Background—Cardiac conduction occurs in an electrical syncytium of excitable cells connected by gap junctions. Disruption of these electrophysiological properties causes conduction slowing or block. Depending on the location of affected cells within the heart, this has the potential to result in clinical syndromes such as atrioventricular block. With a view to developing gene therapy strategies for repairing cardiac conduction defects, we sought to establish whether the phenotype of fibroblasts can be modified by gene transfer to produce cells capable of electrical excitation and coupling.

Methods and Results—High-titer lentiviral vectors encoding MyoD, a myogenic transcription factor, and connexin43, a gap junction protein, were produced by established methods. Human dermal fibroblasts (HDFs) were efficiently transduced (>80%) transduced at a multiplicity of infection of 50. HDFs transduced with the MyoD-encoding vector underwent myogenic conversion, as evidenced by myotube formation and detection of muscle-specific proteins. Importantly, calcium transients indicative of membrane excitability were observed in MyoD-induced myotubes after loading with a calcium-sensitive dye and electrical stimulation. Transients from adjacent myotubes displayed different excitation thresholds, indicating an absence of coupling between cells, consistent with skeletal muscle biology. In contrast, simultaneous transduction of HDFs with MyoD and connexin43-encoding vectors resulted in the appearance of transients in adjacent myotubes with identical thresholds, indicative of electrical coupling. Notably, dye transfer studies confirmed gap junctional intercellular communication.

Conclusions—Fibroblasts can be genetically modified to produce excitable cells capable of electrical coupling. These observations strengthen the prospect of developing gene-based strategies for repairing cardiac conduction defects.

Key Words: gene therapy • arrhythmia • conduction

Advances in gene transfer technology and molecular cardiology make the prospect of using genetic manipulation to favorably modify normal physiological and pathophysiological processes within the heart increasingly plausible. For example, in the context of cardiac impulse generation and propagation, focal gene transfer has recently been reported to result in both the creation of biological pacemakers and conduction slowing through the atrioventricular (AV) node. The essential physiological properties of cardiomyocytes supporting efficient myocardial impulse propagation are an excitable cell membrane and capacity for gap junctional intercellular communication (GJIC). We demonstrate for the first time that the phenotype of fibroblasts can be modified by lentivirus vector–mediated gene transfer to produce excitable cells capable of electrical coupling. Membrane excitability was acquired by forcing myogenesis through expression of MyoD, a skeletal myogenic determination factor, and the capacity for GJIC through expression of connexin43 (Cx43), a gap junction protein. Further refinement of this technology may provide the basis for development of gene-based therapies targeting clinically significant arrhythmias caused by pathological processes that slow or block cardiac conduction.

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Methods

Vector Production

The cDNAs for human MyoD (Sonia Pearson-White) and rat Cx43–green fluorescent protein (GFP) fusion gene (Dale Laird) were subcloned into the third-generation lentiviral vector construct pRRLsin18.cPPT.CMV.eGFP.WPRE (Inder Verma). Vector stocks encoding MyoD (LVMyoD) and Cx43-GFP (LVCx43GFP) were produced as described. Transduction titers (transducing units/
ML) were assigned on human embryonic kidney 293 cells (Microbix) in the presence of Polybrene 8 μg/mL (Sigma).

Cell Culture
Early-passage, pediatric human dermal fibroblasts (HDFs) were cultivated from skin samples taken during diagnostic muscle biopsy and grown in Ham's medium and Dulbecco's modified Eagle's medium (DMEM, Gibco; 1:1, vol/vol) with 20% fetal bovine serum (JRH Biosciences). The 293 cells were cultured in DMEM with 10% fetal bovine serum. Medium for inducing myogenic differentiation consisted of DMEM and Ham's medium (1:1, vol/vol) with 2% horse serum (CSL) and 1% insulin-transferrin-selenium (Sigma). Feeder layers were produced on circular 22-mm glass coverslips (Matsunami) as described.10 Fibroblasts for genetic modification were transduced with LVMyoD alone or in combination with LVCx43GFP and plated onto the feeder layer at 2×10⁴ cells per coverslip. Growth medium was changed to differentiation medium after 2 to 3 days and subsequently changed every third day. The clonal cell line designated HeLaCx43GFP was produced by transducing communication-incompetent HeLa cells with LVCx43GFP and plating gene-modified cells at low density, followed by selection of single colonies on the basis of GFP fluorescence.

Immunohistochemistry
To assign titers to LVMyoD stocks, assess HDF transduction efficiency, and label for Cx43, cells were fixed with 4% paraformaldehyde (BDH) and permeabilized with 0.1% Triton X-100 (Amresco). MyoD was detected with an anti-MyoD primary antibody (Dako) and a Cy3-conjugated secondary antibody (Jackson Laboratories). After differentiation, myotubes were fixed as described earlier and permeabilized with 0.1% saponin (Sigma). Staining for sarcomeric myosin heavy chain was performed with the MY32 primary antibody (Sigma) and a Cy3-conjugated secondary antibody. Cx43 was detected with an anti-Cx43 primary antibody (Zymed) and a Cy3-conjugated secondary antibody. Cell nuclei were identified by counterstaining with 4',6-diamidino-2-phenylindole dihydrochloride (Sigma). Cx43-GFP expression and subcellular localization were assessed by GFP fluorescence. Cells were visualized by wide-field fluorescence microscopy (Olympus).

