Arrhythmia/Electrophysiology

Cell Therapy for Modification of the Myocardial Electrophysiological Substrate

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Background—Traditional antiarrhythmic pharmacological therapies are limited by their global cardiac action, low efficacy, and significant proarrhythmic effects. We present a novel approach for the modification of the myocardial electrophysiological substrate using cell grafts genetically engineered to express specific ionic channels.

Methods and Results—To test the aforementioned concept, we performed ex vivo, in vivo, and computer simulation studies to determine the ability of fibroblasts transfected to express the voltage-sensitive potassium channel Kv1.3 to modify the local myocardial excitable properties. Coculturing of the transfected fibroblasts with neonatal rat ventricular myocyte cultures resulted in a significant reduction (68%) in the spontaneous beating frequency of the cultures compared with baseline values and cocultures seeded with naive fibroblasts. In vivo grafting of the transfected fibroblasts in the rat ventricular myocardium significantly prolonged the local effective refractory period from an initial value of 84±8 ms (cycle length, 200 ms) to 154±13 ms (P<0.01). Margatoxin partially reversed this effect (effective refractory period, 117±8 ms; P<0.01). In contrast, effective refractory period did not change in nontransplanted sites (86±7 ms) and was only mildly increased in the animals injected with wild-type fibroblasts (73±5 to 88±4 ms; P<0.05). Similar effective refractory period prolongation also was found during slower pacing drives (cycle length, 350 to 500 ms) after transplantation of the potassium channels expressing fibroblasts (Kv1.3 and Kir2.1) in pigs. Computer modeling studies confirmed the in vivo results.

Conclusions—Genetically engineered cell grafts, transfected to express potassium channels, can couple with host cardiomyocytes and alter the local myocardial electrophysiological properties by reducing cardiac automaticity and prolonging refractoriness. (Circulation. 2008;117:720-731.)

Key Words: action potentials ■ electrophysiology ■ gene therapy ■ ion channels ■ mapping

Cardiac arrhythmias account for significant worldwide morbidity and mortality. Traditional antiarrhythmic therapies are aimed at modifying the abnormal electrophysiological substrate by focal injury (surgery or radiofrequency catheter ablation),1 implantable devices,2,3 or pharmacotherapy. The latter approach is hampered by the relatively low efficacy of most antiarrhythmic agents and by their global cardiac action that may result in life-threatening proarrhythmic effects.4

Clinical Perspective p 731

Gene therapy has been suggested as a novel antiarrhythmic strategy.5,6 The feasibility of this innovative approach was established in several in vitro and in vivo studies demonstrating that manipulation of the expression of different ion channels or their modulators can be used to alter myocardial electrophysiological properties, providing a possible future therapeutic strategy for both tachyarrhythmias and bradyarrhythmias.5–12

In the present study, we evaluated an alternative approach that focuses on the transplantation of genetically engineered cell grafts. Using detailed in vitro, in vivo, and computer modeling studies, we demonstrated that fibroblasts, transfected to express different potassium channels, can couple with host cardiomyocytes and alter the local myocardial electrophysiological properties. This effect was manifested by the ability of the cell grafts to suppress cardiomyocyte automaticity in coculture experiments and to prolong the local myocardial refractory period (RP) in in vivo transplantation studies.

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Methods

Expression System and Electrophysiological Recordings
The NIH-3T3 fibroblast line was stably transfected to express the voltage-gated potassium channel Kv1.3,13 the inward-rectifier potassium channel Kir2.1, or enhanced green fluorescent protein (eGFP).

Details regarding the creation of the lines and their electrophysiological characterization can be found in the expanded Methods section of the online Data Supplement.

 Coculturing Experiments
To assess the effects of the transfected fibroblasts on the electrophysiological properties of cardiac tissue, we initially performed coculture experiments using primary cultures of neonatal rat ventricular myocytes.13 Details regarding the methodology of these experiments, immunostaining analysis, calcine dye transfer,14 and multielectrode mapping13 studies can be found in the expanded Methods section of the Data Supplement.

In Vivo Animal Studies
All studies were approved by the Animal Board and Safety Committee of Technion (Haifa, Israel).

Rat Model
Male Sprague-Dawley rats (200 to 250 g) were anesthetized (ketamine/xylazine), intubated, and ventilated. After left thoracotomy, 6×10⁶ fibroblasts (nontransfected, n=8; or Kv1.3 expressing, n=10) were transplanted into the left ventricular myocardium with a 28-gauge needle (volume, 300 μL). A small suture was created at a nearby site to identify the site of transplantation. Cells were labeled with DAPI (Sigma, St Louis, Mo) or with 3,3′-dilinoleyloxacarbocyanine perchlorate or 5- (and -6) -carboxyfluorescein diacetate (Molecular Probes, Carlsbad, Calif). To prevent graft rejection, all animals received cyclosporin (10 mg·kg⁻¹·d⁻¹) and methylprednisolone (2 mg·kg⁻¹·d⁻¹).

