. Figure 7 gives a schematic summary of this study and the possible cross talk interactions that can take place between cardiomyocytes and the various ADSC used. Our findings that ADSC influence electrophysiological properties of NRVM corroborates previous studies, both in vitro and in vivo, that demonstrated that stem cells influence electrophysiological properties(5,6,31,32). However, in this study we specifically studied three different species sources of ADSC namely rat, human and swine. Human and porcine ADSC where chosen to investigate their arrhythmogenic potential and to see whether porcine
ADSC react differently than human ADSC. RADSC were chosen to model allogeneic stem cell application. We have shown that cells of the same species as the monolayer cause similar conduction slowing as xenogeneic stem cells. Although NRVM and human cardiomyocytes differ, the use of NRVM (cultured on MEAs) has been established as a reliable model for electrophysiological studies (33-35). In comparison with adult models the RMP and CV values obtained in this and other studies are rather low (7,36,37). In NRVM monolayers therefore sodium channels are partially inactivated. In view of these and our own observations, we assume that propagation in the monolayers of NRVM subjected to ADSC is, (due to depolarization, also) partially carried by the calcium current resulting in relatively low conduction velocities. The advantage of the in vitro model of ADSC transplantation is that it allows a controlled application of stromal cell number and conditioned medium to a 2D model excluding the influence of confounding factors.

Conclusions

Our results show that ADSC cause (heterogeneous) conduction slowing when co-cultured on a monolayer of NRVM. Paracrine modulation and intercellular coupling between these two cell types contribute to the formation of a potentially pro-arrhythmic substrate. We have generated a paracrine based pro-arrhythmic cell model with pADSC and an electrotonic based pro-arrhythmic cell model with hADSC. The study shows that adipose stromal cells from different species may interfere with host cardiomyocytes via different mechanisms. We have also demonstrated that the arrhythmic potential of stem cells is maintained even when cross-species transplantation is used. Our study was designed to address potential adverse electrophysiological effects of ADSC based therapies. Although the question whether the excreted soluble factors are ‘beneficial’ (e.g. the potential hemodynamic benefit seen in a more clinical setting), is outside the scope of this paper, we have shown that conditioned medium from hADSC alone does not cause conduction slowing and could thus potentially be used for the possible beneficial soluble factors it contains without having the adverse effects of the interactions these cells can form with cardiomyocytes.

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SUPPLEMENTARY MATERIAL

Methods

**Isolation and culturing of neonatal rat ventricular myocytes**

Neonatal rat ventricular myocytes (NRVM) were isolated as follows. One to two days old Wistar rats were decapitated and the hearts were rapidly explanted. Atrial tissue was removed and ventricles were dissected into pieces and left to rotate overnight at 4 °C in HBSS (Gibco, Den Haag, Netherlands) containing trypsin (1 mg/mL; Becton Dickinson BV, Breda, The Netherlands). The following day the enzymatic effect of trypsin was inactivated with culture medium (M199 medium; Gibco; supplemented with 10% heat inactivated fetal bovine serum (FBS; Gibco), 1% HEPES (Gibco #5630-0-80), 5000 U/L penicillin-G (Sigma, #P7794), 2 mg/L vitamin B12 (Sigma, #V2876), 3.5 g/L glucose, 1% non-essential amino acids (Gibco, #11140-050), and 1% L-glutamine (Gibco, #25030-081)), ventricles were enzymatically dissociated in HBSS containing collagenase type 2 (1 mg/mL, Worthington Vollenhove, The Netherlands, 230 units/mg) at 37 °C, centrifuged at 160 g, 5 minutes and cells were re-suspended in culture medium. To separate fibroblasts from cardiomyocytes, cells were pre-plated in a polystyrene treated T175 cell culture flask at 37°C in 5% CO2, 95% humidity and 21% O2. After two hours, non-adherent cells, i.e. predominantly NRVM, were collected and were seeded at 1.4 x 10^5/cm² onto microelectrode arrays (MEAs; Multi Channel Systems MCS GmbH, Reutlingen, Germany). This array has 60 integrated extracellular electrodes aligned in an 8 by 8 matrix at interelectrode distances of 0.7 mm. MEAs were coated with fibronectin (125 µg/ml BD Biosciences, Breda, The Netherlands) at least two hours prior to NRVM seeding. NRVM were cultured at 37 °C 5% CO2, 95% humidity and 21% O2 in culture medium, which was switched to 2% FBS two days after cells were seeded on the MEA. The day after seeding, NRVM were washed twice with HBSS (Gibco) and fresh culture medium was added. Light microscopy was used to determine if a confluent monolayer had formed in each of the cultures.

