DR. ROBERT GULDBERG: Thanks, Marc. I want to thank Marc, Ralph and Gordana[?] for organizing this really exciting and timely workshop. This topic has been discussed as absolutely critical to evaluating and translating regenerative medicine strategies.

I was asked to talk about advances in micro CT imaging for regenerative medicine, and this is something that's a relatively mature technology. It was actually developed about 25 years ago, and I was fortunate to be training in one of the labs that had one of the first custom-built systems, at the University of Michigan, and it was really originally designed to look at structural flaws in non-biological materials – actually, at one of the automotive companies. And it, in fact, still had some application in that area for regenerative medicine, so we're interested in looking at the architecture of scaffolds, for example, and how that relates to tissue ingrowth and biomechanical

properties. Micro CT is also very useful for that.

But its original application in tissues was really to look at bone, of course, because of the radio density of bone; and it’s really become the standard for looking at age-related and disease-related changes in the architecture of – [unintelligible] – bone. And so all the pharmaceutical companies own these micro CT systems that are working on osteoporosis drugs, for example. And that was helpful, because it created a market and, therefore, commercial entities that now create these systems; and that’s made it a readily available technology for laboratories as well.

The advantages are that it’s quite efficient. We’re working, as Joseph talked about, screening a number of different technologies. We’re working with a lot of pharmaceutical companies using this as a very efficient way for analyzing the outcome

of preclinical studies. It provides three-dimensional information. You really get two sorts of information coming out of micro CT. You get quantitative morphology from the reconstruction algorithms and morphometric software. You also get composition. It's related to the attenuation of the x-rays on a point-by-point basis. So, the – [unintelligible] – density has meaning. In bone, of course, that's the density of the bone, the mineralization. When you're using contrast agents and soft tissues, it has a different meaning, which I'll talk about.

Very high resolution. The original systems were more around 50 micron resolution. The newer systems that you can buy commercially are submicron. You can do in vivo scanning. In fact, there are clinical systems that are being used to assess changes in microstructure in the calcaneus and in the radius. As I mentioned, this is now readily available technology.

There are disadvantages, of course, in terms of exposing to ionizing radiation; and it's been primarily limited to demineralized tissues, but I'm going to talk about some contrast agents here used in regenerative medicine. I also want to point out a recent reference that has been published on some standards and guidelines for micro CT that you may find useful.

So, in the sense of looking at bone, then, of course, it's natural to think about looking at bone regeneration. And so we have primarily done this in this model system, which is a rat large segmental defect model that's 8 millimeters. This is quite a bit larger than it needs to be to not heal if it's not given any treatment. And we've designed this to be compatible with doing in vivo micro CT, so this is a polymeric fixation plate that we can monitor the ingrowth of bone into these defect regions.

And just as an example today, I'm going to talk about a technology in which we are trying to deliver bone morphogenetic protein in a more sustained manner to overcome some of the challenges associated with this current clinical use. Well[?], we use a nanofiber mesh to surround the defect region and an injectable hydro gel that slowly releases the BMP over about ten days.

And the analysis we are using are radiographs – micro CT – to get 3-D bone ingrowth histology and biomechanical testing. So, you can see the implanted construct. This is a perforated version of the implant, and part of what I'm going to talk about today is the use of micro CT to assess different designs for this regenerative strategy.

And so this is just one of the representative studies that we've done recently, where we looked at whether the mesh alone was able to heal the defects, or the mesh plus the hydrogel; and then delivering BMP either in a nanofiber mesh that

does not have perforations in the side, or where we included perforations. And as I mentioned, you can do this longitudinally, so you can do in vivo scanning of the animals. And so this is a[n] in vivo scan at week four in which you can see the design for Group 4 provided accelerated bone regeneration relative to any of the other groups, including the other BMP group. So, the inclusion of the perforations had a positive effect on the bone regeneration response. If we'd just looked at 12 weeks, we wouldn't 've seen that, because the two BMP groups at that point are equivalent.

Importantly, the imaging correlates well to biomechanics, so when we do the biomechanical testing, you can see that there's very consistent results relative to the imaging, with Group 4 significantly better than any of the other groups and, in fact, not statistically different than intact bone. And so using this regenerative approach, we were able to actually almost

completely restore the biomechanical function of these huge defects in just 132 weeks.

We could also look at dose dependency. So, these are micro CT images looking at different doses of BMP delivery. And one of our goals here was to be able to see how low we could go in terms of delivering this protein because it has some complications in terms of inflammatory responses and other complications in the clinic. And so delivering a lower dose that could be safer is important.

