Heart Regeneration in Humans: Are We There Yet?

Chuck Murry: My title is Are We There Yet? Of course, the answer is no. We're not there yet. I didn't think anybody would believe that we were actually there. What I wanted to convey was this sense of impatience and urgency that I feel, at least, it's like, for heaven's sakes, why aren't we there yet? What is it that we need to do to really make this happen? I think from the standpoint of thermodynamics, we should be able to regenerate the heart. We should be able to do regenerative biology in lots of things.

But it's been harder than we thought so we'll talk about some of the impediments and maybe some paths forward. These are my disclosures. I am a serial entrepreneur, which is a pathological condition. But nothing leaps out of the academic world into the healthcare sector without a process of commercialization and so we keep trying.

I have some equity disclosures to bring up. This is the basic scheme that we are working with. The heart is essentially the least regenerative organ in the human body. It will make neuroscientists cringe when I say that, but I got the podium. There's not a stem cell population in the heart that begets new myocytes. The cardiomyocytes are very tightly locked out of the cell cycle. Smart people like Sylvia Evans keep working on this. Go, Sylvia, figure this out.

But I mean, it's tough. Essentially, after one has large scale loss of cardiomyocytes, the body is not up to the job of replacing them. The heart heals by scar formation, and there's a substantial drop, basically stoichiometric with mass loss in mechanical function of the organ. You might imagine it would be like a square wave and we're down a step, but we'll hang in there, but we don't. It becomes a progressive illness. In that sense, I think the word degenerate, it's like a degenerative illness, if you will. We don't know why the heart runs down, but it does. It's like this downward spiral of decompensation. Eventually, a patient gets to the point where their heart function is not sufficient to support the blood flow demands for the body, and we call this heart failure. This is quite deadly. You have on average five years to live once you're diagnosed with systolic heart failure, so it's worse than most forms of cancer.

Our approach is simple to understand. It's a disease of cellular deficiency. We can now grow almost limitless amounts of cardiomyocytes in the laboratory, we should be able to re muscularize the heart. Other people are working with other approaches. I mentioned reactivation of the cell cycle, so really promising things happening there with hippo pathway signaling and other kinds of stuff. I'm rooting for all the folks there. People are trying to reprogram and trans differentiate cells. That's also a very challenging but potentially promising area. My thought is that I don't know if this will be the best way, but probably the fastest way that we can get large amounts of new muscle into a human heart and see what that does is by cellular transplantation so that is what we have focused.

The first thing is you've got to get the cells, and with apologies to the developmental

biologists in the audience, we had to take these beautiful interacting loops of activation and inhibition circuitry that underlie development of the heart and turn it into a linearized manufacturing scheme. It turns out to be all about wind, stupid, and I'm the stupid one in this case, because it took me five years to figure this out. We kept asking simple questions like, well, is wind pro or con for making cardiomyocytes? This turns out to be the wrong question because the answer was yes.

That's because you have to first activate wind to make mesoderm, and then you have to repress the wind pathway to commit the cells to forming cardiomyocytes. It was collaborations with Randy Moon, going back to zebrafish embryos, where we finally figured this out, where we had spatial temporal control over the pathway using fish genetics. Once the embryo taught us the lesson, we were able to then systematize this. Other people in bioengineering, Sean Policek, for example, figured out how to do this with small molecules, agonists and antagonists. We had to get away from these awful protein reagents that would fail at least half the time while we sent the people's children to college. It now works. We can teach undergraduates to do this in two weeks, though it took us five years to get this figured out. We have brought this into pharmaceutical grade manufacturing. These are people working in a clean room under good manufacturing practice conditions.

We can do this at scale. We've gone from monolayer culture to three dimensional stirred tank bioreactors where we grow spheroids of cardiomyocytes. We can do this up to three liters. You can see a paddle wheel that turns, we stop the paddle wheel and it's like a snow globe and you can watch it start to settle. It's a very expensive snow globe. A three liter run costs about $80,000 just in reagents alone. It gets expensive to do manufacturing science inside of an academic setting.

But we can produce cardiocytes at large scale, 8 billion. A typical infarct that causes heart failure would cause a deficit of about 1 billion cells, just for reference. Then we get these little cardio balls or cardiac spheroids or organoids, or whatever you want to call them. These are some that we pulled out when they're young. It's a little hard to see them contracting. But in the video advances, we put in a calcium indicator gene called G camp, and you can see each little spheroid of a couple thousand cells will flash in synchrony, but each little spheroid, like a miniature heart beats to its own drummer. When these cells come out, they all have pacemaking type action potentials. By the way, all the myocytes in the heart start out life as pacemaker cells, and then pacemaking with maturation becomes restricted to specific foci, like the sinoatrial atrioventricular nodes. Our cells have pacemaking properties.

This comes back to be important later. We took cells with these G camp transgenes, transplanted them into the hearts. Here, we worked our way up the food chain from mice to, you're not supposed to say food chain anymore, but from mice to rats to guinea pigs. I thought we were going to die before this ever got to humans. The cause of death would be listed as incrementalism. We made the leap to a non human primate, and you're looking at the apex of a primate's heart here. These gray streaky things are grayscale image of the G camp fluorescence. I'll turn the video on.