Swine Model
Five pigs (20 to 30 kg) underwent right thoracotomy, after which 6×10⁶ cells (in 300 μL medium) per injection site were transplanted in 7 different right ventricular locations. To prevent graft rejection, animals were treated with cyclosporine (10 mg·kg⁻¹·d⁻¹), methylprednisolone (3 mg·kg⁻¹·d⁻¹), and azathioprin (50 mg/d).

In Vivo Electrophysiological Study
The myocardial effective RP (ERP) was determined by epicardial ventricular pacing. To localize the stimulation effect to the site of cell grafting, we used custom-made intramyocardial thin electrodes (0.01-in electrodes, 1-mm tip-length exposure) with a bipolar distance of 1 mm. The stimulation protocol consisted of a train of 20 consecutive stimuli (S1) at a fixed cycle length, followed by up to 3 extrastimuli. The stimulation amplitude was set to twice the capture threshold, and ERP was determined as the average interval of the extrastimuli). The stimulation protocol consisted of a train of 20 consecutive stimuli (S1) at a fixed cycle length (400 or 300 ms), followed by up to 3 consecutive premature stimuli (S2 through S4). The coupling interval of the 3 extrastimuli was reduced by 10-ms steps until ERP was reached.

Histological Examination
Details can be found in the online expanded Methods section.

Computer Simulation
A 1-dimensional numeric model (based on the Luo-Rudy model16 for action potential simulation) was used to simulate the effects of the engineered fibroblasts on coupled myocytes. Details can be found in the online Methods.

Statistical Analysis
All results are expressed as mean±SEM. For the coculture experiments, we used general linear model 2-way repeated-measures ANOVA to test the hypothesis that changes in the average beating rate over time varied among the 2 experimental groups (Kv1.3 fibroblasts and nontransfected fibroblasts). The model included the effects of treatment (representing the nontreated factor), time, and treatment-by-time interaction. The Greenhouse-Geisser adjustment was used to correct for the inflated risk of a type I error. The Bonferroni correction was used to assess the significance of predefined comparisons at specific time points. For the in vivo rat and swine experiments, repeated-measures ANOVA was used to assess the effects of fibroblasts injection at different time points and after MTx application. For the in vivo swine studies comparing the effects of different engineered fibroblasts on ERP, 1-way ANOVA was used.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

In Vitro Studies
Patch-Clamp Studies
Voltage-clamp studies of the transfected fibroblasts confirmed the robust expression of the Kv1.3 channel and its sensitivity to selective pharmacological blockade (Figure 1A and 1B). Because we hypothesized that the Kv1.3 fibroblasts would generate significant electrotonic interactions during the late repolarization and early postrepolarization phases and therefore could modify myocardial refractoriness and automaticity, we also analyzed the tail current properties of this channel (Figure 1C). Using a specific protocol, we noted a significant tail current for up to 100 ms after hyperpolarization. In contrast, we could not detect any significant currents in wild-type or eGFP-expressing fibroblasts (Figure 1D).

Connexin Immunostaining
A prerequisite for the ability of the engineered fibroblasts to modulate the electrophysiological properties of neighboring cardiomyocytes is the establishment of functional gap junc-
To assess for this ability, we first performed immunostaining analysis in fibroblast-cardiomyocyte cocultures. As can be seen in Figure 2A, the connexin43 (Cx43) immunosignal could be localized to the junction between the Kv1.3 fibroblasts and neighboring cardiomyocytes.

**Calcein Dye Transfer**

We next loaded the engineered fibroblasts with calcein and cocultured them with rat cardiomyocytes. Calcein is an intracellular dye that is diffusible only through gap junctions, and its transfer between cells is a standard technique for studying gap junction communication. Sequential microscopy revealed that by 3 hours a fluorescent signal could be observed in the cardiomyocytes adjacent to the loaded fibroblasts (Figure 2B, right), indicating the formation of functional gap junctions between the 2 cell types.

