

Hello, I'm Eduardo Marbán. I'm the Director of the Johns Hopkins Reynolds Center and Chief of Cardiology at Hopkins. Today I'll be talking about gene and cell-based therapies for cardiac arrhythmias.

The current armamentarium, therapeutic armamentarium for cardiac arrhythmias, includes pharmacotherapy, the use of drugs, which are variably effective, but often bedeviled by pro-arrhythmia; that is, sometimes drugs that are intended to treat an arrhythmia actually make it worse. Radio frequency catheter ablation in which fluoroscopically placed catheters are used to selectively destroy bits of the endocardium, are potentially curative, particularly for inborn wiring errors such as Wolff-Parkinson-White syndrome, but are less reliable for the more common arrhythmias, including ventricular tachycardia.

Implantable devices, pacemakers, and implantable cardioverter defibrillators are palliative for bradyarrhythmias, that is, an electronic pacemaker can save a person from an excessively low heart rate, but the person is just as sick as he or she was if the device is removed, or fails, and lifesaving for tachyarrhythmias in the case of an implantable cardioverter defibrillator, but these devices terminate rather than prevent the arrhythmia.

Either modality involves a lifelong commitment to repeated procedures at a minimum battery changes and potentially lead extractions and system revisions, a very significant expense, upwards of \$50,000 for each of these modalities, and potentially catastrophic complications, including pulmonary collapse, hemorrhage, chronic bacterial infections, and even death. So, for these reasons, we contemplated the use of either gene therapy or stem cell based therapies for the treatment of cardiac arrhythmias.

Today, I'm going to tell you about four goals of ongoing work at the Johns Hopkins Reynolds Center, and particularly in my group. The first is to test the idea that gene therapy can be used to treat a real cardiac arrhythmia, atrial fibrillation in vivo; secondly, to create a genetically engineered pacemaker; third, to develop a genetic calcium channel blocker; and, finally, to render skeletal myoblast non-arrhythmogenic.

Atrial fibrillation is a common arrhythmia due to chaotic and rapid activation of the atria which produces a rapid ventricular rate due to overload of the AV node. The rapid and irregular heart rate lead to breathlessness and decreased exercise tolerance, producing symptoms in over 2 million people in the United States, including a full 12 percent of those over age 80 that are afflicted by the arrhythmia.

There is some controversy in the literature as to whether rhythm control versus rate control using AV nodal blocking agents is preferable. The three recent randomized clinical trials point to the superiority, or at least the equivalence, of strategies that are simply designed to slow the heart rate by blocking the impulses through the AV node, partially using calcium channel blockers or beta blockers. Nevertheless, all therapeutic strategies involving drugs have a high incidence of side effects. The target tissues are small, whereas the channels and receptors that are being targeted are present throughout the body.

The drug intolerance leads to a rather radical need for AV nodal ablation and the implantation of a permanent pacemaker simply to control the heart rate in a full 25,000 patients a year, pointing out the need for new types of therapeutic approaches.

In our studies, we chose to target the AV node directly for gene delivery, and the reasons for this were, first of all, that the AV node, if modified to slowly conduct, would be capable, perhaps, of controlling the heart rate during atrial fibrillation without the side effects associated with systemic drug delivery. Secondly, the fact that a dedicated coronary artery is supplying the AV node allows for the possibility of isolated deliveries locally to the AV node using a permeablizing agent and selective cannulation and delivery transvascularly of a genetic construct. Third, the phenotypic changes can be readily quantified simply by taking the pulse, or an electrocardiogram. And, finally, the gene transfer-induced changes can be rescued by conventional electrophysiological methods, that is, normally available clinical methods, ablation and permanent pacemaker, can be applied if the therapy is inadequate or excessive. And this distinguishes this form of gene therapy from others in which there is no apparent rescue strategy.

I'll be talking about two vectors today — adenoviruses and adeno-associated viruses. Adenoviruses are ideal for proof of principle studies; they are relatively easy to make, have a very excellent transduction efficiency in the heart, and can intensely express the transgene, but the expression is transient, and the fact that these agents are somewhat pro-inflammatory limits their use in long-term studies. So, for long-term studies of safety, efficacy and durability, we use adeno-associated viruses, so-called AAV. These are single-stranded DNA viruses that enable long-lasting expression in striated muscle. They're somewhat harder to make and to grow, but once we use adenoviruses to define what it is that we want to achieve, and to select transgenes, we then are motivated to do the somewhat more difficult long-term experiments using adeno-associated viruses which are well-suited for durable applications.