**Isolation and culture of adipose tissue-derived stromal cells**

ADSC were isolated and cultured as described previously (1). Adipose tissue was dissected from rats’ inguinal fat (male, Wistar, 7-8 months), porcine (male, 3-4 months old, kindly provided by the department of experimental surgery of the AMC) or human subcutaneous abdominal fat (donated by healthy patients with body mass index below 30; Bergman Clinics, The Netherlands) and stored at 4 °C. Within 24 hours, the adipose tissue was minced and washed extensively with PBS. The tissue fragments were incubated in an equal volume of PBS with 0.1% Collagenase A (Roche Diagnostics, Mannheim, Germany), containing 1% bovine serum albumin (BSA; Sigma-Aldrich, Boston, MA) at 37 °C for 1 hour while exposed to continuous shaking. The enzymatic activity of collagenase was stopped by adding PBS, 1% BSA and digested tissue were filtered through 70 µm filters. The collected cell suspension was subjected and centrifuged at 600xg for 10 min. The supernatant was discarded and stromal vascular fraction (SVF) was incubated with an erythrocyte lysis buffer at 4°C for 5 min. Then, SVF pellet was collected by additional centrifugation. Cells were suspended in culture medium that consisted of DMEM (Lonza Biowhittaker, Verviers, Belgium), supplemented with
10% FBS (Thermo Scientific, Hemel Hempstead, UK), 100 U/mL penicillin, 100 mg/mL streptomycin (Gibco, Invitrogen, Carlsbad, CA) and 2 mM L-glutamine (Lonza Biowhittaker, Verviers, Belgium). The ADSC were seeded at a density of 4 x 10^4 cells/cm^2 and cultured at 37 °C, 5% CO2, 95% humidity and 21% O2. Culture medium was refreshed every two days till 80-90% confluence was reached. ADSC were propagated at a 1:2 ratio and used from passage 3 onwards for the experiments. Cells were referred to either rat ADSC (rADSC) or human ADSC (hADSC) or pig ADSC (pADSC). For conditioned medium; confluent flasks of ADSC were cultured in NRVM culture medium containing 2%, after twenty-four hours medium was collected, filtered through a 0.22 µm filter (MILLEX® GV SLGV033RS) labelled Cme-ADSC and stored at -20 °C until use.

**Experimental conditions**

Monolayers of NRVM containing ADSC were prepared by treating ADSC with mitomycin-C (Sigma M4287-2MG) and were labeled with CDFA-SE (Invitrogen Vybrand® CFDA SE Cell Tracer Kit) according to manufactures protocol. ADSC were collected using accutase (Gibco, A11105-01 StemPro® Accutase®), and centrifuged for 5 minutes at 160g. Supernatant was removed and the pellet was re-suspended in NRVM culture medium containing 2% FBS. ADSC were then added to monolayers of NRVM in cell ratios of NRVM: ADSC 1:1. Monolayers of NRVM serving as controls received fresh NRVM culture medium containing 2% FBS. Two days later electrical mapping was performed. Medium from NRVM monolayers and the monolayers containing the different species of ADSC was collected after two days, filtered through a 0.22 µm filter (MILLEX® GV SLGV033RS) and stored at -20 °C until use. This medium was referred to as conditioned medium (Cme) and labeled as follows; Cme NRVM, and Cme NRVM: ADSC. For the transwell setup monolayers were created in fibronectin coated T12 wells, as described above. On day 4, transwell inserts were placed inside the well and ADSC were seeded into these inserts. The ratio of NRVM: ADSC was 1:2 as the surface area of the inserts is smaller than the T12 wells. Two days later medium was collected, filtered, labelled Cme transwell ADSC and stored at -20 °C until used. In the experiments investigating the paracrine effects, conditioned medium was added to monolayers of NRVM only, two days prior to electrical mapping.