**Microelectrode Array Mapping Studies**

On the basis of the encouraging Cx43 immunostaining and calcein dye transfer results, we continued to assess the ability of the transfected fibroblasts to modify the excitable properties of cardiac tissue. Initially, possible changes in the spontaneous beating frequency of neonatal rat cardiomyocyte cultures were assessed after seeding of the Kv1.3 fibroblasts. Two-way repeated-measures ANOVA indicated that the treatment-by-time interaction effect was significant ($P=0.04$), reflecting the decrease in beating rate in the Kv1.3 fibroblast cocultures (Figure 3A). Although the spontaneous beating rate did not differ between the control (nontransfected fibroblasts; $n=6$) and study ($n=6$) groups at baseline (before fibroblast seeding; Figure 3A, day 0), we could start detecting a significant reduction in the beating rate in the Kv1.3 fibroblast cocultures by day 2. This negative chronotropic effect was maintained throughout the experiment (Figure 3A). To dissect the contribution of the Kv1.3 channel to the electrophysiological outcome, we applied the Kv1.3 blocker CTx. Application of CTx had a dose-related effect and resulted in complete reversal of the negative chronotropic effect when given at a dose of 100 nmol/L (Figure 3B). In contrast, CTx application had only minimal effects on the beating frequency in the control group.

We next assessed the spatial extent of the electrophysiological changes induced by the engineered fibroblasts. To this end, we performed coculture studies in which the Kv1.3 fibroblasts were labeled with a fluorescent tracer (to follow their location within the cocultures) and seeded as cell patches on top of primary cardiomyocyte cultures (Figure 3C).
In Vivo Studies

We next continued to evaluate the ability of the Kv1.3-expressing fibroblasts to modify the in vivo myocardial electrophysiological properties. To this end, possible changes in the ventricular ERP were evaluated in rats. Figure 4 depicts changes in the local ERP before and 1 week after cell grafting. Note that the ventricular ERP (S1–S1, 200 ms) was 60 ms before cell transplantation (Figure 4A) and increased to 170 ms at the site of cell grafting 7 days later (Figure 4B). Interestingly, this increase was partially reversed (to 100 ms) 60 ms before cell transplantation (Figure 4A) and increased to 170 ms at the site of cell grafting 7 days later (Figure 4B). Note the conversion of the inverse logarithmic relationship into a straight line (Figure 3D), reflecting a greater increase in electrogram amplitude at sites closer to the fibroblasts.

Figure 2. Fibroblast-cardiomyocyte coupling in cocultures. A, Left, Identification of the Kv1.3 fibroblasts (green) and cardiomyocytes (red) by anti-Kv1.3 and anti-cTnI antibodies, respectively. Right, Positive Cx43 immunosignal (purple; superimposed on the differential interference contrast image) at the junction (red arrows) between fibroblasts (white arrow) and neighboring cardiomyocytes (yellow arrows). B, Calcein dye transfer between the preloaded fibroblasts (FB) and cocultured cardiomyocyte.

In Vivo Effects in the Pig Ventricle

Because of the fast beating rate of the rat heart, we could evaluate the effects of cell grafting only at rapid stimulation rates (cycle length, 200 ms). To assess whether the grafted cells could increase refractoriness at more clinically relevant slower ventricular rates, we injected the Kv1.3 fibroblasts into sites in the pig right ventricular myocardium. Immunostaining of these hearts with anti-Kv1.3 antibodies (Figure 5C). Gap junctions could occasionally be recognized at the interface between the grafted fibroblasts and host cardiomyocytes (Cx43 immunostaining in Figure 5B and 5C). Interestingly, we noted an increase in Cx43 expression in fibroblasts located close to host cardiomyocytes.

increase in the local ERP at the site of cell grafting (from 73±5 ms to 88±4 ms; Bonferroni adjusted for 2 comparisons, P<0.05; Figure 4D). This increase in ERP, however, was much lower than that resulting from the transplantation of the Kv1.3 fibroblasts (154±13 ms; P<0.01; Figure 4D). As expected, this value did not change after administration of MTx (P=0.98; Figure 4D).

