Advancing Epigenetics:
Novel Drug Discovery Strategies for Epigenetic Targets
Webinar
17 Oct 2012

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Sean Sanders: Hello and welcome to this *Science*/AAAS audio webinar. My name is Sean Sanders and I'm the editor for custom publishing at *Science*.

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The field of epigenetics has exploded in the last several years and significant advances have been made in understanding many of the basic mechanisms involved in epigenetic modifications. Researchers are coming to appreciate the critical roles that these modifications play in the development and progression of disease and are increasingly focusing on the identification of clinical treatments for aberrant epigenetic states. Given the increasing interest and investment in drug discovery for epigenetic targets, there is a clear need for robust methodologies to identify candidate therapeutic compounds. This webinar will focus on current techniques and technologies that are enabling researchers to identify and validate potential targets for clinical intervention.

We have an exceptional panel of experts on the line today. They are Dr. Manfred Jung from the University of Freiburg in Germany, Dr. Margaret Porter Scott from Epizyme based in Cambridge, Massachusetts, and Dr. Ji-Hu Zhang from the Novartis Institutes for BioMedical Research also in Cambridge. It's a great pleasure to have you all with us today and I'm looking forward to the discussion.

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Before we get started, I have some information that our audience might find helpful. Note that you can resize or hide any of the windows in your viewing console. The widgets at the bottom of the console control which of the windows you see. Click on these to see the speaker bios or additional information about technologies related to today's discussion or to download a PDF of the slides.

Each of our speakers will talk briefly about their work. After which we will have a Q&A session during which our guests will address the

questions submitted by our live online viewers. So if you're joining us live, start thinking about some questions now and submit them any time during the webinar by typing them into the box on the bottom left of your viewing console and clicking the submit button. If you can't see the box, just click the red Q&A widget at the bottom of the screen. Please remember to keep your question short and concise, as this will give them the best chance of being put to our panel.

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Finally, thank you to PerkinElmer for their sponsorship of today's webinar.

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Now, I'd like to introduce our first speaker for today and that is Dr. Manfred Jung. Dr. Jung holds an undergraduate degree in pharmacy and a Ph.D. in pharmaceutical chemistry, both from the University of Marburg in Germany. He carried out his postdoctoral studies in Canada at the University of Ottawa and was a lecturer and researcher, and then a senior researcher, in the Department of Pharmaceutical and Medicinal Chemistry at the University of Münster in Germany. In 2003, Dr. Jung moved to the University of Freiburg, where he is currently a professor of pharmaceutical chemistry. His field of research is chemical epigenetics, focusing on drug discovery and assay development with inhibitors of histone deacetylases, NAD+-dependent histone deacetylases, histone acetyl- and methyltransferases, and histone demethylases. A very warm welcome to you, Dr. Jung.

Dr. Manfred Jung: Thank you very much, Sean. Hello everybody. I would like first to give a brief introduction into epigenetics.

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So first genetics, the genome is the sum of all the information that is stored in the DNA, the blueprint for the formation of an organism. But if you look at one of those bees, they have a complex phenotype so all the cells have the same genes but the liver cell only uses a different subset of the information than a muscle cell, although they have the same whole genome.

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So now, epigenetics is responsible to maintain this regulation. So if a liver cell divides, although it has the complete genome, it only becomes a liver cell again or two liver cells. But epigenetics is also involved in phenotypic switches. So in development for example if you come from the bee larvae to the bee, we have the same being, we have the organism, the same genome, there's no change but obviously the phenotype is very drastically changed without any genetic alteration. The same switch may also occur just from healthy cells to diseased cell without any genetic damage but epigenetic damage.

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Because epigenetics shows plasticity as a response to the environment and we have biochemical manifestation of environmental signals, these maybe chemicals such as drugs but also stress or nutrition. So again an example here, if we have these bee larvae and we treat them with pollen, you get a working bee and if you treat them with gelee royale in an identical twin of another larvae, we get the queen bee. So the queen and the working bee, they're actually twins. They have the same genetic information, but epigenetic is responsible for the different phenotype but in response to external signals. This is to some extent threatening but also shows great potential for epigenetic drug discovery.

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So what is the biochemistry behind epigenetics? What is the epigenetic machinery? We have different mechanisms. We have DNA methylation, methylation of cytosine residues. We have small noncoding RNAs and the focus of today's presentations are the histones.

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Both are basic proteins, the protein part of the chromatin DNA is wrapped around the histones as you can see here and those histone tails that protrude from this balloon-like depicted histones they are rich in amino acids especially arginine's and lysines and these are subject to enzymatic modifications. In this talk, we will focus on just a subset of these many modifications on acetylation and methylation, on lysine residues.

