TRANSCRIPT

Epigenetics in Hematologic Malignancies: Pathogenesis and Therapy

December 9, 2011

Slide 1. Title Slide

Dr. Rick Winneker:

My name is Dr. Rick Winneker. I'm the Senior Vice President for Research at The Leukemia & Lymphoma Society. I want to welcome everyone to this continuing medical education symposium, focused on Epigenetics in hematologic malignancies. A very exciting area.

The Leukemia & Lymphoma Society is not only a leader in providing up-to-date information about emerging blood cancer treatments to patients and also to professionals, but we're also involved, of course, in funding the research that's needed to advance these new therapies.

Last year alone we invested over $76 million in cutting edge research around the world, across all hematologic malignancies and for all phases of investigation, from basic research through pivotal registration trials for promising new drugs. We're also engaged in policy initiatives to help create a future in which all blood cancer patients have access to the best treatments available. I'm sure that you'll all agree that this is extremely critical, now more than ever.

So before turning the podium over to Dr. Irv Bernstein, I'd like to thank Millennium Pharmaceuticals Incorporated, Celgene Corporation and Allos Therapeutics for providing the grant support for this symposium, as well as our sponsors, Robert Michael Educational Institute and the Postgraduate Institute for Medicine.

So now I'd like to introduce our moderator. Dr. Irv Bernstein is Chief of the Division of Hematology-Oncology at Seattle Children's Hospital, Director of the Division of Pediatric Hematology-Oncology at the University of Washington School of Medicine, and Head of the Pediatric Oncology Program at the Fred Hutchinson Cancer Research Center, and a very long time advisor to The Leukemia & Lymphoma Society, and currently the Chair of our professional education subcommittee. Dr. Bernstein.

Dr. Irv Bernstein:

Thank you. We're really pleased that a number of us on this committee, part of the Medical and Scientific Affairs Committee of the Society, each year strives to develop this symposium that really focuses on some of the cutting edge areas, to really present a program that goes from really fundamental aspects, all the way up through translational aspects. And in that order.

Slide 2. Overview of Epigenetics and its Role in Malignant Transformation

So at this time let me introduce our first speaker, who will really provide a background understanding of epigenetics, one of the leaders in creating the field, Dr. Steve Baylin. He's Deputy Director of the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins and Research Professor Oncology. He'll now present an overview of epigenetics and its role in malignant transformation.

Dr. Stephen Baylin:

Thank you. I'd like to thank the organizers for inviting to me to what I think will be a great session.

My role really is sort of to provide a background and to perhaps provide substrate for things that are going to be revisited by each of the speakers in this complex area of epigenetics as it relates to the fundamental role of what we call the cancer epigenome, perhaps in the pathogenesis of cancer, and to the very exciting potential translational and therapeutic opportunities that the knowledge is giving us.

Slide 3. Disclosure of Conflicts of Interest

So if I could have my first slide change. Really for this talk I don't have any conflicts, but I do have the following affiliations that are noted here on the screen.

Slide 4. Epigenomics

So for those of you who don't work in the arena, let me first just give a simple concept of what epigenetics really is. The hard drive for everything that our cells can do, either right or wrong, is DNA, which obviously has all the information in it to encode for all cellular functions and cellular responses, but a hard drive can't work without a software package or packages to really tell it how to function and what to do and in a sense this is what epigenetics really is. It's the way we package our DNA with this elegant structure of the nucleosome as a central architect for this around which we wrap DNA, modifications of the tails of the histones that are fundamental components of those nucleosomes, and the way we move the nucleosomes around for higher order structures.

So what's shown here is a gene perhaps which is in a ready state to be transcribed or being actively transcribed with an open nucleosomal confirmation here, if that same region harboring around that gene is changed into a more closed and higher order nucleosomal structure, shown here, that gene can be suppressed to be available or in an off state.

Now since the hard drive didn't change to do that, if that were fundamentally abnormal switch, say, to a repressive domain in a cancer, the therapeutic hope would be that by modifying that we could bring the gene back to normal function. One example of that is that you can have abnormal DNA methylation at cytosines next to guanasines, which is a transcriptionally repressive sort of component in the main, and we have drugs that can take that methylated cytosine back to acytosine and this could theoretically revert that abnormally repressed gene back to an open status. This relates also to the histone modifications and the tails you can hear about. You could have abnormal individual regions of the genome or in general have that same situation for abnormal histone modifications in drugs that could revert those aspects as well.

Slide 5. Altered Chromatin Status in Cancer

This is a cartoon, a very cartoonish way of depicting the normal versus the cancer epigenome. Our normal genome is actually packaged away in a large part in repressive chromatin of higher order nucleosome structures, histone modifications of a repressive nature, and is shown by the black lollipops here, most of the candidate cytosines next to guanasines in those regions are actually DNA methylated.

And at the same time there are genes, particularly those that have so-called CpG islands surrounding their transcription start site, that are maintained in a more open confirmation, are transcription ready, and fundamentally most of those genes, this CpG rich area is not DNA methylated. So that's the normal landscape.

Cancer looks very imbalanced with respect to this. You have many of the areas which should have

DNA methylation that appear to have lost it and may be in a more open confirmation, and the very same cells, as I will show, you can have hundreds of genes with DNA methylation being abnormally imposed or other modifications in abnormally repressed states. So we have this fundamental imbalance that we look at.

Slide 6. Cell Culture

Let's look at the spectrum just for DNA methylation for CpG islands in cancer. And this is the data that the Cancer Genome ATLAS Project is compiling for just the tip of the iceberg in the way you would screen for epigenetic abnormalities, but this is the Infinium platform from Illumina. It's annotated on the heat maps I'm showing here, only for CpG island regions in proximal promoters and most of these are protected from DNA methylation as I told you. And you can see this because the green on this map means a lack of DNA methylation at the probes being queried in this hybridization assay. And all the green right here are normal cells, ranging from embryonic stem cells to adult progenitor cells to differentiated cells. And all these genes for these probes are green because they're not DNA methylated in these regions. The red, which is maximal methylation, you can see peppered through hundreds of genes here in these 50 cell lines, which are represented right here, which are different forms of cancer, which cluster differently according to patterns. These are leukemias right here, which have a lot of this particular abnormality change. The blue is lung cancer, pink breast cancer and so forth.

Now you can take the probes that identify these changes in these cell cultures, take those same probes and go back across hundreds of primary samples, DNA, in this case let's take the breast probes here, go across the primary breast cancers in the Cancer Genome ATLAS Project and you can see that many of these same genes, again as shown by the red, are DNA hypermethylated here, in colon cancer, here's the normal, more green here, and so there are hundreds of these genes. We don't know which of all those hundreds may drive tumorigenesis in each state, some may be just passengers, but it shows you the distribution of this particular epigenetic change in cancer.

Slide 7. Considerations Key to Probing the Functional Significance of Epigenetic Abnormalities in Cancer

So what are our challenges here as we go through? Well, as I'll show you, genetic changes and epigenetic changes are seamlessly being matched through latest studies and I'll talk quickly about that. There may be vulnerability of groups of genes to undergo particularly changes by their location in the genome and in nuclear architecture. I'm going to talk briefly about that. Understand the epigenetics of development, what happens during embryogenesis and how normal epigenetic changes go really are becoming ever more fundamental to understanding the things that cancer cells may do wrong. And I'm going to point to some of that. This may even help us learn about the origins within particular cell renewal systems in adults, about where particular tumors start out and how much plasticity for movement in those tumor cells there is.

And we must think not only as individual genes, but groups of genes that become altered, and this sometimes challenges the mentality for interpreting the changes and it's really fundamental to thinking about the ways that we target the epigenome therapeutically as well.

Slide 8. Familial Cancer Genes

So there are driver genes that get abnormalities in epigenetics that are fundamental to their loss of function for tumor suppressor genes. These are listed here, a group. These are genes that can have mutations in the germ line and cause family cancer syndromes. They can be mutated in somatic tumors. Or

they can have hypermethylation of DNA around their promoters, which is an alternative to the mutations for loss of function. We understand these, people accept these as tumor suppressor genes. Those other hundreds of genes are more the question.

Slide 9. Typical Gene Methylation Pattern Within a Partially Methylated Domain in Cancer

I want to show you now some of the ways people are using latest technologies to measure DNA methylation changes in cancer and what these are teaching us across the genome because they're becoming extremely important to what we understand.

The work I'm drawing on here is from ___ working with Peter Laird at University of Southern California, just out in Nature Genetics. And what it shows is the use of sequencing virtually every cytosine next to a guanasine across the genome for the distribution in a couple of colon cancer samples versus normal colon samples. And I want to show you what sticks out in this kind of analysis.

So what you see here is a region on chromosome 15 of 5 megabases, in which the normal colon cancers here and the colon cancer versus the normal is being repaired, and what you see here in this bottom line analysis is the drop in the green means loss of DNA methylation in the tumor, which around this gene right here is right in proximity with the gain of DNA hypermethylation in the CpG island or around the promoter, and then loss on either side. And what they've fundamentally shown is these imbalances or what appears to be an imbalance between losses and gains, are often in the same regions next to one another in megabase stretches of DNA across different chromosomes. So what we thought was kind of a willy-nilly loss in gain is really tied fundamentally regionally to certain areas of the genome. And this is becoming extremely important.

Slide 10. Partially Methylated Domains in Cancer

Here's another example, more broadly, across chromosome 3p, but the same kind of a thing. Loss is here and now because you're looking at the whole region, you see these little upwards ticks in red, which are surrounded by the losses in methylation in green. Those are CpG islands for genes that reside in those loss areas. They gain it at the promoter, while the regions surrounding lose it. And it turns out that these are primarily regions that they've attached to late replicating regions of the DNA during DNA replication and cell cycling.

Now why is that important? Well, this is based on ___, which relates to human as well. These late replicating regions of the genome in embryonic stem cells harbor a group of genes, which I'm going to mention, which have a histone modification called polycomb marking, and a particular silencing histone modification that I'll mention in a moment that goes with that polycomb marking. At the same time they have a balancing active histone modification at their start site. And this is something that our chairman's so, Dr. Brad Bernstein at Harvard actually described as bivalent chromatid. It's a chromatin that holds genes in embryonic stem cells in a poised low transcription state, sort of awaiting signals during cell fade as to what to do. And it turns out that these are the genes, a large body of which are vulnerable to this abnormal DND methylation, as I'll mention in a second, that we get in cancer cells. So they're tied to these regions.

Slide 11. Genomic vulnerability

There are also critical proteins that protect against DNA methylation that reside at the start sites of these genes in this region. And the fundamental message I'm sending you is these may be vulnerable areas of our genome for certain kinds of epigenetic changes in cancer.

Slide 12. A View of the "Cancer Epigenome"

The next thing we have to do is come back to that imbalance in cancer epigenome and relate this to a really important theme that's coming out in cancer from the Cancer Genome ATLAS Project and individual laboratories that are doing deep sequencing analyses of tumors. Because increasing they're describing mutations in those very genes that maintain the epigenome. The genes that move the nucleosomes around, the nucleosome remodeling genes, genes that lay down DNA methylation or like the Tet proteins that you're going to hear about in IDH1 mutations that protect against DNA methylation potentially, and genes that modify our histones, put down the modifications as well, such as the gene EZH2, which ___ that polycomb mark in the bivalent chromatin that I talked about.

So we have to now consider something that's very important, is that the epigenome may be tremendously important in its abnormalities, as some of them lie downstream from genetic mutations. And that has a fundamentally, a therapeutic concept, is that it may be much easier to target reversal of the epigenetic abnormalities downstream of those mutations that it is to go directly after the mutation itself.

Slide 13. Dynamics of CpG island DNA Hypermethylation - the 2011 View?

Examples, which you're going to hear more about, are mutations in IDH1, which are mutually exclusive in leukemia to mutations to Tet, probably because the IDH1 mutation lead to inhibition of the Tet enzymes. And that theoretically leads to a loss of protection against DNA methylation. You're going to hear about that. And one can raise the question is that kind of a mutational spectrum targeting these vulnerable areas of the genome, where the gene's likely to harbor ___ reside.

Slide 14. Evidence for an Instructive Program

Coming back to this bivalency, I wanted to show you a little bit more what I meant by that. So the bivalent mark that polycomb lays down is a modification on lysine 27 of histone H3 of trimethylation. And that's the polycomb mark. And it's balanced by the presence of an activating mark, methylation on lysine 4 of histone H3. Bivalency. And a couple of years ago, few more than, our laboratory, Peter Laird and his colleagues, ___ and colleagues and then ___, a number of laboratories have shown this high prevalence for the genes, for example, in colon cancer, greater than 50% of them that get DNA hypermethylated to harbor polycomb and/or this bivalent mark.

Slide 15. Easwaran, Van Neste, & Johnstone

We have a group with Hariharan Easwaran leading the way, that's looked at it even more deeply, looking broadly at both histone modifications and DNA methylation changes across cancer genomes. And I'll just show you the bottom line fundamental thing that we find.

Slide 16. Mapping the Chromatin Lineage History of Genes DNA Hypermethylated in Cancer

The orange bar here shows you the number of genes that are DNA hypermethylated in our search and these different kinds of lung cancers that have bivalent chromatin, and you can see it's 80% of them in embryonic stem cells. But what I think is really important is that we find that 70 to 80% of genes in hematopoietic stem cells, normal hematopoietic stem cells isolated by various markers, also harbor these genes in a bivalent context. And that gives you an idea that starting even with adult stem cells and progenitor cells we can learn where those vulnerabilities are that may dictate a large group of genes that fundamentally becomes abnormally DNA methylated in cancers. If you take the 50 top frequency DNA

hypermethylated genes we found from all of these different tumors and map them back, here are the embryonic stem cells and here are the hematopoietic stem cells. So this is a relationship in adult cell renewal system as well.