The presence of the transplanted cells at the pacing sites was confirmed in histology. The grafted cells were labeled before transplantation by the fluorescent tracer 3,3′-dilinoleylxocarbocyanine diacetate or 5-(and-6)-carboxyfluorescein diacetate (green cells in Figure 5A) or DAPI (Figure 5B) and could later be detected in vivo. Alternatively, the grafted cells also could be detected using anti-Kv1.3 antibodies (Figure 5C). Gap junctions could occasionally be recognized at the interface between the grafted fibroblasts and host cardiomyocytes (Cx43 immunostaining in Figure 5B and 5C). Interestingly, we noted an increase in Cx43 expression in fibroblasts located close to host cardiomyocytes.
Figure 3. A, Changes in the beating frequency in the nontransfected (●) and Kv1.3 fibroblast cocultures (○). *Bonferroni adjusted for 3 comparisons, P<0.05 vs baseline values (day 0) in the Kv1.3 fibroblast group; †P<0.01 for between-group comparison. B, Effect of escalating doses of CTx on the relative beating frequency of nontransfected (●) and Kv1.3 fibroblast (○) cocultures. The difference at baseline between the groups (⁎P=0.015) was fully reversible after application of 100 nmol/L CTx (P=NS). C, Changes in electrogram amplitude as a function of the distance of the recording electrodes from the Kv1.3 fibroblast patch. Left, The fluorescently labeled Kv1.3 fibroblasts were identified (arrow) as a fluorescent (white) patch. Other white spots in the image represent autofluorescent artifacts of cell debris. Middle, Superposition of the electrograms recorded from the area of interest. Right, Plot depicting electrogram amplitude as a function of the distance from the fibroblasts patch (note the inverse relationship in the logarithmic fit). D, Changes in electrogram amplitude as a function of the distance from the Kv1.3 fibroblasts before (●) and after (○) CTx application. Middle, Examples of electrograms recorded at different distances from the fibroblast patch. Right, Plot depicting electrogram amplitude as a function of the distance from the fibroblasts patch.
at 3 different stimulation drives studied (S1 cycle lengths, 350, 400, and 500 ms) compared with nontransplanted areas (Figure 6D). The increase in ERP was reversible after local application of MTx ($P<0.05$; Figure 6D).

To assess the arrhythmogenic potential of this procedure, we performed programmed ventricular electrical stimulation (up to 3 premature stimuli) in 4 animals. In none of these animals could we induce any ventricular arrhythmias.

We next assessed the electrophysiological effects of 2 additional types of genetically engineered fibroblasts. To determine the possible contribution of the transfection process per se, we established a fibroblast line that overexpresses eGFP. Similar to the wild-type fibroblasts in the rat studies, transplantation of the eGFP-expressing cells in pigs had only a mild effect on ERP (Figure 7C).

Because the Kv1.3 channel may affect different phases of the action potential (because of its rapid opening kinetics, lack of inactivation during phases 2 and 3, and significant tail current), we chose also to study fibroblasts overexpressing a potassium channel that will be active mainly during late phase 3 and phase 4 of the action potential (in a manner similar to the Kv1.3 tail current). We therefore created a fibroblasts line overexpressing the Kir2.1 channel. Voltage-clamp recordings from these transfected fibroblasts confirmed robust functional expression of this channel (Figure 1E).

Grafting of the Kir2.1 fibroblasts significantly prolonged the local ventricular ERP from a baseline value of 219±9 to 260±7 ms ($P<0.01$). Figure 7A and 7B depicts examples of the ERP measurements at the site of cell grafting before (Figure 7A) and 7 days after (Figure 7B) transplantation of the Kir2.1 fibroblasts. Immunostaining analysis confirmed the long-term survival of the cells, the continuous expression of the Kir2.1 transgene, and the formation of gap junctions with host cardiomyocytes (Figure 5E and 5F).

Figure 7C summarizes the effects of the different fibroblasts used in the swine study. Note that transplantation of the Kir2.1 fibroblasts and Kv1.3 fibroblasts resulted in the greatest prolongation of the local ERP and that these values were significantly higher than control remote sites and sites transplanted with the eGFP fibroblasts (all $P<0.01$).
Modeling Results

To attain further mechanistic insights into the electrophysiological changes induced by the transfected fibroblasts, we used a 1-dimensional numeric mathematical model simulating action potential propagation along a chain of 5 cardiomyocytes in which myocyte C is connected to the engineered fibroblast (online Figure II). The RP in these simulations was defined as the minimum interval between 2 consecutive stimuli applied to myocyte A that could be propagated to myocyte E. In the setup without a connecting fibroblast, the calculated RP was 375 ms (Figure 8A); it increased slightly to 425 ms (Figure 8B) when a nontransfected fibroblast was used. When myocyte C was connected to the Kv1.3 fibroblast, a larger increase in the RP was noted (500 ms; Figure 8C).

