DR. JOSEPH WU: So, good morning, everyone. It's a pleasure to be here. I want to thank Gordana and Ralph for inviting me here.

I'm going to talk about pluripotent stem cell biology and how we link regenerative medicine with imaging. To start out, this is basically a classic background slide talking about iPS cells. As you know, iPS cells have really become a paradigm shift in stem cell biology. This was mainly started by Shinya Yamanaka in 2007, publishing a paper showing that you can take human skin cells and reprogram them with Oct4, Sox2, Klf4 and cMyc, and make them into IPS cells. These cells can then be used to model disease on a dish and also for drug screenings and potentially for cell therapy. As follow-up, a similar kind of approach was published by Jamie Thompson using a slightly different cocktail of factors.

I'm a cardiologist by training, and so most of the work that I'll show you is what our effort has been to push ES cells and iPS cells for cardiac drug screening, for cell therapy and also for understanding disease modeling. For each one of these areas, I'll

show you examples of how we use imaging to figure out what’s going on.

This is a slide showing that heart disease is the number one cause of morbidity and mortality. For men and women, this is actually the number one cause of death compared to cancer and other kinds of diseases here.

In terms of the iPS cells and ES cells, there are three main applications. One is disease modeling. The other one is drug screening, and then the third one is cell therapy. For disease modeling, I’ll give you an example of what we’ve been doing. We go after large diseases; for example, this is a case of a large family with familial dilated cardiomyopathy. It’s one of the most common causes of heart transplantation in infants and adults.

Back in the 1980s, about 5 percent of patients with dilated cardiomyopathy were initially thought to be idiopathic, but were later diagnosed to have familial dilated cardiomyopathy. Idiopathic is just a fancy term that doctors use when they actually don’t know

what’s going on. But by 2005, up to about 35 percent of these patients with idiopathic has been confirmed to have familial dilated cardiomyopathy, and this is due to advances in next-generation sequencing, as more and more genes are discovered.

This is a family that I saw in the clinic. You can clearly see at the top left corner an echocardiogram of a boy who has a poor contractile function compared to his brother, which has a pretty normal contractile function. The imaging is done with echocardiography, a very common imaging modality that we use in the clinic.

We asked the whole family to show up and basically did echocardiograms with the whole-family screening to figure out. In the beginning, it was thought to be idiopathic, but now we know it runs in the same family, the diagnosis has then been switched to familial. And then the question is, if it’s familial dilated cardiomyopathy, what exactly is the gene that’s causing the mutation?

So, we did whole exome DNA sequencing and were able to confirm that the mutation exists in the troponin T mutation with the arginine to tryptophan switch, and it was confirmed by genomic PCR DNA sequencing.

This figure shows the large family, and this is the boy who had the disease. His brother has no disease. Father has the disease, uncle has the disease, and grandma has the disease.

I asked the whole family to show up, get the skin biopsy, hit it with the four reprogramming genes and made oPS cells out of all of them. These IPS cells are pluripotent, and they can become teratomas in animals. We then differentiate these cells to cardiomyocytes. This is an example of a beating embryoid body on top of a multi-electrode array. These are 64 channels right here.

When the embryoid body beats, you can actually record something similar to EKG right here. So, this is an action potential pattern of a control patient, compared to the pattern of somebody with dilated cardiomyopathy.

Once you have these beating embryoid bodies, you can then challenge them with different kind[s] of drugs that you want to simulate. In this case here, we gave the patient norepinephrine. For a control patient, if you give norepinephrine, the heart rate goes up and stays up. No problem at all. The norepinephrine is a catecholamine drug that we give to a patient in the Coronary Care Unit, or in the Intensive Care Unit. Basically, it's involved in the fight-or-flight response. The heart rate goes up. The blood pressure goes up. Normal people tolerate it, but for people who are sick, you can use it transiently; but if you use it over a long period of time, it's actually quite toxic to the heart.

As you can see here, for patients with dilated cardiomyopathy, the heart rate goes up, and after about two to three hours, the heart rate starts pooping out.

Now, if we expose these cells for about a week, you can clearly see that in normal patients, there's some mild disintegration of the myofibrils, but in a

dilated patient, you can clearly see the significant disintegration of the myofibrils on a single-cell level. This is actually quite drastic in terms of what we're observing.

The other thing you can do is model this for what we use clinically. In the clinic, we oftentimes treat these patients with beta blockers. This is a current clinical trial using SERCA2A, which increases the intracellular calcium to boost the cardiac contractility. This is an example using atomic force microscopy, whereby the cantilever sits on a single cardiomyocyte. As the cardiomyocyte beat[s], the cantilever moves, and then you can measure the amount of contractile force generated by each cardiomyocyte.

