using a long catheter. We are using a short needle with directly the serum hooked to the needle, but if you are using a long catheter, concentration may make a big difference.

Finally, revascularization is occasionally being done in the same area as the area where cells were put in, which completely confuses the results.

This is, for example, the Spanish study, what you see is that, what they call the untreated segments, that it is segments which had just bypassed, the wall motion score went from 1.2 to 1.1 and 1, but really, this is almost normal motion, so obviously, it makes it easier to demonstrate that in the other segments which have bypass surgery and cells, the improvement was greater.

This is a summary of our data from the Phase I trial. We had an improvement in the functional status and an increase in ejection fraction. These results are meaningless because these patients had associated bypass surgery.

So, we rather looked at the number of scarred segments, and I remind you these were akinetic segments without viability on dobutaminic echocardiography without any possibility for

revascularization. So, we looked at the changes in the contractions of these segments which have been grafted with cells.

So, initially, obviously, there was no motion since it was one of the inclusion criteria, and afterwards we had, at two different time points, approximately 60 percent of segments regaining some function.

I am not saying that these segments were normally contracting, they were not. There was a slight and modest improvement. This was a blinded assessment, in other words, we blinded the dates of the echo tapes and asked independent echocardiographers to review them and to grade them. There was a modest improvement, not normal contraction, but it was sufficient to push us to move forward to the Phase II study.

I just show you a couple of examples. This is a flat exterior wall, no motion at all, and this is the same wall with the systolic thickening following myoblast transplantation. This is the MRI study which does not project on the screen. I have it on the computer, but not on the screen.

You see here the interior infarct which has been grafted, and you can appreciate an

improvement in wall motion in the postoperative period. This is an exterior infarct. You see the thin wall here, which has been grafted, and this is the post-op pattern with a thickening of the wall.

I add intentionally that these patients also had bypasses in the left system. I don't like the slides where you see pre-transplantation, post-transplantation, just omitting that in addition, there was either bypass surgery or balloon angioplasty.

This is another example of an interior infarct pre-transplantation and bypass to the posterior descending coronary artery and the post-op, with an improvement in the wall motion.

So, now, can it be due to the revascularization of the PDA? It is unlikely, but it cannot be eliminated.

So, basically, this is the design of the MAGIC, the Phase II trial which has been initiated now in Europe, in different countries in Europe. It is targeted to include 300 patients in different countries, and to emphasize what Dr. Ruskin was mentioning earlier, it is a placebo-controlled study. In other words, patients following randomization have a muscular biopsy and they have

eventually injection of a placebo solution in addition to their bypass surgery.

There are three arms, one control and two treated groups, one having 400 million, the other having 800 million cells. The production of cells, and this is probably important, has been centralized in two sites, one in Paris and one in Boston, and it is exactly the same technology which is used in the two sites.

The primary endpoint is the improvement in the contractility of the segments which have been grafted with cells in the core lab and in a blinded fashion. In addition to that, we are obviously looking at major adverse cardiovascular events at the one-year follow-up time.

I would like to move on now before finishing to some clinically relevant perspectives which may have really clinical implications in the near future.

First of all, so far we have been talking primarily of ischemia cardiomyopathy, but as mentioned by Dr. Perin, there are other causes of heart failure in particular non-ischemic, globally dilated cardiomyopathy.

So, we have been interested in assessing

myoblast transplantation in this particular context, and use a particular genetic strain of hamsters which develop a non-ischemic dilated cardiomyopathy, and randomize the animals to receive either autologous skeletal myoblasts, because phenotypically, these myoblasts are free from the disease, or culture medium.

To make a long story short, you see that there is a definite improvement in function which correlates with a major engraftment of cells in this non-ischemic myocardium. I think it just brings another piece of evidence that maybe something good is occurring.

The second problem is cell death. Regardless of the cell type, cell death is extremely high, 80, 90 percent of cells are dying shortly after the injections for a variety of causes, in particular, apoptosis, but also ischemia. It makes sense since we are injecting cells in scar areas which receive very little vascularization. So, even if myoblasts are fairly resistant, they die nevertheless.

So, now there are several studies suggesting that the co-induction of angiogenesis may be an effective means of improving survival of

the cells, and ultimately, of improving function of the animals.

This is a study comparing transplantation of fetal cardiomyocytes, injection of fibroblast growth factor, or a combination of both. As you can see, function is improved when you combine the two therapies.

Recently, we have duplicated this study except that we used myoblasts and another growth factor, and we found exactly similar results.

So, there are different ways of inducing angiogenesis, and I know Dr. Epstein is going to discuss that, but the point I wanted to make, this is, you know, the difference in cell survival between myoblasts alone and myoblasts plus an angiogenic growth factor.

The point I would like to make is that probably in the future, you will have to deal with proposal of studies trying to combine cell transplantation with some form of angiogenesis just to optimize cell survival and potentiate the benefits of the intervention.

A third point regards cycling. This is the muscular biopsy of the patient who died. I previously talked about this patient who died from

a stroke. Initially, in this biopsy, and this is not unexpected, you find fast skeletal myosin and slow type myosin. You don't find fibers, virtually no fibers co-expressing fast and slow.

When we looked at the heart of this dead patient, we found approximately 30 percent of cells co-expressing fast and slow myosin, which means that although once again these myotubes remain myotubes and do not turn to cardiac cells, it seems that some of them may incur some phenotypic changes in response to their new myocardial environment and start expressing slow myosin, which as you know is a fatigue-resistant myosin.

So, this is important and should be put in parallel with this study showing that if you co-culture myoblasts in cardiac cells, cardiomyocytes, this is the green myoblast, this is an antibody against a cardiac troponin and against another cardiac marker, some of the myoblasts, as you can appreciate here, will express some cardiac markers.

Now, what is shown here is that if you stop the beating of the co-culture fetal cells, there is no myoblasts which can acquire cardiac cell characteristics. Conversely, if you subject

the preparation to a cyclic stretch, then, the stretch makes some of these myoblasts able to express the cardiac markers.

In other words, it is quite possible that in vivo, the cyclic contraction of the neighboring cardiomyocytes leads to the expression of some cardiac markers and leaves the slow myosin in the grafted cells, and the practical implication could be that maybe combining cell transplantation with ventricular stimulation, resynchronization could actually improve the extent by which the grafted cells express slow myosin and become fatigue resistant.

So, once again, because this is a clinically used modality, biventricular resynchronization, in the future, we may have to deal with studies trying to combine ventricular resynchronization with cell transplantation.

Finally, a few words about the routes of delivery, I have talked about epicardial injections, we are also looking at the scaffolds, which are just put on top of the infarcted area. We are currently working on polyurethane, as well as collagen patches. Obviously, it is less traumatic and maybe it could reduce a little bit

the extent of cell death. This is a pattern after a couple of weeks.

Now, the catheters, I know that this issue will be discussed this afternoon. I just want to say that from a surgical perspective, I am amazed by the fact that many clinical studies have been initiated in spite of the fact that we had few data on cell retention, functionality, cell viability is not the only issue.

It is not because you see myoblasts, that they are going to turn into myotubes. You really have also to assess the functionality, the ability for these cells to become myotubes, long-term engraftment, as well as the possible interactions between catheter materials and the cells.

Most of the studies published so far in the preclinical setting have dealt with technical feasibility rather than functional efficacy, and the various routes have not really been compared.

Having said that, we are very interested in the percutaneous routes, and in our group, we have interventional cardiologists working in that. I must say that we have been primarily interested by the transvenous cell injection through the coronary sinus, and this is the summary of the

study which was presented last week at the ACC, which is a functional study that is a sheep model of myocardial infarction in which we injected cells through this catheter.

You can appreciate that it allows a real delivery of the myoblasts, which turn into myotubes, and this correlated with a significant improvement in function. So, this is a not a feasibility study, this is a true efficacy study, which is encouraging at least with regard to this particular catheter.

So, these are maybe the challenges of the future, in the setting of bone marrow, the famous MAPS, the mesenchymous adult report in cells, which feature distant advantages, possible disadvantages. We are currently working on the cells, cardiac progenitors, and I am sure Michael Schneider will have a lot of things to say about that. Also, possible embryonic stem cells.

What is also important now is to compare cells between them, and not exclusively with controls. This is true, for example, with the bone marrow.

I just would like to show you a recent study that we have done comparing skeletal

myoblasts, CD133 progenitors or culture medium in a randomized study, and the result is that there is virtually no difference between the CD133 and the myoblasts in terms of function.

If you look at histology, it is easy to find the myotube. It is co-expressed, you know, specific markers like myosin heavy chain. It has been extremely difficult to identify the CD133. We have to rely on PCR to find some of them, which means that probably very few are still present after one month.

So, I think it is important to compare the cells, and to some extent, given the amount of data which have accumulated over years, skeletal myoblasts may provide a sort of benchmark for testing other cell types.

A similar study is being done in Doris Taylor's lab showing basically that there was no difference between skeletal myoblasts and mesenchymous cells.

Once again, we are back to the question which was raised by Michael Schneider. This is the setting of chronic heart failure, and in this particular setting, current evidence will rather favor skeletal myoblasts.

This is a completely different setting from acute MI in which bone marrow cells seem to generate impressive results, but they are different patient populations, and it is quite possible that the acute stage of the MI, the bone marrow cells receive appropriate signals which allow them to improve function. The setting may be quite different where you are dealing with heart failure patients and old scars for which apparently, skeletal myoblasts look more suitable for improving function.

So, I just would like to close by two general slides summarizing a little bit what we have learned from our 10-year experience in the field.

Regarding preclinical issues, it is clear that screening experiments have to be done in rodents, but I think it is critically important to validate that in large animal models before arriving to clinical trials.

We have a good example of that with the combination of bone marrow cells and JCSF. You are aware of the initial study by Orlig's group showing a regeneration of mouse myocardium by combining JCSF and bone marrow cell transplantation.

This mouse study could now be duplicated by two independent groups including Orlig's group in primates, and then we have the Lancet paper last week showing that there was a higher rate of restenosis in patients receiving these two therapies.

So, this jump from the mouse to the man without an intervening large animal model seems to be maybe a little questionable.