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So is there some sort of an epigenetic code that's equivalent to the genetic code? Well here, you just see an already simplified snapshot of one of the four different histone proteins, histone H3 and you see

part of the amino acids of the tail and different modifications that could occur on this tail. You already see that this forms a complex pattern of modification. It's not just a binary on/off code if we look at one modification, a methyl group is there or not, but there could be all kinds of different modifications. They are interactive. They might be mutually exclusive and although we have to start sequencing epigenomes and start to extract the information, we have to always bear in mind that the meaning of this complex pattern might be context dependent. We cannot decipher it without ambiguity as we can decipher the genetic code.

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So what about the enzymes, what about the drug targets actually in epigenetics? So we have enzymes that establish the modifications the so called writers, different transferases.

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We have enzymes that remove the modifications like deacetylases or demethylases.

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And as a third part of the epigenetic machinery, we have the so-called reader proteins. They are able to detect the modification and actually then propagate the signal. From all of these three classes, these targets are identified for drugs discovery and drug discovery programs are currently ongoing.

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In my talk, I will focus on reversible lysine acetylation. Here you see a lysine residue and a histone and at the bottom deacetylated lysine. Histone acetyl transferases establish the modification and histone deacetylases remove the acetyl groups. Among the deacetylases, we have 18 human subtypes and the important ones are 11 that by their mechanisms are zinc dependent aminohydrolases. We have to bear in mind that especially the histone deacetylases have many non-histone substrates other proteins so the effect of drugs against this enzyme will also affect non-epigenetic, non-histone targets.

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First, an overview about the acetyl transferases very briefly. Biologically, they are very well characterized. It has been shown that fusion proteins are associated with many diseases especially cancer, but unfortunately we do not have clean drug-like histone acetyl

transferase inhibitors so we do not have clinical candidates. So there's still a great demand in looking for these inhibitors.

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So now coming to in vitro assays, so here's the picture again. To the left, there's the lysine substrate in the protein or in many in vitro assay, we actually use oligopeptides then the histone acetyl transferase uses a co-substrate acetyl-CoA and transfers the acetyl group to the substrate and the byproduct coenzyme A is formed.

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So what we can do for an assay is use radiolabeled acetyl-CoA and then monitor the transfer of the radioactivity to the peptide or substrate, a protein substrate.

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We can quantitate the thiol group and the byproduct coenzyme A or as with all or many posttranslational protein modifications, we can use antibodies. We need specific antibodies that can distinguish the modification from the unmodified version and then detect the antibody on a secondary assay.

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So now coming to the histone deacetylases, the zinc dependent enzymes 11 subtypes, they are the ones that the most efforts are targeted against currently. Here we already have validated targets as two histone deacetylase inhibitors are already approved for human treatment for cancer cutaneous T-cell lymphoma and there's many exciting preclinical data that HDAC inhibitors might also be interesting for neurodegeneration, metabolic disorders, species-selective inhibitors, also for parasitic diseases.

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I told you that there are 11 subtypes of the zinc dependent enzymes and one of the big issues in the field is do we need subtype selective inhibitor or rather pan inhibitors? So far, we cannot really answer that question. It seems from preclinical data that subtype selective inhibitors might have a benefit, but still there's a lot of effort to discover new inhibitors also with selectivities that are not so well established yet. So also, there's a big demand for screening and drug discovery.

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So what about the HDAC assays? Here in contrast to pretty much all other epigenetic enzymes, one can actually use small molecule substrates and usually tripeptides or as you can see depicted here, even just acetyl lysine derivatives are used for in vitro screening and those get also deacetylated. We have some knowledge on modifying the structure of these small molecule substrates to fine tune the reactivity. For example, adding a trifluoromethyl group to a certain histone deacetylase instead of an acetyl group. So if the enzyme converts, this upper substrate deacetylates it, there's no change in the fluorophore so we need a detection step.

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And what we do is add a protease, usually trypsin, which does not recognize the acetylated lysine but recognizes the deacetylated metabolite and then the fluorophore is cleaved off or sometimes also in a variation, a substrate for luciferase is formed and then we have the signal that we can monitor and use for in vitro screening.

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It's very interesting that we can also use this for cellular screening because it has been shown that the small lipophilic substrates can penetrate living cells and are converted within living cells. This can be done from cell culture, from animal samples or even blood samples from patients that undergo clinical trials with HDAC inhibitors and we can monitor HDAC activity from these samples and there's a lot of interest now whether we can use this as a surrogate biomarker for clinical studies.

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With this, I would like to end my talk. I'd like to thank my collaborators and my funding agencies.