You can focus at the brightest spot, which is right up here and watch it flash. Then you start seeing the whole apexes flashing. What that says is, this is several months after

transplantation, that the cells are alive, they're well, they're beating, they're in sync with one another. If we then correlate this with the electrocardiogram, here's three different regions of interest where we grab the fluorescence intensity. You can see all these three different regions are all synced up one with another, and that's what your eyes told you as well.

But what you can't see is the electrocardiogram of the heart, which says that the sinoatrial node is calling the pace here, and the cells are following like good soldiers. This is years ago that we did this work, but this said, it seems to be we're on the right track. This is what regeneration should look like. We then went on to do an efficacy study in non human primates, where we infarcted the animals first, came back a couple of weeks later and surgically delivered using a needle and syringe the human cardiomyocytes into the infarct region. just to what you're looking, this is a confocal micrograph at low magnification. Note the size scale at 5 millimeters in the lower right. Red is monkey myocardium. In blue, a little hard to see is an image stained for collagen. This is the scarred region, so you're looking at the interface between the ischemic and non ischemic vascular beds.

In green is the human myocardium that we have transplanted into it. You can see that while the architecture is not perfect, the mass is substantial, at least in this section that I've chosen. You might imagine this would be able to induce a better squeeze to the chamber. If we use magnetic resonance imaging to quantify that using ejection fraction's our principal metric. That, by the way, is just the amount of blood that's squeezed out of the left ventricular chamber with each beat.

You can measure this. Each line here is a single animal. At baseline, the non human primates, like human primates have ejection fractions of about 65% or so with some variation. We infarct these animals, and they lose about 25 points here, what we call baseline. In the natural history group, the control that received just a vehicle injection, there's no spontaneous improvement. That is the natural history of disease, no regeneration, no improvement in function. In the animals in whom we placed the cardiomyocytes, we get 8-10 points back at a month, and at three months we get about 20 points back. We're back up to the normal range. Even though these data were done several years ago, it is still the thing that gets me out of bed in the morning. It says, if the human cells can do this in a monkey, they should be able to do this in a human.

This has been the thing that has kept me up, which is that the human cells, I told you that they follow the sinoatrial node. What I didn't add was the word eventually. When we put them in, there's this period of electrical chaos that ensues, and we've come to call this engraftment arrhythmia. This is a rhythm strip from one of the first primates in which we discovered this. They were on telemetry, so we had 24/7 rhythm monitoring. You can see a nice steady cadence here, and all of a sudden chaos ensues, and the cardiologist called this a wide complex ventricular tachycardia. It turns out that this is the cells taking over the pacemaking of the heart with a wild and fast rhythm, in this case of about 250 beats per minute. We tried very hard to make this not due to ourselves. We hadn't seen this in mice, rats, or guinea pigs.

Why should this happen? But it's the cells. The natural history of this complication goes like this. The animals are infarcted here where the dots start along. There's four different

animals shown here. Then the vertical line is where we came in and gave the cells, and you can see very soon temporally so there's good temporal association. These arrhythmias ensue, and in some of the animals, it's a lot. A good fraction of the day is taken up by ventricular tachycardia. The monkey tolerates this pretty well. Their hearts are about the size of a plum. They're a large animal, technically, but smallish large animal. Their heart rates are naturally about 120 beats a minute. We tried this in pigs, and the pigs were quite unwell. Some pigs reached euthanasia criteria from their hearts being so rapid that they couldn't properly fill empty and they went into heart failure. We did a lot of physiology studies.

I'll just summarize that here that I was wrong again. I thought this would be a problem of conduction disorder where we got this rhythm called reentry. It's like people doing the wave in a sports stadium where itself stems going around and around. That is not the physiology. The physiology is like an ectopic pacemaker that's going ping, ping, ping, ping as an impulse generator. I was lamenting this at a meeting where I was talking with a colleague named Kathy High, somebody I admire greatly. She's a hematologist, was at Penn and Children's Hospital in Philadelphia. Kathy was the first person to get a gene therapy approved in the United States, an AAV gene therapy for retinal blindness. I said, I've got this electrical problem. I'm a pathologist, not an electrophysiologist. This is, etc. [Laughter] Kathy just looked at me and said, put your big boy pants on, Chuck. Do you want to make a therapy?

If you want to make a therapy, you have to own every problem that comes your way. You can't say, I'm not an electrophysiologist. You have to deal with it. That was annoying. But you know how things sometimes are annoying because they're true. It stuck with me, and I finally like, yeah, I got to learn this electrophysiology and figure out what to do about it because it didn't seem like anyone else was rushing into it. We were ready to go to the clinic, and this was like a proverbial fork in the road. Do we go to the clinic when I know that there is this arrhythmia problem, or do we stop and figure it out. By the way, science is competitive and multiple other groups are doing this, and wouldn't it be great to be first? I'm competitive, right? Other people I suspect are competitive. We decided to push pause and sort this out, and other people went, it actually went on into the clinic.