It is also important that this preclinical study be designed just like clinical studies with appropriate controls and blinded assessment, but having said that, we must be aware that all these models have serious limitations at what point we are not able really to model the very complex situation of heart failure patients with a long-standing coronary artery disease. A good example is that in our preclinical work, we have never seen any arrhythmia in any of the animals.

Regarding clinical issues, it is important to have a well characterized cell therapy product. I am not sure that once the feasibility has been demonstrated in small pilot trials, it is necessary to multiply this 10-patient studies, because the amount of information that can be collected from

these small studies is indeed limited once visibility has been established, and I think it is rather important to move on to larger clinical trials focusing in efficacy and safety, and allowing to draw more meaningful conclusions, safety, the arrhythmias with the myoblasts and possibly instant restenosis with the bone marrow, and efficacy obviously is left ventricular function and major inverse cardiovascular events.

So, we are really now at very early stage, as you know, in the field. We have some evidence that the myoblasts, among others, may improve function, but we still have a lot of basic questions to answer, and in the meantime, I don't think we can make any progress without the implementation of well-designed clinical trials more or less resembling those which have been designed for drugs with appropriate controls, randomization, blinded assessment, and so on, because this is the only way really to know whether hosts will be matched or not.

I would like to acknowledge obviously all those who have participated in this endeavor with a special thanks for those who really did the work.

Thank you very much.

[Applause.]

DR. RAO: We will be open for questions. Go ahead, Dr. Borer.

Q&A

DR. BORER: First of all, Dr. Menasché, I have to tell you I think that was one of the most exciting talks I have heard in a long time. That was really wonderful.

I have some specific questions. I will only give a couple of them, so that everybody else can talk, and then maybe ask a few more later.

During your many years of preclinical studies, I am sure you made efforts to determine whether there were aspects of the preparation that could increase the plasticity of the myoblasts, so that they would manifest themselves more as cardiomyocytes than as myocytes.

If you did, number one, did you find anything that altered the character, that increased plasticity, because if it did, that suggests that maybe the current preparation isn't the end of the line, maybe one could do better.

With that in mind, you mentioned that you saw increased evidence of differentiation into cardiomyocytes or more cardiomyocyte

characteristics in the beating setting.

So, I wonder--I am sure you thought about it--but I wonder if you did culture any of the cells, rather than on flat plates, on flexor cell plates where periodic stress was applied in the culture phase, so that you could perhaps generate some of these cardiomyocyte characteristics before injection.

That is one set of questions, and a second question I would like to just put in here, because it's a one-word answer, if these cells are electrically isolated, as you mentioned, how is it that they were caused to be in concert with the rest of the heart?

DR. MENASCHE: Regarding your first question, to be honest, we have not found any trick during the cell culture process which could really increase the transdifferentiation of these myoblasts into cardiomyocytes, and really, I don't think--I am thinking of the works of Chuck Murray, for example--I think no one has really shown that changes in the culture conditions could really make them turn into cardiomyocytes.

The only evidence that can acquire some cardiac-like characteristics is this expression of

slow type myosin. We are currently exploring the possibility maybe of increasing the expression of these slow myosin isoform by implantation stimulation of the cells, but these are experiments which now are going to be done, and this was the reason why I was mentioning ventricular stimulation as a potential additive in the future to the clinical trials.

But to summarize, no, we have not found any particular intervention, although maybe we have not found the right one, which could increase the proportion of cardiac-like skeletal myoblasts. Now, the mechanisms, I don't know; from scratch, I don't know.

There are different possibilities. One is a limitation of remodeling. I am not sure if there is a predominant mechanism because in our patients, we have had some evidence of improved systolic function, but we have never seen a reduction in left ventricular diastolic dimensions.

Another possibility is that GAB junctions are not the only ways for electrical impulses to travel across the heart, and as you have seen on the film, these cells retain excitable properties. In order words, if you excite them, they will

contract.

So, it is not completely impossible that in areas where physically, they are very close to the neighboring cardiomyocytes, they may be directly excited by electrotonic currents, and there is a third hypothesis we are currently exploring, and which is the paracrine hypothesis.

It is quite possible, and it has been shown for bone marrow, for example, that these cells secrete various growth factors or cytokines, and so on, that can positively affect the function of the host cardiomyocytes.

For example, in our studies we have found that myoblasts and myotubes from patients secrete very large amount of IGF-1, which has important effects on tissue regeneration. So, maybe it has nothing to do with their countertype properties, but rather with the fact their behavior, small factors releasing good factors for the heart.

So, we are currently playing with all these hypotheses, but I don't have any definite answer.

DR. RAO: So, it is pretty clear, like Dr. Schneider pointed out earlier, that mechanism is an issue that is still not clear.

DR. MENASCHE: No, it is not clear at all. The only thing is I don't think that you could infer from the lack of connexin 43 expression, that improvement in function is not possible. I think both should be dissociated.

DR. RAO: Dr. Kurtzberg.

DR. KURTZBERG: You mentioned studies with bone marrow derived AC133 cells. I wonder what the rationale behind the selection was and why you thought there would be an advantage to using selected cells over whole bone marrow.

DR. MENASCHE: The reason is that we first did a large animal study with whole bone marrow in the sheep model of myocardial infarction, once again, a chronic infarct. So, we injected the whole bone marrow and we didn't find anything, no improvement in function, no limitation in remodeling, no evidence for transdifferentiation of cells.

So, we said, well, maybe the whole bone marrow is not the appropriate medium for this particular setting, let's try to purify the cells, and we went to the CD133. The results were slightly better in that. There was some improvement in function compared with controls, but

the improvement was not greater than that we got with the myoblast.

Currently, we are comparing now myoblasts with AMAPCs, so we have tried to pick the different populations, in a stepwise approach, test all of them.

DR. RAO: Dr. Cannon.

DR. CANNON: Thank you for your talk, it was most interesting. I am Richard Cannon from NHLBI.

My question is in any of your preclinical animal work, did you ever inject your myoblast culture preparations or cell suspensions into the circulation to see where they might end up and what toxicity they might cause.

This may not be an issue with an intra-operative injection into scar, but I would imagine with a catheter-based approach, it is conceivable that despite the operator's best efforts, some of these cells might be injected into systemic circulation.

Do you have any data on where the cells end up, do they lodge in the brain or the kidneys, do they cause any toxicity or injury to other tissues?

DR. MENASCHE: Well, in the preclinical studies we have done, we have not found evidence for, first of all, all the injections were direct intramyocardial injections, so it may be difficult to find them in the brain or in the liver.

We have not found them disseminated throughout the body, but I must say that maybe if we had done more extensive studies, autopsy studies of the brain or the lungs, or any other organ, maybe we could have found some of them.

We have never injected intentionally the cells intravenously just to see what was happening. I don't believe it is a real issue because even when you are injecting them intraoperatively in humans, it is clear that some of them are escaping through the lymphatic system or in the venous system, and so far we have never seen any evidence for unexpected or unusual complications.

But I agree with you that if you are expecting some leakage of the cells in the systemic circulation, this is probably a point that should be addressed more extensively than we have done.

DR. RAO: I have a practical question. Did you, when you looked at the cells, ever look at BRD incorporation to see whether cells continue to

divide at any time?

DR. MENASCHE: Yes, absolutely, including in the human trial. In the human trial, we always keep aliquot, initially, we kept aliquots of cells and just let them grow, and this is why we have been able to show that these cells were differentiating into myotubes including in heart failure patients.

DR. RAO: Did you ever take your samples, and look at freeze/thaw? You know, you grow them in cell culture, can you freeze these cells and do they behave the same way when you send them to another site like you are planning in the Phase II trial, for example?

DR. MENASCHE: Absolutely, we have done that and we have validated that after thawing, they retain their ability to differentiate into myotubes.

DR. RAO: Have they been done in any transplant paradigm, or has it only been done by looking at they are forming myotubes in culture?

DR. MENASCHE: Both. We have several preclinical studies in rats and in sheep, in which we have used cryopreserved and thawed cells with apparently functional outcomes similar to those we

had with fresh primary myoblasts.

This is the reason why actually we got permission to freeze them should they become necessary for logistical reasons in the Phase II.

DR. BORER: You mentioned that about 95 percent of the cells that you inject have CD56 characteristics. That suggests that there is some alteration in some of the cells or perhaps a different cell line is growing in parallel, in the cultures that you are using, so I wonder, number one, how many passages do you use before administering the cells, and, number two, is the reproduction error rate increased with passage in any meaningful way, and does it make any difference?

Obviously, a lot of these cells that you inject are nonviable. When you inject them, they don't survive. I don't know which ones are surviving and which ones aren't. But it seems to me that the number of passages employed may affect the ultimate outcome of the injection, and I wonder if you have some data on that from your preclinical work.

DR. MENASCHE: We used three to four passages, but it has been shown by Chuck Murray

that if you multiply passaging, you may end up with a population of differentiation of effective cells, in which case you might end up with some unexpected overgrowth without any functional benefit.

So, it is probably important not to multiply passaging too much. With three to four passages, we have been able to reach the target numbers of cells, 400- or 800 million of cells. We don't go beyond that.

DR. TSIATIS: In your randomized clinical trial, do you actually have formal stopping rules for either safety or efficacy, and, if so, what are they, or what is the general philosophy for monitoring?

DR. MENASCHE: It is primarily based on the judgments of the DMSB given the type of surgical population we are dealing with. It is difficult to have stopping rules, just as you can have with drugs, for example, for each adverse event is reviewed by the DSMB, and based on that, they would decide whether the study has to be stopped or not.

DR. RAO: Dr. Neylan.

DR. NEYLAN: Thank you.

I have another preclinical question. I

was wondering if you had an opportunity to compare the morphology and functionality of the myoblasts when these are injected either into the akinetic areas or perhaps into an area resected, and thus undergoing a normal reparative milieu, and whether perhaps under that milieu, there might be a different behavior of these cells or expression.

DR. MENASCHE: Really, basically, our model has been the model of, you know, coronary ligation creating myocardial infarction, so you really end up with an akinetic scar. I cannot answer this question.

DR. NEYLAN: You never had the chance to maybe resect that, maybe adhere to the natural surgical tendency of cutting things out.

DR. MENASCHE: I try to refrain from that. I have cardiologists as bodyguards, so it would just refrain you from doing that.

DR. MULE: You had mentioned that the vast majority of cells that are injected will die, and clearly, there is room for improvement with perhaps increasing angiogenesis, and so forth.