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I'd like to thank my group for their enthusiasm, excuse me, and you can find more information on our work on our website and now I hand over to Margaret for her talk. Thanks a lot.

Sean Sanders: Great. Many thanks, Dr. Jung.

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Our second speaker for today is going to be Dr. Margaret Porter Scott. Dr. Porter Scott received her B.A. from Oberlin College in Oberlin, Ohio, and her Ph.D. from the State University of New York in Stony Brook. She is currently director of lead discovery at Epizyme

responsible for enzymology, high throughput screening, and compound management. Prior to Epizyme, Dr. Porter Scott held scientific positions at Millennium, Neogenesis, Vertex, and Pfizer. Most recently, she spoke on biochemistry and screening approaches for histone methyl transferases at the 2011 Society for Biomolecular Screening and 2012 Society for Laboratory Automation meetings. Welcome and thanks for being with us, Dr. Porter Scott.

Dr. Margaret Porter Scott: Thank you very much. It's really a pleasure to talk to you in the webinar today about protein methyl transferases as inhibitors and as personalized cancer therapeutics.

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So at Epizyme, the goal of all our research is to positively impact the life of cancer patients. We feel that we can do this best by creating drugs for focused patient populations with genetically defined cancers. The genetic definition of disease allows us to more quickly get drugs to patients and at a lower cost. It allows us to have a concise development path, a rapid proof of concept and a clear clinical path forward.

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So there are of course many enzymes that act on chromatin. At Epizyme, we have decided to focus on protein methyl transferases as the target class due in part to the clear understanding of their active site of mechanism. So one reason that reversible methylation of lysine and arginine residues appear to play such a paramount role in gene transcription is the diversity of chemical states that are afforded by the various levels of methylation that these enzymes can achieve.

So lysine can exist in four distinct chemical states with respect to methylation. So there's an unmethylated or zero methyl state, mono, di and trimethyl states for lysine. The methylation is of course on the side chain amine. Likewise, for arginine residues, they can exist in four distinct states. They can exist in an unmethylated or a monomethylated state and then there are two forms of dimethylated arginine, you can have an asymmetric or a symmetric methylation that confers additional specificity. So each of these unique chemical states can serve as a distinct recognition element for the binding of components of the transcriptional machinery and thus this leads to very distinct biology downstream.

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So I don't have the introduction, but Dr. Jung was great. I don't have much biology in my talk, but I wanted to set the frame for the way that we think histone methyltransferases functionally impact disease. And so in the unmethylated state or the methylated state is maintained by the reciprocal action of methyltransferases and of course demethylases.

When you have too much methyltransferase activity, you can get a hypermethylated state and then this is what leads to transcriptional dysregulation, transcriptional changes that can lead to the phenotypic changes and oftentimes unfortunately, these phenotypic changes lead to human disease.

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On my next slide, I have a basic graphic to really show our approach for impacting these kind of diseases. Epizyme is really focused on the creation of small molecule inhibitors that will block methylation of chromatin in these cases where it is linked to disease.

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And the whole family of protein methyltransferases is collected on these two trees. So there are two distinct classes of protein methyltransferases. There are 51 lysine methyltransferases and 45 arginine methyltransferases. Some of the known inhibitors are illustrated here on the tree. While many of them are very good tool molecules, we have yet to see human proof of – it's an exciting time in the field where we're awaiting the first human proof of concept for a histone methyltransferase inhibitor and of course, many of these enzymes do have clear implication in human disease. On the next slide, I'll show you a collection of the genetic observations of protein methyltransferases as potential drivers of cancer.

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So there's really a growing body of evidence to suggest that dysregulated methyltransferase enzyme activity is linked to uncontrolled cell proliferation. In fact, specific methyltransferases have been found to be genetically altered in particular human cancers that are listed here in ways that confer a unique dependence of the cancer cell upon the enzymatic activity of the histone methyltransferase – on the activity of the histone methyltransferase for that population.

For example, the enzymatic activity of the histone methyltransferase DOT1 is required for the proliferation of MLL rearranged leukemia due to a specific chromosomal translocation associated with that disease. In my next section of my talk, I'll focus on how mutations in EZH2 can confer a selective requirement for EZH2 in non-Hodgkin's lymphoma.

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So before we even knew about these mutations in EZH2, it was well known from many, many diverse inputs by many publications on many different kinds of cancers that EZH2 activity was implicated. In fact, the clearer collection of these papers was really focused on the methylation of K27. So any way that leads to hypermethylation of K27 is something that has been shown to be associated with hyper proliferative or even a cancer phenotype.