You can see here the control cell has this amount of force. The dilated cell has much less force. When you treat the dilated cells with adenovirus SERCA2A, you can rescue the force right here. Likewise, when you treat these cells with metoprolol, which is a beta blocker, you can significantly cut down the amount of disorganized myofibrils in these cells right here.

These are two common modalities that we use to treat patients, and this slide basically refers to the SERCA2A clinical trial. This clinical trial was started by Roger Hajjar's group. In their clinical trial, they're showing that by giving patients adenovirus SERCA2A, you can improve the New York Heart Association heart association class, the six-minute walk test, and the maximum oxygen consumption.

Just to cut a very long story short, what we've been able to show is that we generated iP cell-derived cardiomyocutes, from patients in a dilated cardiomyopathy family carrying a point mutation defined by whole exome sequencing at TNNT2. Compared to the healthy controls, the disease cells exhibited the altered regulation of calcium, decreased contractility, and abnormal distribution of the sarcomeric alpha actinin.

And if you treat them with metoprolol, or a genetic overexpression of SERCA2A, you can improve the function of the dilated iP cell–derived cardiomyocyte, recapitulating the results from large beta blocker trials and the recent Cupid trial.

We're doing the same thing with other disease phenotypes, such as hypertrophic cardiomyopathy, which is the most common cause of sudden cardiac death in young athletes. And because of time, I won't go into this topic.

To shift gears a little bit, the second phase that I'll show you are examples of how we're trying to use these ES cells and IPS cells for drug screening. As you know, there's a lot of healthcare investment being pumped into by the pharmaceutical companies for coming up with new drugs. At the same time, there's a lot of revenue involved, which is about $500 billion estimated, combined for the top 19 pharmaceutical companies. Pfizer is number one. Novartis is number two, and Merck is number three. The annual R&D is about $70 billion for these pharmaceutical companies.

The FDA right now requires a mandatory preclinical drug testing for cardiac toxicity, and this is mainly due to some of the drugs that have been withdrawn from the market.

When I was a housestaff a while back, I used to give this medication, cisapride, which is a medication we would give to any diabetic who have gastroparesis, and to improve their gut motility. It turns out in 2000, this drug was withdrawn from the market – it was actually a $1 billion market drug – because of prolonged QT and increased cardiac death in some of the patients. I think, in retrospect, I'd probably given out about 400 or 500 prescriptions of cisapride at that time.

So what is the limitation of the current cardiac toxicity screening assay? If you look at how pharmaceuticals screen for drugs, they use CHO cells or HEK cells transfected with the Herg channel. The CHO cells and HEK cells are actually not human cells; they're basically hamster ovarian cells and transformed embryonic kidney cells. Therefore, they're not – quote, unquote – "beating" cardiac cells.

Because of the lack of the complex channel interactions in these transfected cells, I think is part of the reason why we failed to detect the actual

QT prolongation effects, and that's some of the causes for the false negatives as well as the false positives.

The other reason is that, if you look at the action potential here at the cardiomyocyte, there are four phases. Phase 4, 0, 1, 2, 3 right here. The HERG channel only accounts for phase 2 and 3 right here. It does not really account for the calcium channel. It does not really account for pacemaker currents, sodium-calcium exchange and sodium potassium ATPase right here.

What we've been trying to do is, again, using IPS cells to show that the iP cell-derived cardiomyocite can be used as a substitute for drug screening. We take normal iPS cells and show that they're very similar to control ES cell-derived cardiomyocytes and screen them for common drugs, including cisapride, which has been withdrawn from the market; nicorandil, which is an anti-angina medication; verapamil, which is a calcium channel blocker used for hypertension; and nifedipine, another type of calcium channel blocker used for anti-hypertensive.

The goal, then, is to create this biorepository of about a thousand cardiac iP cell lines for drug screening over the next five to ten years. So that you know, before 2020, we do clinical trials on patients. Post 2020, instead of doing directly on the patients, the pharmaceutical company will come up with a top 10 list of compounds, test them on animals and then screen them on these iPS lines. At the end of the day, we could tell the pharmaceutical company that, “Hey, your drug screen fine on men and women,” “Your drug screen fine on young kids and elderly,” “Your drug screen fine on Asians, Caucasians, Hispanics. However, your drug causes prolonged QT in patients with dilated cardiomyopathy,” or, “Your drug has a negative inotropic effect on patients with hypertrophic cardiomyopathy,” and things like that. So, this is where we’re going.