About the kinetics of myotube formation in the ischemic areas, is it a dynamic process, in other words, once you inject the cells, myotubes

will form over a given period of time, and then no more tubes will form, or additional tubes are generated over a prolonged period of time, and do those tubes, when they are formed, remain viable for the extension of the observation period?

DR. MENASCHE: It is difficult to tell you. The kinetic studies indicate that although a substantial number of cells die, the remaining ones obviously proliferate in different shape over a period which seems to extend, say, two to three weeks.

At least in patients when we have seen improvements, we have never seen improvements before one month, and in animals, it is difficult to see any improvement before two weeks.

Now, afterwards, the longest follow-up we have is 14 months in animals, but we cannot know whether the myotubes that we found at the end of the experiments were present since the beginning or whether they have been continuously regenerating.

The interesting observation, however, I don't know whether it really answers your question, is that these myotubes harbor new myoblasts, so when you look at them with electron microscopy, you clearly see, on their basal lamina of these

myotubes, newly formed myoblast cells, so they are able to regenerate their own pool of precursor cells.

Now, whether these cells participate in the formation of new myotubes, I don't know.

DR. RAO: The last comment, Dr. Noguchi, and then we move on.

DR. NOGUCHI: I am sorry to have prolonged this, but it is just fascinating. Of course, FDA always loves these controls, but to follow up on Dr. Mulé's question, is it a question of liability, do the cells have to be alive, or you have a myotube has some structure, and then I just recall there is, in tumor biology, an old effect called the reverse effect where if you have a few viable cells with a lot of dead cells, you can actually get tumors developing from one cell where normally, you might need a million or 10 million.

I am just wondering if you have done any mixtures of dead and live cells to really see how much is viability, how much is surrounding stuff.

DR. MENASCHE: No, we have not done that intentionally. We have just completed a study in which we have looked more carefully at the patterns not only of cell death, but also of cell

proliferation.

So, we know that we have this mix of dead cells and living cells, but we have not done an intentional mixing of them to see whether there was any tumor formation.

Regarding oncogenicity, we have learned a lot from our colleagues working in the field of dystrophic myopathies, and it really seems that these cells have a very low tumor-retaining potential.

In the newt mice in which we have injected our human myoblasts, we have never seen any tumor in spite of the fact that many of these cells expectedly died.

DR. RAO: Thank you, Doctor.

We will move on to our next speaker, Dr. Epstein.

Bone Marrow Cell Therapy for Angiogenesis: Present and Future

DR. EPSTEIN: It is really an honor to have been asked to speak to this very august group.

I wanted to emphasize because I really do think it is important in this, an ever-growing field to make sure that disclosure is presented, and I have a number of potential conflicts of

interest, which I hope in no way influences what I will be talking about to you for the next 20 or 30 minutes.

I will talking about bone marrow cells and angiogenesis. I wanted to start out and make a careful distinction. Dr. Schneider brought this up in his earlier questions, but basically, what we are considering today is really the use of bone marrow cells, stem cells, progenitor cells for myogenesis, but also for angiogenesis.

It is critically important to understand that these are very distinct targets with undoubtedly different mechanisms and certainly very, very different issues, and therefore will have a profound impact on how the FDA I think judges whether or not a particular proposal is meritorious.

For example--and you have heard this very eloquently discussed by Dr. Menasché--for myogenesis, the transdifferentiation of adult progenitor cells or skeletal myoblasts is a critically important issue. Maybe you don't need transdifferentiation into cardiac myocytes to improve myocardial contractility, but nonetheless, it is a very important issue to consider.

Also, if you think about how many cells are present in a large myocardial scar, the issue of adequate numbers of cells to replace the scar to cause a significant biologic effect has to be considered, and you have heard a very eloquent presentation and some demonstration relating to this.

Now, the issues relating to angiogenesis are, as I indicated a moment ago, different. Transdifferentiation is really not an important factor, because what has been recognized most recently is that cytocrine secretion, exerting a paracrine effect can induce proliferation and remodeling of existing vessels.

So, it is not necessary, although it may happen, it is not necessary for the cells that you are injecting to turn into blood vessels. They could induce the development of already existing blood vessels, and the adequate number of cells relating to angiogenesis is not nearly of similar concern secondary to these paracrine effects that have an amplifying activity of the individual cells that are injected.

So, these are very different issues, each are very important. I think the path to myogenesis

is going to be a longer one. I think that there are a lot of problems that still have to be solved, and I think the issue of angiogenesis, we have gone along that path for probably a longer period of time, and my sense is that we are closer to pivotal clinical trials even when one considers cell therapy, but I will be focusing my remarks on angiogenesis.

The first thing I wanted to point out, which is obvious to anyone who is involved in the field now, is how complex the molecular and cellular mechanisms are that are involved in collateral formation.

This is a slide I always like to show. This is a cartoon showing different genes expressed, either increased expression or decreased expression, four different times, different amounts, two actually wind up with a collateral.

So, there are multiple, multiple genes that are necessary to actually form a new collateral vessel. Just to illustrate the importance of interactions between different angiogenic cytokines, I wanted to show you the results of a study that we did a couple of years ago using a rabbit ear.

So, here is the ear. It is supplied by three major vessels. This is a laser doppler image which is color coded for velocity. Red is highest velocity, green is intermediate, and blue is low velocity. If you tie off two of these three vessels, you have a marked decrease in flow, and the nice thing about the rabbit ear is that you could observe this hourly if you wanted, and you could with this laser doppler do repeated analyses of the changing flow with time.

What you can also do is focus on a particular area of interest and measure tissue perfusion and the change in tissue perfusion that occurs with time.

Here is the tying off of these vessels, resulting in a profound decrease in flow, which gradually recovers over several weeks, and in this particular model, it is quite interesting. It plateaus off below normal flow, so this is a model of chronic hypoperfusion, which makes it kind of interesting.

In this model, we looked at what happens with endogenous VEGF levels, and VEGF is a key angiogenic molecule, so we measured VEGF by western blot before the induction of ischemia, and there is

essentially no VEGF present, however, if we measure VEGF levels throughout the course of this, and even at the end, there is a low level of VEGF present. So, this is further indication that we are dealing with a chronically ischemic preparation that has a background of VEGF present.

The next issue that we wanted to document, we took the model during the period of chronic ischemia, and we take that now as our starting point for this experiment, where we had an angiopoietin-1 gene within an adenoviral vector, so that is the transgene, which we inject intradermally in the region of hypoperfusion in the ear.

We inject it and we see over the course of time, a major increase in collateral flow and tissue perfusion, but remember there is background VEGF present. If we coinject with the adenovirus expressing angiopoietin-1, an inhibitor of VEGF, and this is a soluble VEGF receptor, so it sops up and inactivates whatever VEGF is present, it obliterates the collateral-forming effects of angiopoietin, so it just is an example of how you need multiple factors to develop your collaterals.

If any one of these is perturbed, you

could seriously influence the course of collateral development.

Now, just as background for the cell therapy, there have been a number of adequately powered, randomized studies that have been performed using individual cytokines for angiogenesis, and basically, these are either basic FGF or VEGF used in the coronary circulation or the peripheral vasculature, either the protein was injected or a gene encoding the protein were injected.

As of the moment, there have been no definitive and robust beneficial results. There is trends, there is some encouragement, there is some early positive results, but nothing to really get excited about.

Of course, as I indicated, all of these randomized studies to date have involved a single agent to promote collateral development, and it was these considerations about four or five years ago that provided the impetus for developing and testing a second generation of angiogenesis strategies, which is the use of cell therapy, which had the potential to deliver multiple collaterogenic cytokines.

I just wanted to show this slide. I am not an expert at all in stem cells, progenitor cells, but I just wanted to indicate to you what has been used in clinical trials or in late-stage preclinical trials. So, hematopoietic stem cells characterized by positive CD34-133, which do progress to endothelial progenitor cells and then to endothelial cells, which have been shown to lead to an increase in collateral flow.

Now, more recently, monocyte lineage cells have been demonstrated. These are characterized by the lack of CD34, but having CD14 and 45 MAC-1, these monocyte lineage cells have been shown, not to produce endothelial cells directly, but nonetheless, are capable of inducing collateral formation.

We have used freshly aspirated bone marrow cells that have been filtered and directly injected. These are autologous into pig ischemic hearts, as well as patients. You have heard about monocyte-derived bone marrow cells. Dr. Perin used these in his study.

We have been also working now with mesenchymal stem cells or stromal cells. There are multiple terms that have been used to describe

these. These are CD34-negative, 45-negative cells, and these have been shown to produce collaterals.

Now, I won't get into this in any detail, but you should be aware of the fact that some of these cells are believed to incorporate into developing collaterals, with that being a major mechanism by which they enhance the development of collaterals, whereas, other interventions are not believed to have that as a major mechanism, but the major mechanism being the secretion of all sorts of cytokines and growth factors that lead through a paracrine effect to the development of either new collaterals or the enhancement of existing collaterals.

What I will be talking about, because all of our recent work has been done with these mesenchymal stem or stromal cells, I will be talking about that for the next few minutes, and these we refer to MSCs.

So, these MSCs, just to start or justify our further studies, were cultured in vitro and assayed. The conditioned medium was assayed, and here is our control cells which produce small amounts of VEGF MCP-1 and FGF, but the MSCs produce really quite large amounts of these angiogenic

cytokines.

So, we were very excited about that, thinking that they could be little factories that might enhance collateral development. So, this is the mouse hind limb model. This is laser doppler imaging, as I showed you with the rabbit ear. Here is the mouse's tail and the two hind limbs, and the femoral artery is ligated at day zero, and this is followed now every few days, and you can see there is some return of function under control conditions.

This is just injecting media that had not been exposed to cells, and here is what we see with media alone and with the control cell. This is mature aortic endothelial cells.

But then when we inject into the hind limb MSCs, we see a quite marked improvement in perfusion, and this can be quantitated as shown in this slide. So, this was a very exciting demonstration for us, which was repeated multiple times in different experiments.

Now, I won't belabor the number of studies that have been done here. It is in your handout that was distributed, I think it is page 20 and 22, but I will just go over a couple of the highlights.

There have been 7 or probably 8 published studies in either chronic ischemia--and this is angiogenesis studies, no myogenesis--in chronic ischemia or in acute myocardial infarction.