Slide 17. Developmental Pathways and Nature of Genes DNA Hypermethylated in Cancer

And what are these genes that are in these groups of bivalent genes and marked? Well, they set up an important hypothesis because if you look at the genes that are bivalently marked that get this abnormal DNA methylation change in cancer, they are really hugely grouped not only to developmental genes, but developmental genes which are transcription factors, DNA binding genes, types of genes that could be very important. And this sets up a hypothesis that I think is worthy and getting a lot of testing. Because if these genes are bivalently marked, that's a plastic marking, they're available, they can respond to signals coming in to go up or down, determining on the lineage of the cell and what the cell needs to do.

Slide 18. Model for Molecular Progression to DNA Hypermethylation of Many Genes in Cancer

The DNA methylation mark tends to be associated with much tighter silencing and raises the question of these 70, 80% genes and their patterns in different tumor types, is this abnormally tight silencing, preventing those genes from coming on, a way that we can lock cells in more primitive states than they should be with loss of maturation capacity or retain self-renewal than they should have, and of course, those steps are fundamental to tumor genesis, an important question.

Slide 19. Dynamics of CpG island DNA

Another thing that our laboratory has recently published on that I think is important to consider is what are the environmental changes, what are the things that we do to our genome that might target those vulnerable areas. And we've done some work recently relating to inflammation that suggests that this is a process through either myelodysplasia in the setting of having exposure to previous chemotherapies, environmental changes of many types for solid and liquid tumors, how might this play a role.

Slide 20. Molecular Determinants of Cancer- Cell Stress

And Heather O'Hagan and Wei Wang in our lab have led an exercise in mapping some of these chromatin changes across the genome after exposing cells to an induced reactive and oxygen species increase by giving hydrogen peroxide to these cells. And I'm just going to give you the bottom line.

Slide 21. Transcriptional Consequences of ROS Induced Stress

What this shows is an array of protein binding across chromosomes. This is chromosome 21. And the up-ticks mean that the proteins go to a certain region, this being the more telomeric region of chromosome 21. And you can see the critical proteins for establishing DNA methylation, ___ 1 and 3B, the damage that the reactive oxygen species causes in this particular chromosome, and I'll show you polycomb constituents, all seem to move 30 minutes after that exposure of the cell to this telomeric region of the chromosome, which harbors ___ richness and CpG island. It's a little differently distributed on every chromosome. And this is where the CpG islands would be.

At the same time the yellow shows you where the damaged product for reactive oxygen species goes. So it's targeted to these genes with CpG islands, but not so much to this gene, which is not a CpG

rich gene. And what you can see for this kind of targeting, which includes the EZH2 component of polycomb, is there's a transcriptional ramification for this transiently. Even inactive genes like CMIC, during that first 30 minutes, gets a down regulation of this active mark for H3K4 trimethylation, an up regulation of the polycomb mark, and a down regulation of another active mark.

So what we're saying is if you were pounding on the genome in this way and particularly these late replicating regions, if you were to leave some of that machinery in place, would you set up the abnormalities. And evidence that this may be the case is in these late replicating genes, which are not DNA methylated in this cell line that we're working with, but as a function it seems almost of decreasing basal expression, that 30 minutes after reactive oxygen species, you can actually see the onset in the sequencing for DNA methylation, of an increase in the number of CpGs in the start site of that gene, starting to go up. So again, it raises the hypothesis that vulnerable areas matched with assaults like inflammation and the kinds of changes we're showing you in machinery, could be a factor here.

For the last few minutes let me just turn to some fundamental aspects of the therapeutic possibilities for a change like DNA methylation. And you're going to hear this revisited and I'm just going to give you some preclinical data that I hope will set the stage and which we think is important.

So many new drugs are coming into the arena for targeting individual epigenetic abnormalities, you're going to hear about some of those, or for potentially targeting in a broad way this cancer epigenome that we talked about.

Slide 22. Epigenetic-mediated gene silencing in cancer

In our Stand Up to Cancer project that I co-lead with Peter Jones, a challenge, we're trying to take old drugs that are out there, which Peter Jones showed like 5-azacytidine can erase DNA methylation with cell replication, histone deacetylase inhibitors, but use them in a way that stresses not their cytotoxic effects, but their lower dose related non-immediate cytotoxic effects, raising the question can they reprogram this epigenome. And so in trials we're using DNA methyltransferase inhibitors at low dose along with HDA inhibitors, the concept being can you take abnormally closed chromatin, like this, with DNA methylation, and return it to an open state that would get more normal function for particular groups of genes.

And our challenge is not to just figure out how they do in trials used this way, but what are the mechanisms, what is underlying that efficacy.

Slide 23. Pre-clinical Studies of Low Dose Aza and DAC

So in work with my colleague Cindy Zahnow and her lab and her post-docs Huili Li and my graduate student Hsing Tsai, we have taken the following approach in the laboratory to try to look at what turns out 5-azacytidine and deoxyazacytidine may actually be remarkable drugs in terms if they're used at doses that are proper.

Slide 24. Memory Effects of Low Dose 5-Aza-cytidine

The approach we've taken is to try to get very low doses of these drugs for leukemia and for solid tumors, expose cells either in culture or now working with primary cells in leukemia and breast cancer, to very low doses over three days and then take the drug away and the cells never see the drug again. And we look for a lack of acute cytotoxicity after that first three days in terms of cell cycle check, DNA damage and apoptosis. And then we can rest the cells and give them various in vitro assays, put them back into mice xenographs and increasingly we can do things like enrich in these breast cancer cells for self-renewing populations and sphere assays. Take the drug away, passes these cells in the sphere assays, look

at the effect on their ability to grow back and self-renewal assays here, or put them after that back into orthotopic locations to see what their phenotypes do.

So what we first know, for instance, in leukemia, 5 to 10 nanomolar doses of deoxyazacytidine will cause anti-tumor effects and at the same time if you look back at this Infinium platform, you can see here's the peak for abnormal DNA methylation on the histogram here, here's following 10 nanomolar ___, so they do reduce the amount of DNA methylation. Our challenge is to associate those changes with the efficacy we're seeing.

They also induce, and this is the ___ platform if we're looking at gene re-expression, not only do they induce gene re-expression, but for days after withdrawal of the drug some of these genes will stay up consistent with the type of reprogramming we'd like to see. And then we can match that with phenotype and what do we see?

Slide 25. DAC (10nM) "Memory" Blunts AML Tumorigenicity

Here are cultured Kasumi leukemia cells exposed to 10 nanomole of the drug. These cells, their CD34 population will reconstitute leukemia in an immunocompromised mouse. The CD34 negative will not. Both those populations have marked blunting of their ability to clone in methylcellulose after 10 nanomolar exposure. If you put those cells back into a mouse, don't treat the mouse, you can see this marked blunting here of the ability of those cells to cause the leukemia in the mouse.

If we do this with primary cultured leukemia cells that have never seen plastic, and look at the effects of this kind of transient exposure on that methylcellulose cloning, you can see that, for example, I guess there's six lines – five of the six lines here, this one particularly, are really blunted again for that ability to clone in soft agar.

Slide 26. A "Memory" Effect for nM 5AC on Sphere Assays of Primary Breast Cancer Cells

Now this, coming to the final, is a breast cancer experiment, using a mammosphere assay for self-renewal. And what's been done here is to take primary cells from four individual women from their pleural effusions, they have luminal disease, luminal breast cancer, do the transient exposure and look at the ability to form these spheres. And you can see in the primary assay a marked reduction in the ability of these cells to form the spheres. You can then passage these a second and a third time and you can see that they maintain or remember that depletion of that cell population or its ability to come back remains quite suppressed throughout three or four passages of these cells.

Slide 27. Pathway Transcriptional Changes After Low Dose 5-aza-cytidine

So what subserves this change? This is our challenge. We're learning, we don't know completely, but in the last two slides I will show you that these changes, after you ___ the drug and grow them in drug-free media for a few days, at the time they're put back in we can find decreased activity of mitotic activation pathways, cell motility and invasion pathways, increased cell maturation, activation of a myriad of immune changes, which are really interesting, too, it's not just antigens put back on the surface, but the whole immune pathway and cytokines that could be very important to the responses that we see in patients, or decreases in key signaling pathways such as ___.

Slide 28. "Memory" Effect on Cell Cycle events

This is an example. Here are two ____ samples. One that has a DNA methylation drop after the transient exposure in the red line here. Just upstream actually of a non-methylated CpG island in this case,

P21. The other does not. The P21 is activated in this cell line and concomitant to that a target of P21, the whole FoxM pathway, multiple transcripts in this pathway, which is necessary to go into cell cycle, and when blocked can also sensitize cells to subsequent cytotoxic therapies, all are coordinately down regulated in this sample here. And we have multiple examples of this.

Slide 29. "Memory" Effect on AML, CD34+ Maturation

And finally, maturation of cells I mentioned. Here's the MetaCore pathway for a particularly, I believe this is the Kasumi cell line of AML, in which all the red ticks at multiple time points after drug administration here, mean up regulation of a series of genes, some are early myeloid progenitor genes, and these oxidative burst enzymes here actually go up in mature myelocytes. And you can test that in the laboratory. Have we produced cell maturation.

Slide 30. Epigenetic-mediated gene silencing in cancer

So here's a primary leukemia cell, same protocol for treatment, and you can see marked up regulation of the mature marker CD11 on the surface of these cells. Meaning that may be one mechanism by which we get an anti-tumor effect.

So I'll stop and leave this slide here. I hope it's been an introduction for you. There's a myriad of potential and possibilities that you're going to hear about at a fundamental level and a therapeutic level from the speakers to come.

And again I thank the organizers for inviting me.

Dr. Irv Bernstein:

Why don't we take some questions after each talk and we may have a panel discussion after, but if people have burning questions this would be a great time.

Dr. Stephen Baylin:

Glad to take them or entertain anything that people want to know.

Audience:

I'm just curious whether you think the sensitivity to these drugs is going to be differential among, let's say, cancer stem cells versus the bulk population and to what extent that complicates interpretations.

Dr. Stephen Baylin:

It could complicate interpretations and yet we're excited about the fact that we find as sensitive as any in the populations. And in fact when we enrich for the stem cells in breast cancer, we're seeing A, better results when we do these tests in primary cells, and B, we've been enriching four of those self-renewing populations in those cells. So our preliminary data might suggest that this is a very good way to target those so-called stem-like or whatever your term for it is. So we've considered that a plus, but I guess there is a complication in interpretation that needs much more work, no question.

Dr. Irv Bernstein:

And extension of that question, you would also expect changes in normal cell counterparts as well as non-targeted cell populations. Would you expect that these would be corrected or this is potentially oncogenic in itself and to what extent can you combine different pathways in addition to ...

Dr. Stephen Baylin:

This is also a great question because you'll read in the literature breast cancer, for example, Bob Weinberg's group, Nelly ___, they've seen cell lines that seem to get a growth enhancement of it. And I think it very much relates to where the cell starts and we're very cognizant in our breast cancer trials, we have a few lines of breast cancer that look to be in that short transient assay up-regulated for their growth. And many luminal lines that are down-regulated. And we're trying to put pathways together and understanding. Our breast cancer trial has started. So far the first nine or ten patients have tolerated it very well, but we're looking very carefully for anything that correlates to enhancement of growth. So other targeted therapies have the same situation, so this is something that you have to watch for very carefully.

Slide 31. Drug Discovery for Epigenomic and Transcriptional Targets in Hematologic Diseases

Dr. Irv Bernstein:

Thank you. For those of you who don't know Jay, he's an Attending Physician in hem-onc and Assistant Professor of Medicine at the Dana-Farber Cancer Center at Harvard Medical School. He's doing some very exciting work, so we're really pleased that he was able to make it here and I think you'll find waiting for it worthwhile. He'll discuss Drug Discovery for Epigenomic and Transcriptional Targets in Hematologic Diseases.

Dr. James Bradner:

Thank you.

And thanks to the organizers, and particularly The Leukemia & Lymphoma Society for including our research in today's – what I've learned by email and text pages – historic session.

As was mentioned, I run a discovery chemistry lab at Dana-Farber Cancer Institute and the principal focus of our research is the discovery, chemical optimization, clinical translation of novel small molecule modulators of gene regulatory targets.

Slide 32. Disclosure of Conflicts of Interest

I will mention to these conflicts of interests during the course of this lecture, but it's a good time to point out that a major clinical ambition of our research is to deliver new, hopefully creative prototype drugs to the clinic for therapeutic translation and we can't do this alone. So these are the companies that we've worked with to do that.

Slide 33. Targeting Gene Regulatory Complexes

So there are really three discreet areas of interest for our lab and they resonate with what is going on more broadly in the space of chromatin and transcriptional drug discovery today. That is to target the dominant oncogenic transcription factors themselves, the so-called master regulatory proteins, to target chromatin-modifying enzymes, and what I'll focus on here is a new area of interest for our lab, which is to abrogate protein interactions, critical to chromatin-dependent transcriptional signaling, by interfering with the recognition of chromatin by histone-binding domains.

Now really there are four ways in which our lab can make contributions we think. First, to create platforms for ligand discovery. You might know that there really aren't many drug discovery platforms for these types of targets. You can't order the bromodomain assay kit from Promega yet. Secondly, is when we use these platforms and identify novel molecules, we have the ability to freely distribute these to academic and industrial researchers worldwide, to study various aspects of disease biology. And then we're able to team up with translational researchers, and so much of the work I'll talk about today is a

collaboration with Constantine Mitsiades, also from the Farber here today, we can define a rationale that creates a very clear path forward for therapeutic development. And I'll try to use the rest of this time to justify that we do any of this.