We were able to show, actually, compared to the clinical standard, we can deliver a dose that’s about one fifth that used clinically in a different delivery system and achieve the same result.

Now, normally you can’t see – as was mentioned in the previous talk, you can’t use CT to look at blood vessels, but if you use profuse contrast

agents or circulating contrast agents in vivo, you *can* look at the vasculature. And we began looking at this a few years ago, using a hindlimb ischemia model as the initial test bed. So, you can see here the hindlimb of a mouse at 10 micron resolution. On the one side, we've ligated and excised the femoral artery, and we used this to analyze different therapeutic angiogenesis strategies for restoring function to this ischemic limb.

This is using a radio – [unintelligible] – contrast agent. This is an ex vivo analysis in which we perfuse and polymerize a contrast agent, but there are circulating contrast agents now that have a long residence time in the bloodstream, that allow you to do in vivo imaging as well. Again, you can use some of the advantages of micro CT to look at a large number of different parameters of the vascular network, including the volume of the vascularity, the number of vessels; their average separation; the connectivity, which

actually correlates quite well to blood flow function; average thickness and size distribution.

So, although we initially quantified this or developed it in the ischemia model, we're using it now to look at a very important aspect in regenerative medicine, which is vascular ingrowth during tissue regeneration.

It was important to look at the issue of size resolution and how that compared to what sort of vessels you could see. At this time, we were using a system that only went down to about 8 micron resolution. Here you can see a transluminated histologic section. They're showing the vessels, and then we re-scanned that at various resolutions – there's[?] – [unintelligible] – sizes – to see the sort of vessels you could detect. You could see at 36 microns, you're really only detecting the arterial size vessels, but down to 8 microns, you're

capturing most of the vessels that – that are apparent in the histologic section.

When you compare this to histology – and, again, this is doing it in the hindlimb ischemia model, where we're looking at the control relative to the ischemic limb. You get very similar results to the histologic stain technique.

So, back to why we saw an advantage of the perforations and the bone regeneration example that I showed. We were able to use this vascular imaging technique to show that the perforations in the side of these nanofiber meshes was facilitating vascular ingrowth from the surrounding musculature – and, in fact, that there was a distribution difference from the proximal end to the distal end in terms of vascularity. And this is helping us now to design some graded delivery techniques to help overcome this limitation.

One of the key issues in regenerative medicine, of course, that we always talk about is how the endogenous environment, or niche, interacts with these different strategies for regenerating tissues; and we've used these techniques to also look at one of those types of scenarios where we're interested in looking at how the mechanical environment en vivo influences the regenerative response.

So, this is a study where, again, we're using the nanofiber mesh and hydrogel system to stimulate bone regeneration either at a dose that we would expect *not* to heal, or at a dose that we would expect to heal the defect. And we're doing this in two different variations, where we have – we either use a – a stiff plate in which the fixation plate is locked for the entire time, which is our standard configuration. Or, we unlock the systems that allows only axial motion at any time during the experiment. And in this case, we activated it at the very beginning of the experiment. And you

can see that this very early loading inhibits vascular ingrowth and also bone regeneration. Vascular connectivity is substantially reduced, and bone volume as well. And what appears to be happening, actually, is the nascent vessels that are at this interface are being disrupted because of the mechanical environment not allowing bone regeneration to proceed.

Now, conversely, though, if we wait just four weeks before we allow that mechanical stimulus to be applied, we actually get the opposite results. You get a stimulation of the bone regeneration response and a vascular remodeling response, where there's a thickening of the vessels that are growing into the defect region.

Okay. So, again, using standard micro CT, you can't see non-contrasting tissue. So, you can't see blood vessels. You can't see cartilage. You can't see the growth plate, or the articular surface in this rodent femur. You can't see the

intervertebral disc in the spine. But a few years ago, we set out to try and be able to look at some of the soft tissues – in particular, cartilage. If we look at the rabbit distal femur and scan without a contrast agent, all you see is the bone structure. With using an equilibrated contrast agent, however, you can detect the soft tissue overlying the joint, including some scalpel marks and insertion sites in the joint.

Importantly, we were able to design the contrast agent such that you could segment the bone and the cartilage and then isolate the articular surface. And, again, one of the advantages of micro CT is you get these very nice morphometric parameters that can be calculated. So, on a point-by-point basis, we can calculate the thickness of the articular surface and map that back onto the three-dimensional image.