Figure 8D shows the simulated action potential morphology of myocyte C when connected to the nontransfected and Kv1.3 fibroblasts. Also shown are the changes in membrane potential of the coupled fibroblast. Note that depolarization in myocyte C leads to depolarization of the connected fibroblast and opening of the Kv1.3 channels. The resulting outward current hyperpolarizes the fibroblast. The potential difference generated between the 2 cell types leads to the development of electrotonic currents (the fibroblast acting as a sink) that ultimately modulates different phases of the cardiomyocyte action potential. As can be seen in Figure 8D, these electrotonic interactions result in a reduction of the slope of phase 0, in reduced amplitude of the action potential, in shortening of the action potential duration (APD), in hyperpolarization of the resting membrane potential (RMP) during the early diastolic period (Figure 8D, right), and in an increase in the RP (Figure 8C). The latter 2 events stem from the Kv1.3 tail current, which acts to hyperpolarize the RMP and prevents initiation of a new action potential. Similar hyperpolarization of the RMP also was identified when simulating the effect of the Kir2.1 fibroblast (Figure 8E).

According to the aforementioned paradigm, the electrophysiological changes induced by the transplanted cells depend on the active (biophysical properties of the transfected channels) and passive properties of grafted and host cells. To assess the latter, we modeled the effects of changes in the fibroblast surface area (affecting cell capacitance; Figure 8F) and the degree of fibroblast-cardiomyocyte cou-
Figure 6. A through C: Measurements of pig ERP at a control remote ventricular site (A), at the site of Kv1.3 fibroblast transplantation (B), and after MTx administration (C). Top, Shortest S1–S2 interval that still elicits a ventricular activation; bottom, the following S1–S2 interval that fails to capture the ventricle (ERP). D: Summary of the changes in the ventricular ERP before and after MTx application at the sites of Kv1.3 fibroblast grafting and at control remote sites. Measurements were performed using 3 different cycle lengths (350, 400, and 500 ms). Bonferroni adjusted for 2 comparisons, *P<0.025 vs control sites; † P<0.025 vs MTx treatment.
pling (affecting intercellular resistance; Figure 8G). These studies demonstrated that by increasing the fibroblast surface area or improving the fibroblast-cardiomyocyte coupling, the fibroblast acts as a greater sink, further prolonging RP.

Discussion

In the present study, we evaluated a new approach for the modification of the myocardial electrophysiological properties using genetically engineered cell grafts overexpressing specific ionic channels. Our results using fibroblasts transfected to express different potassium channels (the voltage-gated potassium channel Kv1.3 and the inward-rectifying Kir2.1 channel) demonstrate the validity of this concept by showing the ability of the cell grafts to reduce excitability and to prolong local refractoriness in ex vivo, in vivo, and computer simulation studies.

The observed changes in the local ventricular electrophysiological properties indicate the ability of the engrafted engineered fibroblasts to survive, to integrate with host tissue, and to modulate local excitability by generation of electrotonic currents with neighboring cardiomyocytes. The possibility of electric interactions between cardiomyocytes and fibroblasts was previously demonstrated by Rook et al,18 who showed morphological and functional coupling in cardiomyocyte-fibroblast pairs in vitro; by Fast at al,19 who reported on electric connections in cultured cardiomyocytes harboring fibroblasts; and by our previous report showing the ability of transfected fibroblasts to create local conduction blocks in

Figure 7. A and B, Measurements of pig ERP values at baseline (A) and 7 days after transplantation of the Kir2.1 fibroblasts (Fib; B). Top, The shortest S1–S2 interval that still elicits a ventricular activation; bottom, the following S1–S2 interval that fails to capture the ventricle (ERP). C, Summary of the ventricular ERP values after transplantation with the different cell types (Fib-GFP, Fib-Kv1.3, Fib-Kir2.1, and nontransplanted remote sites). *Bonferroni adjusted for 4 comparisons, \( P<0.01 \).
Figure 8. A through C, Measurements of the RP in the simulation without a coupled fibroblast (A) and with a nontransfected (B) or Kv1.3 (C) fibroblast. D, Simulated action potential morphologies in myocyte C (MyoC) and the attached fibroblast when connected to a nontransfected or the Kv1.3 fibroblast. Right, Magnification of the boxed area depicting the more hyperpolarized RMP of the cardiomyocyte connected to Kv1.3 fibroblast. E, Simulated action potential morphologies in myocyte C and the attached Kir2.1 fibroblast. Right, Magnification of the boxed area. F, Effect of the fibroblast surface area (Sfib) (normalized to the myocyte surface area [Smyo]) on ERP in the in vivo rat studies (as also predicted by the modeling results; Figure 8B). Heterologous gap junction–mediated fibroblast–cardiomyocyte coupling has the potential to reduce cardiomyocyte excitability by direct membrane depolarization or by an electric sink effect.21,23,24 For example, Miragoli et al24 showed in a coculture study that myofibroblasts can significantly reduce cardiac tissue conduction velocity and maximal upstroke velocity, most probably because of gradual depolarization of the cardiomyocyte RMP. Similarly, Kizana et al21 showed a reduction in the spontaneous beating rate of cardiomyocyte cultures when grown on top of wild-type fibroblast cell layer but not if cultured on connexin knockout fibroblasts. An alternative explanation may be structural changes induced by the grafted wild-type fibroblasts (fibrosis, volume effect of the grafted cells themselves, etc) that may lead to interruption of the normal myocardial architecture and consequently to changes in the local electrophysiological properties. Such structural changes may be the underlying mechanism in a recent report showing changes in the atrioventricular node properties after transplantation of transforming growth factor-β-simulated fibroblasts.25