Slide 34. Transcription and Chromatin Factors

So if you look around the space of gene regulation, there really are some fantastic cancer targets. And perhaps only owing to the perception that these targets are "- undrugable", which in the parlance of our field is as yet not drugged, that there really have not been coordinated efforts in drug discovery for gene regulatory proteins. In dark font are some of these genes that express gene regulatory proteins, which are primarily activated, as you heard in Ross's and other talks today, in blood cancers. And then in gray, outside of these more obvious oncogenes, are an ever-growing list of cancer dependencies, that maybe underlie tissue specification. Of course, to be prostate cancer you have to first be prostatic tissue and thus androgen receptor is a great target. And what we're learning from the developmental biologists is that there are a lot of really fantastic targets that underlie the hardwiring of the actual blood lineage in which this cancer is evolved.

Now beyond the transcription factors, which I'll admit are very difficult to drug, there is a growing list of chromatin factors that are very important for cancer pathogenesis. And I'll talk today about two bromodomain proteins, BRD3 and BRD4.

Slide 35. Pharmaceutical Inhibitors of Gene Regulation in Cancer

Now where available, small molecule modulators of gene regulatory proteins are some of the most impactful substances we have in the treatment and prevention of cancer. You think of all-trans retinoic acid in acute promyelocytic leukemia or the first targeted therapy, tamoxifen, modulating the estrogen receptor. But beyond these sort of transcription factor inhibitors, there is a growing list now of chromatin-modifying enzyme inhibitors, inhibitors of DNA methyltransferases as has surely been discussed in some length by Dr. Baylin and others here already.

Slide 36. Platforms of Ligand Discovery

One of the problems for approaching a new gene regulatory target is that there really isn't a biochemical capability of studying that target in a small miniaturized 384 or 1536 well plateassay: a suitable platform for the discovery and then subsequent chemical optimization of an advanced therapeutic agent. And so I cut my teeth in discovery chemistry, learning how you might create discovery platforms with gene regulatory targets in mind and we printed small molecules in microarray format to wash these largely binding proteins as they function in the cell, over these arrays, looking for novel interactions, that then the molecule that binds might actually prove to be an inhibitor.

Or for panels of enzymes like the HDACs and now lysine methyltransferases and lysine demethylases, we create robust platforms of chemical optimization with miniaturized homogenous assays and where available, we have found structural information to be particularly useful to guide medicinal chemistry to improve the fit of our molecules into active sites.

But unlike a lot of chemistry labs, we are sort of equal opportunity employers of chemistry. We don't care so much what the molecule looks like, but rather how it functions. And consequently molecules that are natural products or products of what's called diversity-oriented synthesis or even these perfect small drug-like pharmacofores are all quite appealing to us.

Slide 37. Chromatin Chemical Biology

Now I do want to make mention that a major area of interest in our lab is to create what we call next generation histone deacetylase inhibitors. There was a perception that the molecules that were first delivered to the clinic were very non-selective agents. And what we learned by studying their biochemistries in very sophisticated assays is that they're actually quite selective, just redundant for hitting histone deacetlyases HDAC1, 2 and 3. And that's what you would get if you develop molecules to hyperacetylate chromatin, but there are of course 18 of these enzymes. And we have tried to open up the therapeutic window of HDAC inhibitors by creating very soft HDAC inhibitors, molecules that you can put on your skin for cutaneous T-cell lymphoma in early stage disease. Patients in the dermatology clinic will never get sick enough to warrant feeling sick on an HDAC inhibitor. And you could accomplish very high local exposure by programming the molecule chemically, the positioning of this simple ester bond, to undergo rapid pre-systemic hydrolysis by these ancient esterases in the bloodstream, butyrylcholinesterase in particular.

And when we started treating mouse models of cutaneous T cell lymphoma with very advanced disease in Tom Kupper's lab, hair started to grow, at which point we thought we're rich. But it turns out that the reason the hair was growing is that the cancer underlying it was going away and that this is a useless model of male-pattern baldness.

But this compound is now open and enrolling in a multi-institutional clinical trial as a first on-man clinical trial with support from The Leukemia & Lymphoma Society at Shape Pharmaceuticals.

Also to expand this therapeutic window, we've been developing isoform-selective inhibitors, hitting individual HDACs that we think are really important for certain biology, in this case HDAC6, which is unfortunately named a histone deacetylase because it lives in the cytoplasm, where tubulin is, and that's one of its principal targets. But by inhibiting HDAC6 and combining this type of molecule with bortezomib or other proteasome inhibitors, we can accomplish extraordinary synergistic killing of multiple myeloma cells, cells leveraged for sensitivity to inhibition of protein degradation pathways because, of course, they elaborate such large amounts of immunoglobulin. These molecules are open now and enrolling in prospective clinical trials, supported also by The Leukemia & Lymphoma Society and Acetylon Pharmaceuticals.

And we won't have time really to talk about it here today, but Erica Esrick from the Ebert Lab has a spectacular poster elsewhere in this conference, talking about our work to develop very selective inhibitors of HDAC1 and 2, without inhibiting 3, there's no effect on this mitotic spindle, and we can de-repress fetal hemoglobin for application in sickle cell disease, research supported by the Doris Duke Charitable Foundation.

But I want to tell you about instead is something new and sort of exciting in our lab and that is this idea that one might be able to inhibit the Myc oncoprotein, either directly or through its function by targeting upstream and downstream gene regulatory cofactors.

Now you know Myc and Max, when they bind to this Ebox DNA, they turn on the the whole of the cellular growth program. And Myc exists in our body, dangerous as it is, to allow a child to grow to full height and for tissues to re-expand over time. But cancer invariably co-opts Myc, either by direct amplification or activation of upstream oncogenic signaling pathways. And it's fair to say that it's perhaps the most desirable target in all of cancer drug discovery, yet we have no Myc inhibitor. And the reason for this is that when a discovery chemist looks at this helix-loop-helix leucine zipper structure, there's no obvious pocket into which one might position a bioactive small organic molecule.

But the opportunity is so important because as a master regulatory protein it really is one stop shopping for all of the hallmark phenotypes of cancer.

Slide 38. 1983

And it was actually at this conference several years ago that as an attendee I saw the crystal

structure Steve Blacklow presented of the Notch transactivation complex. And here notch in T cell acute lymphoblastic leukemia is the driver of Myc. And what I got excited about was this idea that underneath the private co-activator here, mastermind, there was a little pocket and here maybe you could put a bioactive small molecule.

So we started thinking about inhibiting Myc as targeting the immediate upstream transcriptional driver or the immediate downstream transcriptional effector, which is what led us to these bromodomains that I'll talk more about.

Slide 39. Direct Inhibition of the Notch Complex

And in the second publication from my academy laboratory we teamed up with Greg Verdine at Harvard Chemistry to create a series of really chemical probes of this interaction, walking the surface of this extended alpha helix with these constrained alpha helical peptides. But by loading these peptides up with arginines, we increased their positive charge and that drives them into cells. And what we found, working with Gary Gilliland, is that these molecules induce a proliferative arrest in genetically engineered mouse models of T cell leukemia and do so by down-regulating Myc.

Slide 40. Direct Inhibition of the Notch Complex (Cont.)

Now these were potent compounds at about 100 nanomolar, but through this research we developed capabilities in synthetic chemistry around proteins, which now we apply to the study of histone tails. But also we learned that a very important experiment is to treat cells with a molecule and then like a psychiatrist, step back and say what are you experiencing? And the cell reports back with its transcriptional signature, as probed, with the whole of the molecular signature's database, it says notch is off. And the second most down-regulated signature was Myc. And to us this was very gratifying because this molecule then, we presumed to be quite selective. And as you'll see, this is a strategy that we've employed significantly moving forward.

Now it's going to be very difficult to deliver a constrained alpha helical peptide to the nucleus of a cell and so we handed this project off to Aileron Therapeutics, who are very gifted in the art of constrained alpha helical peptide chemistry.

But back at the ranch, we've now whittled down this large protein interface to something quite a bit more tractable, validated with the small constraint peptide, and there we begin to study small organic molecules.

Slide 41. Chromatin-Mediated Transcriptional Signaling

But the way that we're thinking that one might approach gene regulatory pathways is not this sort of transcription versus chromatin field battle that's been blazing in the academic world for a long, long time, but rather a unified theory of what we call chromatin-dependent transcriptional signaling. The idea is that Myc and Max bind up at upstream E-box enhancer elements, but they don't act alone. They recruit what's called the mediator complex to trigger to polymerase here, to turn on a growth gene shown here. But that also through the recruitment of histone modifying enzymes, they decorate the local chromatin environment, establishing a sort of memory that this gene must remain on. As a cancer cell, of course goes through mitosis, it winds up and unwinds its chromatin, how does it remember it's cancer. There are numerous negative marks and those have been discussed at great length today, namely cytosine methylation.

But there are also positive marks. Local acetylation of side chain lysines. And to these marks might bind bromodomain proteins. And as they localize, they establish a post-it note to remember that this

gene, the growth gene, should remain on.

Slide 42. Chromatin-Mediated Transcriptional Signaling (cont.)

Now from a chemist's vantage point these post-translational modifications of chromatin each represent a list, and I should say an expanding list, as they're recognized with modern mass spectrometry to be increased in complexity structurally, as great drug discovery opportunities. And it's fair to say that pharmaceutical firms are well aware that this low hanging fruit, the enzymatic writers and erasers of these marks, are very tractable and desirable targets.

But at the time we started our lab there was considerably less attention paid to the readers of these marks. Proteins that bind through a protein-protein interaction, but only in the context, as we'll talk about here, of side chain acetylation of lysine residues. And those are called bromodomains and I like them because it kind of looks like they stand for Bradner.

The reason I like them actually is because as a discovery chemist you look at this protein and you get very excited because here there's a pocket. The bromodomain is a bundle of four anti-parallel helices and this ZA helix has a ZA loop and the BC helix has a BC loop and in between these loops is a greasy pocket into which this acetyl lysine is presented like a finger in glove.

Slide 43. Bromodomain Inhibition

Now what's interesting is that the acetyl lysine binding event is quite non-potent. It's about a 10 micromolar to 100 micromolar binding interaction or as the chemists at Harvard say, an interaction so weak even a doctor could inhibit it. And at the time that we started this work it was clear that translocations observed in cancer commonly involve bromodomain proteins, CBP and P300 we think of as HATs (histone acetyltransferases), but in fact they both have bromodomains. MLL we think of as a lysine methyltransferase, but it too has a bromodomain.

And we like this BRD4 because BRD4 had been linked through research in the field of virology to the recruitment of polymerase to cause it to elongate, which is an important functional transition that coordinately activates a growth program.

But one of the things that we liked best about the bromodomain was this, and that it actually, unlike Myc and Max, unlike so many gene regulatory proteins, including notch, here there was a deep hydrophobic invagination. Number two, that acetyl lysine is actually a poor fit for this pocket. And number three, the side-chain acetylated lysine binds into a relatively surface exposed conserved asparagine. Meaning that nature did not take full advantage of the opportunity in binding this pocket. And so we thought potentially we might make molecules that had better what we call shape complementarity to fit inside this pocket.

But at the time we started this work, there were really no platforms of discovery for bromodomains and so we teamed up with a really brilliant crystallographer at Oxford University and through the miracle of Skype we built out a platform capability to study bromodomains together.

Slide 44. Illustration

For afficionados in the audience, it was initially differential scanning fluorometry and ultimately then fluorescence polarization and then finally a bead-based proximity assay called alpha screen technology, from Perkin-Elmer, where acceptor-donor beads are brought into proximity by a synthetic histone tail binding to a recombinant bromodomain.

Now this assay is so powerful because it works with very small protein inputs, rendering it a perfect system to look for what you initially find are weak binders.

Slide 45. Bromodomain Biochemical Platforms

But what would be the chemicals that we might start with? We started end-acetylating numerous types of molecules and ultimately we found in the Japanese patent literature a patent for Mitsubishi Tanabe Pharmaceuticals. Suggesting that compounds of this sedative class, the thienobenzodiazepines, maybe some of you took some Xanax on the airplane over from Europe or Valium or midazolam at a colonoscopy, these sedatives were linked to bromodomain inhibitory activity.

Slide 46. Retrosynthetic Analysis

Jun Qi, a synthetic chemist in my lab, worked out a retro-synthetic analysis for this scaffold that gave us an opportunity with simple commercially available chemical building blocks to build out a big library of these molecules, sampling each of these sites to create what we call structure activity relationships. Jun is a brilliant chemist. It was fair to say he was learning about chromatin in this project.

Slide 47. Synthesis of (+)-JQ1 and (-)-JQ1

And there's one stereo center in this molecule and what that means is that this compound, that we name affectionately for Jun, JQ1, it comes in two flavors. A left hand and a right hand.

Slide 48. Figures and Graphs

And what we learned in this research is that actually the bromodomain pocket is a left-handed glove and that this molecule is quite selective across the molecular phylogeny of 41 bromodomain-containing proteins, it inhibits only four, BRD2, 3, 4, and BRDT.

We were quite surprised to learn how potent this molecule was and he's made 500 or 600 of these. I don't intend to make it sound so simple. But it was a double digit nanomolar inhibitor of the bromodomain interaction and by isothermal titration calorimetry, we learned how absolute the preference is for this left hand configuration.

As Wells and MacClendon showed in the medicinal chemistry literature, inhibiting protein-protein interactions is really hard. And as you start to try to piece away at a big interface, molecules tend to get bigger and bigger and greasier and greasier. And for chemists in the audience I will tell you that Jun's molecule has about the highest ligand efficiency of any inhibitor of a protein-protein interaction, something only I and his wife find impressive. She, too, is a chemist.