Here you can see just a comparison. The technique we call – call is “equilibrium partitioning of an

ionic contrast agent," and I'll explain what that is in just a moment. Other people in the field are calling this "contrast-enhanced micro CT." It's basically all the same technique, very similar to the DeGemerick [phonetic] technique for MRI. And basically, it's using either a negatively charged or positively charged ionic contrast agent to make the cartilage visible to micro CT.

And you can see here the difference between micro MRI and epic[?] micro CT in terms of typical resolutions that can be achieved. We're actually, in our mouse studies now, going down to about 3 micron resolution to be able to detect the articular surface in mice.

So, the principle of the technique is that it uses the negatively charged charges of the proteoglycans and assumes that, if we have an ionic contrast agent, that it will equilibrate inversely proportional to the proteoglycan

concentration, such that if you have a high proteoglycan concentration, you would expect to have low-contrast agent concentration. And conversely, if you have less healthy cartilage in which the proteoglycans have been depleted, you would expect to see an increase in the concentration of the contrast agent.

There’re a number of contrast agents that are now being used. We primarily used one called Hexabrix [phonetic], which is a negatively charged ion that’s – it’s used clinically for GI imaging and has six iodines per iron providing the contrast.

And just initially, to look at how well this worked, we looked at explants of cartilage. And to orient you, this is bovine cartilage – just circular explants. This is the surface zone of the explant, and this is the deep zone, where we’ve taken a virtual cut through the circular explant. And here, we’re following it over – over time in vitro, either in control media, or exposed

to interleukin 1, which is known to deplete the proteoglycans. And so you can see in the controls over time, you maintain a relatively low attenuation level, which is indicative of high proteoglycans; whereas, in the explants exposed to interleukin 1, as expected, we began to see an increase in the attenuation around the periphery as the proteoglycans are depleted.

You can quantify this, of course; and we can show that, you know, over time there's a significant increase in the overall attenuation in the interleukin 1 explants. And most importantly, if you do biochemical measurements of the S-[unintelligible] content, you get a very nice, inverse linear correlation between proteoglycan content and this nondestructive assessment of the attenuation.

And so we validated, then, that this epic micro CT technique is a strong predictor of the S-[unintelligible] content. So, as I said at the

beginning, micro CT provides you very nice morphologic information. It also can provide you compositional information in terms of the Vo-[unintelligible] attention. And in this case, instead of mineralization for bone, it's indicative of the S-GAG[?] content in cartilage.

So, we've gone on to show that in looking at intact joints, and primarily in the rat articular cartilage model, we have an equilibration time of about 20 to 30 minutes. We get very nice reproducibility of the technique and high sensitivity to detect small differences – or, small changes in the attenuation of the joints.

We also had to validate their ability to look at the morphology, and so for this, we just looked at some different-age animals. We looked at rats that were four, eight and 16 weeks of age. And here – here we used two different techniques to validate the thickness of the joints. We either used a needle-punching technique, which there was

a nice linear correlation, or, again, use histologic measurement techniques and again found very nice agreement between micro CT and histology.

So, the last thing I want to talk about is then using this to evaluate OA therapies, and we're – this is a growing segment in my laboratory in which we're looking at regenerative strategies either to prevent or slow down the progression of osteoarthritis, or to regenerate a degraded articular surface. We've used a number of different models, but I'm primarily going to show results from the rat medial meniscal transection model, where we destabilize the meniscus in the rat and are using this contrast micro CT method to nondestructively quantify the morphology and the composition. And you can see just one section of a typical image, where you can see both the bone and the articular surface.

So, this is a sham[?[-operated knee at three weeks. Again, we're just looking at one slice here, so this is the Gray[?] scale image. You can see the cartilage, which would not be visible without the contrast agent. And in this model, at three weeks, you begin to see lesions forming in fibrillation sites. So, we define lesions as anything greater than 50 percent of the cartilage thickness, and a fibrillation area is less than 50 percent.

If you look at the entire surface of this joint, you don't see any differences between the controls and the experimental. And, in fact, there's only about two different – two lesions and two fibrillation sites that develop per animal. So, you can imagine if you're doing histology to detect these and to analyze it. You have to do a lot of histology. You have to section through the entire joint, and that can take many months to do. And so the pharmaceutical companies are developing

therapies for O.A. are very excited about this as a higher throughput method of analysis. 06:52:44

We can also look at osteophytes. And I apologize. This image got rotated, but here's the osteophyte down here. And so not only can you look at degradation in the articular surface, but we can also look at changes in terms of the formation of these o- -- these osteophytes, which are one of the hallmarks of the progression of the disease. And, actually, it's very sensitive. You can see these differences as early as two weeks. And we think with a larger sample size, we may be able to go down to one week.