The electrophysiological changes induced by the Kv1.3 fibroblasts, in contrast to those of the control fibroblasts, were much more pronounced and were manifested by a significant reduction in the spontaneous beating rate in the coculture experiments.13 More recent studies showed the ability of fibroblasts to propagate electrical signals over extended distances (300 μm) in coculture experiments20 and to reduce the spontaneous beating frequency in coculture experiments21 and suggested an important in vivo role for cardiac fibroblasts in modulating the electrophysiological properties of the sinoatrial node.22

Interestingly, grafting of nontransfected fibroblasts in our study resulted in a mild but statistically significant effect on ERP in the in vivo rat studies (as also predicted by the modeling results; Figure 8B). Heterologous gap junction–mediated fibroblast–cardiomyocyte coupling has the potential to reduce cardiomyocyte excitability by direct membrane depolarization or by an electric sink effect.21,23,24 For example, Miragoli et al24 showed in a coculture study that myofibroblasts can significantly reduce cardiac tissue conduction velocity and maximal upstroke velocity, most probably because of gradual depolarization of the cardiomyocyte...
ular RP in the rat, pig, and modeling studies. The crucial role of the transfected potassium channels was demonstrated not only by the significantly greater effects achieved by the transfected cells (compared with nontransfected fibroblasts) but also by the reversibility of these electrophysiological changes after administration of the specific Kv1.3 channel blockers (CTx or MTx).

We have chosen to use the Kv1.3 channel in these proof-of-concept studies for several reasons: the robust expression of this channel in the transfected cells, the absence of this current in cardiomyocytes, the high affinity to specific inhibitors that allows selective blockade of this current, and the rapid opening and relatively slow kinetics from opened to closed states (the latter property resulting in the significant tail current) that allow modulating effects during various phases of the action potential.

The mechanism underlying the observed electrophysiological effects stems from electrotonic interactions formed between the grafted and host cells. In the nontransfected fibroblast scenario, the fibroblast transmembrane potential closely follows that of the coupled myocyte (because of its relatively low capacitance), thereby limiting the magnitude of the electrotonic interactions. In contrast, depolarization of the Kv1.3 fibroblast leads to opening of these channels. The resulting fibroblast hyperpolarization leads to a sustained voltage differences in the myocyte-fibroblast pair throughout the action potential, consequentially modulating the cardiomyocyte electrophysiological properties through electrotonic interactions. Further evidence to support this mechanism can be found in the coculture studies (Figure 3C and 3D), showing that the electrophysiological effects are not limited to the fibroblast-attached myocytes but extend (through electrotonic interactions) to a distance several cells away from the seeded Kv1.3 fibroblasts.

Although the modeling studies demonstrated shortening of the cardiomyocyte APD, both the in vivo and modeling studies showed significant prolongation of the local ERP after Kv1.3 fibroblast grafting. Such a mismatch between the shortened APD and prolonged RP is the result of the significant hyperpolarizing tail current of the Kv1.3 channel. This tail current clamps the myocyte membrane to negative potential during the late phases of the action potential and early diastolic period, thereby preventing the initiation of a new action potential. The same early diastolic hyperpolarization mechanism may also underlie the suppression of automaticity in the coculture experiments.

To further test the hypothesis that the hyperpolarizing tail current of the Kv1.3 channel is responsible for the prolonged refractoriness, we created another transgenic fibroblast line expressing the Kir2.1 channel. This inwardly rectifying potassium current (I_Kr) is physiologically active at the end of phase 3 and during phase 4 of the action potential and is the main current in ventricular cells during diastole. Inhibition of this current in guinea pig ventricular myocytes resulted in prolongation of APD, depolarization of the RMP, and development of spontaneous pacemaking activity. In contrast, overexpression of this channel significantly shortened APD, accelerated phase 3 repolarization, and hyperpolarized the RMP. In the present study, transplantation of the Kir2.1-expressing fibroblasts resulted in a similar prolongation of ERP, further strengthening our hypothesis that hyperpolarizing electrotonic interactions during the immediate postrepolarization stage may prolong local refractoriness.