Imaging of Calcium Transients
Myotubes were loaded with fluo3-AM (Molecular Probes), a fluorescent calcium indicator, 11 to 13 days after the initiation of differentiation. Coverslips were positioned in a custom-made chamber containing HEPES-buffered physiological solution. Myotubes were stimulated (1 Hz, 1 ms, 0 to 50 V) with an extracellular electrode positioned immediately adjacent to the intersection point of the myotubes of interest. With stimulation, the resulting localized electrical field evoked calcium transients that were imaged in line-scan (x-t) mode with inverted confocal laser-scanning microscopy (Leica). To determine whether adjacent myotubes were electrically coupled, stimulus strength was gradually increased to observe whether calcium transients had identical or different thresholds.

Microinjection Studies
GJIC between myotubes was assessed 11 to 13 days after differentiation by microinjection. Borosilicate micropipettes were loaded with 2 fluorescent dyes consisting of tetramethylrhodamine-dextran (gap junction impermeable, Molecular Probes) and Lucifer yellow (gap junction permeable, Molecular Probes). Microinjected myotubes were assessed for dye delivery and transfer by inverted confocal laser-scanning microscopy.

Dye Transfer Studies
Calcine–AM– (membrane tracker, Molecular Probes) and DiI (membrane tracker, Molecular Probes) labeled HeLaCx43GFP cells were added to confluent monolayers of target HDF cells at a donor-recipient cell ratio of ~1:10. Cocultures were established with and without the gap junction inhibitor α-glycyrrhetinic acid (Sigma).

Results
Lentiviral Vectors Force Efficient Myogenesis and Cx43 Expression in HDFs
LVMyoD and LVCx43GFP were packaged at titers exceeding 10⁸ transducing units/mL. A multiplicity of infection (MOI) of 50 was required to achieve efficient myogenic conversion of HDFs. In duplicate experiments at this MOI, LVMyoD and LVCx43GFP transduced 82% and 79%, and 94 and 91%, of HDFs, respectively (Figure 1A through 1D). Myogenic conversion of MyoD-expressing HDFs was achieved by culture in low-mitogen medium and was evidenced by the appearance of myotubes within 5 to 7 days (Figure 1E). The extent of myogenic differentiation was established by immunostaining for myosin heavy chain.
allowing visualization of sarcomeric striations, characteristic of mature skeletal myotubes (Figure 1F). Myogenesis in HDFs transduced with both LVMyoD and LVCx43GFP was indistinguishable from MyoD-forced myogenesis alone. Interestingly, the majority of myotubes displayed 2 equally prevalent patterns of Cx43 subcellular localization: linear accumulation between myotubes (Figure 1G) and focal accumulations, possibly indicative of formation of gap junctions with the underlying feeder cells (Figure 1H). These feeder cells express Cx43 (Figure 2A) and functional gap junctions, as evidenced by dye transfer from donor to recipient cells (Figure 2B through 2F).

**MyoD-Induced Myotubes Display Noncoupled Calcium Transients**

Eleven to 13 days after initiation of differentiation, MyoD-induced myotubes were stimulated, and calcium transients were elicited in ~1 in 4 visible myotubes examined. Two separate experiments were performed, each consisting of the analysis of at least 20 low-power fields (LPFs; ×16 objective), with 4.80±0.36 excitable myotubes detected per field (n=41 LPFs). Importantly, in these 197 excitable myotubes, identical thresholds were never observed in adjacent myotubes (Figure 3A and 3B), indicating an absence of gap junction–dependent electrical coupling.

**Cx43 Expression in Myotubes Results in Coupling of Calcium Transients and Intercellular Dye Transfer**

In contrast to myotubes lacking Cx43 expression, concurrent expression of this molecule resulted in identical stimulation thresholds in 8 of 54 adjacent excitable myotubes, indicating electrical coupling (P<0.001, 2-sample t test, compared with MyoD-only myotubes; Figure 3C and 3D). The addition of Cx43 also resulted in a 7-fold reduction of the proportion of excitable myotubes to 0.68±0.09 (n=79 LPFs; P<0.001, 2-sample t test, compared with MyoD-only myotubes). These observations were made in 2 separate experiments, each consisting of at least 39 LPFs. Gap junction formation between myotubes and feeder cells might explain the observation of reduced excitability.