Slide 49. Illustration

And what we found here in the high resolution crystal structure, created by Panagis Filippakopoulos, is that this molecule fits with perfect shape complementarity. And if you look at this pocket you can see that in three dimensions this chlorophenyl ring rests its head back on the BC loop and the dimethylthiophene cuts like a knife between these two looping regions and it binds right into this conserved asparagine. We could even understand why in yellow the R-enantiomer would clash, throwing an elbow for these surface leucines.

Slide 50. Figures

Now we've studied at great lengths with an undergraduate in my lab, is this induced fit, are these

loops flapping around and when they see the molecule they lock in, and the answer was no, by molecular dynamical simulations, crystal-graphic B factors, hydrogen-deuterium exchange mass spectrometry. What we found is the bromodomains are like rocks. Why is this helpful?

It tells us that these still images that you get from crystallography are actually quite useful for evolving a second generation compound, which hopefully I'll get to tell you about at the end of this talk.

Slide 51. Direct Inhibition of Oncogenic Fusion Proteins

Now where available, a direct-acting inhibitor of an oncogenic translocation is really attractive and nobody appreciates that better than the American Society of Hematology. Because there have been all-trans retinoic acid which binds to the PML-RAR-alpha oncoprotein and Gleevec® which binds to the BCR ABL dominant oncoprotein, both are very impactful targeted therapeutics for cancer.

Slide 52. NUT Midline Carcinoma

And so we first chose to study this molecule in a rare and nearly uniformly lethal malignancy called BRD4-NUT or NUT midline carcinoma. This gene we poorly understand called NUT rearranges into the twin bromodomains of BRD4 and occasionally BRD3, and it causes a very aggressive solid tumor in the mediastinum and head and neck, that actually as we've become sort of a center of excellence for this rare disease, many institutions treat as leukemia, it is that poorly differentiated. But it's poorly chemo-sensitive, poorly radio-sensitive and there's at this point now only two long-term survivors.

Slide 53. NUT Midline Carcinoma (cont.)

It was described by Jonathan Aster and Christopher French at Brigham and Women's Hospital and our pathology group, and so we asked them could we bring in these special cell lines that they had been using to study this molecule. And what we found is that they're very sensitive to this compound in the nanomolar range, but when these small round rapidly proliferating little blue cells see drug, and this is all to scale, they differentiate. Sort of as a memory of how APML cells were once found to differentiate into promyelocytes.

Now this is preceded by a G1 arrest and is followed by extraordinary apoptosis. And after seven days it's like a crud you can peel off the bottom a petri dish.

But at the time we were doing this research there was no mouse model of this cancer and this is around the time you start to get very excited about treating mice.

Slide 54. Graphs

And so I was caring for a 29 year old firefighter at Brigham and Women's Hospital, who was very much at the end of life with a significant burden of disease encasing the vital structures of his left hemithorax. And we approached him as a collaborator and asked could he provide us a sample of this precious material draining from a chest tube that we would throw out every nursing shift. And Andrew Kung effectively grew this in appropriately immunocompromised animals and by PET scanning and biopsy, looking for the pharmacodynamic response of keratinization, it became very clear that animals that received this molecule, at least until we ran out of it on day 18, enjoy a long and healthy life. Animals that don't, rapidly succumb to their disease.

Slide 55. Barriers to Clinical Translation

Now this first in mouse clinical mouse clinical trial of a bromodomain inhibitor was actually very eye-opening because what it taught us is that for whatever reason, BRD4, which when knocked out is early embryonic lethal, that inhibiting BRD4, is actually very well tolerated by laboratory animals. And we started talking to drug companies, professional investors, our own administration at the Farber, would anybody be interested in developing this as a targeted drug for this rare cancer. And this was the feedback we received. It's too rare. There's only ten of these people and most of them are children, how much are you going to charge these people for this drug. We need to find something else to do with it.

Secondly, it's just a chemical probe. If there's a chemist in the audience, you appreciate, we actually left the protecting group on as sort of a way just to tell you, yeah, we get it, it's not a drug, it's for everybody to use as a chemical probe.

Slide 56. Years of Diagnosis

And so we actually created through like social networking and the internet an online web registry, where patients could actually bring themselves to us as maybe having midline carcinoma. And what we learned in this index report that we've just prepared for publication is that the number one risk factor for having this rare cancer is living near us in Boston. You see, because Chris French diagnoses it. You know it's never been diagnosed in the city of San Francisco, where there are a lot of really good medical centers. But yet it's been diagnosed four times in Italy because Juan Rosai, the brilliant surgical pathologist lives there.

And the proportion of adult patients is rising, as the numbers are rising, and we think this is because well, when you perform fluorescence in situ hybridization on an adult patient with squamous cancers – but what we've learned is that it's a horrible disease. And that NUT positive staining, as now we have a simple immunohistochemical test for this cancer, is the poorest prognostic factor in squamous carcinoma with a 6.7 month median survival and no response has been fortuitously identified in this retrospective analysis amongst common chemotherapeutic agents.

Slide 57. Chromatin-Mediated Transcriptional Signaling

So what else to do with the molecule? Well, it really comes back to Myc. The idea here was that Myc and Max might require BRD4 as an adapter between this complex and this complex. The signaling complex and the receiving complex polymerase.

Slide 58. Multiple Myeloma

And we chose to study Myc in the context of multiple myeloma as Dana-Farber is a center of excellence for this disease and robust platform that Ken Anderson's developed over so many years. And also that in Constantine Mitsiades we had a very knowledgeable and expert collaborator on the biology of Myc in myeloma, which owing to the long longitudinal of study of Myc by Kuehl, Bergsagel and others, many people have identified that myeloma has amongst blood cancers one of the most common and highly expressing Myc phenotype, next only to Burkitt's lymphoma.

Now Myc is actually commonly rearranged in multiple myeloma as shown here. And Myc amplification is the most common amplification event in this cancer, which really just makes it like all of human cancer.

Slide 59. BET Bromodomains in Multiple Myeloma

Now what we learned is that BRD4 is actually expressed at increased levels comparing normal

plasma cells to smoldering myeloma and that it's always expressed in myeloma cell lines. The majority of human samples from myeloma patients, captured from the MMRC, actually amplified the BRD4 locus, but that's owing to broad amplifications and redundancies in the short arm of chromosome 19.

As you know, myeloma proliferation is a seed that grows in the context of the micro-environmental soil and Constantine's lab has studied this for many years. And what we learned is when incubating myeloma cells with stromal cells, BRD4 levels actually increase. So even if you didn't think that Myc was connected to BRD4, it looks like a pretty good target.

Slide 60. BET Bromodomains in Multiple Myeloma (cont.)

So we did a very important experiment. We treated three different myeloma cell lines, each with a different genetic lesion activating Myc, and we just sat back and said what is JQ1 doing to these cells. And when you plug in all of the signatures available through the signature database at the Broad Institute, the dominant signature inhibited is that of Myc in this gene set enrichment plot.

Next only to E2F. And Myc and E2F are, of course, co-factors for one another in a cell cycle progressing transcriptional signaling system.

Slide 61. BET Bromodomains in Multiple Myeloma (cont.)

This is unique amongst all transcriptional networks in myeloma. NFkB, implicated in the pathogenesis of this disease. Glucocorticoid receptor, XBP1, this tissue-specifying factor. None of these were influenced by JQ1.

So we look to the oncogenes that commonly drive Myc, using a platform capability from Nanostring, and we treated cells for one, four and eight hours and looked at the transcriptional output and to our surprise the oncogene that was most effective was Myc itself. That for whatever reason, Myc expression in myeloma is addicted to BRD4 and, remember I told you that right hand molecule is inactive, well, it was inactive in down-regulating Myc, supporting this as an on-target effect.

Now this was a dose and time-dependent phenomenon that Myc would be down-regulated and at the level of chromatin, after two hours, the Myc oncoprotein is no longer detectable genome-wide on chromatin with this ELISA assay, whereas NFkB and other transcriptional oncoproteins still are.

Slide 62. BET Bromodomains in Multiple Myeloma (cont.)

So there was this selective Myc effect, but why? We had a University of Delaware student with us for the summer and we tasked him with trying to develop methods to identify Myc genome-wide in myeloma cells. And what we found is that the immunoglobulin enhancer region, that in the MM1.S cell line is rearranged adjacent to the Myc gene in a derivative chromosomal insertion, that BRD4 is massively enriched at these enhancer sites. And in the presence of JQ1 it is depleted, providing some mechanistic rationale for why JQ1 is so effective at down-regulating Myc.

Slide 63. BET Bromodomains in Multiple Myeloma (cont.)

Jake Delmore and Constantine's laboratory examined a panel of myeloma cell lines. They're uniformly sensitive to JQ1 with and without the bone marrow stroma. This is true, that Myc is down-regulated in every myeloma line we've looked at. And we love this, this is a structurally dissimilar compound from our competitor-slash-collaborator, GlaxoSmithKline, and what we found is that their molecule, also a very stunning BRD4 inhibitor, has the same effect, albeit at a slightly lower potency.

Slide 64. BET Bromodomains in Multiple Myeloma (cont.)

Ghayas Issa, a hematologist in our group, then further phenotyped and what found is that this G1 arrest is actually not followed by apoptosis, but rather is followed by cellular senescence, which the work of Gerard Evan and Dean Felsher and others over the years has shown us when you turn Myc off, cells tend not to die, they tend to go to this weird type of cellular sleep. And that's evident by beta-galactosidase staining and at the transcriptional level.

Slide 65. BET Bromodomains in Multiple Myeloma (cont.)

But thinking translationally about this as a drug for myeloma, we did some informative experiments. Patient-derived samples, down-regulated the Myc oncoprotein, many of them produced an anti-proliferative response in the nanomolar range. Three models of myeloma were pursued, an orthotopic decimated xenograft model, where these glowing mice demonstrated a prolonged survival. A plasmacytoma model, where this drug was also effective. And importantly, Marta Chesi and Leif Bergsagel developed a very elegant model, the V kappa Myc model of myeloma, in which this molecule as a single agent provoked a complete response, which as they will report at this meeting, and referred at the last, amongst the 60 or more molecules tested in this model, there's a short list of about three that have ever produced a CR and the other two are FDA approved substances used in the treatment of this disease.

Slide 66. Non-Hematologic Malignancies

Now JQ1 affects Myc in myeloma, but there are numerous cancers that depend on Myc. And I should say that because we are not a drug company, we can sort of just give the molecule away to lots of people to study and in real time when the post-docs ask for it, which is when you want to get it in their hands. And so we sent it to Mass General and the Broad Institute and the NCI and the data coming back was all the same. That amongst every type of cancer, there's a subset of about 10%, at least in these cellular models, that are very sensitive to bromodomain inhibition.

Slide 67. Clinical Translation

But we needed a molecule that we could bring to the clinic and so with some internal support from the Farber's Accelerator Program, we did medicinal chemistry. We worked our way around the molecule using iterative synthesis through a year of 6 in the morning conference calls with Shanghai to improve the potency, solubility, drug-like properties, ease of synthesis, cost of synthesis of this compound. And now have now a stable of orally bioavailable compounds with single digit nanomolar activity in cells that have durable half-lives. And these compounds are being developed with a partnership with a new company called Tensha Therapeutics, a Japanese word that means to transcribe.

Slide 68. Open-source model of drug discovery

Now I will say this research taught us something about our own academic capabilities, that by taking a horizontal cut through our local and really international biomedical community, we could very rapidly advance molecules from the prototype stage to validated, pharmacologically validated therapeutic use in cancer, prompting human clinical investigation. And for all these reasons we think that academia is a really fertile environment for drug discovery.

Slide 69. Distribution of Chemical Probes

And this slide is out of date. We've given the molecule now to over 140 laboratories worldwide and this includes six pharmaceutical companies, four foreign governments, really anybody that'll ask for it. And although it's only been a calendar year, the interesting thing is that people call us back with the data. They're under no special obligation. Although they agree to do is not eat it, which is important, because one person wanted to eat it.

And what we've learned in this year is this incredible velocity of knowledge about BRD4 that has really driven home the idea to me that these chemical probes are so important if you get them out there, for advancing science across numerous disciplines, certainly well beyond the blood cancers that we in our lab exclusively care about.

Slide 70. Acute Myeloid Leukemia

And this is best exemplified by a very lucky email I received from Christopher Vakoc at Cold Spring Harbor Laboratories, formerly at the University of Pennsylvania. In an abstract Chris Vakoc presented at last year's ASH, a hairpin screen was conducted inMLL leukemia, and Chris had an excellent presentation about the role of the polycomb complex in MML leukemia that emerged from his hairpin knock-down screen performed with Scott Lowe at Cold Spring Harbor.

Slide 71. Graphs

But actually the top hit in his screen was BRD4. And when he read our paper he reached out and said could we have some of your molecule, and he's now the world's leading user of JQ1. And what we found is that in this cancer, like in that midline carcinoma, these rapidly dividing leukemia cells forget their leukemia and they trigger differentiation of that into a more macrophage or monocytic like phenotype that's evident at the transcriptional level, as stem cell signatures and Myc signatures are down-regulated. And there again Myc is inhibited at the transcriptional level.

Slide 72. New Bromodomain Inhibitors

We are not the only people now working on bromodomains. It's fair to say that this has become regrettably very competitive, but we've learned actually through research ongoing at GlaxoSmithKline with new types of compounds, in this case at least , that these compounds are active also by down-regulating BCL2, research from the Huntly and Kouzarides labs, recently published in Nature. And Constellation Pharma was kind enough to re-synthesize our compound and study it in some of these same diseases and found that twice daily dosing of our drug has an even better effect than once daily dosing. It's pretty rare for a company to make your molecule and then publish a paper about how awesome it is. But everything is different now, I guess, in the space of chemical biology.