We did something I would recommend no one do, which was a crisper screen in pigs. We couldn't model this disease in culture because we didn't know what was causing it, and small animals don't get it. We begin to gene edit the action potential to try to discover which genes coded for the ion channels that carried the arrythmogenic current. Typically, what would happen is we would make an edit or a series of edits in a cell line. We'd grow up large amounts of cardiomyocytes. We'd transplant them into the pig. Does it get in graft an arrhythmia? It still does. We got to the point where, and this is all during the pandemic and it was crazy. We began to wonder whether does DNA really go to RNA, go to protein? Are we missing something here? It turned out it just was complicated, which is to say, it seems to have been caused by three excitatory channels that are present on the sarcolemma that are part of the pace making circuitry in the absence of an inhibitory or hyperpolarizing potassium channel that normally that's not present in immature cells, but is in adult.

We deleted these three activating channels and over expressed this inhibitory channel,

and we converted the action potential waveform from something with a pacemaking potential to a more hyperpolarized one with stable, resting membrane potential. Schematically we turned engraft arrhythmia back into sinus rhythm. You can see this shown quantitatively here, that the arrhythmia burden, while not quite perfect, it's 95% reduction. That told me at least we identified what the targets were. Maybe these cells would be a therapeutic. Maybe that's not the way to go, but at least we felt like we've got a handle on this thing now. When you don't even understand what you're dealing with, it's just very uncomfortable, at least for me. In parallel with that, we started using pharmacological approaches to find an antiarrhythmic drug or cocktail that would inhibit this.

We'd switched, by this point from non human primates because there's essentially no throughput to a primate model. They're wonderful for discovery, but so we'd switched to pigs, and we brought in pigs while they had engraft arrhythmia and did short term infusions of various FDA approved drugs. Then we did some things in combinations as well. Lots of things didn't work, but we found two FDA approved compounds called amiodarone plus ivabradine. The ivabradine targets one of the channels called HCN4 that we had to edit in order to make the rhythmic go away, so that's physiologically making sense to us. Amiodarone ostensibly is a potassium channel blocker, but it's a poor molecular probe because it's very non selective bit all things. As a drug, it's useful for that reason because it's like a tonic, in a way. Cardiologists and surgeons use this a lot.

But importantly, so this is the average heart rate in the pigs with engraft arrhythmia, and we brought it down to essentially normal with the amiodarone berdine cocktail. We learned this it isn't the fact that the heart was being paced from a funny location. It's how fast it was going. When a large heart is going that fast, it can't fill well, it can't empty well, and it can't perfuse itself. That's what causes this downward spiral. Then if I show you the Kaplan-Meier survival curves, the effects of this drug treatment was very impressive, at least to us. What you can see, each one of these steps down is one of the pigs reaching euthanasia criteria predefined with our veterinary staff. You can see these pigs were quite unwell while they had this.

We lost over the course of this experiment with a large dose of cells, two-thirds of the animals. The pigs were well, they were happy, and we had 100% survival with this drug treatment. We thought, we've got two approaches. Now we've got a pharmacological approach to deal with this, and we've got a gene editing approach. Problem solved. I just showed you pacemaking channels were required for this. You can target them with gene editing or with drugs. I don't know how it is with you. We were throwing high fives, and we solved it. We understand the mechanism. You're happy for a little while, then wait, there's these little anomalies that you all have heard of Occam's razor. Everyone knows Occam's razor. Do you know about Occam's broom? That's the thing you use to sweep under the rug, the little things that don't quite fit.

These little anomalies started to emerge. For example, these cells have the same ion channel repertoire when they're in culture, but they go about 40 beats a minute. Why are they going 250 beats a minute after transplantation? Because that's partly why I was very glib about this before. I didn't think this would happen. I also oversimplified when I described the targets that we edited, because I said I got rid of all the excitatory

channels from the fetal heart. But we had to do one other channel or ion exchanger that is robustly expressed in the adult heart, and that's the sodium calcium exchanger, which is an electrogenic. It uses the sodium gradient to extrude calcium and let sodium in. It's a calcium response to calcium overload intracellularly. Thinking about this, it was like, I don't think we found a key set of nodal points in this causal chain, but there had to be something upstream that was driving this.

Where was the calcium driver coming from that was running through this sodium calcium exchanger? The only thing I could think of was that we had to have a calcium leak somewhere. There had to be calcium getting into the cytoplasm either from outside the cell or from inside the cell, that was the driver of this arrhythmia. We went back to some RNA sequencing databases that we had. Today, we do this with spatial transcriptomics, but back when we did this in the previous decade, we had done this with laser capture microdissection and zapped out graphs from human myocardium in the infarcted rat heart. We'd done RNA sequencing libraries and informatically deconvolved the human and the rodent sequences and so forth. We watched our cells over a time course as they mature. We had focused a lot on ion channel gene expression that was at the sarcolemma. That's how we prioritized our CRISPR editing list.

What we hadn't thought as much about was another part of the cell that's very important actually in ion fluxes, and that is this intracellular membrane. The end plasma reticulum of the cardiomyocyte is called the sarcoplasmic reticulum or the SR. We had a bunch of genes that had interesting dynamics there that we hadn't paid enough attention to.