It we look at the localized attenuation, we can see that there are differences between the SHAMs[?] and the MMTs, so as we would predict, there was an increase in the arthritic animals in terms of the attenuation, and this is indicative of a loss of the proteoglycans in that [unintelligible]-ticular surface. And you can see

that best if you look at a sagittal view. Sao, this is [a sagittal view of the intact joint and then the isolated image of the articular surface. And, again, you can see very clearly the increase in the equilibrated contrast agent that is indicative of the loss of the prot-[unintelligible].

If we compare this to histology, you can see a couple different sites of fibrillations and lesions here that compare very well between the – [unintelligible] – stained histologic sections and just a corresponding slice of a three-dimensional slice of a micro CT image.

And if we look at the incidence of the focal[?] lesions, we see that in the arthritic animals, you have, again, about two focal lesion sites per animal and two fibrillation sites; whereas, in the control animals, you have none. And these focal lesions, again – since this is a three-dimensional imaging technique, we can quantify things like

lesion volume, that’s not possible using histologic methods.

And then just to show one regenerative medicine application for this, we’re doing a study in which we’re delivering therapy derived from extracellular matrix – that I can’t go into the details on, but you can see the results of the treatment here in which the arthritic joint is developing lesions at three weeks; whereas, the joint that’s receiving this interarticular injection of the ECM treatment has a lower level of lesion formation and a lower level of attenuation changes. So, if you look at the average number of fibrillations, they’re significantly decreased. If you quantify things like the lesion volume, there’s no lesions, actually, that form in the treated animals; whereas there’s a finite level of lesion volume in the arthritic animals.

And also, there's a change in the attenuation, so there's also changes in the proteoglycan content in the arthritic animals – which is improved in the animals that are treated with the ECM.

So, this is part of sort of an integrated imaging strategy that we're using for testing different OA therapies, either for slowing down the progression of OA, or treating already arthritic joints. You can use micro CT, of course, for just for looking at changes in the bone structure. And there are changes that occur. I didn't talk about that today, but there are changes that occur in the subchondral[?] bone. Using the contrast agent, you can look at changes in the articular surface. We're also using some reactive oxygen species[?] imaging techniques to look at inflammation in the joint, and then using profused contrast agents, or circulating contrast agents. You can look at changes in the vascularity.

And then, of course, we also include the imaging methods with some functional assessment – in this case, catwalk gait analysis to look at pain and function.

So, to summarize, then, I think – you know, micro CT is a very mature technology. It's obviously become the standard for looking at osteoporosis drugs, but I think it does have a great role in looking at quantifying and comparing different regenerative strategies because of some of its advantages in terms of being very high-resolution, nondestructive. It has its limits for in vivo uses, but there are – you can do this in preclinical studies as long as you don't do it too frequently. And you can look at both morphology and composition in the tissues.

And so I'll end there and be happy to take any questions. I just want to go ahead and acknowledge my group as well as our collaborators, Mark Levinson, who helped develop the contrast –

[unintelligible] – cartilage imaging technique, as well as our funding sources.

Thank you very much.

[APPLAUSE.]

MODERATOR: We've got time for questions.

Q: I have two questions. One is a technical one. The first one is – I mean I remember you showed the – [unintelligible] – dimensions, and I was wondering. I mean is this the same – I mean is this is more a — you know, 'cause the – [unintelligible] – machine, the 40 micron, the – [unintelligible] – 40 is what you used for it – right? And so I'm wondering, like, I mean is this actually more – like, a resolution that is more – that the software allows you to achieve, because the resolution of the machine is about 40 microns, if I'm right? So –

DR. GULDBERG: No.

Q: -- [crosstalk] – or is there something that I misunderstood?

DR. GULDBERG: Yeah. So, the original systems were around 40 or 50 microns. The systems that came out maybe ten years were down, at the highest resolution, about 6 to 8 microns. And then the latest systems, I think it's micro C – for scan codes, the micro CT 50, I believe, has a 700 nanometer voxile[?] size as its maximum size. So, as you probably know, there's a difference between resolution and voxile[?] size, but those are the voxile[?] sizes that are now available with the current commercial systems.