**Electrical mapping and microelectrode measurements**

The MEAs containing the different cultures were positioned in a temperature controlled (37 °C MEA holder (TC01/02 Multichannel Systems MSC GmbH). Each MEA harbored 60 electrodes which had terminals in the core portion of the MEA (Supplemental Fig.1). On every day of experimentation two monolayers of NRVM from the same cell isolation served as controls. All cultures were stimulated from at least two stimulation sites using a bipolar extracellular stimulus electrode (twice diastolic stimulation threshold, 1 ms or 2 ms rectangular current pulses). Unipolar electrograms were recorded with a 256-channel amplifier (BioSemi, ActiveTwo, Amsterdam, The Netherlands, 24 bit dynamic range, 122.07 nV LSB, total noise 0.5 µV). Signals were recorded with a sampling frequency of 2048 Hz (filter setting of the amplifiers DC – 400 Hz (-3dB point). The recordings were made with respect to the integrated reference electrode of the MEA. Conduction velocity (CV) was determined
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from activation maps constructed using the maximum negative dV/dt as activation time (AT; relative to the time of earliest activation) with the use of a custom made program (2) based on MATLAB R2006b (The MathWorks, Inc., Natick, MA, USA). CV was determined along lines perpendicular to isochronal lines by dividing the distance by the difference in local activation time. Lines had a length of at least 4 electrode distances. Local activation times in the figures are color coded in classes of 5 ms. CV was determined at a basic cycle length (BCL) of 600 ms or during spontaneous activity if pacing was not possible. Based on the method described by Lammers et al. (3) we quantified the heterogeneity in conduction as a measure of arrhythmia vulnerability. Maximum AT differences between each adjacent electrode quartet in the grid were obtained and the total range of maximal AT differences was plotted in a histogram. Microelectrodes were pulled from glass capillaries (Harvard apparatus GC100F-10) and filled with 3 M KCl. An AgCl covered silver wire was used as a reference electrode. Following activation mapping, action potentials were recorded during pacing at BCL 600 ms. Resting membrane potential (RMP) was taken as the highest negative membrane potential recorded, upstroke velocity was taken as dV/dt max.

Immuno-staining and fluorescence imaging
For immunofluorescence, cells (1.25 x 10^5 cells/cm²) were plated in 12-well plates (MP Biomedicals) containing fibronectin-coated (125 µg/ml BD Biosciences) coverslips and cultured under the same conditions as cells on MEAs. Separate cultures were made as immuno-fluorescence could not be performed on MEAs. On the day of the electrophysiological recordings these cultures were fixed with 4% PFA for 10 minutes, permeabilized with 0.1% Triton X-100, and blocked with 1% BSA (Roche BSA fraction V #10735094001). Cells were stained with primary antibodies (mouse anti-sarcomeric-actinin primary antibody; Sigma 1:1000), mouse anti-human monoclonal CD44 primary antibody (Lifespan Biosciences, LS-B1862; 1:250), rabbit anti Connexin 43 (Invitrogen 574366A; 1:200), and rabbit anti Connexin 45 (Santa Cruz Biotechnology, sc-25716, 1:100) in 1% BSA overnight at room temperature, washed three times with PBS, and then incubated with secondary antibodies (Alexa Fluor-647 goat anti-mouse IgG (Life Technology, A21235; 1:250), Alexa Fluor-488 goat anti-mouse/rabbit IgG (Life Technology, A11008/A21222; 1:250), for two hours in 1% BSA. Cover-slips were washed an additional three times in PBS and incubated for an additional 10 minutes with either cyto*orange (Life Technology, S11368, 1:1000) or DAPI (Sigma, D9542, 1:40000), washed again with PBS and embedded in 50% glycerol/50% PBS. Examination was performed by Leica SPE confocal laser scanning and Leica Application Suite Advanced Fluorescence (LAS AF) software.
Suppl. Fig. 1. Layout of the 60 electrodes in the MEA. Each electrode has a diameter of 100 μm and an interelectrode distance of 700 μm. Numbers 1-4 represent stimulation positions.

Suppl. Fig. 2. Effect of Cme rADSC and Cme hADSC on monolayers of NRVM. Bar graphs illustrating the effects on A: conduction velocity and B: conduction heterogeneity in monolayers of NRVM cultured in condition medium obtained from rADSC and hADSC cultures.
Suppl. Fig. 3. Micrographs of the various cultures. Transmitted light and immune-fluorescent micrographs. A–D: Transmitted light micrographs of NRVM monolayer and NRVM monolayers co-cultured with the different ADSC. Black dots and lines are the electrodes in the MEA. E–H: Immune-fluorescent micrographs of NRVM monolayer and NRVM monolayers co-cultured with the different ADSC. Please note that these are not the same monolayers as panels A-D.