The points to be made are, number one, all of these studies have shown safety, they have shown feasibility, and they have all showed positive trends, they have been encouraging, but as Dr. Menasché very carefully pointed out with his own myogenesis studies, when you are dealing with such small numbers of patients, none of these studies was randomized, double-blinded. There is no way you could draw any conclusions regarding efficacy.

So, it is encouraging and it certainly would indicate that additional studies are necessary, but we can't make any inferences whether the strategies that work in an animal model very reproducibly necessarily work in humans.

Now, I want to point out, and I think we have to be aware of this upfront, and any investigator who is involved in the field has to be aware of it, that there are potential problems with any angiogenic strategy including cell-based strategy.

For example, genetics. Here are some

beautiful studies done by Birgit Kantor in collaboration with us. This is microscopic CT imaging of two different strains of mice. This is the femur, tibia, and this is the femoral artery, and the femoral artery had been ligated, and you can see that the C57 black 6 mouse has an extraordinary capacity to develop collaterals, however, about C. mouse, same ligation site, has a paucity of collaterals. Well, clearly, the same thing must relate to humans.

Another thing that was raised earlier is the enormous variability amongst patients to respond to angiogenic interventions. These are not patients, these are mice, and this is the typical experiment that I showed earlier, looking at laser doppler perfusion.

The mouse has the femoral artery ligated, and there is a gradual recovery of flow in young mice, however, it you look at knockout mice that have high cholesterol levels, their capacity to develop collaterals is significantly impaired.

Now, if you take an old mouse--these are mice about 18 to 20 months of age--they are really having trouble developing collaterals, and then if you take an old mouse who has high cholesterol

levels, they are really in bad shape.

Now, no one has demonstrated this relationship in humans, but I am certain that it occurs. So, there is going to be different capacities of different individuals to develop collaterals, and undoubtedly reflecting different potential to respond to angiogenic interventions.

Now, here is something I would like Dr. Schneider to look at, because he said this has not been published before, but it has been published. This is in I think JACC in 2002, but when we did our clinical study, injecting autologous filtered, freshly aspirated bone marrow cells into ischemic myocardium of patients, we took an aliquot of these cells and cultured them, and looked at VEGF production, as well as other cytokine production, and over the course of time, one sees an increase in VEGF production, so these cells do have the capacity to produce different angiogenic cytokines including VEGF, but that is the mean data.

If you look at the individual data, there is marked patient-to-patient variability in the capacity to express VEGF, so here is a patient who really has a great capacity to produce VEGF, whereas, this is a patient who hardly can produce

VEGF at all, and it is not a great stretch to think that this patient may not respond as vigorously to cell therapy as the patient whose cells have a great capacity to produce VEGF.

Now, we didn't look at enough patients to be able to make such correlations, but I am sure that this is an issue that has to be addressed, as Dr. Schneider really pointed out before.

Now, let's look at the cells we are injecting, and this was also raised earlier, so we are looking here at a HIF-1--I will just get into that in a moment--but it is a transcription factor that is a key modulator of the cells response to ischemia, so let's take it for the moment that it is a key angiogenic factor, so this is a Western showing HIF levels, and the first thing I want you to concentrate on is under normoxic conditions, young and old, HIF is not present, it is mostly absent as a matter of fact, in the absence of hypoxia.

Now, in the young mice, if you expose these cells to hypoxia over 12 hours, there is a major increase in HIF protein, and then that has important compensatory effects on the cells' response to hypoxia, however, cells derived from

old mice have a markedly impaired ability to form HIF in response to hypoxia, so there are age-related changes in the capacity of cells--these are MSCs--to perform in a way that we would expect them to if they were going to have a potent effect on collaterals.

So, HIF is a master switch gene in the presence of hypoxia, a heterodimer is formed, HIF-1-alpha, and HIF-1-beta, which attaches to the promotor of many genes and turns these genes on, and amongst the genes are multiple genes related to angiogenesis, VEGF, VEGF receptor, FGF, et cetera.

Now, just to show the biologic effects of what I just showed you before, that is, the inability to increase HIF protein in response to hypoxia, here are young and old mice, and we are looking at VEGF levels. These are cells, MSCs growing in culture, and here is the intrinsic VEGF production.

When we expose young cells to hypoxia, there is a major increase in VEGF production, mediated mainly by HIF-1 reduction, but old mice not only have the lower levels of HIF-1, but they have a lower target production of HIF-1, that is, VEGF production.

So, these are real phenomenon I think that we have to be aware of and begin to start thinking about when we are dealing with any angiogenic intervention, but certainly with the cell therapies.

So, these considerations provided the impetus to test another generation of angiogenic strategies, and this relates again to one of the earlier questions of the panelists, and we are very much involved in genetic manipulation of these MSCs to see if we could further enhance their ability to secrete angiogenic cytokines and so to improve collateral flow.

This is a construct of Genzyme. They have been very helpful in working with us in this. In the absence of severe hypoxia, these two dimers of HIF, HIF-1-beta and alpha, there is no heterodimer formed because HIF-1-alpha is rapidly degraded.

So, to overexpress HIF-1-alpha, so that the heterodimer can form, we transfect these cells with an adenoviral vector that has the HIF-1-alpha transgene and that has a deletion insertion, putting on a herpes sequence VP16, which stabilizes the protein under normoxic conditions, so we are able to overexpress HIF-1-alpha, the heterodimer

can be formed, and the genes can be transactivated.

So, we then looked at the capacity of this intervention to cause these MSCs to secrete angiogenic cytokines, so this is VEGF. The cells now are exposed to just hypoxia, and you can see there is about a doubling of the amount of VEGF present, but when we transfect these cells with the HIF-1-alpha, there is a huge increase in VEGF production, and the same thing is true for fibroblast growth factor.

So, this was really exciting to us because we saw that we could genetically manipulate these cells to make them at least in vitro more like a better collateral enhancer.

We then went to our mouse ischemic hind limb model to test this concept. Here are our control cells, mature aortic endothelial cells. Here are our non-transfected MSCs, and here are our transfected MSCs. So, not only do we see an effect in vitro, but we see what would have been predicted from the in vitro effects in vivo.

I think that this is probably something that we have to think about given the effects of various risk factors on the ability of cells to achieve their desired effects.

I did want to point out for the panel that down the line, we are not only going to be talking about cells, but cell-derived products in cardiovascular therapy, and I will just spend a moment on this, and mention the effects of conditioned medium on collateral development.

As I showed you before, if you grow cells in culture and allow them to produce whatever goodies they are producing, and then you take the media and you inject that media into the ischemic hind limb of mice, you get--well, that will be the next slide--but here is what I showed you before, so these in the media contains more VEGF, more MCP-1, more FGF, and multiple other gene products that we haven't tested, but when we put this conditioned medium into the ischemic hind limb of the mouse, here is the control again, here is our control.

Here is the injection of the MSC conditioned medium. We see that the media alone has the capacity to increase collaterals. So, this undoubtedly is going to be something that you are not going to see an application to this, I don't think in the next few months, but in the next six months or a year, I think that the cell products is

another very interesting way to use the angiogenic potential that bone marrow cells have.

Just to show that this is biologically important, we looked at the number of collateral vessels the media increased, the number of collateral vessels, the strength of the leg as an ambulatory score, the media increased that, and also the amount of atrophy that occurs in the calf as a result of ischemia, and the media injection decreases that, so it was a biologically relevant intervention.

I just wanted to mention safety concerns, and I was interested in Dr. Menasché's comment about this. There are multiple well-known ones that are usually tracked, and I won't get into this, but I would just alert you to something that is more theoretical than proven, but I think you have to be aware of it and at least consider it when you are considering the safety of angiogenic interventions.

That is--and this is a general rule that I have come up with--whatever induces angiogenesis, induces atherogenesis, and I refer to this as the Janus phenomenon. Janus was a Greek god with two heads, so that when he looks out one way, he is

also looking at the other. That goodness can also be badness, there are no free lunches.

So, I would think that any angiogenesis intervention, one of the potential side effects that one should look for is the acceleration of the atherogenic process.

This is just a slide that is still in development, but basically, it shows that when you induce ischemia in a hind limb, you have decreased PO2. This activates cytokine release, which activates bone marrow cells, splenocytes, many inflammatory cells.

We now know that inflammatory cells are critically important to the development of collaterals. Macrophages have been shown to be critical. We have shown that both CD4, t-lymphocytes, and CD8 t-lymphocytes are critical to collateral development, but these same factors, these same inflammatory factors also have been well described to lead to, much longer than angiogenesis, an acceleration of atherosclerosis.

So, if it causes angiogenesis, think very hard as to whether it might be worsening the atherosclerotic process.

The conclusions: single molecule-based

strategies to improve collateral flow, although effective in animals, have yet to be proven efficacious in patients. Cell-based strategies have great promise because of the ability of bone marrow derived progenitor cells to secrete multiple collaterogenic cytokines.

However, cell-based therapies also have the potential of achieving suboptimal effects because of the effects of aging and other risk factors on cell function. The optimal strategy has yet to be identified, but genetic manipulation of cells would appear to hold great promise, and use of cell products, such as conditioned medium derived from cells, will also undoubtedly be explored as a therapeutic strategy in the near future.

Thank you.

[Applause.]

DR. RAO: Thank you, Doctor.

We have time for a few quick questions.

Q&A

DR. SCHNEIDER: Steve, as an exploratory tool, conditioned medium for angiogenesis makes a lot of sense for the reasons that you articulated, but as a therapeutic product, that would be true

if, and only if, conditioned medium contained products that could not be identified or could not be added combinatorily from defined factors.

So, it seems to me it will be especially useful in those conditioned medium experiments to test the effect of specific blockers and find out, at a reductionist level, what the components are. If it were as simple as angiopoietin and VEGF, one could use angiopoietin and VEGF.

DR. EPSTEIN: Right, it's a very good point. My own feeling is, having been in this field now for 12--more than that--14 years, it is so complex and the number of factors that are involved in collateral development are not 4 or 5, but they are dozens, and maybe even more than that, that I personally will not waste time trying to figure out what two products are enough or what three products, I don't believe that, but the cells, they know how to develop collaterals, I mean they are doing it all the time, so I will go with cell therapy, and I will allow someone else to look at what combination of three factors might be optimal.