So showing on this slide are all the different ways that can lead to hypermethylation of K27. So PHF1 or sorry PHF19 is a processivity factor for EZH2 and tends to lead to higher levels of trimethylation. I'll show you in my next slides the mutations that we found, change of functions in non-Hodgkin's lymphoma, many reports of amplification of PRC2 subunits in cancer, and then of course the demethylase activity, loss of UTX is another hallmark in many cases that's associated with cancer.

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So what I'd like to share with you now is the story of how we discovered these EZH2 mutations that were identified in – well, sorry we didn't discover the mutations. We actually discovered the function of the muted protein. So the mutations were discovered by genetic sequencing and published in this Morin paper that I referenced here. It was shown that in several different subsets of non-Hodgkin's lymphoma that there's a tyrosine residue that was mutated and it could be muted to several different amino acids. So what was surprising to us when this paper came out is that despite what I told you from all the other inputs that lead to hypermethylation of K27, this mutation at first appeared to be inactivating based on the biochemistry, the initial biochemistry that was done. So that graphic there shows the differential activity of all the mutants relative to wild type and showing wild type having very good activity and all of the tyrosine mutants being inactive.

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So we took this in hand, these mutants in hand and tried to study them ourselves. The very first thing we did was we were able to reproduce very clearly the findings of these authors that in fact when you take this peptide and used these mutant and wild type proteins against it, here showing all the mutants that we had, we don't see any activity on methylated peptides.

So because we're focused on histone methyltransferases and we have a large substrate panel in-house that includes many of the other posttranslational modifications, we were able to quickly test against the monomethyl peptide. Now we started to see more equal activity of the wild type and the mutant peptides. Taking it one step further and looking the dimethyl peptide, now we see that in fact these mutants have much, much more activity in fact on the ability to take dimethyl peptide and methylate that to the trimethyl state than wild type does.

So we were able to further probe these mutants with our compound, which I'll show you on the next slide. But I think the further point that I want to make here is that if you imagine the heterozygous situation where you have one copy of the wild type and one copy of the mutant, you now have the complete enzymatic activity that you would need. You have the ability to take unmethylated and drive it to mono or dimethylated and then you also have the ability with the wild type to take dimethylated peptide and drive it quickly to trimethylated. So we think that the heterozygous situation, the wild type and mutant actually acts synergistically too and actually it's published in this paper you see referenced, they act synergistically to lead to hyper trimethylation of the substrate. So in fact this mutation was not inactivating, we just had to really look at the correct substrate to see, to find this activity.

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So we recently had been able to publish a potent and selective inhibitor of EZH2. It's a nanomolar inhibitor in an enzyme assay and it's a SAM competitive inhibitor as you can see in figure C and not competitive with nucleosome. So a very clear mechanism, SAM competitive mechanism and a potent compound in vitro.

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The selectivity of this compound is illustrated. We've taken the tree and put bubbles on it to show the potency of the compound and so you can see that for EZH2 and all of its mutants, those biggest bubbles represent nanomolar potency. For EZH1, we have about a 2-

log shift in potency so we're 2 logs more potent against EZH2 than EZH1 and in fact we're much, much more potent than that against all the other HMT. So in fact, we have, you know, quite good 2-log selectivity of EZH2 and greater than 3-log selectivity against all the rest of the HMTs. So this gives us confidence going into cell based assays that we'll be seeing the on target activity of our compound.

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In fact, EPZ005687 selectively kills lymphoma cells that bear these EZH2 mutations and what we always like to do is correlate the change in methyl mark to the change in proliferation. So you see in figure A the clear diminution of the methyl mark in the presence of the compounds and you can also see in figure B how selective it is for the K27 methyl mark over any other methyl mark. Then the proliferation, studies are shown on the right-hand panel. So the wild types cell line is relatively refractory to killing by 5687 whereas the two mutant cell lines we showed there, the WHU and the Pfeiffer, their proliferation is potently inhibited. In fact, they're even killed by 5687.

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So this is, you know, the example that I can show you of potent inhibitors that we have and we have taken this kind of approach and following on this kind of success and really decided to prosecute many, many other protein methyltransferases and we're doing this with a cross-screening approach. So we have a panel of 20 enzyme assays as they're shown here on this tree and we screen every compound in our library against this entire panel every month and it's been a very productive approach for us.

We're finding new leads for programs out of our own library very often and I can show you a way that our tree looks here. We have good coverage over the whole protein methyltransferase ome. There are some branches here where the enzymatic activity is not well understood and it's certainly an active effort on the part of us and others to understand those, but for the branches where enzymatic activity on the protein substrate is well understood, we have really nice coverage.