There are numerous compounds coming forward, not just for this bromodomain, but for others, and we think this is a very exciting time to be studying the epigenetic readers, even beyond lysine acetylation, Stephen Frye's work, to target methyl lysine binding proteins.

Slide 73. Credits

And I just want to leave you with this. My appreciation for those who've collaborated with us around this research, in particular Constantine who's here and Ghayas Issa who'll be looking for an oncology fellowship soon enough, and Jun Qi, JQ1 himself, for his bravery as a talented synthetic chemist of joining our fledgling operation. These are the groups that we've been working with to push these compounds forward to the clinic.

And I really appreciate your patience in waiting for me on this long travel day and will gladly answer any questions. Thank you.

Dr. Irv Bernstein:

Great. Let's grab a few questions.

Audience:

Great talk. I was curious, one thing struck me in the solid tumor work that you mentioned so far, that it was 10%. Myc is so broadly up-regulated, so many, does that mean that many other things other than BRD4 are subset of Myc target? How do you see that?

Dr. James Bradner:

I think that, hopefully you heard the question, it really is one of the new areas of discovery in our group, is to understand, number one, why a subset of cancers that all depend on Myc are sensitive to BRD4, not all of them. And secondarily, how do these cancers become resistant to this molecule, we're not curing them. And these things are linked. When we do genome-wide __ for BRD4, we identify it at enhancer sites. And so really BRD4 is an enhancer cofactor as we think about it, and in myeloma, sustaining Myc transcription by the positive reinforcement of, in this case, in this cell immunoglobulin heavy chain enhancer region, is then very BRD4 dependent. Where in other types of cancers, where Myc is an end-effector of the EGFR signaling pathway or the like, it is perhaps then less essential that these enhancer, dominant enhancers, are driving BRD4. This is our thinking. But that's R01-aim level speculation. What we can say is that the other flavors of Myc, like N-Myc, are also exquisitely sensitive to JQ1. So there is some sense of coactivator relationship. But I'd be the first to say that one of the take-home messages of all of the recipient labs of JQ1 that could care less about Myc in many cases is that other master regulatory transcription factors are also co-oped in BRD4, which makes this molecule block adipogenesis. And so these tissues-specifying or phenotype-defining master regulatory proteins, if their transcription factor is a function through enhancers, BRD4 may well be important. But it is not a direct Myc inhibitor, so consequently other Myc-dependent cancers do not respond.

Slide 74. Research and Epigenetic Influence in Myeloma and Lymphoma

Dr. Irv Bernstein:

Our next presenter will be Dr. Jonathan Licht, who will talk about research in epigenetic influence in myeloma and lymphoma. Dr. Licht is Johanna Dobe Professor and Chair, Division of Hematology-Oncology and Associate Director of Clinical Sciences at the Robert H. Lurie Comprehensive Cancer Center at Northwestern University in Chicago. And we're delighted to have you here.

Dr. Jonathan Licht:

Thank you very much, Irv, and I'd like to thank Richard and The Leukemia & Lymphoma Society for their organization of this meeting and their long-term support of ours and many of our programs in the audience.

What I'd like to talk to you today about is to amplify on Dr. Baylin's talk, a particular aspect of epigenetic changes in hematologic malignancy.

Slide 75. Disclosure of Conflicts of Interest

First of all, I'd like to disclose my conflicts of interest.

Slide 76. Hypothesis

And I'd like to say our basic hypothesis is that mutations in epigenetic regulators in cancer, in hematologic malignancies, can cause global shifts in chromatin modification, structure and function.

Slide 77. Histone Post-translational Modifications

Now towards this we're particularly interested in modifications in this talk of the histone tails. This is a crystal structure of the histone octomer. And the histone tails can be modified in manifold ways. They can be methylated, acetylated, phosphorylated, ubiquitylated, and other modifications as well.

Slide 78. Histone Lysine Modifications

Amongst the most diversely modified histone tail residue is the histone lysine tail, which can be in this case acetylated, ubiquitylated, methylated, sumoylated and these modifications are mutually exclusive and each one of these modifications has a meaning. These modifications are written by specific enzymes and then read by other enzymes.

Slide 79. Histone Modifications are "Read" By Specific Modules

For example, the H3K9 trimethyl mark is read by a domain called a chromodomain, which is in many transcriptional repressive proteins. By contrast, the H3K4 trimethyl mark is associated with gene activation and is read by a different module found in proteins associated with gene activation. So in this way there's a regulatory logic to the chromatin modifications. This is tightly regulated across development, across differentiation, and you can see that if there's some mutation that might upset the balance of these modifications, there can be quite profound changes in gene regulation that might lead to malignancy.

Slide 80. Different Chromatin Modifications and Enzymes

Let's talk about some general rules. In general gene activation is associated with acetylation of histones, trimethylation of histones on lysine 3, residue number 4. There's an absence of H3K9 and K27 methylation. K27 done by the polycomb group that Dr. Baylin mentioned. By contrast K9 methylation and 27 methylation is common in transcriptional repression. And those repressed genes often are absent the K4 mark, and then tend to be deacetylated.

Slide 81. Histone Lysine Methylation in Evolution and Disease

Now these histone lysine methylation marks are made by sets of enzymes. Almost every one of these enzymes has a domain called the set domain, which stands for the names of three proteins, . These are evolutionally conserved across evolution and they tend to make specific marks. And what you can see in the far right column is that disorders of these types of histone lysine methyltransferases can be associated with specific disease syndromes.

For example, in leukemia disregulation of the MLL histone methyltransferase for H3K4 methylation is common. In prostate cancer, EZH2 tends to be over-expressed and in hematologic malignancies it can be deleted. We'll go into this in more detail in a few moments.

Slide 82. Global Chromatin Anomalies in Lymphoid Malignancy

So what I'd like to talk about now is some syndromes of global chromatin anomalies in lymphoid malignancy.

Slide 83. EZH2 and H3K27

One of these to come back to is EZH2, the major enzymatic component of the polycomb group proteins. This is mutated or lost in myeloid malignancy and if this quick time picture worked you would see that in cell lines that are devoid of EZH2 functional protein, there is a lack of histone 3 lysine 27 methylation.

Slide 84. UTX Mutation

In another type of mutation that was recently uncovered initially in diseases as multiple myeloma, but also in other diseases, is mutations in a histone demethylase called UTX. This is a methylation, demethylase, specific for H3K27. It's an alpha keto glutarate dependent enzyme, a type of enzyme that might be affected by IDH mutations that will be discussed by Dr. Levine. This can take a fully methylated histone – methylated histone, and demethylate it completely. UTX tends to be specific for K27. Again you'll see that K27 is often disregulated in hematologic malignancy. EZH2 loss, UTX, loss of function, can be associated with problems.

Slide 85. UTX Mutation (cont.)

And here in this set of myeloma cell lines you see that the lines with a UTX mutation, there's a hint that there may be increased K27 trimethylation, compared to cell lines that do not have a mutation. We're currently analyzing these cells by mass spectroscopy to get more quantitative information, to see if this holds true.

Slide 86. EZH2 Point Mutation in Lymphoma

Now another mutation that was uncovered about two years ago by the group in Vancouver and subsequently investigated by a number of other groups, is the EZH2 point mutations in lymphoma, found in both germinal center lymphoma and follicular lymphoma, but not activated B cell types of lymphoma.

Slide 87. Structural Model of SET Domain

This mutation was always in the same spot and was found in the heterozygous state, suggesting that this was a gain of function, not a loss of function mutation. And in fact, if we model the crystal structure of the set domain and you see where that mutation occurs, it occurs right in the center of the pocket, right here, and you lose a bulky tyrosine in this pocket.

Slide 88. Y641

And this would allow some extra room in this pocket, so if a histone tail inserts into the pocket to be catalytically enzymatically converted and methylated, there's room not for a single methyl or two methyl, but as many as three methyl groups. So this pocket is opened up and this methyltransferase can now do trimethylation quite avidly.

Slide 89. Gain of Function

And what you can see here is two cell lines that are lymphoma cell lines without the EZH2 mutation and with the EZH2 mutation and you can see by this anti-histone 3 lysine 27 methyl antibody, increased methylation. This is confirmed by mass spectroscopic analysis. These are the EZH2 wild type cells. And seen over here at the high bars here are EZH2 mutant cells. There's an increased number of histone tails showing the histone 3 lysine 27 trimethyl mark.

Slide 90. EZH2

When you see this by immunoblot, when you see this about four or five-fold change by mass spectroscopy, this means this is a global abnormality. It's not happening necessarily in any one region. There could be some lumps and bumps to the mark, but it's probably distributed widely. And we're trying to discover now how does this type of chromatin mark lead to changes in gene expression.

Slide 91. B cells infected with EZH2

We can do this by taking B cells and infecting it with mutant EZH2. You'd express wild type EZH2 all you'd like and you can show it's over-expressed, but there's no change in K27 trimethylation. But if you infect with one of two different EZH2 point mutants, you get an increase in the trimethylation and a decrease in the dimethylation. There are no changes of EZH2 component SUZ12 and EED in this system.

So over-expression of EZH2 per se doesn't change into globally, but point mutation does.

Slide 92. Mutant EZH2 Stimulates Growth in Agar

This has biological consequences in that EZH2, if we take a B cell line that does not grow in soft agar, seen right here, and infected with a retrovirus expressing wild type EZH2, no is formed. Whereas either one of these two, EZH2, point mutants, can encourage growth in soft agar. So this does seem to have some transforming activity at least in this assay, and the genetic basis of this, what genes might be affected to cause this, is currently under investigation.

Slide 93. An Epigenetic Disorder in Multiple Myeloma

What I'd like to turn to in the bulk of my talk is an epigenetic disorder in multiple myeloma. Now the pathogenesis of multiple myeloma was quite obscure for many years until the work of investigators such as Mike Keel, a pioneer at this at the NCI, and others, who showed in the late 90s and early naughts that there was a number of recurrent chromosomal translocations found in multiple myeloma. These represented abnormal recombination of the immunoglobulin locus, linking the immunoglobulin locus to a number of putative oncoproteins. And one of these is known as MMSET, located on chromosome 4.

Slide 94. t(4;14) Myeloma - A Global Chromatin Anomalies

So MMSET is a rather large multi-domain protein with a number of domains that are implicated in binding to chromatin and modifying chromatin. Like the other enzymes I described

earlier, such as EZH2, it contains a set domain and is a putative histone methyltransferase.

Slide 95. The MMSET Protein

MMSET is expressed broadly and it is normal for normal embryonic development. Complete knockout of the gene in mice leads to a lethal phenotype and in heterozygous state there's quite significant fetal anomalies.

Slide 96. MMSET Overexpressed in t(4;14) Myeloma

But MMSET is expressed at a modest level in any cell type, including these cell lines without the 4;14 translocation. In 4;14 translocated myeloma cell lines or specimens, you see a significant up-regulation of MMSET. They're somewhat different sizes in different cell lines and patients because the break point of the immunoglobulin gene and its invasion into the MMSET gene can occur at somewhat different places at the . But all of these over-expressed protein species would contain the histone methyltransferase domain.

Slide 97. MMSET in Myeloma

So what we'd like to know, is MMSET important for these types of myeloma and if we understand the mechanism of action of this protein, could we at some point understand the disease pathogenesis and devise new therapies.

Slide 98. MMSET Effects on Growth Properties

Is it important for growth, using transient as well as stable knock-down assays? In this case we're using a tetracycline-inducible short hairpin knock-down. Depletion of this long isoform of MMSET, which contains the histone methyltransferase domain, leads to a cessation of growth of these myeloma cells and an increase in G1 arrest and induction of apoptosis. This work, and several other groups have done the same, show that this form of myeloma seems to be dependent for its continued growth on this histone methyltransferase MMSET.

Slide 99. Hypothesis

By contrast if you do the same experiment in other forms of myeloma, without the MMSET translocation, there's no effect. So these forms of myelomas may be molecularly addicted, if you will, to this histone methyltransferase.

Slide 100. MMSET: *in vitro* Methyltransferase Activity

MMSET is a histone methyltransferase. We can show this in vitro, making the protein in vitro and incubating it in the presence of a histone substrate and radioactive methyl group donated by . You can show in this control that the gene 9A histone methyltransferase methylates histone 3 only, but MMSET by contrast in vitro can methylate histone 3 and histone 4. So at least in vitro it has a rather promiscuous activity.

Slide 101. In Vitro- MMSET Can methylated Many Different Histone Sites

In fact what it likes to do most in vitro is actually methylate itself much better than it methylates histone 3 or histone 4.

If you probe these reactions, which we have taken recombinant histones and incubate it with MMSET and then probe with antibodies against specific histone lysine methylation marks, we can find that a variety of histone methylations occur. K36, K27 and histone 4 lysine 20. So again pretty promiscuous in vitro.

Slide 102. KNOCK OUT SYSTEM FOR MMSET

But what does it really do in vivo? What does it do in these myeloma cells?

For this we used a very nice system developed by Josh Lorring and colleagues at Johns Hopkins in which they've either knocked out the wild type MMSET allele in these myeloma cells or by homologous recombination knocked out the rearranged allele of MMSET. If you knock out the rearranged allele, here seen as TKO, there's a big drop in the RNA levels in MMSET and protein levels as well.

Slide 103. MMSET Knock out (KO)-Global Effects on Chromatin

When we look at these cells, which have had a global knockdown of MMSET to basal levels, we see a striking loss of one particular mark, histone 3 lysine 36, di and trimethylation. At the same time we see a big increase in K27 trimethylation. Again a recurrent theme. Something going on with histone 3 lysine 27, seems to be a recurring theme in hematologic malignancies.