It may be that you could find such factors, but don't forget, not only do you have to

know what factors are present, but you have to know in what concentrations, and so on.

I think the cells, they are eliminating so many of the issues that if we were going to look at individual cytokines, we would have to explore for years, so it is a good point, but I think that the practical issues, given the huge complexity of this, would be overwhelming.

DR. SCHNEIDER: To follow up on your comment, which I would share, that it is extremely likely that engineered cells will outperform naive cells, I would like to ask the participants from FDA what additional hurdles are seen in the consideration of gene-engineered cells to be applied to these therapeutic situations.

DR. NOGUCHI: I think we can answer in general that actually, we have a fairly rich experience with gene-modified cells that have been given to individuals for a whole variety of diseases, not too many for cardiovascular, but I wouldn't expect that we would have very much difficulty in really being able to handle that.

DR. RAO: Dr. Mulé.

DR. MULE: Combining your presentation with Dr. Menasché's, I was sitting here wondering

what is known about, if one takes whole bone marrow cells and perhaps Dr. Kurtzberg can add to this, with Dr. Menasché's studies, if there are cells within the marrow that can give rise to myotubes, and you combine that with a population of cells that could be responsible for collaterogenesis, the issue is are they the same cell or are we at a period in time where we can identify two subsets within the marrow that conceivably could be combined to overcome some of the issues of viability that Dr. Menasché has talked about.

If the answer is no, we are not there yet, then, it begs the question if one were to use an adenovirus to introduce a gene to improve collaterogenesis into the cell population, that is identifiable for producing myotubes, the issue is does that manipulation adversely impact the ability of that cell to create myotubes.

DR. EPSTEIN: Well, those are sensational questions, and I have never thought of this last one before, but it is certainly--you know, it is so easy to do harm, and it is so hard to do good, so I mean your question is very apt, I mean does the very expression of the cytokines that enhance angiogenesis, might it interfere with myogenic

potential, I don't think anybody has done that experiment. Hopefully, the answer will be no, but it certainly is an experiment that has to be done.

The MSCs that we are deriving from the bone marrow do not differentiate into myoblasts, and it would be a very interesting experiment to take Dr. Menasché's approach and mix these in with the skeletal myoblasts to see, because I can't understand how, if you have a scar, and you are injecting cells into the scar, and you do nothing about the blood flow, why those cells won't turn into scar. The blood supply clearly was demonstrated to be inadequate because you have got scar.

Of course, Dr. Menasché is actually doing some experiments now using the same molecule that we are using to induce collateral formation, so it certainly is a very, very important strategy to test.

DR. KURTZBERG: In answer to the other question raised, I don't personally think we know which subsets are important yet, or whether subsets are more important than whole cell preparations. I think that all should be the focus of questions going forward.

Evan Snyder has an interesting model of spinal cord injury and repair. It's a rat model. They ligate, take a hunk of spinal cord and then inject allogeneic cells and look at repair, and they see repair and re-formation and re-connection of nerves, but when they went back and looked to see what cells did it, it turned out they were host cells that were facilitated by something that the allogeneic cells brought to the table, although they don't know what.

To me, that just points out how much we don't know and how complex the process is, and how much more we need to study.

DR. RAO: One last question. To me, and I am somewhat naive in this field, there is a difference between new vessel initiation and collateral formation of regrowth in terms of the factors which had acquired, and so on, and it seems to me in some models of cardiac ischemia, what we are looking at are completely ischemic regions and long term, which there is no regrowth, and if you had to do anything, it would be new vessel formation.

Would it be fair to say that we can't extrapolate from the current models that you talked

about in terms of the religation and revascularization, or is it reasonable to be able to extrapolate from those models to what you think might happen in a cardiac model?

DR. EPSTEIN: Well, I think it is reasonable to extrapolate because we demonstrate that we are able to improve perfusion, but your question is really a very interesting one, and that is, there used to be a major emphasis that increased perfusion is just due to angiogenesis or the development of new capillaries.

Well, I think most people involved in the field would agree at this point that capillaries don't increase flow. They facilitate the distribution of flow, and what you need is an increase in conductance vessels or arteriogenesis to truly produce an overall increase in flow.

However, we have some preliminary data to suggest that both processes are real, that angiogenesis is a part of arteriogenesis, and that you need the development of capillaries, that the development of new vessels, capillaries, can remodel to form collaterals.

That is why I don't use the term anymore of angiogenesis. I say "collaterogenesis," because

it gets away from the mechanistic aspects, which are critically important, and we still don't have the answer what cytokines produce angiogenesis, what are important in terms of arteriogenesis, and are both important to actually optimize the development of collaterals, so we still have a couple of years I think to go to answer that question.

DR. RAO: One last question

DR. HARLAN: When you showed the adenoviral HIF transfected cell lines and showed that those were more efficient at elaborating cytokines and inducing vessel growth, it adds another question, that then becomes, however, potentially anyway, a less well refined product, the cell-conditioned medium from those cells, and in view again of what we heard when we started today, as we move forward with thinking about delivering products to people, you want them to be defined, and I wonder if you would comment on that.

DR. EPSTEIN: Well, if this field is to move forward, I think that criteria is going to have to be eliminated because there is no way you are going to be able to define--maybe I am exaggerating--the hundreds of molecules that these

cells are producing. We don't even know how to measure them.

But I am sure the FDA allows the infusion of serum and plasma from one individual to another. Do you know what is in that serum?

DR. HARLAN: I am not the FDA.

[Laughter.]

DR. EPSTEIN: So, there is a precedent for not knowing what you are injecting. To be honest, we are injecting cells, and we know a few of the molecules that they are secreting, but we have no idea of the concentration, and whether it is going to vary from one patient to another, and if a patient has diabetes or has hypercholesterol, so believe me, if you going to be compulsive and say we have to know the concentration, when, over the course of time, those molecules are up, and what is their interaction, we have to stop this field right now, it can't move forward, and it is too bad. I mean you would like to know everything, but this is not a characteristic of cell therapy.

DR. RAO: On that note, we will break for lunch. We broke a little late, so we will try and come back a little bit later, but not too much, so we will shoot for 1:00.

[Whereupon, at 12:15 p.m., the proceedings were recessed, to be resumed at 1:00 p.m.]

A F T E R N O O N P R O C E E D I N G S

[1:12 p.m.]

DR. RAO: Good afternoon.

Before we begin with the talks, I would like to introduce three more members of the committee who have just joined us. I like to let them do it.

DR. HIGH: My name is Katherine High. I am on the faculty at the University of Pennsylvania. I am a hematologist with an interest in gene transfer for hematological disease.

DR. BLAZER: My name is Bruce Blazer. I am at the University of Minnesota in the Department of Bone Marrow Transplantation with an interest in immunobiology.

DR. RAO: We also have Dr. Grant from the FDA.

DR. GRANT: Hi. I am Steve Grant. I am a cardiologist. I am also a clinical reviewer within the Office of Cellular Tissue and Gene Therapies.

DR. RAO: We will continue with the series of talks that were scheduled.

The next speaker is going to be Dr. McFarland.

Cellular Therapies for Cardiac Disease

DR. McFARLAND: Thank you, Dr. Rao, and welcome back from lunch.

[Slide.]

This slide is intended to remind me to answer the implicit question which may have been raised, and the question is: Isn't the FDA putting the cart before the horse?

The answer is, well, yes, in a way. We thought that it would be good to focus and give people a peek at what is in the cart below those flat-screen monitors, I suppose, before we spend the afternoon dealing with the horse which is pulling the cart, the draft horse of product development being preclinical studies and product characterization.

[Slide.]

As Dr. Rao said, I am Richard McFarland, and I am in the Office of Cell Tissue and Gene Therapy in CBER.

[Slide.]

What I am going to do, I have been charged with providing a perspective, FDA perspective to the preclinical and manufacturing issues of cell therapies for cardiac diseases.

First, I am going to describe the general

framework in which the FDA conducts our science-based assessment of safety of novel cellular therapies prior to allowing clinical trials to proceed.

Second, I am going to describe the goals of preclinical testing, safety testing in general, and then a little specific about how that applies to cellular therapies for cardiac disease, and finally introduce the speakers for the rest of the afternoon.

[Slide.]

FDA review is product-based, and it parallels prudent product development. This is in contra-distinction to the NIH grant process, which is more based on diseases and organ systems, which is illustrated just in the administrative structure of the two agencies, NIH being primarily divided by institutes, and FDA being primarily divided by products that we regulate.

That means that our FDA review is dependent on the characteristics of a specific product, and the preclinical studies are designed to support the use of specific products, and the clinical trial design that we review is designed to be supported by manufacturing and preclinical data.

That product-based review is framed by regulations. I think I am the one designated to get ready for regulations from the FDA.

[Slide.]

These are selections from the Investigational New Drug regulations. I want to highlight a few things, that being that regulations stipulate there is adequate information about pharmacological and toxicological studies, that the sponsor has concluded that it is reasonably safe, and that the kind, duration, and scope of those required tests vary with the nature of the proposed clinical investigations.

[Slide.]

A little further down in the regs, for each toxicology study that is intended primarily to support the safety of the proposed clinical investigation, a full tabulation of data suitable for detailed review should be submitted.

This is kind of critical to the way that we do the review in that we need to get enough data to do a detailed review.

[Slide.]

Let's back up for a minute and go to preclinical evaluation in general. What are the

goals of preclinical evaluation with a perspective of supporting trials?

One is to provide a rationale for the proposed therapy, discern mechanism of action, identify "at risk" patient populations, recommend safe starting doses and escalation schemes for humans, do a preliminary risk/benefit assessment, and to identify parameters for potential clinical monitoring.

[Slide.]

I will talk a little more specifically about use of preclinical models for cellular therapies. Preclinical models are used to provide the scientific rationale with the cellular product intended for clinical use, to understand cell function, trafficking, and differentiation as all these factors impinge on safety, as well as modeling of routes of administration.

[Slide.]

If we had an ideal animal model for cardiac cell therapies, it would have a similar pathophysiology to humans that would improve the predictability of human risk from the models, similar anatomy to humans, which would allow us to use various routes of delivery including catheters

of various types, with actually the clinical catheter, it would also allow us to do extensive dose exploration of cells, and it would be immune-tolerant to human cells, so you could actually use a human cellular product.