The transgene we chose is the Gi alpha protein, which is shown here as an orange oval. The rationale for over-expressing this normal component of the signal transduction machinery within the AV node lies in the fact that adenylate cyclase tunes the frequency of opening of calcium channels in the AV node. The higher the activity of adenylate cyclase, the higher the phosphorylation level of calcium channels and the higher the AV nodal conduction. By over-expressing G alpha I sub-unit, we chose to effect, by mass action, a suppression of adenylate cyclase akin to that produced by digoxin, but in a receptor independent fashion.

Our next task was to evaluate the efficiency of gene transfer to the AV node for which we used a reporter gene construct of adenovirus as expressing the beta galactosidase transgene which turns cells blue. One can see here in this growth specimen in the top left from a pig heart that had received ad betagal six days earlier that the area of the AV node denoted by the arrows intensely stains positive for beta galactosidase whereas the growth specimen shows no obvious beta galactosidase staining outside that region. This was in a pig to whom the gene had been delivered by focal intracoronary gene transfer into the AV nodal artery. The top right

panel shows a photomicrograph of the AV node, illustrating that about 40 percent of the AV nodal myocytes have taken up and expressed the reporter transgene.

What about the actual therapeutic transgene in experiments designed to over express AdGi? The bottom panel shows western immunoblots in which each lane alternates a control, Ad beta gal versus an over-expressing transgene AdGi animal, and the region of the AV node has been ground up and subjected to an antibody that is selective for the inhibitory G protein alpha sub-unit. And we can see about a six-fold over-expression on average in the animals that have received AdGi for the Gi protein in the AV node relative to the control reporter gene animals.

We find in a model of chronic atrial fibrillation in conscious pigs, it's been newly developed, that we can achieve great control after AV nodal gene transfer of cGi, in this case, a point mutant of Gi that's been rendered constitutively active. This graph shows the change in heart rate from baseline, and day zero here represents a time point in which the animals have already been in atrial fibrillation for two to three weeks. They've developed a pacing-related cardiomyopathy, and so they have left ventricular dysfunction due to the very rapid heart rate achieved in the pig during atrial fibrillation. And we either administered a day zero beta galactosidase using an adenovirus into the AV nodal artery, or the cGi construct using the same vector and same delivery route.

Now we can see that there was no change over the two-and-a-half weeks of observation in the beta galactosidase group, whereas the cGi group had a fairly prompt and sustained response of about 50 beats per minute in the heart rate during atrial fibrillation. The heart rate in these animals during atrial fibrillation is about 180, so a decrease of 50 beats per minute is substantial. We verified that this is not only substantial but also meaningful therapeutically by observing the ejection fraction in response to the change in heart rate in these animals and verified that, indeed, there was substantial recovery of the heart rate of the ventricular function during treatment with cGi.

We don't have any data available yet demonstrating the long-term efficacy of AAV delivery of cGi, but what we have demonstrated in collaboration with a drug company Exigen and its licenses technology that AAV can efficiently deliver genes to the AV node. You can see in the graph on the left that a dose of ten to the tenth adeno-associated viral particles delivered to the AV node creates about a 60 percent transduction of the cells in the AV node which is actually comparable, if not a little better, than the previously demonstrated data with five times ten to the ninth particles of beta galactosidase using an adenovirus.

So these data show that we can deliver AAV to the AV node; we now have to do the long-term safety, efficacy and durability studies to show that this therapeutic strategy, in fact, is sustained over time and is effective in controlling the heart rate when given to the AV node transvascularily.

So, in summary, for genetic modification of the AV node, I've shown you the feasibility of in vivo gene transfer either by adenoviruses or by adeno-associated viruses to the AV node by intracoronary perfusion. I've also shown you some new efficacy and durability data in non-sedated animals with chronic atrial fibrillation showing that the control of heart rate is possible with administration of a constitutively active version of Gi alpha sub-unit to the AV node. And taken together the data proved the general principle that gene therapy is a viable option for the treatment of a common cardiac arrhythmia.