Now, as I showed you earlier, we do quite a bit of whole exome sequencing on these patients, so our goal is to not only make the lines, but also to genotype them – and also to do phenotype. And what I mean by “phenotype,” means a lot of imaging on these patients.

For a lot of the patients that we do at Stanford, we actually do echocardiogram, carotid ultrasound, abdominal ultrasound, and also measure the endothelial function on these patients right here to assess their vascular tone. This is an example of where we're coming from, combines iPS cells with genotype, but also with clinical imaging on these patients right here.

For the rest of the talk, I want to move to cell therapy and discuss what we've been thinking about and what are the major hurdles for cell therapy. When you think about what we want to do, which is ES cell or iPS cell therapy, there are significant hurdles that need to be overcome and how imaging can be used to address them.

Let's take the example of iPS cell therapy. We need to first figure out exactly what kind of cell type that we need to use. What kind of reprogramming strategies. How do we differentiate them to cardiomyocytes? How do you make sure that there is no tumor? And how do you immunosuppress these patients, especially if we're thinking about allogeneic therapy?

How do you demonstrate in both preclinical mouse model and also a large-animal model, which is oftentimes required by the FDA? How do you show safety and efficacy? And how do you demonstrate that there's a commercialization interest? I'll quickly go over some of these.

At Stanford, we have an interest in using fat cells because, as my collaborator Mike Longaker says, it's basically "liquid gold." That's what he likes to say, because all of us have this "liquid gold," and we can easily go into the patient and isolate the fat and basically start the reprogramming process. Twenty-four hours after we get rid of the fat, isolate the adipose stromal cells, we can start the reprogrammign. The reprogram efficiency is very, very high compared to the skin cells, and it's also twice faster compared to the fibroblasts. And you can also derive them feeder-free without any contaminating feeder layers. This is something that is quite important for commercialization of these cells.

The other technique is then to reprogram them using a non-viral, non-integrating strategy. Instead of using

a lentivirus or retrovirus, we have come up with this minicircle vector that allows you to reprogram these cells. The technique is still very, very inefficient compared to some of the methods out there. However, this technique provides you with non-integrated iPS cells, and we're trying to optimize this technique as well at this point. But the bottom line is that, instead of using a regular plasmid – it's basically a regular plasma inserted with two intramolecular recombination sites here. You can activate it with arabinose, and then it undergoes intramolecular recombination to pop off the reprogramming gene and get rid of the bacteria backbone. Because the size of the plasmid is smaller, the transfection efficiency is much higher; and, therefore, it gives you a higher yield compared to typical plasma.

I think the third issue is that once we get the iPS cells that are non-integrating, we need to differentiate these cells to cardiac cells. There are several techniques out there. But I think the process of differentiating these iPS cells to cardiac cells is no longer a major issue. This is an example of a dish

full of beating cardiomyocites, and this technique will get better and better over time.

I think the major issue, then, comes down to potential tumorgenicity and immunnogenicity. This is an example of a patient who had fetal neural stem cells injected into the brain because he had the ataxia telangiectasia, which is a balance disorder. About four years later, he stated developing more problems, and the physicians scanned the head as well as the spinal cord, and it showed that there are actually tumors on the brain as well as in the spinal cord that came from the fetal neural stem cells injected. Obviously, one of this kind of occurrence in ES or iPS cells is probably going to shut down the whole field.

What we've been doing is trying to figure out ways to assess this. One way to assess this is to use a simple, old HSV-TK reporter gene and suicide gene approach. And in this case, you have HSV-TK, you can image it by using PET reporter probe F18-FHBG. If you see teratoma, you can come in with a suicide gene approach by giving high dose of ganciclovir to wipe out the teratoma right here. Compared to the control,

you give saline and there are more and more teratoma formation, and the animal eventually succumbs to it.

One of the drawbacks of reporter gene imaging is that the cells need to be genetically modified. As you know, when you use lentivirus to introduce the genes to modified cells, you're getting random integration hits. You really have no control over what happens to the cells, and this is a stickler for the FDA in terms of approving this kind of therapy.

The alternative approach is to use a non-integrating approach. In this case here, what we've done is to show that the teratoma express high levels of alpha-V Beta-3 integrins, and you can image the teratoma by using RGD peptide that binds to the alpha-V Beta-3 integrins. You can use a DOTAlinker, link it to copper 64, and you can image the teratoma *de novo* here. In this case here, the cells are not genetically modified with this imaging reporter gene. The clinical implication is in the future patients come in and get the stem cell therapy. Three months later and six months later, we come in with the PET-CT imaging. If anything lights up, we probably need to

chase after it some more, because that suggests there's high levels of alpha-V beta-3 integrins, which could be teratomas in that case.