From cells in culture to one week, there was marked up regulation of the ryanodine receptor and triadine two important parts of this. Further on by one month, calsequestrin, CASAQ-2, etc. It wasn't just maturation at the plasma membrane, but intracellularly, there were these dynamics at the sarcoplasmic reticulum that we had basically just not paid attention to. This led to the hypothesis that what our problem maybe the upstream causes an intracellular calcium leak coming out of the sarcoplasmic reticulum into the cytoplasm and then running through the sodium calcium exchanger and contributing depolarizing current. We went back and decided to tackle this. Fortunately, a postdoc named Andy Wescott joined my lab. Andy had trained with John Lederer in Baltimore and was really well versed in the biophysics of calcium and he'd worked in mitochondrial metabolism in cardiomyocytes. He was keen to take this on. To have somebody come in with really good microscopy, biophysical skills was super useful here.

We made a cell line that would be useful for this, which we took a line from the Allen Institute that had red fluorescent protein targeted to the plasma membrane. Then we popped the latest and greatest version of this calcium indicator protein GCaMP into the cells so that we now had cells that were double positive, and we popped this in at the clavicoccus using what I think it was the chick beta actin promoter. We transplanted these cells into the hearts and then during the vulnerable period at one week and from one week to four week, we took the hearts out and enzymatically dispersed the hearts to recover the grafts. This is harder than it might sound because these cells they don't like this.

But Andy eventually acquired a very nice data set where we could do living cell calcium dynamics to see if we could see evidence for this calcium leak. What did we find? These cells are not happy during this vulnerable period. The next several panels, I'll show you a cell that's spent its whole life in vitro, a cell that's been in the heart for a week during the vulnerable period, and then cell that's been in for four weeks when it's coming out of the woods.

These are the GCaMP fluorescent signals that you see. Again, the cells are being paced here at a half a hertz. It's hard to see this quantitatively, but they have pretty respectable calcium transients with a rapid upstroke and a reasonably brisk decay. Beforehand, we transplant them a slow upstroke, a blunted peak, and a really slow decay with a long tail. These, by the way, look like they're just thick lines, but they're actually really tiny standard errors because there's a bazillion cells that were done in this. This is data we have quantitative confidence in. Then four weeks later, this is normalized again.

That made sense. Calcium handling was mongered up in these cells. We also looked at cell clusters because we weren't sure which would be the more informative single cell because it's obviously multicellular tissue in vivo. We looked at cells that came out in clusters too, and we did the quantitation. Calcium dynamics are a little more normal in the cluster than they are in single cells. But again, we saw the same differences. I'll show you quantitatively what this looks like.

This is the amplitude of the transient. Pretty decent before we put them in, bad after they've been in for a week and then a rebound. Same things with clusters. We didn't do them in vitro. If we look at the time to peak, so long time to peak is a slow transient upstroke, and you can see it's fast in vitro, it's slow after a week and it comes back down, same for clusters. Then we also looked at the ability of the cells to be externally paced by field stimulation, and the ability to capture the cells goes down quite a bit at four weeks, either in single cells or in clusters. There's a lot of dysfunctional stuff.

But here's the thing that surprised me. I thought we would see a maturation profile where the cells would be and it could be maturation, but it's not just maturation because things get way worse before they get better. I showed you gene expression. It shows maturation, but physiology shows pretty healthy, very unhealthy, healthy again. There is a stress response that induces there's something about the transplantation that we have done that causes cellular injury that's manifest as bad calcium dynamics.

The next thing was that we wanted to look for this spontaneous leak of calcium that we hypothesize. There is this thing they called calcium sparks, where these calcium release channels in the sarcoplasmic reticulum are called the ryanodine receptors, and they often plaque up to form these cooperative multi protein hundreds, I'm no expert in this. But like hundreds of these guys dock up in intercellular rafts, and then they fire cooperatively. When one of those units fires, it releases a little spark of calcium so that you can see that as well as the calcium transient. This is what an in vitro cell looks like. Just for reference, then we're not pacing. We're letting them go spontaneously. This is what they look like at one week, and this is what they look like at four weeks. What you can see, if you just watch this middle guy, there's the big calcium transient there, and then there's all these little funky things that are going on in the cytoplasm.

If you look at this in a quantitative way, you draw a region of interest and you just monitor it, yes, so this is a calcium transient, and these little unitary things are calcium sparks. There's way more of them in the cell at one week, and then they're basically gone or they're markedly reduced by four weeks. We've got this quantified here. I think, these little unitary releases of calcium, I think, are the culprit, that this is leak dumping calcium from the intracellular store into the cytoplasm, and that goes through the sodium calcium exchanger and triggers the action potential, and that triggers the arrhythmia.

I think I just told you this. Yes, there's immaturity, but it's not just immaturity, there is a lot of gene expression dynamics that encode transcripts for SR proteins. But there's also this fact that there's an nitrogenic element to this, in other words, a physician induced element, basically, where we stress the cells out through this process of transplantation. This completely alters their calcium dynamics and induces a calcium leak. I think it's this calcium leak that we've induced that is triggering it. It takes a combination of immaturity plus stress to happen. That, and after a month, the maturation is up, stress is down, and that probably is what reduces makes the engraftment arrhythmias go away. Some of what I've told you is inferential. It's not like we've got genetic evidence so, I consider this evidence not proof, but this is the way I'm currently putting it together. I'm going to pivot with my remaining time and talk about what I consider to be the biggest scientific barrier to regeneration in many areas of stem cell based medicine, which is the immune system.