Study Limitations

The present study has a number of limitations. First, we did not record action potential morphologies directly but used an indirect approach of measuring ERP. Second, the electrophysiological effects were assessed during a relatively short time period (7 days); a clinically relevant effect would require longer follow-up. Thus, ensuring the long-term survival of the grafted cells and the continuous expression of the transgene is crucial to achieve long-term sustained effect. Third, our study used a fibroblast line and an immunosuppressive regimen. To overcome these drawbacks, autologous fibroblasts should be used. Fourth, although programmed electric stimulation failed to induce ventricular arrhythmias in the Kv1.3 fibroblast–transplanted hearts, the true arrhythmogenic potential of this strategy needs to be assessed in further studies.

Finally, as shown in the modeling studies, the Kv1.3 fibroblasts may induce both prolongation of the RP and shortening of the APD. One concern is that the shortened APD may alter calcium handling and reduce contractility, as demonstrated previously when the Kir2.1 transgene was expressed directly in cardiomyocytes. A potential solution for this problem may be to design potassium channels that will have minimal effects on phases 0 through 3 of the action potential but will retain the significant tail current properties, thereby continuing to affect the immediate postrepolarization phase.

Summary and Potential Clinical Application

The present study provides proof-of-concept evidence for the ability to perform a targeted modification of the local cardiac electrophysiological properties using a combined cell and gene therapy approach. In contrast to most other cell therapy procedures, which aim to replace dysfunctional tissue, the present approach seeks to modify the localized function of the tissue and thus may be more analogous to pharmacotherapy, but it may overcome some of the shortcomings of the latter approach by inducing a localized rather than a global cardiac effect.

In the present report, we have provided proof-of-concept evidence for the ability of fibroblasts transfected to express potassium channels to modulate 2 important electrophysiological properties that may be important in developing future antiarrhythmic strategies. These include the ability to prolong the local RP by a mechanism that does not involve action potential prolongation (potentially reducing the proarrhythmic risk associated with many antiarrhythmic agents) and the ability to reduce local automaticity. The potential of using cell grafts transfected to express different ionic channels may be expanded to other cardiac fields such as for the treatment of bradycardias (generation of a biological pacemaker) and to other excitable tissues such as nervous system.
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Disclosures
Drs Feld, Marom, and Gepstein are founders of GeneGrafts, a Technion University incubator company. Dr Bressler-Stramer is a former employee of GeneGrafts. The other authors report no conflicts.

References

CLINICAL PERSPECTIVE
Cardiac arrhythmias account for significant worldwide morbidity and mortality. Preventive antiarrhythmic therapies are aimed at modifying the abnormal electrophysiological substrate by either focal injury (surgery or radiofrequency catheter ablation) or pharmacological therapy. Antiarrhythmic drug therapy has been hampered by often low efficacy, global cardiac and systemic actions that often lead to poorly tolerated systemic side effects, and most important, life-threatening proarrhythmic effects. In this study, we present a novel combined cell and gene therapy approach for the modification of the myocardial electrophysiological substrate. Using fibroblast cell grafts genetically engineered to express specific potassium ion channels, we aimed to perform a targeted modification of local cardiac electrophysiological properties. Detailed in vitro, in vivo, and computer modeling studies demonstrated the feasibility of this approach by showing that the engineered cells could couple with host cardiac cells and that this modified their electrophysiological properties. The cell grafts modulated important properties that are of interest for future antiarrhythmic strategies. These include prolongation of the local refractory period by a mechanism that does not involve action potential prolongation (potentially reducing the proarrhythmic risk associated with many antiarrhythmic agents that prolong the QT interval) and reduction in local automaticity (decreasing the spontaneous firing rate).

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Figure II
Online Expanded Methods Section

Expression system and electrophysiological recordings

The NIH-3T3 fibroblast line was stably transfected to express either the voltage-gated potassium channel Kv1.3, the human inward-rectifier potassium channel Kir2.1, or eGFP.

The plasmid pRC/CMV/Kv1.3 was kindly provided by Dr. I. Levitan. NIH 3T3 fibroblasts were grown in Dulbeco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS, 4 mM L-glutamine, penicillin and streptomycin. Stable transfection was achieved by electroporation with a single pulse of 200V, 960μF delivered from a gene pulser transfection apparatus (Bio-Rad). After 48 h, 400μg/ml of G-418 (Gibco) was added to select for cells expressing neomycin resistance. Two weeks later, colonies were picked and tested for channel expression. Colonies demonstrating expression of the channel (by patch-clamp recordings) were further expanded using neomycin selection.