Slide 104. MMSET Global Effects on Chromatin

When we take a look at this complicated immunoblot of the 4;14 positive and 4;14 negative myeloma cell lines, we can simplify it to this. All of the 4;14 myeloma cell lines have high levels of MMSET, high levels of K36 dimethyl in particular, and a deficit of K27 trimethyl.

Slide 105. MMSET-H36me2/H3K27me3 Switch

So a big switch here. When you see this again by immunoblot, this means that all the nucleosomes throughout the cell, all of these histone octomers are modified in this way.

Slide 106. MMSET Expression Causes Global Changes in Histone Modifications

We can look at this by mass spectroscopic analysis, where we actually take the histones and put them through very powerful mass spectroscopic magnets. We can measure simultaneously up to 84 different histone modifications. We're focusing here on histone 3 lysine 36 and 27.

What we see here, seen at the bottom best, is the K36 dimethyl mark is up by a factor of 8 to 10 fold in these MMSET positive cell lines. And the K27 histone mark is down by an equivalent amount.

So immunoblots just show relative amounts. This shows there's a 10 fold switch in the overall chromatin characteristics of these cells.

Slide 107. MMSET Re-Addition Reverses the Switch

We can then show that MMSET is directly responsible for this by doing a re-addition experiment. We add MMSET in its wild type form or MMSET that has mutations in the histone methyltransferase domain, predicted to be in the histone binding or the binding pockets.

This just simply shows the modeling of where these mutations are occurring in the enzymatic domain.

If we take – this is our TKO, these are knocked out cells which have low levels of histone 3 lysine 36 tri and dimethylation. If we add back MMSET, we lose that K27, we gain K36. This point mutant is actually very similar to the EZH2 unit. It's right in the pocket and it gives you more K36 trimethylation. This doesn't occur in patients, but it is an interesting exercise here, showing this principle that this pocket can be modified in size to indicate whether or not you'll di- or trimethylate a residue.

This mutant is very useful because it is completely dead. It is expressed well, but it does not methylate histone 3 lysine 36 and it does not lead to de-methylation of K27.

Slide 108. MMSET Methylation Activity Critical to Myeloma Growth

What we find is if we take these MMSET knock-out cells and we add back wild type MMSET, or the super active mutant, they grow pretty well. But the HMT histone methyltransferase inactive mutant does not grow well at all, indicating that this methylation activity is one component critical for myeloma cell growth. Again, suggesting that if we could inhibit that histone methyltransferase activity, we might have a therapeutic compound.

This process we find is even more complicated. If we reinfect these cells with a particular mutant of MMSET in which one of the PHD domains is depleted, we actually find that we get the K36 dimethylation, but we do not get a complete loss of K27 trimethylation, seen here by immunoblot and corroborated by mass spectroscopy.

What is going on there, it turns out that MMSET also binds to an H3 K27 dimethylase D3, binds it, but MMSET deleted for two different important domains does not. So this says that MMSET is causing global chromatin changes by two mechanisms, but active methylation and active demethylation.

Slide 109. HMTs Gone Wild!--K36/K27 Switch

So our model would be this. Our model is HMT3s have gone wild, so we think MMSET may form a complex with D3, to remove the H3 K27 methyl mark and to add, so this mark was on there, it disappears and we think that it's replaced globally by the K36 methyl mark.

Slide 110. Hypothesis

And this global change in chromatin may lead to manifold changes in gene regulation.

Slide 111. Which Genes are Affected by MMSET Expression?

What genes does it affect? We can do this by comparing and contrasting gene expression arrays from cells in which we deplete MMSET or add back MMSET or add back MMSET with a point mutation. What we find is a number of genes involved in apoptosis, DNA repair. This is important because in recent papers linking MMSET in normal cells to DNA repair, as well as to cell cycle control.

Slide 112. The HMT Activity of MMSET Yields Gene Activation

So we find that many of these genes are dependent in their activity on the set domain. And

these genes will not turn on very well if the HMT activity of the enzyme is deficient. Which exact genes are involved in gene pathogenesis is something we're still trying to elucidate, but there probably will be several pathways involved.

What we can find in general is that when MMSET is expressed in cells, we look at all full gene expression changes, we see a skewing to high level expression. MMSET can turn some genes on by a factor as much as 50 or 60. If we add MMSET into a cell with its enzymatic activity disabled, we only get about plus or minus two to four fold changes. So we think that this HMT activity of MMSET tends to boost gene expression.

If we compare genes that were regulated by both wild type and mutant MMSET, there's a positive slope of this curve indicating that genes that are regulated by both mutant and wild type tend to be up-regulated more by the wild type. So the HMT activity is important to give an extra boost to gene expression.

Slide 113. Which Genes are Affected by MMSET Expression?

Here's an example of a gene affected by MMSET expression, known as JAM2. It's involved in cell adhesion and we note that these myeloma cells, which manipulate MMSET levels, have drastically altered cell adhesion properties and in other systems seem to be altered in their ability to invade.

MMSET knock-down, JAM2 goes down, MMSET knockout, JAM2 goes down. We add back MMSET and JAM2 goes up. The histone methyltransferase mutant will not turn it back on. The active mutant will turn it on at least to some extent. So this is an example of a gene regulated by MMSET.

When we take a look at MMSET binding across this gene, it's not bound in a typical transcription factor type of way. It seems to be down both in the five prime promoter region and throughout the gene body when MMSET's high. At the same time K36 dimethylation and trimethylation is quite high all across this gene. K27 methylation is higher when MMSET is absent. When MMSET is present, K27 methylation in the promoter goes down. So this is an example of one of the genes affected by MMSET.

Slide 114. ChIP in MMSET knockout cells- GLS2

When we do genome-wide profiling by chip seq analysis, we get results from the computer indicating that MMSET may be bound both in promoters and distal sites and , but I think this has to be a caveat to this, is that when we look at these chip seq tracks across a gene, we see peaks being culled across a sea of MMSET all across the genome. What we think is actually the whole genome is being blanketed by this enzyme and it's a pretty high level. There may be some peaks above this high water level, but it's really what strikes us the most is that there are these global chromatin changes throughout, both in terms of MMSET binding and K37 dimethylation changes.

Slide 115. MMSET- Genome Wide Profiling

So despite this increase in K36 dimethylation, only about 5 to 10% of all genes on our arrays or more recently are changed. And some of the genes that are bound by MMSET are not necessarily regulated or methylated by on K36. So we need to develop some new epigenetic

rules.

Slide 116. MMSET Targets

We used to have a rule your favorite transcription factor binds, brings in your favorite enzyme, modifies a promoter, turns a gene on or off. These global abnormalities, I think there's a different set of rules. You're affecting chromatin throughout the genome and you're affecting its – we don't know the rules of which genes will turn on and which will turn off yet. So this is a challenge for us in the coming years in the field.

Slide 117. HMTs Gone Wild- Non Transcriptional Effects?

Lastly, does MMSET have effects outside of transcription? What are some of the other processes that might be affected when chromatin was altered in this global way?

Slide 118. MMSET effects on chromatin accessibility

Well, we thought of two. One was DNA replication. We have some nascent information that there are some abnormalities in start sites of replication in these cells. The other thing we thought about was DNA damage. One of the reasons we thought about this is we thought that the histone modification by MMSET might actually globally alter chromatin accessibility.

Slide 119. yH2AX in Knock out system 30min after radiation

This is a micro accessibility assay. You take a bare nucleus of a cell, treat it with and look for cutting in a nucleosomal pattern as you see right here. So this chromatin is open. If we knock down MMSET, the chromatin completely closes. The nucleus no longer can digest. If you reverse the knock-down, you see the pattern reemerge. This means the physical chemical properties of this chromatin are dramatically different when MMSET is at a high level.

So could DNA damage be different? We noted at baseline MMSET high myeloma cells had a basal level of gamma H2 X staining, which was accentuated by gamma irradiation to a greater extent than genetically matched cell lines in which MMSET was deleted.

We found, though, despite this increased DNA damage, we found that MMSET high cells, treated with higher doses, higher, higher doses of melphalan, the drug commonly used to treat multiple myeloma, survived better than MMSET depleted cells. So having high levels of MMSET allowed you to survive this DNA damage insult.

Slide 120. MMSET- An Effect on DNA Damage Response

What we found additionally is if you treat MMSET low cells with melphalan, and this is a pretty high dose, these cells undergo cell cycle arrest and so have stayed there. By contrast, MMSET high cells continue to enter the cell cycle, as seen by bromodeoxyuridine incorporation. So this is a bad thing. You have some higher damage at baseline, you survive DNA damage better, you don't go into cell cycle arrest, this sounds like maybe an explanation of why these patients don't respond quite as well as others to melphalan.

Slide 121. MMSET+ Cells Fail to Undergo Cell Cycle Arrest of DNA Damage

What we actually find here is that there's another paradoxical thing occurring. There's

more DNA damage at baseline, but we find that MMSET high cells repaired DNA damage better. This is a comet assay in which we monitored DNA breakage by virtue of cells streaming through an electro field and if there's broken DNA it streams out the back as a comet. So the longer the comet, the more DNA damage.

Slide 122. Comets after DNA damage induction (melphalan)

We can see right here that at baseline when we add MMSET into a cell, we tend to see more DNA damage. The tails here are longer than over here. But if we wait 24 hours after removal of the DNA damaging agent, we find that the tails get shorter. In fact, they get short of the control, or they shorter than a MMSET unit without histone methyltransferase activity. So this suggests that this DNA – that MMSET expressed at these high levels might allow myeloma cells to recover from their DNA damage, go back into cell cycle, and grow again. Whether or not this is high fidelity DNA repair, we don't know.

Slide 123. Hypothesis

Lastly, can MMSET be inhibited? We and our colleagues and industry are trying.

Slide 124. BIX 01294

There's a gene 9A inhibitor known as Bicks 1294 and there's another inhibitor known as kitocin, and in this antibody-dependent assay we see that kitocin can inhibit histone methyltransferase activity by MMSET and so can this drug Bicks 1294.

Slide 125. HMT Inhibitor Killed MMSET+ Cell More Readily

In fact we found that the Bicks 1294 compound could kill MMSET high compounds more effectively than it killed MMSET low cells. MMSET high cells were more killed than MMSET low cells. So that motivates us to look for even more specific inhibitors of MMSET activity.

Slide 126. Mass Spec Based Screen for MMSET Inhibitors

Towards this we've developed a mass spectroscopic screen in which we've performed 384 wells of histone methyltransferase activity reactions.

Slide 127. Self-Assembled Monolayers (SAMs) on Gold

We dot these onto little metal plates in which histone tail residues are ligated to a gold backbone and they grow these little gold chains here as you can see.

Slide 128. Gurard-Levin-Mrksch Lab

These are then put into a mass spectroscopic instrument and we actually positively detect the histone methylation by a 14 Dalton shift, indicating a methylation.

Slide 129. High-Throughput Mass Spec

This is this done in a high throughput manner in this machine here, looking for this 14 Dalton Shift.

Slide 130. MMSET Inhibitor Screen Summary

And at least initially we got out of 10,000 compounds, about a half dozen hits, which are undergoing more validation at this point.

Slide 131. Validation of Compounds 1-7

In addition, we're about ready to start doing another 90,000 in this assay.

Slide 132. Conclusions

So what I'd like to conclude here is that disorders of histone methylation are a recurring theme in hematologic malignancy. And histone 3 lysine 27 is a mark that's frequently disregulated, whether it be EZH2 gain of function, EZH2 loss of function, UTX loss of function, or MMSET gain of function, K27 is a very important mark.

Alterations in histone methyltransferase activity alters cell growth. We show that both for EZH2 gain of function and MMSET gain of function, both of which seem to have growth promoting activities.

HMT dysfunction may have effects on other chromatin-dependent processes such as DNA repair, as I mentioned, DNA replication. And we think HMT inhibitors might restore chromatin function and restore normal growth control in the future. And that's something we and others in the audience aspire to.

Slide 133. Credits

So to conclude I'd like to thank my lab seen here in a quick time – well, anyway. So much for Mac-PC conversion.

Slide 134. Northwestern University

I'd like to thank my lab at Northwestern University, including several people here today, and collaborators across the country and our funding sources, some of whom – fortunately this one came out, right? So I'd like to thank the audience and I'd be happy to answer any questions.

Dr. Irv Bernstein:

We have a few burning questions. One question I had was it seems that if you had a selective inhibitor, a key aspect would be titrating the doses precisely. Is that something realistic biologically?

Dr. Jonathan Licht:

I think one question would be is can inhibitors be made that, in the case, for example, EZH2, can an inhibitor be made that's particular for the activated form and will leave the normal form alone. I think given the fact that the histone lysine binding pocket is different in the mutated form, it's possible. I would imagine it's possible. For the case of MMSET inhibitors I think it would be important to titrate. We don't know what depletion of that K36 mark will do in normal cells, so I think we have to empirically determine that.

Audience:

My question has to do with study systems. Much of this work is done with cell and don't necessarily mimic in vivo physiology and anatomy. And I wanted to hear your comments. An excellent talk, by the way. Whether analysis of these myeloma cells in close proximity with stromal cells or other study systems that might more mimic the marrow micro-environment, might render different results.

Dr. Jonathan Licht:

I think that the results in terms of the biological outcome will change. I don't think – it's known, by the way, that growing cells under different stromal environments can actually change global chromatin modifications as well. I do think that these are likely to be rather dominant mutations, not to say that other chromatin modifications might occur if you grow cells in three dimensions or on stroma. But I think that these – my feeling is, though I don't have the primary data to say it, is that these will dominate those. But I agree with you, one problem in the field is that other than Dr. B elegant model of Myc rearrangement in activation of lay B cells, we don't yet have genetic models in mice to see what these oncogenes do in mice at this point.

Audience:

I've got three questions. They're mainly mechanistic questions. So in vitro we know that these methyltransferases are pretty promiscuous. Did you do your methyltransferase assays with nucleosome substrates?