I'll start with one book. We'll go from bookend to book end. We're going to start with autologous cells. Remember, back in, gosh, 2000 and it's 20 years now since Yamanaka first discovered IPS cells. Remember, we all thought, this is the answer. We're going to take our own cells, we'll make- We'll make stem cells out of them, and we'll put them back into us, and we'll get rid of rejection. That was considered to be the answer, at least, that's how I thought about it. Then scientists came along and started slaying beautiful hypotheses with data.

What happened was mostly from mouse studies, people were transplanting into what should be syngeneic animals, and they were getting rejection. Not all the time, but sometimes. This hypothesis came about called the neoantigen hypothesis. Where there are new proteins that are present in these stem cell derived parenchymal type cells, and that is leading to immune rejection. Certainly like for mitochondrially encoded proteins, there was people correlated mutations in the mitochondrial genome with altered peptide sequences for mitochondrial encoded respiratory proteins and so forth. It seemed like, yeah, this new antigen thing is a thing.

Since we had this primate model, we wanted to see whether autologous cells would work in the heart, and so we collaborated with the intramural program set of scientists at the National Heart Lung Blood Institute, Cindy Dunbar and Manfred Boehm, two really terrific scientists who had great groups. Their chief lieutenants were So Gun and Yongshun. Then from my group, it was Kenta and Lauren. This was another pandemic project that was crazy, where we're flying back and forth across the country, and so forth. The timeline went sort of like this. We made autologous, we started with these rhesus monkeys. We made IPS cells out of their CD34 blood derived stem cell or progenitors from them, and then we genedited them to put in a reporter gene for pet imaging. I'll

talk about that in just a minute. Quite a bit of work goes into each animal. Then once we had the line and the cardiomyocytes manufactured, we came in, we infarcted the animals with a balloon catheter.

Then we waited a long time. We waited about four months to mimic what would happen in a patient with an autologous situation because it would take at least six months to have something ready to treat a patient. Then we flew back across the country and surgically transplanted the cells into the heart, and then we waited and we longitudinally monitored the animals. Then we terminated the study at 8-14 months after that.

Imaging is awesome, and you learn things with longitudinal tracking that are so hard to see with cross sectional data sets because of animal to animal variance. The reporter gene was the sodium iodide symporter. This is a gene that codes for a protein that sits in the plasma membrane and is involved in iodide transport, brings iodide into the cell using the sodium gradient. This is the basis for doing thyroid scans in clinical medicine. Then we used a clinically active pet probe called 18 fluorine tetrafluoroborate. When you do this in a healthy animal prior to infarction or excuse me, prior to cell injection, what you see is this, this is a total body pet CT image, and you can see the butterfly shaped thyroid gland. You see the distal stomach proximal duodenum, and then passive collection in the urinary system.

But in the thorax, which is zoomed in here, there's basically just background. Here is looking 10 days afterwards, and this is the pet image, and then it's superimposed on a CT cross section here, you can see the anterior wall where we transplanted the cells were present at 10 days, present at one month, present at two months, present at 12 months, and if we quantify it, you can see that this is a single animal, but we see a graft that goes up, plateaus 2-4 months and then holds steady. Doesn't look like neoantigen driven rejection, it looked like a graph that expanded either through proliferation or hypertrophy and then plateaus. At the study terminus, we took the hearts out and we looked for a couple of different things. First, just to orient with the histology. The first question is, well, if they're autologous, how do you find them? Then we used insight hybridization for the sodium iodides importer gene because that's the transgene that we'd popped in as a reporter.

This is the infarct region along here in this heart and the troponin just identifying it. Then, This is the graft region with the sodium iodide symporter shown in red. You can see it also histologically, they integrate well, but they are not quite like the adult myocardium either. This was a graft that was 14 months post-delivery. One of the questions we had was, will these grafts fully mature? What I'm going to show you is the heart from an eight-month animal, and a 14-month animal. We're going to be looking at the graft host interface. Again, the sodium iodide importer is the key for identifying that, and then we wanted to, what's going to be the index of maturation that we'll use? We'll use this troponin switch that happens. It's one of the late events. From the slow skeletal troponin I to the cardiac troponin I is about the last thing that I know that flips during maturation.

Cardiac is the mature one, and you can see at eight months, the host is much brighter than the graft. The graft has some, here's the immature one in purple. It's a 50/50 mix. It's on the way, but it's not fully matured. But by 14 months, just a scant few cells

express the immaturized form of troponin I, and it's really quite robot. The cardiac troponin I is essentially equivalently intense to that of the host. It takes a while. It surprised me how long it takes, but they eventually get there. It seems like there's no intrinsic block.

But we really did this to study the immune system. My clinical work has been as a transplant pathologist, and I work with a heart transplant program in diagnosed rejection. We looked at these hearts with the eye of what would we do for a transplant patient. We looked for the infiltration of T cells, B cells, macrophages, and things like that in the graft itself. Again, looking at the graft host interface by hematoxylin and eosin, this would be looking for little blue cells. Blue is bad is one of the rules of pathology. If we don't see any badness here and looking for T cells and B cells, there's essentially none to be followed. Just the occasional surveilling cell, but really no accumulation. This is whistle clean from that standpoint. That's interesting.