Q: The second one is – I mean you showed the images from the ECM treatment. You said, obviously, that it's something proprietary; but did – I mean was I correct in noticing that – I mean while you see that the cartilage lesion, or, you know, the

inflammatory – the depletion of proteoglycan is not as much –

DR. GULDBERG: Right.

Q: -- but there are some changes to bone structure. Is that an artifact that you see, or – ’cause the bone density seems to be different. Is it something that you comment on?

DR. GULDBERG: Yes, we did look at changes in the subchondral[?] bone. There were not significant differences between the control and the experimental group. So, the only differences we detected were a decrease in the attenuation, which meant better retention of the proteoglycans, and a complete lack of lesion formation – which was very exciting.

So, what I can say is it’s ECM particle technology that’s being delivered in a solulized[?] form.

And it gets sequestered to the synovial membrane and seems to be having a positive effect on OA.

Q: Okay. Thank you.

DR. GULDBERG: Um-hum.

Q: Hi. This is really nice. About the measurements of the vascular flow[?], say, during the bone regeneration, what –

[INFORMAL COMMENTS.]

Q: If you look into establishment of the blood profusion during bone regeneration, what kind of parameters can you measure? Is it just the presence of blood, or can you look into, perhaps, some of the profusion velocities or some other quantitative parameters?

DR. GULDBERG: Yeah. For those that didn’t hear the question, it’s how does the morphology of the

blood vessels from micro CT correlate with the function – right?

Q: Right.

DR. GULDBEG: And we haven't – we actually haven't done that a lot with the bone studies. We did it extensively with the ischemia studies, and what we found was – using laser Doppler profusion imaging, was that the volume of the vascularity didn't necessarily correlate with improved function; but other parameters, such as the connectivity of the vascular network, did correlate quite well. So, we needed to look at more than just vascular volume, but if we included that with the vascular connectivity, then that would correlate with reprofusion to the lower – [unintelligible].

Q: Okay. And a related question. Are you also planning to look into co-localizing vascularization with the bone development, fusing

the two images together and understanding how the two are developing in – [crosstalk]?

DR. GULDBERG: Yeah, yeah. That's a great question. So, the question is, you know, how does – probably both temporally and spatially, how does mineralization and vascularization relate? And, you know, we have an NIH grant to do just that, so we've done a lot of analyses both spatially and temporally to look at that. And one of the nice things about this technique is when you do the profusion, you get an image of both the vascularity and the bone. You can then take that same sample and decalcify it, and you get an image of just the vascularity, so you can look at both.

Q: So, can you give us a sense on the temporal resolution of the micro CT? For example, you know, if you're interested in imaging coronary arteries, I assume in a mouse it's going to be difficult; but if you – once you get down to, for example, guinea pigs, in which the heart rate is

down to about 150, 200, how good are you in terms of defining the coronary anatomy?

DR. GULDBERG: You're saying gating for the –

Q: Yeah, that's right.

DR. GULDBERG: -- so, yeah. That's certainly an issue -- motion artifact associated with the heart beating and so forth. For a lot of our studies where we're doing analyses on either the spine or the limb, we don't have a lot of problem with that. If you're more in this region, then you would have that issue. We haven't had success doing it in the mouse. There are gating functions on these commercial systems, but I think you'd have to go to, as you say, a larger animal to be able to do that.

Q: So, I have a quick question. In this bone defect – this large defect model – you delivered BMP for five days, but it took 12 weeks, if I remember –

DR. GULDBERG: Right.

Q: -- to heal the gap. Was there a reason for the choice of five days?

DR. GULDBERG: Yeah.

Q: Or, is that just the length that your delivery vehicle would sustain?

DR. GULDBERG: Yeah, that’s a good question. So, that’s what our delivery vehicle was able to sustain. There are some studies out there looking at longer term and, in fact, tethered osteoinductive factors. And what it seems like is that some initial burst release probably is beneficial, and so there’s probably some in between. I think having too long a sustained release is probably not good, because what you’re doing is you’re influencing the migration of cells into that defect, and then their differentiation.

And then you see the subsequent bone regeneration. So, prolonging the inflammatory response at that site is probably not a good thing.

The other thing that the system does is it helps to retain that. So, I didn't really talk about that, but the bone regeneration is only in the defect region, and so we don't see that ectopic mineralization that's one of the complications that you see clinically.

MODERATOR: Okay. Let's thank our speaker again.

[APPLAUSE.]