I'd like to move on to review our efforts to create genetically-engineered pacemakers. The cardiac conduction system involves the origin of the heartbeat in discrete areas of the heart known as pacemakers. Normally, this is the sinoatrial node depicted here by SA. The impulse travels through the atria to the AV node which also have some indigenous pacemaker activity albeit at a lower rate than the SA node, and then throughout the His and Purkinje system into the ventricular myocytes. However, the atrial myocytes and ventricular myocytes, AM and VM, respectively, themselves lack spontaneous activity. Their activity is triggered simply by the spread of impulses originating in higher pacemakers.

In patients that require an electronic pacemaker, either the sinoatrial node is defective or, typically, the AV node is blocked by some kind of disease such as fibrosis, necessitating the origin of an impulse within the ventricular myocytes, or the atrial myocytes, or both. So, what we sought to do here was to change the phenotype of ventricular myocytes to resemble those of sinoatrial node cells by rendering them spontaneously active in part of the heart, thereby originating a ventricular pacemaker without the need for implantable hardware.

The strategy that we used to do this involved the selective knockout of one current component in the heart known to cellular electrophysiologists as  $I_{K1}$  and known genetically as the Kir2.x gene family. And the idea here is that this gene is richly expressed in ventricular myocytes, and in atrial myocytes, but not expressed at all in nodal cells. We reasoned that, perhaps by knocking out this gene, using a dominant negative construct of Kir2.1, we would disable the  $I_{K1}$  current but leave all the other currents that depolarize the cell, which are on top of the action potential here, and which repolarize the cell which is shown on the bottom part of the slide, intact. Perhaps they would suffice to unleash latent pacemaker activity in a ventricular myocyte.

Our work has shown that, indeed, this is the case and that we can create a biological pacemaker by selective inhibition genetically of the Kir2.x gene family. Panel A here shows a ventricular myocyte in a control cell, and the normal action potential configuration without any phase for depolarization. The action potential that's seen originates simply because the cell has been subjected to an external stimulus. Otherwise the membrane potential would have been flat and negative.

In panel B, at a five times slower time base, you can see the spontaneous electrical activity of a ventricular myocyte that's been engineered to over-express Kir2.1 dominant negative constructs to disable the  $I_{K1}$  current, and now we can see that these myocytes

actually exhibit spontaneous pacemaker depolarization very similar to that seen in a normal sinoatrial node cell.

That's in ventricular myocytes in which we could verify expression of the transgene by co-expression of a reporter gene. What happens in vivo is shown in panels C and D. The guinea pig electrocardiogram looks very much like that of a human except at a faster time base with stereotypical P waves representing atrial activity, QRS complexes, and then T waves, and that's the normal progression of the excitatory impulse throughout the heart.

However, in panel D, you can see in the arrows labeled with red V's that there is an impulse originating in the ventricle which is happening at a rate that is somehow faster than the atrial impulse and is controlling the overall heart rate for the animal, as expected, if we had engineered a ventricular pacemaker.

This is in a guinea pig in which the gene was delivered throughout the heart, and we actually were able to get the desired pacemaker activity somewhat by chance rather than by design, as we did not administrate it focally. However, in these biological pacemakers, we were, nevertheless, able to isolate cells and ask whether the engineered pacemakers remain responsive to indigenous signal transduction. This would be one theoretical benefit of biological pacemakers over electronic pacemakers which have to be rendered somehow responsive to rate by unnatural mechanisms.

Cells that contain their indigenous beta receptors, for example, should be able to respond to exercise appropriately if a biological pacemaker has been engineered, and we took cells from these guinea pigs, exposed them to isoproterenol and found that, indeed, the heart rate of these engineered ventricular pacemakers increases, and it does so in the lower panel significantly and consistently.

As I said, in the guinea pigs, the delivery of the transgene was throughout the ventricles, and it was by chance rather than by design that we were able to get focally occurring ventricular pacemakers. However, we then took the same construct and delivered it focally into the ventricle by a homemade injection catheter in pigs that we had engineered to have iatrogenic AV block using heptanol ablation of the AV node. And in these animals, we then followed the heart rate before and after the administration of isoproterenol, simply to see if we could recruit any indigenous pacemakers.