How about the other end of the spectrum? What about complete immunological mismatch between graft and host? We then went on to do that, and this is some work that's in rejection at various journals at the moment. We wanted to know, because I don't think autologous scales. We wanted to see if we could find an immunosuppression regimen that would work. There's a sociological question as well. What are cardiologists willing to do to patients as well? It's kind of a begin with the end in mind thing.

It turns out this is ignoring transplant cardiologists and just doing plain old cardiologists. We actually hired a survey firm to do a survey with 100 practicing cardiologists and ask them, would you immunosuppress for this treatment if it would give you a 25 point increase in ejection fraction? It turns out cardiologists are very chill about arrhythmias. They don't mind those at all, but they have the HBGBs about immunosuppression. They did not like strong immunosuppression. It just tipped precipitously. If we have to suppress them like for heart transplantation, no way.

But if it was more like a rheumatoid arthritis patient, okay, we'll give it a shot and see how it goes. The idea was to find a regimen that spared steroids and cyclosporine. Which were probably the two most toxic elements of immunosuppression regimens. We used our monkey model to do this. I'll skip through the design. We did the best we could. These are complicated things to do in non human primates. We came up with three different regimens or four different regimens, tacrolimus only, abatacept which is a costimulatory blockade only. The Tac + mycophenolate Mofetil, which is antiproliferative agent, and then Tac + abatacept as the four groups. We did this old school. We use histology to grade rejection when we took the hearts out.

The first thing is there's no magical immune privilege, blah, blah, blah. If you take allogenically mismatched cardiomyocytes, you transplant them in the immune system finds them and rejects them. I told you, blue is bad, and look at all the blue that's in here. These are little blue cells, T cells, B cells, macrophages crawling through and killing off this graft. The graft is marked here with this immature troponin stain, but it's just being infested with these cells. If we left this much longer, the graft would be completely gone.

In a way not surprising, we'd hoped for a break but we didn't get it. Three of the regimens failed miserably, but one worked quite well. That was lucky, I guess,

tacrolimus, where the graphs turn out about as clean as they did in the autologous situation. One had a little bit of rejection out of the four animals. But there's not a lot of little blue cells here by H&E, and a very low burden of T cells and B cells by immunostaining. What have I told you here? Autologous works pretty well. We didn't see this neo antigen problem, and we've gotten them for 14 months, which is like as long as it's practical to test in animal models I think. That allogenic cells, the good news is they're not as immunogenic as a whole heart, but they are immunogenic and the immune system will take them out. We found this immunosuppression regimen that we think will keep them alive long term with the limitation.

We only could do this study for four months, and it avoids side effects and we think it'll be adopted. I'm just going to wind up telling you where I think we're going to go in the last couple of minutes. We're looking to take a shot on goal in patients. One of the interesting things about coming down to USC has been taking advantage of the vast population of the basin that USC is a quaternary referral center for patients with advanced cardiogenic shock, where they come in critically ill and have mechanical support devices put in place. The scheme goes like this. The most common cause they're admitted is for having a large myocardial infarction. They lose a huge chunk, say in their anterior wall, they go into acute shock just due to pump failure. They're brought in, and they are hemodynamically stabilized by putting them on support devices. Here, one of them is called Extracorporeal membrane oxygenation, where blood is taken out of a large vein, say, the femoral vein in the groin, and it's first pressurized in this pump, and then it's run through a membrane oxygenator, and it's pumped back into the femoral artery on the contralateral side.

That turns out not to be enough, you need a second pump in place, a catheter based pump that snakes its way up from an arterial access point and it is placed in the left ventricle, and this is called an Impella, and it takes blood out of the left ventricular chamber and puts it into the aorta. This is standard of care for people with these massive myocardial infarctions, and they remain the most critically ill population in cardiovascular medicine, I think. This is a Kaplan-Meier survival curve from the data at USC, but this is what they look like all over the world. These patients are really sick. The timeline is a little hard to see, but this is just 60 days. Twenty five percent one week mortality, 50% one month mortality.

We've learned ourselves work best in the acute situation, not in the chronic situation where it's burned out heart failure. These are the people we think we'll be able to help. We've got a very complicated scheme of, these are complex patients, but we plan to treat them surgically because we can't put another catheter in while the patients are so heavily instrumented. But it boils down to this basically. We're going to do our thing. We'll grow our cells in bio reactor, we'll co preserve them until we need them. We'll do what's called a dose preparation and put them into an excipient, a very straightforward saline based media that will be used to transplant the cells. We will hand them off to a surgeon. She then will deliver them surgically to a patient and will incubate them and let them do their thing and watch and measure a bazillion different things.

The [inaudible] charts tell us that we can begin this trial in the Q4 of 2027. Wish us luck. We've been on this chase for a long time. The last thing I want to say is more about the culture of science and the way we do things. It was really fun to make this change and

come down to USC. My predecessor, Andy McMahon had built this fabulous department, and it was really built on embryology, and the mission was to learn how tissues form in the first instance. I'm reminded of this quote by Victor Hamburger, who's this fantastic developmental biologist from WashU who's got this quote, "A real teacher has been and still is the embryo, who is incidentally the only teacher who is always right".

I love this quote because it's humble, yet snooty. Anyway, it sticks with me. I want to put a yes end to that. I'm trying to bring in a second pillar of thought and focus to this culture, which is we need another teacher. Here, our teacher is going to be the patient. The patient, by the way, is always right. Because in the end, we have to let the patients teach us how to do this. The embryo teaches us how to make tissues in the first instance, but the patients in the end will teach us how to regenerate tissues.