Dr. Jonathan Licht:

We did that once, but didn't publish it. It has been published by Danny Weinberg's group and on nucleosomal substrates the preferred substrate in vitro, nucleosomal substrate, is histone 3 lysine 36. Just like our in vivo results.

Audience:

And then my next question is related to interaction. So if you knock down and if you suppose that loss of K27 needs to precede of K36, what kind of phenotype do you see in your cells?

Dr. Jonathan Licht:

I think that experiment is in process. It's on the books. So it's a good question, it's one we want to look at.

Audience:

And my last one is do you know whether it's homologous recombination or non-homologous that's abnormal with your MMSET ?

Dr. Jonathan Licht:

In our case we don't know yet, but we're doing those experiments right now. There has been, there's actually one poster or maybe an oral presentation here from another group, suggesting it's non-homologous is abnormal in their system. This is something we're looking at as well. Thanks. Good questions.

Audience:

I have a question relating to if you've done any work with bortezomib in your MMSET models?

Dr. Jonathan Licht:

We haven't done bortezomib in these models. I think it would be very interesting to see if bortezomib as a rather broadly active agent against myeloma cells might have a combination effect with developing histone methyltransferase inhibitors. That's something we and others I'm sure will be looking at.

Audience:

Have the trials using bortezomib not shown selectivity to T 4;14?

Dr. Jonathan Licht:

My understanding of those trials is that whereas if melphalan is initial therapy for 4;14 myeloma, those patients have not done as well. They've fallen out of remission more rapidly. The initial response to bortezomib in the 4;14 positive patients has been as good as in other patients. That's my understanding of the data to date.

Slide 135. Role of Mutations in Epigenetic Modifiers in Leukemia Pathogenesis and Therapy

Dr. Irv Bernstein:

This is a great symposium so far, and to help continue in that direction our next speaker will be Dr. Ross Levine, who will talk on the Role of Mutations in Epigenetic Modifiers in Leukemia Pathogenesis and Therapy. Ross is the Geoffrey Beene Junior Faculty Chair, Associate Attending in Leukemia Service and Associate Member of the Human Oncology and Pathogenesis Program at Memorial Sloan Kettering Cancer Center in New York. We are delighted to have you here.

Dr. Ross Levine:

Thanks, Irv. And I'd also like to add my thanks to The Leukemia & Lymphoma Society, both for this symposium and for their unwavering support of leukemia and lymphoma research and patients.

Slide 136. Disclosure of Conflicts of Interest

So what I'd like to do in the next half hour or so – sorry, my own conflict, my collaboration with Agios, who is working out IBH inhibitors, and I will talk a little bit about those genes, but not about the therapeutic potential.

Slide 137. Two-hit model of AML Pathogenesis

So what I'd like to do in the next half hour or so is focus on a single disease and that is acute myeloid leukemia. And try to ask whether a combination of genetic studies in patients and functional studies might begin to give us some initial insight into how some of the alterations that affect epigenetic patterning might affect prognosis, biology and therapeutic response. And follow up a little bit on what Steve Baylin mentioned, which is this concept that there are a subset of mutations that we know have putative roles in regulating epigenetic patterning. And one might argue that all mutations have some effect

on epigenetic patterning.

So before I do that I'll just begin by talking a little bit about sort of the classical dogma that I think the field has thought is the molecular basis of acute myeloid leukemia and this comes out of really three decades of work by many investigators and is probably maybe more aptly described in this review by Gary Gilliland, my mentor, and Jim Griffin, where they really talk about two different types of mutations that cooperate in acute myeloid leukemia pathogenesis.

And one class of mutations are those that activate oncogenic signaling pathways like the FLT3 tyrosine kinase, JAK2, oncogenic mutations, in the RAS family of genes. And the other set of mutations are in genes like AML1, RUNX1, CEBP-alpha and other genes that we know are very important in myeloid differentiation.

And these two classes I think are based on two important concepts and I'll return to these concepts a number of times in my talk.

One of these is based on human genetics and that is because when you have a patient who has a FLT3 mutation, you virtually never see that that same patient and the same cell has another mutation that activates the signaling pathway, suggesting that by and large mutations in oncogenic pathways are mutually exclusive of each other. And the same generally can be said of mutations in myeloid transcription factors. And so that led to this idea that they form really two separate mutational classes that neither really – and each can cooperate with each other, but they're sort of internally consistent, mutually exclusive groups.

The other data to support that is the observation that expression of either of these classes of mutations in vivo in mouse models is not sufficient to cause acute leukemia, but together, if you express one from column A and one from column B, you very potently get transformation to acute myeloid leukemia. So the human genetics suggest that these classes of mutations exist and the mouse genetics validated that they collaborate in vivo.

The concern, though, is whether this framework is really accurate. Not so much about whether these classes are internally consistent, but rather whether they're all encompassing. And I think this is based on two fundamental observations. The first is that not all patients have mutations in both of these classes. We and others look very hard for mutations that activate signaling in AML patients and they just can't be found in all patients. And that suggests that activating signaling may not be an absolute requirement in every single patient, although it's important in many patients.

The second, though, is that many of the new mutations don't immediately fit either into these classes in terms of the genetics or in terms of what we think about their role in pathogenesis and the pathways they interact.

So the question I think we've been struggling with is might there be other classes, class three, class four, and how do we incorporate new genetic knowledge into sort of a working framework for AML and what does that teach us about biology and prognosis.

And so I think really, and what has been a watershed of information, we've really in the last I would say three to five years, had an explosion of novel disease alleles identified in patients with acute myeloid leukemia. And these have come really out of two sets of studies. One set of studies were really from sort of the agnostic whole genome or whole ___ approach and these include the identification of IBH1 mutations in AML by the group at Wash U. And the identification of DNMT3A mutations by multiple groups, including groups at Wash U and China. And moreover classical or sort of lower throughput approaches, array-based approaches, candidate gene approaches, have led to novel disease allele identification in AML and MDS, MPN, including TET2, IDH2, following up on the first identification of IDH1, ASXL1, PHF6 and then on and so forth.

Slide 138. Discovery of novel mutations in AML patients

And so the starting point for our work in the last couple of years has been the real recognition that none of these mutations neatly fit into those two classes that I showed you on the previous slide. And so the issue really is that the biologic and prognostic significance of these novel disease alleles has not been fully delineated. We don't know they do and we don't know how they fit.

But we know that many of them, and some of these again we already know have clear impact on regulating the epigenome, DNMT3A, TET2, but our theory has been that many, if not all of these, have direct effects on epigenetic patterning.

So the question you have to ask yourself then is how are you going to go about studying all these mutations and trying to uncover how you can understand them in individual patients.

So the approach we've taken is a little bit different than the approach of whole genome sequencing of small numbers of patients, which has largely been a fruitful approach, led by the Wash U and other groups. Our approach has been to say, listen, we're not going to find new mutations, but rather we're going to take all the mutations that have been recently described, now there are about 18 genes that are known to be recurrently mutated in AML, and we're going to profile about 500 patients with acute myeloid leukemia, and we're going to do this with multiple goals in hand.

The first goal we're going to have is as a clinician, is we want to see if any of these new mutations, all the things I showed you on the previous slide, actually have prognostic significance, can they tell us which of our patients will relapse, which of our patients will not do well with therapy, and which of our patients might actually do remarkably well with standard therapy.

The second thing, we were very fortunate, is that ___, close collaborator and friend of mine, had done both methylation profiling and transcriptional profiling on this exact set. So we could at the outset plan to integrate our mutational data with methylation data.

But the most important thing we wanted to do was to understand whether we could use this knowledge to inform AML biology. And I'll talk about that in a few minutes.

Slide 139. Mutational Profiling of ECOG 1900 Cohort*

And then we had a bonus. So it turned out that this cohort that we were studying was from the only positive Phase III trial in AML in adults under 60 in the last 20 years, and that was a trial of standard dose daunorubicin, 45 per meter squared as part of induction therapy, versus high dose daunorubicin, 90 per meter squared, and this was a ___ trial in the New England Journal led by the Eastern Cooperative of Oncology Group, an important advanced. But arguably incremental in that it's a modest improvement in a large cohort in a heterogenous disease. So we wondered maybe there are specific genetically defined or epigenetically defined subsets that might actually benefit – specifically from this increase in anthracycline dose. Meaning that this is not a better therapy for all of our patients, but there might be specific subsets that actually might benefit from this therapy and I'll tell you about each of these.

Slide 140. Mutation Summary

And so when we sequenced these patients we found approximately 600 mutations in the first 400 samples we identified. We now have a slightly larger number of mutations. We found anywhere between two and three mutations per sample in our candidate approaches. Tim Lay and colleagues will report I believe about, in whole genome sequencing, an average of three to four driving mutations in whole genome sequencing. So I don't think we're that far, we're not looking at the tip of the iceberg, I think we're looking at a fair amount of mutational spectra.

Importantly, though, in this subset the number of mutations did not impact clinical outcome. And that's suggests one of two things. The one possibility, again, is that mutations aren't primarily driving

outcome, but the other possibility, in the patients with zero, one or two mutations, there are a few more mutations that we don't know about yet and that's our expectation that we will find a number of additional mutations ___ these patients and that the patients with five, six mutations aren't inherently more genetically complex than the ones with zero, two.

The striking thing, of course, compared to solid tumors and even other hematopoietic malignancies, is how few mutations there are in patients with acute myeloid leukemia. And that really I think is telling us something, that although it's a heterogenous disease in outcome, expression profiling, the number of driving mutations may not be that diverse in AML and I think that's a very interesting observation that I wouldn't have predicted personally five years ago.

Slide 141. Cooperativity Matrix Reveals Marked Mutational Heterogeneity

So this slide now shows the frequency of all the mutations in this cohort. The most common mutation, as in virtually every AML study, is mutations in the tyrosine kinase FLT3, about one-third of patients. Second most common is DNMT3A. And you can go down throughout the list. And you can see many of the mutations, which I'll talk about, including TET2, occur in a significant proportion of patients.

Importantly, this is whole gene resequencing with validation of every mutation in ___ material, which we think is critical here because a lot of these are big tumor suppressors and you need to annotate every single variant in every patient for whether it's inherited or acquired, or you won't be to determine what's a polymorphism and what's an acquired mutation.

So Jay Patel, who's the student in my lab that did all this work, then came up with initial cooperativity matrix and this was an Excel spreadsheet he showed me, about what mutations co-occur with which. And as you can see it's difficult, if not impossible, to read if you stood right in front of it and I think clearly impossible from where I'm standing. And I said to him this is not a good way to show mutational sort of cooperativity in AML. And so he went back to his desk in the lab and used the program, which normally is used for translocations, and then he showed a different way of looking at the mutations and that's shown here. And so this wheel now shows the mutations that occur in AML and who they co-occur with. And I'll take you through this.

And so the width on the wheel of any particular mutation is proportional to the frequency of a particular alteration. Like FLT3 being the largest spoke on the wheel. And then two mutations co-occurring are shown by the bar going across and the width of the bar being directly proportional to the number of patients that have those two mutations together. And we think this really shows you that certain mutations actually very commonly co-occur.

And so I'll show you an example. This is just looking under the very careful view of just FLT3 mutant acute myeloid leukemia and you can see that FLT3 mutations co-occur with virtually every mutation, with the exception, for example, of ASXL1, a gene that my lab's been working on, and we of course now, not seeing this ever co-occur in 500 patients, would never make that mouse model because it's not an informative experiment because these never co-occur in patients. However, one could argue that FLT3 should be tested in mouse models and in patients and in cellular systems with all these other lesions and we think that is telling you something about its promiscuous nature.

Slide 142. Relevance to Clinical Practice

So the question, though, we wanted to ask ourselves is might this information be useful for clinical practice. And then I'll come back to the biology. So wanted to ask a few questions. The first is do any of the new mutations, the things that have been described in the last two to three years, have prognostic value in AML? And importantly, unlike many of the other prognostic studies, this is a large Phase III trial that's homogeneously treated with very good follow-up, so we can feel very good about that, and it's similar to

data from the Germans and the Dutch and SWOG and CALGB. And the question is can we then use this information to make better risk stratification models for our patients? And then I'll come back to the standard versus high dose daunorubicin at the end of my talk.

Slide 143. Effects of Mutations on Overall Survival

So the first thing we did is ask are there any novel mutations that just by themselves have adverse prognostic outcome in AML. And it turns out that a number of them do, including the FLT3 IDT, which we already knew about, two mutations that had not been previously linked to adverse outcome, ASXL1, which I will talk more about today biologically, and PHF6 and of course the MLL partial tandem duplications, suggesting that even in a very large heterogeneous disease, things can have adverse outcome overall in the cohort.

Slide 144. IDH2 R140Q Mutations Associated With Improved Overall Survival

What's fascinating is you can find things that predict for favorable outcome. And the thing that surprised us in this dataset was the observation that IDH2 R140Q mutations, not IDH1 and IDH2 together, not even all IDH2 mutations, just this mutation predicts for favorable outcome in acute myeloid leukemia. This is overall survival and it's about 65% with this mutation. And then this is the other two IDH alleles and they're essentially equivocal.

What's interesting is that this matches very well with the data from the MRC trial, which shows very similar results. And it's completely different, what's been seen by CALGB and by the German groups. And that's because the MRC trial and this trial, patients were treated with autologous stem cell transplantation in CR1. Suggesting in fact that it's not that it predicts for a good outcome, it predicts for a set of patients that might do very well with a very aggressive approach. So a chemo-sensitive disease that we need to hit very hard. Whereas in fact if you don't treat these patients, look at elderly patients, IDH mutations are unfavorable. So it's not to say that this is a good marker, but it's a marker that you need to treat these patients very aggressively and I think that's a very important distinction.