Another strategy is basically to deplete the cells of potential teratoma-forming studies. This study by Irv Weisman's group shows what you can do is use a combination of SSEA-5 low, CD9 low, and CD90 low markers to significantly deplete the number of teratoma-forming cells here. This didn't completely get rid of teratoma-forming cells, but it does cut down the incidence signficantly. For example, if you inject undifferentiated cells into the animal, probably 10 out of 10 will form a teratoma. On the other hand, if you undergo this kind of depletion process, probably only 1 to 2 out of 10 will get the teratomas.

Another issue that we need to figure out is to address the immunogenicity process. For ES cells, obviously it's going to be allogeneic therapy. For iPS cells, it could be autologous therapies in humans, although because of the commercialization issues, it may end out to be allogeneic therapy for iPS cells as well.

We've been working on various protocols for inducing immunotolerance for these animals, and I think in this case here, as you can see by the imaging, the common immunosuppressive drugs that we use for a xenogeneic transplant protocol don't do much. You can see that by day 7, most of these cells are dead right here. Ideally, we inject undifferentiated cells and we want to see a teratoma formation, meaning that cells survive, and they form a tumor in this readout here. Combination tacrolimus and sirolimus actually don't do much right here. This was quite disappointing for us back in 2008. So we went back to the lab and talked to a whole bunch of immunologists and basically come up with a second version of the protocol, which is using a co-stimulatory blockers. It's a combination of anti-LFA1, anti-CD40 ligand, and CTL-4IG. This regimen prevents secondary activation of the T cells and makes the T cells anergic. In this case here, if you take human iPS cells, put them into an immunocompetent mouse, everything gets rejected within 7 days. Put them into an immunodeficient mouse, it forms a teratoma. And put it into an immunocompetent mouse treated with a co-stem blocker, and this is what we're seeing with prolonged survival.

The other thing is that the FDA often asks for 2 different models; for example, one in mouse and other in some kind of large animal. This is an example of what we've been trying to do. We basically took a dog and isolate canine adipose stromal cells, derive the iPS cells, and then take these IPS cells, label it with iron particles and HSV-TK PET reporter genes, and re-inject back into the same dog. This is an example of what we would do in a clinical scenario in which a patient shows up with heart failure. Isolate the somatic cells, make iP cells, differentiate the cardiomyocytes, and re-inject back into the same patient right here.

This is actually very, very difficult to do, and we got humbled by this type of experience. It kind of tells you how difficult it is to do this type of therapy.

The other problem is the cost-effectiveness of the patient-specific therapy, and this slide shows you how difficult it would be to get all this done. As you know, the M.O. of any biotech company is to have high

return on investment. It makes sense to carefully validate a few lines so that you avoid lawsuits, and you can sell to as many patients as possible. This is a slide showing that the biotech company Geron basically went out of the ES cell business because they couldn’t make this a profitable venture.

This is our five-year plan for taking human ES cell-derived cardiomyocites to the clinical trial.

And for the last minute and-a-half, I’ll talk about what’s needed for the ideal imaging agent to track stem cells. You have to be able to image cell survival, proliferation, death and potential tumorgenicity. The imaging agent cannot be toxic to the cells. The imaging agent should be applicable for human imaging.

There are two major types. Earlier I showed you a lot of examples of genetic labeling. For physical labeling, you can use ion particles or you can use radioactive probes. This is much easier, although the information you get is less because of the dissipation

of the radioactivity. For ion particles, you can’t really tell if the cells are still alive or dead.

For the reporter gene imaging, the F18-FHBG probe is actually approved by the FDA as an IND. This is work by City of Hope and Sam Gambhir at Stanford. This is a one patient pilot study. This involves patients with glioblastoma. The FDA is less stringent because these patients are going to die within six to eight months anyway. It will be much more difficult to use this technology in cardiac patients. So we’ve come up with two strategies. One strategy is to use the phiC-31 integrase, which allows site-specific integration of the reporter genes into the human cell chromosome, in collaboration with Michelle Calos.

This is an example of how we tried to do it. In this case here, we knock into the chromosome 19 at the pseudo attP site right here, and we can then image these cells with bioluminescence as well as PET reporter gene.

The second strategy is in collaboration with Fyodor Urnov at Sangamo. This is even more specific, because we can knock into any particular site using zinc finger nuclease technology. Again, at the end of the day, we did not see any significant adverse effects by ZFN integration.

This is the last slide. What we're trying to do is create a biorepository of these iPS cells lines, to do drug safety screening, to link genotype and phenotype, and to use imaging to address all these issues that I show examples of earlier.