In the same animals, which are AV blocked, we can also measure the sinus rate of the atria, and we can see that in the animals that had received the dominant negative Kir2.1 triple A construct, the same construct that we used to unleash latent pacemaker activity in the ventricle of guinea pigs, that they did have an escape rhythm that was higher than that in the control groups in which we injected either beta galactosidase, or wild-type Kir2.1 gene, but somewhat slower than the sinus rate in the same animal. So, we have engineered a pacemaker, but one that may not be as chronotropically competent as that that's present normally in the sinus node.

We did verify that the engineered pacemaker originated in the site of injection by electrocardiographic mapping, and the complexes that are labeled 30 seconds after isoproterenol here in the Kir2.1 panels correspond directly to those that were observed during pace mapping from the injection site at the time of injection.

Well, that involves creating a pacemaker using gene therapy, the injection of a gene to convert the phenotype of a preexisting heart cell into that of a pacemaker cell. An alternative strategy involves engineering stem cells and making them pacemakers and then using these as transplant sources to create a transplanted pacemaker in the heart.

The way we went about testing this general concept was using human embryonic stem cells, HESC's, that are derived from the inner cell mass of blastocyst and can be grown in vitro to create embryo bodies which then exhibit differentiation into various forms of striated muscle and other end organs. We can either do this in taking advantage of the spontaneous differentiation in vitro, or we can genetically engineer these constructs stably using lentiviruses; these are RNA viruses that are modified from human immunodeficiency virus to introduce a transgene permanently into the cell line without perturbing its function.

So, the strategy to create a biological pacemaker using human embryonic stem cells involves, first, stably transducing the human embryonic stem cells with one of a variety of different genes, either a reporter green fluorescent protein typically or, as I'll show you later, several members of the pacemaker channel gene family, and then to differentiate these stem cells in vitro into embryoid bodies. We can then identify visually beating cardiac masses from these human embryonic stem cells, microdissect them out, and transplant them onto an otherwise quiescent monolayer of rat heart cells, and ask the question, "Does this transplanted human cardiac mass entrain and graft onto the quiescent rat heart cell monolayer? And we can actually verify the phenotype by monitoring electrical and mechanical activity using video microscopy or multielectrode arrays, or optical mapping.

We now show you a movie of a GFP expressing human embryoid body in which the cardiac mass is beating on the bottom left as a human derived cardiac mass and entrains the rat cardiac myocytes that are seen elsewhere in the field. Just to repeat that, for ease of viewing, you can see on the bottom left is a human cardiac heart mass that's been transduced green fluorescent protein and, simultaneous of each contraction of that human cardiac mass, there's a contraction of the underlying rat cardiac myocytes. We can verify that the impulse in such a transplanted monolayer originates at the site of the beating cardiac human mass that is stem cell derived by using multi-electric ray mapping, and we could verify that the impulse spreads from that site. If that human cardiac stem cell mass is removed microsurgically, if it's crushed, or if it's uncoupled from the rest of the monolayer using an uncoupling agent such as heptanol, the beating activity ceases, providing further proof that this is, indeed, the site of origin of a cardiac pacemaker.

That shows that we can induce pacemaker activity by transplanting human heart cells that are derived from embryonic stem cells. It doesn't actually show any frequency tuning capability for engineering these cells to provide a different base of frequency. In order to get towards that laudable objective, we use genetic engineering to alter the heart rate, and this shows the effects of transducing embryoid bodies at day zero and then following their heart rates at day three and day twenty in which the embryoid bodies have either been transduced simply with the reporter gene in the squares on the bottom or in the circles on the top with an HCN1 transgene delivered by lentiviruses. HCN1 is the prototypical member of the hyperpolarization activated cyclic nucleotide-gated family of channels so-called pacemaker channeled genes. This shows that when we introduce the HCN1 gene into these embryoid bodies, the heart rate doubles after about three weeks, and you can imagine that using techniques like this we might be able to engineer frequency-tuned pacemakers that are kept in a repository so that different patients requiring different basal levels of pacemaker frequencies can benefit from such genetically altered constructs.