[Slide.]

Well, such an animal model doesn't really exist, so we often use syngeneic animal models of cardiac diseases because they can provide us useful data for assessment of safety.

That would be cells from analogous cell source processed in a similar way from animals, autologous cells in the animal species or syngeneic species, gives rise to potential processing, formulation, and storage differences, and limited product characterization both preclinically and clinically leads to some uncertainty in addition to the uncertainty that is inherent in the modes themselves.

[Slide.]

Add to that, we have added complexity due to innovative delivery systems, many of which have not been tested for delivery of cells. Common delivery systems that we have seen, intraoperative transepicardial injection usually during CABG,

catheter-mediated transendocardial injection, and catheter-mediated via cardiac vein, all of which I think were discussed this morning.

[Slide.]

I only want to present a short scaffold of the animal models that have been published so far, and our speakers this afternoon will much more extensively discuss the data.

There have been data in small animal models, often cryoinjury, occasionally coronary artery ligation to give an ischemic area damaged myocardium. One of the advantages of the small animal systems is that you have them available, at least in mouse and rat, to use a human cellular product to give you an idea of function and safety of those cells. Primarily, it has been in mouse, rat, and rabbit.

[Slide.]

Large animal models, typically, dog, sheep, and pig, and we have seen some of those data this morning, as well, an ameroid constrictor used to generate an ischemic area has been a popular model of disease. They are amenable to catheter administration and more amenable to clinical monitoring modalities, however, you are stuck with

using syngeneic cells in this situation.

[Slide.]

The regulations say that the data should be adequate and extensive. What are the potential sources of data to support initiation of clinical trials?

Well, the gold standard would really be preclinical studies specifically designed to support a specific trial with a specific cell.

We also have data from other potential sources: existing animal studies that were designed to answer other questions, in-vitro studies, clinical trials using the "same" product.

[Slide.]

However, we use data that are published and unpublished. Using published data either from animal studies or human studies as sole support for initiation of clinical trials raises some questions, some complexities, because often these studies are not designed to answer a toxicologic question, and therefore, adequate toxicology endpoints may not have been incorporated into the design.

If they were incorporated into the design, but not in the publication, we need access to those

data. Published reports may provide sufficient information for independent review.

[Slide.]

There are some limitations in using published studies. Protocols in the studies need to be sufficiently detailed. We need to be able to do our independent review as per our regulations. We need to see specifics of the route of administration.

We need to see catheter specifics, such as identity of the catheter, flow rate, pressures, effects of catheters on cells, location of injection in relation to the ischemic area, and protocols, either animal studies or human protocols, we need the control details of the "routine" monitoring and analytical plans.

[Slide.]

The data must be presented in sufficient detail.

In-process and lot-release data from manufacturing need to be presented in sufficient detail for us to know exactly what the product is, and complete study reports for both animal and clinical studies.

[Slide.]

Cellular products used in published reports may not be comparable to the intended clinical product. Often, in published reports, there is insufficient data to allow us to make a comparability assessment, and that is either because the data don't exist or due to editorial constraints of the publication.

[Slide.]

So, given the limitations of the framework or the window at which FDA is required to look at these, and the detail that we are required to look for prudent product development raises some regulatory challenges.

These are rather recurrent regulatory challenges. This is does the submission contain sufficient information to assess risk to the subjects in the proposed trial. It is a question that we ask at the end of our review. Were adequate preclinical studies performed? If they were performed, were the data submitted in sufficient detail to conduct an independent review?

If sufficient data are present, then we get to the question, are the risk to human subjects reasonable and significant?

[Slide.]

That gives you an idea of the framework with which we need to look through to the window of science and what we are obligated to do as we make an assessment.

There is discretion, there is ability to be flexible within the regulations, and what we are asking the committee to do over these two days is to give us an idea of what the state of the science is. It will be reflected in the questions that we will be discussing tomorrow afternoon.

What is the state of the science? What is a reasonable amount of data for us to be looking at?

This afternoon, we are going to have two speakers that are focusing primarily on cells. Doris Taylor from the University of Minnesota and Silviu Itescu from Columbia.

After the break, followed by Dr. Nick Jensen from the Center for Devices and Radiologic Health at the FDA, who will focus on delivery devices and some of the issues that are related to development and testing of delivery devices, which are an integral part of our preclinical development.

Then, Robert Lederman from the NIH will be

discussing some of his experiences from being in the trenches of doing studies with devices, cardiac diseases.

DR. RAO: Thank you.

We will now have Dr. Taylor.

Guest Presentations

Myoblasts: The First Generation Cells for Cardiac Repair: What Have We Learned

DR. TAYLOR: Thank you. I have to confess that if I had seen those previous slides before I had signed my talk, it would be a completely different talk, so bear with me.

I am going to be talking about some of our data and some of the data from the rest of the field, but I think what I really want to focus on is myoblasts for cardiovascular repair and what lessons we can learn from the cells that have been used for the longest period of time preclinically, and I think another way to think about this is gene therapy revisited, are we going to do it all over again.

I think the point that I want to make is that there are a lot of lessons that we can learn from the gene therapy field as we are going forward with cell therapy, and I think it is important to

take those lessons away from this. I will be glad to talk about that in more detail if people have questions later.

I like to start with this because this is what I tell the people in my lab, and I think it is true - make everything as simple as it is, and no simpler, from Einstein.

Philippe will recognize this. This is an image I borrowed from his Lancet manuscript in 2001, showing the first patient into whom myoblasts were actually delivered clinically. I actually use it to illustrate what I think is the salient point here, which is that most of us are dealing with animals as well as in patients, infarcted myocardium, where a process of events has occurred that starts with inflammation, moves to cardiocyte apoptosis, a remodeling and compensation process that you heard about in extreme detail this morning, scar expansion, decompensation, and progression to failure.

The truth of the matter is we are trying to intervene in this with either cells, genes, or devices, but we don't know where in this cascade we are actually intervening, nor do we know where we should be intervening.

I think most of us who got into this field envisioned it, first, as a field where we would intervene early after an acute myocardial infarction to try to prevent the slippery slope here of remodeling, scar expansion, decompensation, and failure, but the truth of the matter is that most of the patients in whom studies have been done are patients who have already progressed to some degree of failure.

Although we initially started to begin to look at prevention and repair of not only cardiac injury, but also vascular injury, we are now really trying to understand whether or not we can move back up this scale in a reverse remodeling way, or to grow new cells, and I think those are the questions that are really out there in the field right now.

The Holy Grail in this field then is that transplanted cells cannot not only engraft, but restore blood flow and contractility to injured myocardium, and all of you know, because you have seen some of the data from Philippe and are fairly well versed in this field or you wouldn't be here, that there is more than 15 years of preclinical data in rabbit and dog, there is at least 1 to 5

years of preclinical data in pig, rat, mouse, sheep, and now hamster, all of which showed in our preclinical models that transplanting autologous skeletal muscle derived cells was safe, effective, and feasible, and therefore, Phase I, surgical and intervascular studies were initiated worldwide.

I think the future that we will probably ultimately all try to examine is what is ultimately the best cell for cardiac repair. I have been asked to focus on myoblasts, so I am going to do that although a little bit later this afternoon, I am going to talk necessarily a little bit about comparisons among cell types because I think it really begins to ask questions about mechanism that we have to address as we are going forward.

Obviously, the best cell may be some autologous bone marrow-derived product. It may be a cell plus or minus a therapeutic gene to either promote angiogenesis or some other signaling cascade that maybe promote cell survival. Ultimately, it may be embryonic stem cells although I would submit at least in this country, we are a number of years away from those cells, not only because we don't understand how to regulate their differentiation, but because we also don't

understand how to make them stop dividing appropriately.

Then, obviously, you can't be from the University of Minnesota without talking about adult-derived stem cells.

So, what are the questions in the field? I am just going to put my opinions out there. I think just keep these in mind as a background as we go forward.

Is there a best cell? I don't think there is a best cell. I think it really depends on the patients, the time after injury, the dose and a number of other factors.

Is there just a better way to get it there? One of the questions that we keep coming back to over and over and over is whether or not cell therapy is just going to ultimately be another local drug delivery problem, and whether or not we are really going to be able to get the cells to where we need them or whether they have the capacity to actually migrate there or home there, and I think that is going to be an important phenomenon to begin to explore.

The other side of that is do the cells go where we want them, or do the cells go where we

want them and somewhere else where we don't want them, and I think we will come back to that.

Should we just use growth factors and forget cells? I am not going to focus on that.

Is there a future for biologic devices, and is the real question dose, timing, and choosing the right patients for the right cell? I would submit that it probably is.

So, where are we in this field? Well, this is a table that I copied from a review by Loren Field, and the table goes on for slides, just to show that in terms of preclinical myoblast transplantation, there is a huge amount of data out there, and what the data really begin to show is that there are a lot of different cells that have been used, there are a lot of different species that have been used from mouse, rat, rabbit, pig, dog, and sheep, that these cells have been injected either into normal heart, cryo-injured heart, hearts where vessels have been occluded, and that surprisingly, most people didn't actually measure improvements in function or in angiogenesis or in survival.

I think that is important to consider as we really try to pull together the summary of data

from myoblast cell therapy.

So, how do we really do this? Well, for myoblasts, you basically take a chunk of muscle and you grow cells in vitro, and you end up with cells in a dish, and then you deliver these cells to the injured myocardium and you measure the effect.

So, you inject them, you deliver them, and you measure the effect. So, I am going to go through the different parameters here, talking primarily about myoblasts and what exists in each of these areas.

So, the cells are typically referred to as myoblasts, but the bottom line is these are muscle-derived cells that contain, not only myoblasts, but also often more fibroblasts than myoblasts, a number of cells called SP or site population cells, and then a whole lot of other cells that we don't necessarily know how to characterize yet.

So, this is a very heterogeneous cell population, and you do take these cells and you grow them in a dish for several weeks, and what that means is that all of us are exposing these cells to serum-containing medium, and that what Arnold Kaplan learned years ago from mesenchymal

cells is that when you are using serum, what is in that serum, FDA regulations notwithstanding, matters, and that the lot number of serum is going to give you a different outcome in terms of the numbers of these cells and what their phenotype is when you are done at the end of the day.