I just want to thank the folks in my lab, as well as my collaborators and the funding support. And thank you very much.

[APPLAUSE.]

MODERATOR: Do we have any questions?

Q: [Unintelligible] – in principle, a way around the teratoma issue is to do direct conversion – [unintelligible]. Would you comment on that?

DR. WU: So I think you're referring to basically taking the skin cells and hit them with cardiac-specific genes and then try to convert into cardiomyocytes. I think for basic science applications, it's fine approach. But for clinical applications it's going to be very, very tough. And the reason is when you think about it, you start with 10 million skin cells, and your conversion rate is only 0.1 percent, or 1 percent at best. You're getting about 100,000 cardiac cells that are very heterogeneous because some of the cells may have multiple copies. Some of the cells have converted completely. Some of the cells have not converted completely.

And then you want to inject these cells back into the patient. First of all, there's not going to be enough cells. Secondly, if you think about it as a

commercialization standpoint, pharmaceutical companies are not going to be interested in that. I mean why would they go through this hassle if they don't know the Q&C of these cells, and plus they can only give it to one patient.

So that's why I think if you're thinking about commercialization, it still has to be iPS cells or ES cells starting with lines that are very well qualified. You could produce tons of these cardiomyocites that are very well qualified and inject them into patients.

Q: Thanks. It was an interesting talk. I was wondering if you have – what kind of data might be available, either in your lab or somebody else's. You know, I'm thinking as you're looking at familial cardiomyopathies and relationships within families, whether iPS cells that you've derived from different family members, for example, accurately reflect the disease and the level of disease of that patient and if you could

distinguish, for example, different family members by their IPS cell activity.

DR. WU: These are very, very good questions, and these are the questions that we as well as others have been trying to figure out. We're not there yet. I think for the iPS cell, most of us go after low-hanging fruit. "Low-hanging fruit" means that these are monogenic mutations that run in families. The polygenic disease is going to be much more difficult to remodel, to recapitulate. "Polygenic" means, for example, diabetes, coronary artery disease, hypertension.

Obviously, one of the goals that we want to do is to show this in these human iPS cell-derived cardiomyocytes. And this is also part of the main reason why we've been going after large families – so that within the same family, we could ask just exactly the question you're asking. Is there a difference between siblings who carry the same mutation, but have different phenotypes.

For the hypotrophic family that I showed you, it's actually a large family of eight kids. Good for us that the parents have eight kids. And out of the eight kids, four of them have the mutation, but two of them have the phenotype. The other two do not have the phenotype. We don't understand what's going on, so we're trying to see if we can recapitulate on the dish.

Q: That should be really interesting. Thank you.

DR. WU: Thanks, yeah. Yes, uh-huh?

Q: I'm very interested in the tumorgenicity of the IPSC's. So, you said that ten out of ten cells would form a teratoma. I was wondering if you had experiments that demonstrated that. And –

DR. WU: Yeah.

Q: -- the next question is, if you – suppose you *could* sort all the cells in a very highly effective manner. Could any of the differentiated cells revert back and de-differentiate to become a dangerous cell?

DR. WU: Both are very good questions. On the first one, let me just clarify. It's not ten out of ten cells. It's ten out of ten animals, meaning that if we inject one million undifferentiated cells, you will get ten out of ten animals that form teratomas. If you inject 1 million cells that have undergone the sorting with SSEA-5 low, CD9 low, and CD90 low markers, probably two out of ten will get teratoma formation.

We've also done dosing studies. For example, if you inject one cell, it doesn't form teratoma. Ten cells doesn't do it. A hundred cells doesn't do it. In the cardiac system, somewhere between 10,000 to 100,000 cells do you start seeing the

teratoma. We do this by bioluminescence imaging, which is very sensitive for that.

The other question you asked was if you inject a differentiated cell type, what happens to the differentiated cells? Do they revert back to teratomas, or do they stay as a cardiac cell? That's the exact question that we've been asking because in a dish we give them a whole bunch of growth factors and cytokines to push them to a cardiac cell. Once we pull them out, they're no longer exposed to the same kind of cytokines. The question in the field is, when you inject, do they revert back? We don't have the data for your question. We're trying to do it by using single-cell PCR, meaning we inject the cells, capture the cells, and sort them and then do single cell using the Fluidigm single-cell PCR to see if they remain a cardiomyocyte, or do they revert back, or do they become more mature cardiomyocyte because they're in the cardiac environment.

Q: Thank you.

MODERATOR: Thank our speaker – [unintelligible].

[APPLAUSE.]