So, to summarize for a biological pacemaker, the introduction of a single gene converts normally quiescent ventricular muscle into a pacemaker. These biopacemakers remain responsive to beta adrenergic stimulation, proving the general principle that responsiveness to signal transduction is retained. I've shown you some large animal data, on focal delivery, that hints that we may be able to achieve the creation of a focal pacemaker in the ventricle using a relevant large animal model. I've also reviewed an alternative strategy that involves genetically engineering stem cells as sources for transplantable pacemakers. That, taken together, these are novel biological alternatives to electronic pacemakers.

I'd like to next go on to review our work to develop a genetic calcium channel blocker. Calcium channel blockers are among the most frequently prescribed drugs in the current armamentarium. They're used to slow AV nodal conduction in the heart rate control of atrial fibrillation. They're sometimes prescribed to improve ventricular function in various forms of hypertrophic cardiomyopathy. Such drugs, when given to animals, have also been shown to improve memory in certain models of senescence.

But their use is associated with numerous side effects related to extracardiac effects — vasodilatation, because of the effects on arterial smooth muscle; constipation by blocking of calcium channels in the colon; and worsening of heart failure by blockade of the calcium channels involved in excitation contraction coupling in the ventricle.

So, our idea was to use calcium channel blockade by gene transfer to achieve regionally specific and durable calcium channel blockade. The calcium channel blockade could be made regionally specific by delivering the gene to only one place, which is what we did. We delivered it in the data shown directly to the heart and not elsewhere to the body, or by choosing appropriate tissues specific promoters. And the durability, of course, would come in the choice of an appropriate vector, such as adeno-associated virus, which can produce persistent expression.

Our experimental strategy was to ectopically express a small G protein called Gem in the ventricle of guinea pigs using adenoviral gene transfer. Gem is a small G protein that was first cloned from lymphocytes and later found to bind calcium channel beta sub-units. These are accessory sub-units of calcium channels that are necessary in order for calcium channels to be functional and reach the surface membrane. So, when beta sub-units are scavenged by Gem, the alpha sub-units never make it to the surface membrane, causing a functional suppression of the so-called L-type calcium current. And, I'm going to show you that, together, this produces a cellular and in vivo phenotype of cardiac calcium channel blockade.

On the top left are patch clamp recordings of membrane currents from control myocytes, ones that received only a reporter gene, and on the top right are records from a cell that had received a reporter gene plus Gem. And you can see that the calcium currents are almost unregistrable in the cell that had received Gem. The current voltage relations that show peak current density as a function of voltage in the lower panel show that the overall suppression of calcium current amounts to about a 90 percent suppression on average in cells that have received Gem relative to control cells.

The rest of the electricity in the cell appears to be relatively unaffected as evidenced by the action potential shown in panel A. The top left panel shows a control cell before and after the administration of a pharmacological calcium channel blocker, nitrendipine, and you can see marked action potential abbreviation associated with the administration of nitrendipine.

In the top right is a cell that had already been transduced with Gem, and nitrendipine had very little further effect on the action potential, as expected, if the calcium channels were already blocked. But also notable is the fact that the upstroke of the cells is normal, indicating indirectly the presence of normal sodium channel activity and repolarization is unimpaired, hinting that potassium channels are unaffected, and we verified the lack of effect on other ion occurrences in selective patch clamp measurements, which are not shown here.

The relationship between action potential duration at 90 percent repolarization and the peak calcium current density is depicted in the graph in panel B. And you can see that this relationship follows a linear relationship, whether one takes advantage of natural variability among the cells in their calcium current density, shown as yellow squares in control, Gem transduction shown as red symbols, or nitrendipine blocked control cells shown as blue triangles.

What happens to the QT interval measured electrocardiographically in vivo in these guinea pigs? Here, we took advantage of the fact that the transduction efficiency using a cross-clamp delivery method into the ventricles is about 25 percent to find that the animals had, indeed, abbreviated their QT intervals appropriately as expected from the cellular action potential recordings. And using hemodynamic recordings, we were able to verify the systolic pressure and the first derivative of systolic pressure, or the first derivative of ventricular

pressure, dP/dT Max, were also inhibited by approximately 25 percent, consistent with the fact that calcium channels are severely blocked in a small fraction of the cells in the ventricle.