In terms of injury models where myoblasts have been delivered, the primary injury models are either acute myocardial infarction, acute being anywhere from a week to one month, and primarily that has been a cryoinjury model or coronary artery ligation.

The question that really arises--and then those have varied dramatically in size, the timing after creation of this infarction to the delivery of cells matters, and inflammation, so are these the same as a clinically relevant injury? Well, the size differs, the timing differs, and the inflammation differs with regard to what is actually seen in patients.

In terms of cells, the myoblasts, primarily how they have been delivered, more than 90 percent of the cells preclinically and clinically have been delivered by surgical approach.

Some preclinical data exists in terms of delivering cells via catheter in a pig, but again there are open questions about dose, about where those cells are delivered, about when those cells are delivered, and there is virtually no data out there about the vehicle in which the cells are given.

Typically, people either say they inject the cells in saline or they inject them in the cell growth medium minus the serum.

As I said up here, lot number matters, so the vehicle differs dramatically.

The next issue that you have to deal with, if you have got cells and you grow them and you inject them into an animal, is how you measure the outcome, and the question of safety is obviously an open one, and none of us really have addressed the safety question in preclinical models.

We didn't really know that there was going to be a safety issue. I was talking at lunch about the fact that, you know, we had a number of animals drop dead over the course of our studies when we were doing these experiments early on, but we assumed it was because we were doing open-chest surgeries on these animals to create the infarct,

to deliver the cells, to measure cardiac function, not that it could have anything to do with a potential electrical effect of the cells in vivo.

So, we had to then go back and evaluate whether or not safety was even at all compromised or relevant in these animal models, so none of us are really measuring safety. There have been two studies reported, one in a pig model of holter monitoring animals, and we just presented some data at ACC in rabbit, monitoring electrical effects of myoblasts, and I will show some of those data in a minute.

The other issue in terms of measuring outcome is function. Typically, in rodents, isolated heart preps have been used to measure function although in some cases, sonomicrometry is used. I have actually put the methods here in order of I believe their ability to actually give you useful information.

I think the isolated heart prep is the least useful because it is subject to a lot of variability, it is subject to baseline drift, it is subject to flow and rate factors, and it is subject to ischemia in vitro.

Echocardiography is obviously used

clinically, as well as experimentally, but it varies dramatically with the operator and the orientation of the probes, so you can make echo tell you just about anything you want.

If you really want to measure work done in a region of the heart, I submit that you have to go back and do sonomicrometry in that area and use crystals to actually measure the ability of that region of the myocardium to move in an electrically and mechanically meaningful way.

Then, more recently, cine MRI has really come to the fore in terms of our ability to make measurements in not only people, but animals, as well.

But measuring function is pretty useless unless it correlates with histology, and we begin to ask questions about angiogenesis and myogenesis, and we don't always correlate histology with outcome, and, in fact, one of the issues that comes up over and over is clinically, as well as experimentally, there is a disconnect between the number of cells we can find in the heart and the functional improvement we see, which begins to ask questions about mechanism.

So, in terms of myoblasts, what are these

cells? As I said, there are myoblasts or fibroblasts and there are SP cells. I propose that the mechanism of repair of these cells depends on the number of cell types that you have present and the percentage of each.

I believe myoblasts know to become muscle, and they are capable of myogenesis. I believe the fibroblasts not only secrete an angiogenic factor FGF, but also act as a growth factor and a mitogen for myoblasts.

That has been known for years. Judy Swain's data, they actually keep myoblasts alive and keep myoblasts proliferating over a fairly extended period of time, FGF does.

SP cells, I believe are more likely to be angiogenic and also possibly to fuse with other cells in the myocardium. That is based on data primarily from our group showing that the more immature a cell is, the more likely it is to fuse.

So, in terms of our animal models, I think the question we have to ask is what do the patients look like first, and in the myoblast trials, those patients have been post infarction, usually greater than one month, up to many years. The average in one of the studies was 6.7 years.

Most of those patients are in need of revascularization, they have an ejection fraction of less than 35 percent, and they have heart failure.

If you look at the European experience, Philippe has already talked about some of this, and I am not going to go over these in detail. This is a slide that was given to me by Peter Smits from Rotterdam.

You look at Spain and Poland, and obviously the French study, and then the U.S. studies, both Arizona heart and Bioheart Mount Sinai study, and then the Bioheart study in Europe, and I should say for the sake of disclosure that I have had a relationship with Bioheart, so take these data with a grain of salt, that all of these patients are heart failure patients.

The average in this study is 6.7 years post-infarction, so these are patients who are already pretty sick, and who have significant electrical abnormalities already.

How do the preclinical patients compare? Well, for myoblast studies, these animals all have acute cardiac injury, either, as I said, cryoinjury or coronary artery ligation. Very few, if any,

studies deal with occlusion reperfusion, which is what happens clinically.

You can open up virtually any artery in the heart now, but nobody is doing preclinical studies where we do ischemia reperfusion. We are just now moving in that direction.

I think the reason initially for at least us, and I believe for other people, was that we wanted to kill everything that was there, so that anything we found was due to something we put in, and so we started with cryoinjury where we applied a minus 70 degrees C probe to the surface of the heart to wipe out that region of the heart, but it raises questions about the inflammatory process, which we are coming to understand is critical in terms of potential homing of cells and the potential mechanism.

Most of the preclinical studies, the cells are delivered two to three weeks post-injury, and there is a one- to three-month follow-up. Clinically, this isn't exactly relevant.

I just told you that every clinical study is at least one month post-injury and sometimes six to seven years post-injury, so we are not looking at the same milieu into which we put these cells.

In term of heart failure models, there are a few models out there. Dan Burkoff's group has recently published a study in dog where they used microspheres to actually create a heart failure model, and have gotten data with myoblasts that actually look very similar to some of the data gathered in earlier models.

None of the studies to date have really used any animals with LVAD support, and yet there are clinical trials beginning to move forward in that, and actually, one has already been completed in that context. So, we would expect that with a completely unloaded heart, we might have very different phenomena.

In terms of cardiomyopathy, there is a hamster model and mouse genetic models that have begun to be used for myoblast transplantation, and Magdia Koob's group has actually published reasonable data in terms of a rat model of adriamycin toxicity. So, we are beginning to get a plethora of models in which we can look at myoblast transplantation.

I put up here actually the little bit of physiology that I could pull together about some of the different animal models just to make a few

points.

One is that when you start looking at these different injury models in mouse, rat, rabbit, dog, pig, sheep, and humans, that there really are significant differences in rodents and the larger animals.

Mouse and rat, you know, the hearts are pretty darn small. Their heart rate consequently is very high. These animals have very few collaterals, and they express completely different contractile proteins than are expressed in the majority of the heart.

Moreover, the action potential in the electrical capacity of the mice and rat is very different. There is no plateau phase in the action potential in mice, and the action potential duration is on the order of 10 milliseconds. In humans, it is on the order of 250 milliseconds.

Rabbit is the first animal model where you begin to get numbers and conditions that resemble humans, and that is why we chose rabbit early on, and I would submit that in terms of feasibility studies, rabbit is a good entry level animal under most conditions except where you are trying to do stem cell work.

When you are trying to do stem cell work, we don't have the markers for stem cells in most of these other species that we do in rat, mouse, and humans, so that is when mice, rats, and humans, or maybe pigs, some of the human cytokines and antibodies cross to pig, but not all of them, so you can begin to do some of those studies in pig.

Nonetheless, in terms of feasibility studies, I think rabbit is a good model, and you move up from there.

So, let's look at each of these. In terms of myoblast in the mouse, you can begin to track the cells because you can use genetic models of where the cells actually express different markers that are unavailable in the animals in which you inject the cells.

You can begin to isolate and characterize stem cells including the stem cells in muscle, but you can't characterize from larger species. You can use immunocompromised mice for human cells, but you are missing an important component, which is the inflammatory component, and we are beginning to understand again relates to homing and perhaps even recruitment of cells.

What is the advantage of a rat versus a

mouse? Well, the main advantage is it is larger than a mouse, so you can do a few more things, but you can still track the cells and isolate stem cells, and you can still have an immunocompromised model.

The rabbit, the bottom line about rabbits is most people make antibodies in rabbits, not against rabbits, so it is really hard to find the tools that you need to do some of the evaluations downstream, but it is still a relatively inexpensive model with cardiac characteristics very similar to humans.

The pig, obviously, the size is good, and the geometry is good for catheter-based studies. One of the points I want to make about delivery of these cells, and I think Philippe showed it when he was talking about his clinical trials, and we have done the same thing in terms of our preclinical studies, surgical studies, is that when you inject these cells surgically, most of us have delivered the cells parallel to the surface of the heart.

We have done that for years because we really thought it was going to increase the number of cells that we could get into the myocardium. Yet, all of the catheter-based studies deliver the

cells perpendicular to the surface of the heart, and it is not completely unexpected that geometry may make a difference in terms of how these cells actually function in the myocardium.

So, I think it is important to evaluate the geometry of the cells in some of these larger animal models.

So, how do you choose an animal model for the myoblast studies? I think feasibility and costs are obviously important, whether or not you are going to do high throughput studies and need to track your cells. Rodent and hamster I think are best for those. Rabbit is best in terms of beginning to be physiologically relevant to humans in terms of heart rate and scalability. I will show some data in a minute in terms of scalability.

The large animal models are obviously much more physiologically relevant. You can get a sense of dose. You can use conventional delivery methods that you would use in humans. You can do the right functional assessments, and the heart size and geometry is very similar to a human.

So, what exists for myoblasts? Well, as I said, the route of administration has been primarily surgical or percutaneous. Intravenous

and intracoronary studies are just beginning although we published some intracoronary data, that was the first thing we published in '96.

Myoblasts are unlike stem cells or unlike bone marrow mononuclear cells. They are very much like stromal cells. They are big. When we put myoblasts in the coronary circulation, what we found is that we got profound ST elevations, and we saw transient ischemia every time we injected these cells.

So, we actually think that with large cells, that the way they are actually having an effect in the myocardium is creating essentially a microinfarct clogging the vessels and then getting out of the vessels as a result of that.

Mononuclear cells are much smaller, and I think don't have the same effect.

In terms of dose, in a mouse, typically, you give about 1,000 cells. Some people go as high as a million, but typically, 1,000 is enough to begin to see an effect.