Human Kir2.1 was purchased from GeneCopeia™ LTD. The gene is embedded in the pReceiver-M01a commercial plasmid, under the regulation of the CMV promoter, and is His-tagged. The eGFP gene was obtained in an expression plasmid (pEGFP-N1, Clontech) under the transcriptional control of the CMV promoter. The NIH3T3 fibroblasts were transfected with the human Kir2.1 or eGFP plasmids, using lipofectin reagent (Invitrogen) according the manufactures instruction.

Whole-cell patch-clamp recordings were performed at room temperature using the Axopatch-200B system and pClamp-9.0 software. The Kv1.3-expressing fibroblasts were clamped to -80mV and currents were elicited by 500ms steps of +5mV (Fig.1A). Next, the susceptibility of the Kv1.3 current to the specific blocker,
Margatoxin (MTx, 10-100nM) was evaluated (Fig.1A). To test for the tail-current properties of this channel (Fig.1C), fibroblasts were initially held at -75mV and then depolarized to 45mV. This was followed by 400ms steps of hyperpolarization to a potential range of -35 to -70mV. To test for the functional Kir2.1 channel expression, the cells were clamped to -40mV and currents were elicited by 500 ms steps of 5mV increments (from -110 mV to +60 mV) (Fig.1E).

**Co-culturing experiments**

Primary cultures of neonatal rat (Sprague-Dawley) ventricular myocytes were prepared as previously described and plated on gelatin-coated microelectrode array plates. Fibroblasts (either non-transfected or Kv1.3-expressing) were then added in a diffuse pattern (for the chrontropic studies) or as cell patches. In some of the experiments, the fibroblasts were pre-labeled with a fluorescent tracer (DiO) in order to mark their location within the co-cultures (to correlate their location with the resulting localized electrophysiological outcome).

The co-cultures' spontaneous beating rates were recorded once-daily at baseline (day 0, prior to fibroblast seeding) and at days 2, 3, and 4 (post-fibroblast seeding) using a microelectrode array (MEA) mapping technique. Multiple electrodes were used to assess the beating frequency. The beating rate was generally uniform in the culture area overlying all the 60 electrodes (~1.5x1.5mm) in all but one culture. During the last two days of the co-culture experiments, the effects of the Kv1.3 blocker, Charybdotoxin (CTx) was also studied. In these experiments recordings were performed for 10 minutes at baseline, to assure stable beating rate, followed by multiple one-minute recordings after administration of increasing doses (0.1-100nM) of CTx.
**Calcein dye transfer**

Fibroblasts were loaded for 45 min with 10 μmol/L Calcein-AM (Molecular Probes), which can diffuse through gap junctions. The Calcein-loaded fibroblasts were added to the plated cardiomyocytes at a 1:50 ratio and dye transfer was assessed by sequential fluorescent microscopy.

**Histological examination**

Hearts were harvested, frozen in liquid-nitrogen, and cryo-sectioned (8μm). Sections were permeabilized with 1%-Triton and blocked with 3%NGS. Immunostaining was performed using mouse anti-cardiac troponin I (cTnI), rabbit anti-Kv1.3, rabbit anti-His-Tag (inserted in the Kir2.1 cassette), anti-Kv1.3, and rabbit anti-Cx43 antibodies (Chemicon). Preparations were incubated with secondary antibodies at 1:100 dilutions and analyzed by confocal microscopy (Nikon and Bio-Rad scanning system).

**Computer simulation:**

A one-dimensional numeric mathematical model was utilized to simulate action-potential propagation along a chain of five cardiomyocytes connected to the engineered fibroblast (see online supplement Fig.2). We followed the L-R model for action-potential simulation and the cable theory for intercellular conduction simulation. The simulation was programmed using Matlab 6.5 software (Mathworks).

Gap junction resistance between two myocytes was set at 10 KΩ/cm and between myocyte to fibroblast at 10/√S [KΩ/cm] (S-fibroblast surface area). The myocyte surface area was arbitrary set to 1 cm². The biophysical parameters of the
Kv1.3 channel were derived from previous studies\textsuperscript{27} and confirmed by our patch-clamp studies. For example, the maximal fibroblast's current amplitude [125nA/(mV*cm2)] in the model was derived from the patch-clamp experiments of the Kv1.3-fibroblasts.