I came up with this term of industrializing regenerative medicine. Part of the trick of making this happen is that we're all doing it in separate laboratories, each person can only work on a little piece, and but industrialization evokes smokestacks and soot and that thing. I don't like it for that reason. But what I mean is doing something at scale that has impact. Think of when we went from DNA sequencing to just know ATGC versus to enumerate, and we don't use it to discover sequences much. We mostly use it to count sequences now, and it's made a huge impact with.

I think we need to do something in regenerative medicine as well and build an engine for discovery. It all will start with stem cell engineering, and we'll learn how to make the cells. But then we have to bring in the host. There are certain things that we started out thinking, let's just regenerate the heart. But really, so much of this is platform technology that's going to be the same for the brain, it's going to be the same for beta islets, for diabetes and so forth. We're all going to struggle with immunology. We're all going to struggle with vascularization. We're all going to struggle with parenchyma specific things, in my case, mechanical and electrical function.

I want to bring this in a way that from every so often, we loop out and test this in patients, take the lessons back in, and use our small scale systems to make new discoveries. I want to go from a single disease to a platform that we are working in. At USC, we're doing what we're good at is heart, joints, kidneys, and CNS. But if this sounds like your cup of tea, you should maybe come talk to us. We're very interested in moving this into the clinic.

I want to build this team with people who want to do something that doesn't quite exist. I don't want to just publish papers anymore. Papers are the currency of what we do. We can't ignore it, we'll die. But we won't die. I want to integrate biology, engineering, and medicine. I wanted to work on problems that are unsolved still, that are high impact, and maybe people who want to see some work touch patients in their professional lifetime. How? That's what we all thought when we got into it, at least, I'm speaking for myself. We have openings and just saying.

This should resonate with anybody in the audience, I'd be happy to chat further. With that, I want to thank everybody who made this possible. This is my crew from when we were in Seattle in 2024. This is our burgeoning group in 2026. We went down with a big crunch and sprang back and I have to thank first, the taxpayers of the United States who have supported us continuously for three decades now, my goodness. Thank you for

paying your taxes. Then a whole bunch of funding sources along the way. We were most recently very fortunate to get a large award from the Marcus Foundation that's going to cover the costs of getting us all the way through this clinical trial that I just described. With that, thanks very much for your attention. If there's any time left, be happy to take a few questions [Applause]

Evan Snyder: Who has the first question out here?

Dan: It's great, Chuck. Really fun to hear and one comment first as a hematologist who does transplant and immunosuppression. Immunosuppression, no problem. Arrhythmias give me the EBG. It's the other way around. How you scale this? Presumably, you can't cryopreserve these cells.

Chuck Murry: Did you say can or cannot?

Dan: It doesn't, cannot.

Chuck Murry: You can.

Dan: Can you?

Chuck Murry: We are able to, yes.

Dan: Because you have this bioreactor, can you just thawn and fuse or do you have to recover?

Chuck Murry: At the moment, we have to wash and concentrate. Sorry. Go ahead, please.

Dan: But that's pretty straightforward because you don't need to have a continual supply.

Chuck Murry: No. We've uncoupled manufacturing from delivery.

Dan: No, that's obviously going to be important to scale and everything else. The other option is then hypo immune cells. If you're looking at those, I know I don't know what Sonia is doing with this, but you got Sonia in town there now. Have you tried with that and is that another option?

Chuck Murry: Part of what Marcus is funding us to do is to work on hypo immune. Just like I didn't, I wasn't an electrophysiologist. I'm not an immunologist, and so we're looking for good collaborators to help find the right way to edit and screen cells and so forth. Transplanters are particularly helpful, Dan, just saying.

Dan: Well, I can give some advice. But I assume you know Sonia.

Chuck Murry: I do. Yes. Sonia and I worked together for three years.

Dan: It would seem to be promising, but looks great.

Chuck Murry: Thank you.

Speaker 1: Hi, fantastic talk. I learned a lot. Sorry, right here.

Chuck Murry: Oh, sorry.

Speaker 1: No problem. I was really struck by your slide that showed the calcium time course of what the cells looked like before, engraftment a week after and then four weeks. I'm wondering at the one week time point, if you guys have looked into where rear to the proteins localize, the different pools and even T tubule formation, because it's my understanding that when we make stem cell derived cardiocytes, the T tubules are pretty immature, and I wonder if maybe in that one week to four week period there's some type of maturation that's happening that has to do with the calcium sparks.

Chuck Murry: To the first part about protein localization, when the cells are starting to get some architectural specialization in vitro. Then when we enzymatically disperse them, the cytoskeleton collapses into a tangled ball of filaments adjacent to the nucleus, and all of the membranes associated with it just are dragged along with it. That whole process has to be rebuilt in vivo after transplantation. That may be part of the iatrogenic stress of the transplant process. Probably happens every time we freeze and thaw and replate ourselves and that thing we just don't think about it. It's a total hot mess. The second part again, please.

Speaker 1: I think I forgot the second part. I was asking about T tubules.

Chuck Murry: T tubules, sorry. By one month, they have respectable T tubules, but not before. Thank you.