To exclude the possibility that cell fusion events might underlie the apparent electrical coupling, Cx43-expressing myotubes were assessed for GJIC-dependent dye transfer. Four of 37 injected myotubes exhibited direct transfer of Lucifer yellow to an adjacent myotube while retaining rhodamine-dextran, indicative of GJIC as opposed to membrane fusion (Figure 3E through 3G). Transfer of rhodamine-dextran was not observed from any of these 37 injected myotubes. These data were obtained from 4 separate cultures.
Discussion
Cardiac electrical conduction requires efficient cell-to-cell charge propagation. The essential physiological properties facilitating this process are membrane excitability and GJIC.5
We demonstrate for the first time that fibroblasts, an electrically inert cell type, can be genetically modified to produce cells capable of electrical excitation and coupling. Forced myogenesis, resulting in the expression of ion channels required for membrane depolarization, and the capacity for GJIC were achieved by transduction of fibroblasts with third-generation lentivirus vectors encoding MyoD and Cx43, respectively. The subsequent detection of calcium transients with the same excitation threshold in adjacent myotubes indicates acquisition of the capacity for membrane excitability and electrical coupling. Importantly, dye transfer studies confirmed the presence of GJIC. Calcium transients occur when membrane depolarization results in the activation of calcium release from the sarcoplasmic reticulum. Depolarizing inward sodium currents generate a charge source, and functional gap junctions allow charge transfer, at low resistance, to adjacent electrically coupled cells with resultant depolarization and synchronized calcium release.

The inherent methodological limitations of the study system used, though not detracting from the validity of the central observation, constrain full assessment of the promise of this approach. The use of calcium transients, a specific but indirect measure of excitability, has the potential to underestimate the proportion of excitable cells. Similarly, equalization of excitation thresholds in adjacent myotubes and dye transfer after microinjection are specific but insensitive measures of GJIC. It is also possible that a proportion of myotubes could acquire an excitable membrane without a functional calcium release system and that microinjection procedures could cause gap junction dysfunction through mechanical disruption or altered channel gating due to changes in intracellular pH and calcium concentration. In addition to these methodological limitations, the possibility exists that some cells acquire the capacity for GJIC without being excitable. In this situation, these cells could act as a current sink and retard impulse propagation.11 Conversely, genetically modified cells may acquire excitable membranes but little capacity for GJIC. In this instance, slow conduction due to reduced coupling may result.11 More elegant study systems for assessing the simultaneous effects of the acquisition of excitability and GJIC on electrical propagation include the optical recording technique12 and the multielectrode array system.13 These methods permit sensitive simultaneous spatial and temporal measurement of electrical activity, including electrical propagation in monolayers, and should prove powerful in more fully assessing the feasibility of the envisaged genetic conduction repair strategy.

The observed reduction in excitability of Cx43-expressing myotubes is intriguing and acts to further limit detection of efficient electrical coupling between myotubes. Two mechanisms can be hypothesized to explain this potentially important phenomenon, both of which are dependent on the formation of gap junctions between myotubes and the underlying fibroblast feeder cell layer. The first hypothesis invokes a GJIC-mediated electrophysiological effect, whereby electrically inert fibroblasts reduce the excitability of myocytes by a direct electrical effect, such as partial depolarization or a sink effect.11 The second hypothesis invokes a GJIC-mediated developmental effect, whereby the intrinsic electrophysiology of myotubes undergoing maturation in the pres-
ence of coupling with fibroblasts may be altered. Gap junctions are known to mediate important effects during skeletal myogenesis; hence, it is plausible that the composition of ion channels suberving membrane depolarization may be altered, with a resultant reduction in excitability. Microelectrode studies with measurements of action potential (AP) and of membrane currents such as I\textsubscript{Na} may offer mechanistic insights.

As is clear from the preceding discussion, the functional consequence of juxtaposition of cells with discrepant electrophysiological properties remains poorly understood. Accordingly, one of the challenges of developing safe and effective gene-based strategies for conduction repair will be defining and controlling boundary effects likely to surround regions of gene-modified cells. For example, AP duration in myoblasts after MyoD-induced myogenesis is likely to be short in contrast to the long AP duration of cardiomyocytes. The juxtaposition of such cells could therefore provide a potential substrate for functional reentry and the generation of arrhythmias.

These results demonstrate the potential of gene-based repair of cardiac conduction defects. Conduction slowing or block underlies a number of cardiac arrhythmias. Heart block, for example, caused by failure of conduction across the AV groove could potentially be repaired by direct in vivo genetic modification of fibroblasts in the AV node or groove. Ex vivo approaches are also possible. Autologous noncardiac fibroblasts could be genetically modified and expanded before reimplantation at sites capable of restoring AV conduction. Similar ex vivo strategies exploiting the inherent biological properties of other cell types also hold significant promise. For example, use of autologous skeletal myoblasts, an intrinsically excitable cell type, would obviate the need for forced myogenesis but still require genetic modification to confer connexin expression and the resultant capacity of GJIC. In the long term, multipotent stem cells with the capacity for cardiac differentiation might also prove useful for restoring cardiac conduction. In that case, any required properties not acquired under defined ex vivo differentiation conditions could be achieved by additional gene transfer.

Our data demonstrate that in vitro gene transfer strategies can be used to confer on a previously inert cell type the physiological capacity for electrical charge generation and intercellular transfer. With further refinement, gene-based strategies raise the exciting prospect of repairing conduction defects in patients.

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References

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Eddy Kizana, Samantha L. Ginn, David G. Allen, David L. Ross and Ian E. Alexander

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