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This has led to the pipeline for Epizyme being very robust. So we have our partnership with GSK and our partnership with Eisai around EZH2 and we have our partnership with Celgene that covers the rest of the HMT ome here. DOT1 I'm able to name here but there are

many other programs for which we have very potent and selective compounds in-house that are now candidates for additional programs.

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So, you know, I'd like to just conclude by acknowledging the team at Epizyme and our collaborators.

Sean Sanders: Wonderful. Thank you so much, Dr. Porter Scott.

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Our final speaker for this webinar is Dr. Ji-Hu Zhang. Dr. Zhang graduated from Nankai University in China and received his Ph.D. in biochemistry from the University of Georgia in the United States. Dr. Zhang worked as a postdoctoral fellow at the University of California-Berkeley and later at the California Institute of Technology. He started his industry career in 1996, when he joined the Affymax Research Institute as a research scientist before moving to Dupont Merck as a principal research scientist in the Leads Discovery Department. Then in 2002, he joined Novartis as a research fellow in the Leads Discovery Center, where he is currently a senior investigator. Dr. Zhang's expertise is in biochemical and cellular assay development and high throughput screening technologies for drug discovery. Many thanks for joining us, Dr. Zhang.

Dr. Ji-Hu Zhang: Thank you, Sean. It's a pleasure to talk in this webinar. As Sean just said, so my talk is focused on the high throughput assay for lysine specific histone demethylases. Let's go to next slide.

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This is the outline of my presentation. First, I'll give a very brief introduction and then I will focus on a site specific methylation sensing assay, which is TR-FRET based assay format for methylation sensing at H3K4 site. I'll use LSD1 as an example and then compare this assay to other assay formats and finally summarize it.

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So thanks to the previous two speakers which make my life easier so I'll keep my introduction part brief. So this slide just to show you, first there's an increasing number of epigenetic regulator, which has been linked to the different kind of diseases including cancers mainly and these modification enzymes are believed to be druggable targets. As a matter of fact, the low molecular weight inhibitors for HDAC some of those are already on the market and for histone

methyltransferases and the histone demethylases, and the small molecule inhibitors are very near to clinical trials or in preclinical development.

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This slide shows some of the histone lysine methylation marks and the modifying enzyme as in proteins as the previous two talks already touched upon. So the histone tails is the place which is subject to different kinds of modifications, which including acetylation and the deacetylation and methylation, phosphorylation and ubiquitination plus there's readers and adaptors, which interact in this area.

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So to get to the challenges about doing assays for small molecule drug discovery and this slide shows the cell based assay for methylation and demethylation. So at first, it's limited knowledge about HMTs and HDMs, the interactomes. There's no specific signaling pathways identified that can be readily translated into proximal cellular readout. This is different compared to kinases. In kinases, a lot of those kinases are known in which signal transduction pathways and here it's much less the case.

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For engineered methylation/demethylation sensors, there's always a high endogenous histone background in all cell lines. This is inevitable. There's no excuse – I mean no escape from these high background interference if you will. So and finally there's lack of tool compounds aiding the assay development.

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So this slide shows the challenge for biochemical methylation, these in vitro assays. And again there's limited knowledge and reagents including active enzymes and the methylation specific antibodies. Also these enzymes again compared to kinases, usually the activities are low and slow and also a lot of times they need a complex enzyme for example the PRC2 complex that Dr. Scott presented earlier and also the substrate sometimes cannot be just thin pole peptide, they have to be nucleosomes.

So currently, many of those assays are based on generic detections, which mean either coupled enzymatic assays or chemically coupled reactions. They go after coactivators or co-products like SAM, SAH, hydrogen peroxide, formaldehyde, etc. for detection and which

assays are – I mean usually not that expensive but limited by assay sensitivity and specificity.

There are of course other assay format including direct mass based detection, which can be detecting both coactivator, cold production or the methylated peptide, but again it's limited by assay sensitivity and also MS have the throughput issues so also lack in tool compounds for helping with the assay development.

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So now, I like to focus on the histone demethylases and about the assay that I'm going to talk about, which is LSD1 assay. First of all, as many of you already know, there are two types of histone demethylases and the first type is ion-containing hydroxylases exemplified here for by Jumanji D2A as shown here. The second type, which is actually discovered first, is the KDM1, which is a mono amino – I'm sorry, which is the MAO so monoamine oxidase. These are not ion containing. They actually have FAD in their active sites and because these two types have different mechanisms so the KMD type, the LSD1 type of methylases actually can only demethylate from dimethylated state down to unmethylated state.