Speaker 1: Thank you.

Evan Snyder: This questioner mentions that certain newborns who have congenital heart disease, for example, anomalous left coronary from the pulmonary artery will have large infarcts but seem to regenerate. Adult heart transplant recipients seem to have at least a little host engraftment into the transplant. Do you really believe the heart has no regenerative capacity, or is it just enough just not enough in adults?

Chuck Murry: We're all born with the ability to regenerate our hearts. I believe that. Surgeons have always told me, pediatric surgeons always told me that the kiddos are different. I think that that's true. I think we've seen that now in multiple species, and we believe that to be true. But it gets pretty tightly locked out. We've looked hard in transplanted hearts with sex mismatches. We've traced extra cardiac progenitors and found that you can repopulate the coronary microcirculation with endothelium quite robustly, maybe 20% or so the coronary endothelium is host derived.

But it's about one in 10 to the minus four for cardiomyocyte, positive nuclei in a female heart. Extra cardiac, there doesn't seem to be much, and then endogenous, I don't dispute there's a little. I think the heart can probably slowly replace a few through mitosis of preexisting cardiomyocytes, but it's just not up to the job for a sudden loss like in an infarct.

Evan Snyder: Cool.

Chuck Murry: That was a good question, by the way.

Speaker 1: Great talk, Chuck. Great to see the update. I look forward to end of next year, seeing it going into patients. I was curious if you can talk a little bit more about the rationale for the trial design, since I think not a lot of people have looked at that patient population. Is it really just because if you do get arrhythmias, they're on a support device. Just wondering if you could talk about why you chose that patient population. What would be a Phase 2 then still?

Chuck Murry: I agree with everything you said, except for the word just. Is it just because it's all about risk benefit. These pumps are great risk mitigators. If they should have an engraft arrhythmia that was not well controlled pharmacologically, they have pumps that can maintain cardiac output in the setting of ventricular tachycardia.

That assuages a lot of my concerns personally about that. It is a safety thing. But part of his biology of the cells that we know that the potency is much greater if we come in in the acute setting than if we come in in the chronic setting. It's hard to see efficacy in a small animal. When we come in, the cells will engraft, but they don't improve function much in rodent and guinea pig models. It's a combination. I think the risk and the benefit tip favorably by going to this population of cells. I just have little enthusiasm to start my clinical work in an area where we don't expect much efficacy.

Evan Snyder: The same questioner who challenged you before about not having any do you really believe in no regeneration also then followed up with great story, well told and now is asking, where would you actually put the cells when you do a transplant? Would you put it into the dead zone, border zone, vascular territory?

Chuck Murry: That's really interesting question also. What we have done so far is try to revascularize the central ischemic region because that's where the greatest deficit is. But I think we also have to pay attention to the border zone because part of the progression seems to be creep of this lateral border zone. There's all this stress biomechanical stress that's there, but there's inflammatory stress and fibrotic stress and all that stuff as well. We at the moment are hitting both the border zone and the central ischemic region. We haven't systematically addressed that as well as I would like. We know that it works pretty well doing it that way, but we haven't tried just one or the other.

Evan Snyder: Cool.

Speaker 2: I have a very basic question about the fibrosis. Every time you injure, the heart will produce fibrosis. When you introduce these drafts, you also are doing a injury. Do you have formation of fibrosis along with your stem cells regenerating? What's the ratio between fibrosis and the new cells that you are generating?

Chuck Murry: I think that's a super important question. I'll tell you what we know and then where we're going with this, if we measure fibrosis using, say, late gadolinium enhancement by magnetic resonance imaging, we see that the cells induce about a 20% shrinkage of the infarct. The scar tissue seems to shrink a little bit through longitudinal

imaging studies. Mostly, we displace the scar. We don't replace the scar by putting this graft in. We don't think this is perfect. I had for a while, a program in fibrosis in the lab, and then it just gets to be doing too many things all at once and not making enough progress on any one thing. My wife tells me, I've got to focus and regenerate the damn heart if we're going to move down to Los Angeles and do all this upheaval that we've done to our life. I'm trying to focus right now, but I think it's extremely important.

I consider that like a 2.0. We've also found if we put stromal cells in, like epicardial cells that give rise to fibroblasts in the heart. That actually markedly enhances engraftment, it enhances remuscularization and improves function. It could cause more fibrosis, but it doesn't. The cells are smart in some way. We'd like to do that also, but from a regulatory standpoint, exponentially more complicated than just a single cell. We're starting with one because it seems to be enough to make things better.

Evan Snyder: We'll take one last question from Zoom. This one is I think is intended to be provocative. It's a good one to end with. If AI could solve one major bottleneck or make something better in heart regeneration, what would you want it to tackle first?

Chuck Murry: The immune system. To me, that's the thing I wake up thinking about all the time. Before it was the arrhythmia problem. I would have loved AI to solve that so that we didn't have to spend five years. But AI wasn't there, and we weren't there, etc. I would put AI on the immune system. But Evan, I'm not sure I even know how to put AI onto it. You have to learn how to use these tools also, and I'm still on the ascending limb of that curve as well.

But I think it's a super complex system, and so that's where I could use to help the most.

Evan Snyder: Well, this has really been a great provocative talk. Let's give Chuck a hand and thank him [Applause]