So, for genetic calcium channel blockade, I've shown you that transfer of a single gene suffices to block calcium channels in ventricular muscle, it abbreviates the QT interval, and decreases systolic ventricular function in vivo. We've also shown that you can achieve non-pharmacologic rate control of atrial fibrillation using this delivery method as an alternative to the cGi construct shown earlier, and that it could also be beneficial in the treatment of hypertrophic cardiomyopathy. We have yet to explore applications of the CNS and other tissues.

Finally, I'd like to tell you about our efforts to render a skeletal myoblast non-arrhythmogenic. Skeletal myoblasts are cells that are derived from resident stem cells in skeletal muscle known as satellite cells, and they can be cultured in vitro to form myotubes, which are not coupled to each other electrically, unlike heart cells. Now, the advantage for cardiomyoplasty for trying to regenerate functional heart tissue is that these are autologous cells that can be harvested from individuals who then come back for clinically indicated coronary artery bypass grafting and, in that context, have their own skeletal myoblast reinjected into the ventricles into the scar or into the border zone. And this is exactly the strategy that's involved right now in some clinical phase 2 trials for cardiomyoplasty, one of which is known as the MAGIC trial.

However, in 10 of 22 patients that were studied in this manner in phase 1 trials, ventricular tachycardia, or sudden cardiac death, were observed. So, it would be highly desirable to somehow genetically engineer skeletal myoblasts in order to render them less arrhythmogenic. However, it's important first to understand the mechanism of the arrhythmia as associated with skeletal myoblast transplantation. So, we chose first to understand the mechanism and, secondly, to devise a genetic strategy for modifying the myoblasts that might render them less arrhythmogenic.

One relevant consideration is the fact that electrical connectivity among cells is produced by connexins that are part of gap junctions, and connexins normally couple heart cells to each other electrically in the syncytium of the heart, but they're absent in skeletal muscle which functions very differently in terms of its electrical excitation contraction coupling pattern. And skeletal muscle cells are normally not coupled to each other electrically and force is varied by the variable recruitment of numbers of functional units rather than by varying the force of contraction of individual cells.

So, one possibility is that the introduction of skeletal myoblasts within the heart creates some kind of barrier to conduction or some kind of alteration of the normal electrical properties because they don't express connexins.

In order to investigate the mechanism of myoblast transplant-related arrhythmias, we used a novel co-culture and optical mapping method that involved co-culturing primary human

skeletal myoblasts with neonatal rat ventricular myocytes in monolayers and then doing contact fluorescent imaging and following the spread of excitation using point or area electrode stimulation. And what's shown here is a schematic for the registration of impulses in 61 sites. The system we used actually now enables us to record from 263 sites simultaneously in these monolayers.

The next slide shows a monolayer in which we grew both cardiomyocytes and myoblasts with the cardiomyocytes on the top half and skeletal myoblasts patterned to grow on the bottom half. And here, what we're measuring is fluorescence as the spread of excitation proceeds from a point on the upper right-hand corner of the monolayer to the center of the monolayer, and we're asking specifically whether it's capable of conducting from the cardiac myocytes into the skeletal myoblasts. And the movie shows that the impulse propagates throughout the cardiomyocyte part of the monolayer but stops abruptly at the skeletal myoblast layer verifying that, indeed, cardiac myocytes and skeletal myoblasts are not coupled to each other electrically. Therefore, the impulse is unable to propagate from one half of the monolayer to the other.

We then investigated the mechanism of the arrhythmias by mixing, in a rather homogeneous fashion, skeletal myoblasts and cardiac myocytes, and investigating both the spread of excitation, as well as the rate of repolarization. On the left panel, we see that the normal spread of excitation from a point source is brisk and that repolarization is equally fast. Contrast that with the behavior of a monolayer in which only 5 percent of the cells were myoblasts. The spread, the conduction throughout the monolayer is markedly impaired and, remarkably, repolarization is very delayed. These manifestations are even more flagrant in cultures that contain 50 percent myoblasts. Here, conduction velocity is very markedly delayed, and repolarization remains impaired. Both of these would be pro-arrhythmic mechanisms by causing both slow conduction and delayed repolarization which is the basis of long QT-related arrhythmias.