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So I'm going to use LSD1 as one example not too much about biology of LSD1 except just saying LSD1 has been involved in transcription regulations of many different types of transcription complexes and it's acting as coactivators in some of the nuclear hormone receptor involved transcription and sometimes acting as company-repressors in some of the other tumor suppressor genes. So it's involved in different kind of cancer.

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So the current status for H3K4 demethylation assay such as LSD1 is mainly coupled enzyme assays or Western blot, ELISA type of detection with very, very low throughput. Also, there is LC/MS radioactive assays and even microfluidic based assays, which have somewhat a higher throughput and used in some of the HTSes.

Our goal here is, you know, to develop high throughput assays, homogenous, sensitive and high sensitivity and amenable to miniaturization and high throughput screening.

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So first, we looked at several possibilities. So we look at particularly something, which can sense the unmethylated site at H3K4 site. We

look at both, some of the reader molecules and the antibodies and the reader molecule is such as the PHD fingers. PHC80 for example is specifically bound to the unmethylated site on H3K4.

In the meantime, we also became aware as the antibody became available, which are also specifically bound into this unmethylated site. So we collaborated with PerkinElmer and labeled the antibody with Europium fluorophore and so the rest of my presentation is on this TR-FRET assay for LSD1.

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So first, we tested this fluorophore labeled antibody against the different H3 histone peptide and different methylation states. From our tests that we kind of confirmed what the literature is saying and this antibody is specifically bound to the unmethylated site of H3K4 and doesn't recognize or almost no activity, no binding activity or very low against the other site. It's also very sensitive to methylation at this site so only the unmethylated gives the bonding event, which is needed for the signal. We thought it's ideal for demethylated assays because it gives a signal increased readout.

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This just briefly shows you some of the data we got from this LSD1 TR-FRET assay and on the left panel, just a different enzyme and the reaction with different times. We used the histone monomethylated peptide as the substrate. So we got a signal to background over 10 and many of you are familiar with TR-FRET assay for this assay format, signal over 10, which means it's a really good signal and also the Z' as I will show is greater than 0.8. Also, yeah, it's very sensitive to LSD1 so it can detect sub nanomolar amount of LSD1, purified LSD1.

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This slide briefly shows you a comparison of the TR-FRET assay with LC/MS mass based detection and this is the same assay running in very similar conditions and one is detected with the TR-FRET assay the other with mass detection. As you can see in TR-FRET, it needs on the left panel, it's, you know, 20 to 50 nanomolar of substrate to give very good readings in mass based assay. It's less sensitive on the low side, so 20 nanomolar or less gives you a signal of very small signal, a little bit different from background but really not very quantitative. You'd need higher substrate concentration.

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However, the LC/MS seems like could give a linear range and the TR-FRET extending earlier and which is easy to understand because in the TR-FRET assay, we use very limited antibody detection, the detection reagent to minimize the cost, which will cause a hook effect if the product I mean is produced too much. So both have its pros and cons I guess but TR-FRET is more sensitive.

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This slide shows you compared TR-FRET with coupled enzyme assay, in this particular case is hydrogen peroxide production with Amplex Red as a substrate. So as you can see, the amount of enzyme needed for the assay is quite different. It's more than one order of magnitude different. So TR-FRET, again it's more sensitive in this case and both can give actually quite linear readouts.

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So TR-FRET compare favorably to both mass based and coupled enzyme assays and we also use some of the known inhibitors to further validate the TR-FRET assay. As you can see here, we used the three, one is 2-PCPA, which is the known LSD1 inhibitor, tranylcypromine, and we also used two other published H3 modified peptide, one is H3M4, one is H3R4. They are more potent than 2-PCPA. As you can see, they behave exactly like what the literature is reported in the TR-FRET assay. On the right panel to show you these two peptides even though they are derivatives from the H3 peptide itself, they actually don't have any effect in the TR-FRET detection.

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So this slide is again just to show you the TR-FRET assay signal robustness and also the data quality. So we got a Z' about 0.8 or so if we used a one-hour reaction in a linear signal range and also the assay seems like very robust to DMSO. It can tolerate up to 5% DMSO in our hands.

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So we used to this assay to run a pilot screening. So we miniaturized this assay to 1536-well format with a total volume of 6 micro liters and then we tested about 14,000 compounds, random compounds. So from the assay plate, we have a Z' factor about 0.8 on average and then we got a hit rate about 0.1 just a little shy of 0.1% at 20% activity. The minus sign here means inhibition. So the assay can identify very, very weak inhibitors even 20% inhibition as you can see here and also a comparison of run 1 and run 2 give very good correlations. So overall it's valid that this data is very suitable for

inhibitor screening and actually two of the weak inhibitors came out from this pilot test. They are actually 2-PCPA analogues. So we got I mean for our confidence for the assay used for inhibitor screening.