In our hands, in rabbit, the lowest dose at which we see an effect is 3×10^7 cells. We tried 10^7, 3×10^7, 10^8, and 3×10^8, and this is the range in which we see the most effect.

In pig, it is about 3 x 10^8, and what we found, you know, pig is about 10 times bigger than a rabbit, you need about 10 times as many cells. Rabbit is about I think about 4,000 times the size of a mouse, and we found that we need many more than 4,000 more cells in a rabbit than we do in a mouse. So, I don't think you can really extrapolate from mouse, but I think you can begin to extrapolate at the size of rabbit and go up.

In terms of cell location and where myoblasts have been injected, you pick a surgical fellow who is doing the experiments, and you will get a different location of injection virtually every time, I guarantee it, and you are not going to convince them otherwise that their way isn't the right way to do it. It is completely ignored in most of the preclinical studies.

There might be mention of one injection or two injections or three injections, but in terms of the exact location, I couldn't tell you, I couldn't find in the literature where the majority of injections occur. I know in my own lab, it is not consistent from study to study.

In terms of timing, myoblasts have been injected two to four weeks post-injury. The

vehicle has been PBS cell growth medium minus serum, or it is completely ignored, there is no mention of it.

So, this is the slide again I borrowed from Philippe's work to illustrate how he did some of the early injections with a bent needle again parallel to the surface of the heart, and also that the injections are done, not just in the center of the infarct, but in the periinfarct region, as well.

Similarly, with a percutaneous approach, and this is another slide from Peter Smits, in the first patient who received cells, and in the majority of cases now with percutaneous myoblast delivery, cells are delivered in the periinfarct region, in the normal region of myocardium, and very few of the injections percentagewise actually end up in the infarcted cell, and that may have an effect on safety, and I will show some preclinical data that support that.

So, the majority of injections are surgical, and you can inject the cells and find them in the center of the infarct. What you get surgically when you inject these cells is one or two things.

On a great day when you are really lucky, you get what looks like a chunk of steak in the center of the heart. On a typical day, you see something that looks more like this, where you have patchy regions of cells distributed through the infarct rather than these large fibers that you see here, and these patchy cells distributed throughout the infarct are not necessarily talking to each other, but they are all oriented with the extracellular matrix.

You can see there are some small vessels here, here. We often see large vessels in the infarct, as well.

These are preclinical data from my group, but they don't look too dissimilar from what you see from Pagani's paper from myoblasts in an LVAD-supported human heart or, in fact, the data that Philippe showed you earlier of the myoblasts surrounded by scar in a patient 17 1/2 months after injection.

I am not going to talk about stromal cells because that is not my job today, but what I am going to begin to talk about is delivery. Assessing delivery requires that we be able to track the cells. In vivo, we have chosen SPECT or

MRI most recently, although I think PET is going to be a good method, as well.

That has to correlate in vitro with histology and appropriate markers. If I don't make any other point today, take home the fact that using desmin, using phospholamban, using GATA-4, using all of these markers that people claim are cardiac markers, are not cardiac-specific markers. You find these markers in other muscle cells, you find these markers in undifferentiated progenitor cells.

If you look in C2C12 skeletal muscle cells, you can see phospholamban, you can see connexin 43, you can see in some cases, in progenitor cells, you see GATA-4. You have got to use markers that are specific for cardiocytes if you are going to call these cells cardiac cells, and the only markers that I know of right now, that I believe are specific for cardiocytes, are channel markers that are actually not expressed in skeletal muscle.

As skeletal muscle matures, it expresses many cardiac-specific proteins, and as skeletal muscle matures, it expresses cardiac markers, as cardiac muscle matures, it expresses skeletal

markers. So, we don't know where in that process we are, so we can't really use those markers.

This is an image showing that we can begin to visualize these cells in the heart. These are indium-111 labeled myoblasts present in a short axis view by SPECT imaging of a rabbit heart showing that we can actually co-deliver tetrofosmin, see perfusion, see the dropoff in perfusion here with the infarct, and then see the indium-labeled cells in the center of the image.

So, we are beginning to believe that we can actually track cells over time now. This is also a cine MRI of a rabbit heart, and these are data that were all gathered at Duke. This is a rabbit heart, so at the level of rabbit, although we can now do the same thing in a mouse, we can iron label our cells in a way that we believe doesn't affect proliferation or viability of the cells, and begin to see them in the center of the infarct region, the infarct region here being contrast-enhanced in white.

So, we can start now to label these cells. We have followed these cells out to four months in this way and can still find them. When we kill the cells and then inject them, the iron label goes

away over about two to three days, so we are fairly convinced that the iron is present in viable cells.

So, the other issues in terms of measuring outcome, I think I have already made this point, safety is an open question, and I think what I take away from the field so far is if you don't look, you won't find it, and that we didn't look, and now I think it is important that we begin to do holter monitoring and other electrically relevant studies, and those are going to require large animals, pig, rabbit. You can't do those in mouse and rat, not at 300 to 600 beats per minute. You are really not going to be able to see a VF or a VT.

In terms of function, I think if the goal here with myoblasts is really to find an ischemia-resistant cell that is electrically compatible with a healthy heart, we have got to also look at electrical activity of these cells over time.

This is again a slide that Peter Smits provided showing clinical data and the number of VPCs per visit in some of the early patients who had cells delivered, and I modified the slide a bit to show times at which patients have actually died after cell delivery.

What you begin to see is that there is a window of time from about a week to a month where there seems to be an increased incidence of electrical abnormalities. When we have done animal studies now, we see that same sort of window from about 3 days to about 3 1/2 weeks, and then it drops off and we don't see the incidence after that period of time.

So, the safety may depend on the cell dose. We have found that if we just look at PVCs in our animal models, that as we increase dose, we increase the number of PVCs, and it may also depend on location.

We have found that if we inject cells in the center of the infarct and we measure PVCs, and this is actually 10^8 cells, 10^7 is not functionally relevant, 10^8 is, 10^9 is, we found that if we inject cells in the center of the infarct, we see PVCs and no monomorphic VT.

If we begin to inject cells in the border zone, we not only increase the number of PVCs we see, but we start seeing runs of ventricular tachycardia. If we inject cells in both the center and the periphery, we see essentially the same thing, and more up-to-data were just presented at

the ACC from my lab.

What is interesting is we began to take these cells back out of heart, what we found is that their action potential duration changed, that initially, the cells had an action potential duration of on the order of 20 seconds, and over time it increased to something on the order of 120 milliseconds, but it is still not compatible with the surrounding heart.

We have also done some modeling data. What we believe is that if you have these cells coupled to each other, that is a good thing in the center of the infarct, but that you don't want them coupled to the remainder of the heart until they are electrically compatible with the remainder of the heart, and yet clinically, very little attention has been paid to location.

Again, it is an issue that we didn't know we were going to have to address, and now we have got to go back and address. In fact, some of the locations of injections could explain why there have been ventricular tachycardia in some of these patients.

The only possibility is, you know, over that window of time, we don't know if these cells

are integrating, dying, or changing their phenotype, we have no idea, and I think we really have to begin to elucidate that.

So, in a standardized model where we know how myoblasts function, we have now got to look at location, dose, and route of administration.

I think I have already said this, so I am not going to really belittle, spend time on function especially other than to say we have begun to collect a lot of data now with a lot of different cell types and a lot of different growth factors, and what we have begun to realize is that virtually anything we put into the heart, cells, myoblasts, fibroblasts, bone marrow stromal cells, bone marrow mononuclear cells, growth factors including VEGF and other growth factors, improve the mechanical properties of the scar, and change diastolic performance.

They do that first, before they have any effect on systolic performance, usually by several weeks. What we figure is that having something alive in the scar is better than having just this dense collagen matrix, and it really doesn't seem to matter what you have alive in the scar, if is vessels, if it is muscle, if it is whatever, you

improve compliance.

But we don't see the corresponding improvement in systolic performance, at least not with fibroblasts in our hand, but we do with myoblasts, we don't with VEGF, but we have now with bone marrow stromal cells and bone marrow mononuclear cells.

These are some data that just came out in Circulation showing that, that if we use crystals to measure regional stroke work in our sham-treated animals, regional function gets worse, but in our myoblast-treated animals, function goes from pretty bad to better, and in our bone marrow stromal cell animals, the same thing is true.

I think that really raises a question about mechanism, but the positive outcome in our hands at least is dose dependent, 10^7 no effect, 10^8 positive effect, sham continues to get worse.

What is interesting is this is not just improvement versus cell number. This is log of injected cells, but this is the percentage of animals that actually improve.

So, what we found is that the percentage of animals increases with cell dose, as well.

So, will myoblast transfer work in

patients? Philippe already has told us that it will, and we have begun to believe that, in fact, that there are different mechanisms of action for these cells.

I think I will just very quickly go through the last couple slides. We believe that myoblasts improve both regional and global function in the heart based on our preclinical data.

If we use cine MRI and actually measure thickness in the wall of the myocardium over time and global wall thickening, so areas where cells were not injected, we use contrast to define where the infarct is, and this is area that has no contrast in it, so the remainder of the heart actually gets better, wall thickening improves in the cell treated, but not in the control vehicle injected animals.

Regional wall thickening where we actually inject the cells gets better to a greater degree, so we only measured this where there was a transmural infarct. We didn't measure it in regions at the periphery of the infarct where there can be tethering going on. So, we use contrast and only measured it in the region.

Diastolic volume decreased, heart weight

decreased, so global indices of failure also improved.

Every cell we and virtually anyone has injected seems to work, which either means the myocardium is easier to repair than we thought or we don't understand what is happening and we aren't looking at the data correctly.

I would like to believe it is this, and I am actually going to posit this in a little bit, but I have a bad feeling. I would also submit that they work despite the fact that we don't know how to get the cells there in large numbers, and we can't always find them histologically, and that we don't really know what to look for.

These cells may be promoting angiogenesis, myogenesis, they may just be unloading the heart, changing wall stress. They may be secreting paracrine factors that recruit other endogenous stem cells to the area of injury, either cells from the heart, if that's your fancy, of cells from the bone marrow, or maybe a combination thereof, or maybe they work because we are lacking long-term follow-up in both animals and patients, and we haven't asked the right questions.

So, I will just stop by saying we have