The abnormalities are so flagrant that, in some cultures, we even saw spontaneous reentry, which is the basis for ventricular tachycardia and ventricular fibrillation, and what are seen here are spiral waves occurring spontaneously in a culture. These are very similar to the kinds of reentrant waves that would be registered on the surface of a human heart during ventricular tachycardia ventricular fibrillation.

So these data point to slow conduction and altered repolarization as important mechanisms in the arrhythmias associated with myoblast transplantation.

As a general anti-arrhythmic strategy to try to decrease the propensity of myoblast transplantation that produce ventricular arrhythmias, we decided to create a construct pLV-Cx43-GFP to co-express connexin and a reporter gene, green fluorescent protein, from a single construct in a lentiviral vector. We were able to take myoblasts and in vitro transduce them to an efficiency of greater than 95 percent with this construct, and we then confirmed the expression of both GFP and connexins by fluorescence, immunostaining, and western blots.

These then were either co-cultured with normal cardiac myocytes, that is connexin-43 expressing myoblasts, or they were compared to myoblasts that were simply engineered to express green fluorescent protein without connexin-43. And optical mapping was used to verify the phenotype.

Immunostaining for connexin-43 verified on the right-hand side that the non-transduced myoblast did not express connexin-43, whereas connexin-43 transduced skeletal myoblasts richly expressed connexin-43 shown by the red punctate dots.

So, the question remains, does connexin expression suffice to suppress these mechanisms of arrhythmias. In the bottom left corner, we see the conduction in a monolayer co-expressing myoblasts that simply contain the reporter gene and neonatal rat ventricular myocytes. And you can see the typical pattern that's somewhat slow conduction and delayed repolarization. Contrast that with the brisker spread of conduction and the connexin-43 modified myoblast co-culture along with the markedly improved repolarization rate. If these effects could be sustained in vivo, they would provide the basis for an anti-arrhythmic effect of skeletal myoblast transplantation.

At this point, I'd like to summarize what I've told you in this lecture today. First of all, for the gene therapy for atrial fibrillation, we've achieved rate control by genetic modification of the AV node. For bradyarrhythmias, in which the heart rate is too slow, I've shown you the genetically engineered pacemakers made either by gene therapy or from stem cells that may suffice to treat these arrhythmias. Calcium channel blockade can be achieved by gene therapy, and this may create a durable and focal drug surrogate. And, finally, skeletal myoblasts can be genetically modified to prevent arrhythmias, and this may be a useful direction for modifying current trials involving these skeletal myoblast sources for transplantation into the heart.

This work is inspired, in large measure, by the pioneering work of Michel Mirowski, who invented the implantable cardioverter defibrillator, the first clinical trials of which were performed in collaboration with Dr. Morowski here at Johns Hopkins. And one relevant parallel is the fact that his technology was greeted initially with skepticism but has now changed the field very drastically.

This development history of automatic implantable cardioverter defibrillators points to the fact that, in the mid 1970's, these were strictly limited to animals, proof of principle studies with extreme technical difficulties, and they were not yet in patients. The first implants were done in 1980 with initial great technical difficulty involving an open thoracotomy. The indications were limited to drug refractory ventricular tachycardia. But throughout the eighties, they gained widespread acceptance for greater indications. By the 1990's, everyone was quite confident of their demonstrated efficacy, and their technical difficulty of the implantation became very, very small. And now, in the twenty-first century, these are first-line therapeutic agents for, not only for demonstrative ventricular arrhythmias, but also for the prophylaxis of sudden cardiac death in general in the population.

I'd like to finish by reflecting on likely clinical development timelines for both AV nodal modification for rate control and for pacemakers. For AV node modification, we now have an established vector, a transgene and delivery method, and an animal model. We need to complete long-term safety and efficacy studies in order to begin FDA negotiations and complete an investigational new drug application to initiate phase 1 clinical trials, which we think may occur as early as 18 to 24 months from now.

For the biological pacemaker, we have an established vector and an animal model of AV block. We're still refining the transgene, as well as new delivery methods. Long-term safety and efficacy studies here are imminent, and we believe we can be in humans with this therapy without any major complications within 36 to 42 months.

I'd like to finish by acknowledging the National Heart Lung and Blood Institute which has provided continued support, the Donald W. Reynolds Foundation which funded the work on skeletal myoblasts shown here in this study, and Excigen for the provision of some unpublished data. Thank you very much.