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So let me just summarize this part of this talk. So first we believe that both cellular and biochemical assays are still in need for high throughput assays especially for proximal or site-specific methylation detection. Secondly as you see that the TR-FRET assay presented here are a good suitable H3K4 site specific methylation sensing and it compares favorably with other assay format like LC/MS and coupled demethylase assays. We think it's ideal for inhibitor profiling and also high throughput screening.

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Finally, I'd like to thank the people who are involved in this assay development and this project. Thank you.

Sean Sanders: Thank you so much, Dr. Zhang, and many thanks to all of our speakers for the really fantastic presentations. We're going to go right away to questions submitted by our online viewers, but just a quick reminder to those watching us live. You can still submit your questions by typing them into the text box and clicking the submit button. If you don't see the box on your screen, just click the red Q&A icon and it should appear.

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So the first question I'm going to put to the panel is going to go to Dr. Jung first. What makes epigenetic targets technically special compared to other target classes like kinases and phosphatases?

Dr. Manfred Jung: Well, excuse me, it's the complexity of the pattern. So we cannot just look at the simple modifications. We have to maybe also take in account the modifications around it. So it's not so easy to follow up the activity and we also have the non-histone substrates, which also add additional complexity to the system.

Sean Sanders: Great. Dr. Porter Scott?

Dr. Margaret Porter Scott: Well so, you know, from a technical standpoint, the in vitro assay if you want to do them on a native substrate, that requires really using chromatin. So either, you know, recombinant methods are available for making nucleosomes, but they are very challenging compared to

other substrates from other classes. Or you can use native nucleosomes, extract native nucleosomes that's actually most of what we've done at Epizyme, but that of course brings all that complexity that Dr. Jung was talking about with it. So you may have marks already laid down in a heterogeneous way and then that's the substrate that also you need to try to work with in high throughput methods to, you know, try to mobilize in plates. It is definitely having a large heterogeneous substrate is something that's quite difficult for in vitro assays.

Sean Sanders: Dr. Zhang, did you have anything to add?

Dr. Ji-Hu Zhang: I will just a little bit. As Dr. Scott has said for in vitro assays also for cell based assays, these HMTs and HDMs, they don't have a specific signal pathway as clearly defined as the kinases. I think that that could be another, I mean, challenge and differences for this type of target.

Sean Sanders: Great. So the next question has come in a couple of different forms and maybe I'll start with you, Dr. Jung, to answer this. This viewer asks whether you can comment on substrate selectivity of epigenetic enzymes. Do you see variation in lead compounds identified from inhibitor screens depending on whether it's a peptide histone or nucleosome substrate that's being used?

Dr. Manfred Jung: There clearly are differences. For some compounds, it might be similar using different substrates, peptides, or small molecules. We have some examples with histone deacetylases. So it's not really to predict whether there is a difference. So it is good to have an additional assay and try to confirm the assays run for example in a high throughput screen and a secondary alternative screen.

Sean Sanders: Excellent. Dr. Porter Scott, did you have anything on this topic?

Dr. Margaret Porter Scott: Yeah. So this is – you know, we actually have -- wherever we can, we've compared these substrates, but I'm afraid the answer may not be – you know, there's not only one answer. So we have seen enzymes where peptides basically reproduce exactly what you see with the full length substrate and in cells and we've also seen cases where the enzyme kinetics and the inhibitor profiles are quite different using surrogate substrates or peptide substrates from the native substrates themselves. So we've seen – you know, sometimes you can get lucky and get away with a small peptide, but what you can never do is go wrong with using the full length native substrate.

[0:55:19]

Sean Sanders: Great. I'm going to stay with you, Dr. Scott, with a question that came in a short while ago asking about personalized cancer therapeutics and they ask whether these cancer cells are isolated from certain clinical patients and will their cells be mutated or not?

Dr. Margaret Porter Scott: Well we've been working with established cell lines in our research so far and then how we are monitoring the – you know, then certainly where the patient samples comes in are really the PD marker, the biomarkers that are used in the clinical studies.

Sean Sanders: Great. Dr. Zhang, a couple questions that came in for you. One is just a quick technical question, this viewer asks whether the H3K4 site-specific methylation sensitive antibody is commercially available or is this something that you developed?

Dr. Ji-Hu Zhang: Right now, it's chemical – I mean it's commercially available. As I said, we searched and we became aware of this antibody and collaborate with PerkinElmer and now it's actually selling as a commercially available reagent from PE.

Sean Sanders: Great. So I'm going to ask a slightly broader question on antibodies since a couple of you have mentioned them. What about the availability of genetic modification specific antibodies, are there enough out there, are they useful, are more being developed?

Dr. Manfred Jung: Well that's one of the major problems of the field not always the specificity that is claimed by the companies is really happening in the assay. So it's good to have peptides to actually probe the antibodies yourself and compare different companies, compare maybe even different batches from the same company to really see whether you get the selectivity that you need.

Sean Sanders: Great. Any other comments, Dr. Zhang?

Dr. Ji-Hu Zhang: Well I think well compared to a couple of years ago, there's certainly more available antibodies. But again, I agree with Dr. Jung, you have to test the antibody for its specificity, selectivity, and also well for depending on what do you use for them, it's actually a lot of times these things are not cheap. It's very expensive.

Sean Sanders: Great. So this is a question about something that Dr. Jung mentioned in his talk about specificity and the viewer asks will not epigenetic

drugs act through the genome and hence be too nonspecific in their actions and have too many adverse effects? I know Dr. Porter Scott, you has a slide up about specificity on your talk as well, but Dr. Jung maybe you can address that?

Dr. Manfred Jung: Sure. They act at the transcriptional level so probably even with a highly specific epigenetic drug, you might affect actually a couple of hundred downstream targets. But manipulating transcription that's a very much established principle in approved drugs. So for example, glucocorticoids in asthma or sexual hormones, they also affect transcription and this can be done safely.

Sean Sanders: Dr. Porter Scott?

Dr. Margaret Porter Scott: Well, yeah, so I think the story is you have to be told on the kind of specificity that Dr. Jung mentioned in vivo. But in vitro specificity that we're able to obtain and monitor for these HMTs actually is very good. I mean they are not as similar to each other as for example kinases and so with good medicinal chemists so far we've been able to find the selectivity profiles that we want to find and, you know, make very selective and potent molecules. And following that up in terms of – you know, I did have that one slide where we showed the selectivity on the methyl marks and so that seems to be possible. I think it was more of a concern when we started and now we sort of proved to our self that our cell selectivity is definitely achievable.

Sean Sanders: Great. So we're unfortunately at the top of the hour so we're just about out of time, but I'm going to pose just a last question to all of you and Dr. Jung I think I'll start – sorry Dr. Zhang, I'll start with you. So this question is about the relevance and importance of these epigenetic modifications relative to the development of DNA methyl transferase inhibitors and the viewer asks which is more clinically relevant. But maybe we can also talk more broadly about the clinical relevance of what's currently being developed and how you see them being applied in the future particularly to cancer. So Dr. Zhang?

[1:00:32]

Dr. Ji-Hu Zhang: Could you repeat the question? Sorry.

Sean Sanders: Sure. So the viewer is asking about comparing the work that you've discussed with DNA methyl transferase inhibitors, what is the relevance of the work that you're doing to DNMTs and also more broadly what is the clinical relevance of the current work and how it will be applied to cancer research?

Dr. Ji-Hu Zhang: Yeah. Well HTMs and HMTs are, you know, two heart of the – just like kinase and phosphatases and they both play roles in transcription regulation and so on and so forth. So I think the will – I mean it's maybe not as much you heard as the HMTs, the demethylases, but I think people – I mean people are working in this area as well. In term of what is the current status in clinical trials, I'm actually not very clear. I don't know if others want to elaborate on that or –

Sean Sanders: Dr. Porter Scott?

Dr. Margaret Porter Scott: Yes, sorry. I don't think I can comment very well on that either.

Sean Sanders: Okay. Dr. Jung, anything to add?

Dr. Manfred Jung: I mean with the DNA methyltransferases we already have two approved drugs, but basically no drug discovery below it. So here, we have a very strange situation. So they are already clinically used and in terms of cross-talk, we have seen that histone modification sort of talk with each other and of course there's also the question how histone modifications and drugs targeting those will cross-talk to DNA methylation. So there's also a big potential for combination therapy of epigenetic histone inhibitors and DNA methyl transferase inhibitors.

Sean Sanders: Uh-hum. Excellent. Well we are unfortunately out of time so it just remains for me to thank our speakers very much for providing great talks and very interesting discussion, Dr. Manfred Jung from the University of Freiburg, Dr. Margaret Porter Scott from Epizyme, and Dr. Ji-Hu Zhang from the Novartis Institutes for BioMedical Research.

Many thanks to our online audience for the excellent questions that you submitted. I'm sorry that we didn't manage to get to all of them.

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Again, thank you to our wonderful panel and to PerkinElmer for their kind sponsorship of today's educational seminar. Goodbye.

[1:03:48] **End of Audio**