Slide 145. Intermediate Risk AML

So we then wanted to look in the set that all of us who take care of leukemia patients struggle with all the time. And this is this group of intermediate risk AML, the group we wring our hands about, should we offer allogeneic transplant, should we do high dose Ara-C, should we do autotransplant, should we do investigational therapies.

And we performed decision tree multivariate analysis and asked are there specific mutations that predict for adverse outcome in intermediate risk AML and in addition to FLT3 and ASXL1 and PHF6 and MLL that we already knew about, were adverse in the overall cohort, the other gene that rose to the top was TET2. And suggesting that within this subset TET2 was an adverse outcome.

Moreover, importantly IDH2 again was favorable and I'll come back to that. CEBP-alpha was also favorable. And you'll note the absence of NPM1, which I'll show you in a minute. And that importantly did not in multivariate analysis predict for a favorable outcome in our cohort.

Slide 146. IDH/NPM1 mutations

So why is that? Because the presence of NPM1 mutations in this cohort, in FTL3 negative patients, do not predict for a favorable outcome in AML. It is the combination of NPM1 mutations and IDH mutations that predict for favorable outcome. NPM1 mutant, FLT3 negative patients without IDH

mutations do as poorly as FLT3 positive patients in this cohort. And this is exactly replicated by the MRC data. And this suggests again that we can't think of mutations by themselves as having prognostic value or biologic relevance. It's who they occur with. And we know in gene expression data done with ___, that this combination has very specific effects on gene expression that aren't seen with either mutation alone. So we think this is important for clinical practice today and is a very important concept. And this is not affected by whether patients are transplanted, it's not affected by the daunorubicin dose. This is clear, regardless of how you treat patients.

Slide 147. Modified risk model for FLT3-ITD

So what if we now look at the two subsets of intermediate risk AML with more clarity. This is the group of patients with FLT3 negative intermediate risk AML and this decision tree analysis, allowed us to break these patients into three groups. One group is that really favorable group of the double IDH NPM1 mutant. Look at that overall survival, 85% overall survival at five years. That's better than 8;21 or inversion 16. This is the most favorable subset of leukemia we've seen.

On the other hand, if you have a TET2 mutation, an ASXL1 mutation, PHF6 or MLL, your outcome is essentially less than 5%, suggesting that even in the absence of FLT3, having these negative mutations are really a bad thing. And this is this concept in the paper by S___, that if you're negative for FLT3, NPM1 and ___, you do poorly. It's because it's these patients that mark out that outcome.

And then other genotypes fall into the middle and this includes CEBP-alpha, for example.

Slide 148. Multivariate Model for FLT3-ITD

What about FLT3 positive disease? This is also viewed by most people as an unfavorable outcome. Well, it turns out it also is not driving it by itself. It is the combination of FLT3-ITD mutations in genes like TET2 and ___, that are associated with bad outcome, or MLL. Suggesting that mutations in epigenetic regulators, when they co-occur with FLT3, are what are driving that outcome. It's not FLT3 by itself. FLT3 is an important part of the picture, but it's not the whole picture, it's who it's playing with.

By contrast, of you're FLT3 mutant and negative for those things, you're as good as if you're CEBP-alpha plus FLT3, which is the one thing we recognize as being a favorable modifier.

And so we think this can really tell us which patients desperately need novel therapies.

Slide 149. Revised AML Risk Stratification Based on Integrated Mutational Profiling

And so we've developed a model based on our data that allows us to integrate this knowledge into clinical practice. And this is this concept, that we need to move from just a chromosomal two, three gene model, to a larger model. And you can really expand favorable risk disease to include these double NPM1 IDH mutants. And we need to expand unfavorable to include all of these other groups, and you can see this highlighted here, here and here.

Well, that's all good and well you can say, and I've had folks who are real experts in the leukemia community say to me listen, every study is different, everybody does prognostic markers, and then the next study comes out and they get the exact opposite, so how do you know that what you've observed is actually not a vagary of multiple testing, with all the caveats that this is multivariate analysis and done for all clinical parameters.

Slide 150. Revised AML Risk Stratification Based on Integrated Mutational Profiling (cont.)

So what did we do? The first thing we did was look at our cohort and ask if we take this now

modified risk classifier, what happens to these patients. So the green and the red curve are patients that either have that favorable genotype or the unfavorable genotype that normally would have been in the intermediate risk group, that are bumped up or bumped down. So I think I can persuade you that these are clinically relevant distinctions in a very large cohort.

But to really convince ourselves is this true, we went back to this trial and we took 110 samples that we had never analyzed, that we didn't even have access to, and we've revalidated the entire schema. And I'm sorry, I don't have a slide here, it exactly validates. So we're able to externally validate this prognostic set on another set of homogeneously treated patients. So we believe this is a robust finding. And we're convinced at our institution that we need to begin genotyping for all of these things quickly, because this has clinical relevance today for our patients. And we think this is not easy to do, lots of and intellectual property issues, it's something everybody is struggling with, but we need to get around these issues and work together as a field to bring integrated genetic profiling to our patients. Because it may be the fastest thing we can do to deliver the best therapies to our patients. Faster than the new molecules. If we could figure out which of our patients need to get standard of therapy and which patients need more aggressive therapy.

Slide 151. Human genetics is always right

So I'd like to now go to the biology and ask ourselves how can we use this knowledge to understand AML biology. And so something that Gary Gilliland taught me is this concept that human genetics is always right. So by studying primary patient samples we can improve our understanding of AML biology and we can do this in a few ways. One way is obviously by finding cooperations that commonly co-occur together or have prognostic significance, we can make new models, test new drugs and do all the things that are really important.

But we think there's something else you could do right away and that is this concept that if we find mutations that never co-occur together, we might find novel mutational classes, that class three, that class four, mutations that we thought ___ have no relationship together, might in fact suggest that they function in a pathway. Or I think alternatively, we have no data to support this, that maybe two mutations that never co-occur together because of this concept ____, and maybe those are novel therapeutic opportunities that we need to think about as a field.

So we hypothesized by studying patients with different mutations, we could understand what they did, and we decided to focus on IDH and TET mutations.

Slide 152. ECOG 1900 Cohort: IDH1/2 mutations mutually exclusive of TET2 mutations

And so with no expectation that we would get this answer, we found that we could not identify a single patient in over 400 with a coexistent TET2 and IDH mutation, with really no, I guess, expectation that these things would be functionally linked. And that suggested to us that these mutations were ___ and might function in a pathway.

And so how might this all work out? So because we'd been thinking about TET2 mutations in my lab, we could I guess arrive at some hypotheses.

Slide 153. Conversion of 5-Methylcytosine to 5-Hydroxymethylcytosine

So what do we know about the TET family of enzymes? ___ now about two and a half years ago identified the TET enzymes as putting a novel mark, a hydroxyl mark, on methylated cytosines and as Steve said, that really protects against the ___, and through what is not fully worked out as a pathway, but probably in many cases through the ___ pathway, although it may be in other cells through a passive

process, for example, in the ___ implantation zygote, results in demethylation of DNA. So the prediction you would have is that if you lose TET2 function and if TET2 is the prominent hydroxy-methylase in hematopoietic cells, you might get an accumulation of methyl cytosine and a loss of hydroxy-methyl-cytosine. And so that was the first question we asked.

Slide 154. TET2 Mutations Are Associated with Hypermethylation

And again, we were very fortunate because __ Melnick had been independently studying the same cohort. And so we were able to give him the data from the TET2 mutations and find in using his assay, that he could identify a set of genes that were hypermethylated in patients with TET2 mutations. So we can now validate this in mouse models, suggesting that we think that there's at least a subset of genes.

One fascinating thing is that it's a very small number of genes that are hypermethylated. It's not the predominant set of genes and we don't I think understand why it's only some and not other genes.

Slide 155. IDH1 mutations define a hypermethylator phenotype in GBM

Now at the same time that we were beginning to work on this, I think one of the seminal papers came out from TCGA and that was the observation in glioma that IDH1 mutations were associated with a hypermethylated phenotype. And we of course paid very serious attention to that. And so the observation that the TET mutations ___ were exclusive, the observation that the TET mutations caused hypermethylation and that IDH mutations in glioma cause hypermethylation, caused us to look very carefully at IDH mutations in leukemia patients.

And we found in fact, just like in the TET mutant patients, there's a hypermethylation, in fact much more dramatic than we see in the TET mutant patients. And we think that's probably because of affecting all three TET family enzymes.

Slide 156. IDH1/2 Mutations Are Associated with Hypermethylation

So we wanted to then ask is it cause or consequence. Because if you read that IDH TCGA glioma paper, the actual discussion speculatively hypothesized that there's an initial effect on methylation that then favors the acquisition of IDH mutations. But our data linking TET and IDH genetically suggested to us that maybe that TET and IDH were the driver and the epigenetic effects were then the consequence.

Slide 157. IDH mutations or TET2 shRNA in primary hematopoietic cells

And so the first thing we did was take primary hematopoietic cells, mouse bone marrow, hematopoietic cell lines, and either knocked down TET2, because those are loss of function mutations, or over-express IDH mutations, so we could show in collaboration with ___ that you get a reduction in hydroxy-methylation and an increase in DNA methylation, replicating what we saw in the methylation data from the patients.

Slide 158. AML patient samples

We have now gone on more recently to take a set of patients from that cohort and do mass spec for overall methylation levels and hydroxy-methylation levels and see the exact same results in primary patients ___, suggesting that the cell line data is supported by what we see in patients.

Slide 159. IDH1/2 Mutations Inhibit TET2-mediated 5-OH-Me-Cytosine formation

So how might this work? So we wondered might IDH mutations, through the production of 2-hydroxyglutarate, inhibit the TET function. And Pat Ward and Chao Lu in Craig Thompson's lab at Sloan Kettering performed I think a critical experiment. And they expressed the IDH mutation in concert with TET2 and showed that you block TET function. And it's subsequently been shown by ___ and colleagues that recombinant 2-HG directly enzymatically inhibits the TET enzyme. So this is not some other effect of IDH. It is 2-HG in vitro. So we think it's a direct effect.

Slide 160. *IDH1/2* and *TET2*

And that leads us to this model, where the IDH wild type allele makes alpha-keto-glutarate, the mutant allele that corrupts the alpha-keto-glutarate to make 2-hydroxyglutarate, which blocks TET function. And that means that whether you gain IDH or lose TET2, you end up with a block here and an accumulation of methyl-cytosine.

But the question is what does any of this have to do with leukemia anyway? How do we link this back to hematopoietic function?

Slide 161. Conditional TET2 KO mouse

So that's something we've worked very hard on, so in collaboration with __ Aifantis, we've developed a conditional knockout mouse where we deleted TET2 specifically in the hematopoietic compartment, using either Vav or Mx-Cre promoters. And we can show that this results in complete loss of TET2 message and protein and despite the fact that TET1 and a little bit less of TET3 are expressed in these cells, there's no compensatory change. And that's a question I can't answer, is why would TET2 be so specially disregulated in leukemias, yet TET1 and TET3 are there. It's not that they're not expressed, and I think that's a fundamental question in the field. Different genes working together and we can talk about that after.

Slide 162. TET2 deletion

So if you look at these cells in vitro they have a very, very dramatic phenotype. The TET deficient cells have the ability to serially replate and they're immortalized and will grow as long as you let them. This is what MML does to cells. So this is something suggesting that you're getting an increase in self-renewal. Clearly and easily demonstrated. Hairpins or deletions of TET2 in our case.

Importantly these are not stem cells. And we think this is a really important fact from the hematopoietic perspective. These cells have the immunophenotype of myeloid progenitors. They're CD34 positive, they're FC receptor gamma positive, they're GR__ negative. And I think suggests possibly, and we don't have all the definitive data, that in fact what TET loss is doing is taking a more committed cell without self-renewal and when you lose TET2 you're giving self-renewal to a non-stem cell-like population. And that fits again with what MML's been shown to do, although we need to formally test that and I think that's something that we're doing in vivo right now. And we think it suggests again that this may be the susceptible population for leukemia.

So to show whether there's actually an enhanced stem cell function, though, you need to go in vivo. And so the critical experiments are to do competitive transplants. And it turns out that TET deficient cells have an incredible advantage in competitive transplant __, suggesting they have very much enhanced stem cell function.

Slide 163. TET2 KO cells

If you look at these knockout cells, again, they look more like progenitors than stem cells. Their gene expression is much more like the common myeloid and granulocyte macrophage progenitors than they are like hematopoietic stem cells and they, with the exception that there is a self-renewal program, including genes like EVi1 and Meis1, that's up-regulated. And importantly we can show the exact same gene signature is highly enriched in primary patients __ TET2 mutations. So whatever we're seeing in the mouse models is true in patients.

Slide 164. Loss of a single Tet2 allele

One important I think phenomenon is that patients don't delete all of their TET2. They mutate one copy, usually leaving the other intact. And so we went to our mouse model and asked might haplo-insufficiency for TET2 actually be sufficient to cause the phenotype. And it recapitulates the whole thing. It's a little more sluggish, takes longer to develop, but the cells replate, they ___ in stem cells and the mice get sick with chronic myelo___ leukemia, suggesting again that just reducing TET is sufficient to cause increased stem cell function.

Slide 165. Cooperativity Studies in Vivo

So what about AML models? I'll just show you two tantalizing slides that we've now developed AML models. The first is using oncogenic __, we've been able to take that in the TET2 knockout mouse and show you get impaired survival. And importantly a secondarily transplantable disease into irradiated recipients, suggested that this is an acute myeloid leukemia phenotype. So RAS doesn't cause AML by itself, TET2 doesn't, but together we get AML.

Slide 166. Niccastrin deletion

And __ Aifantis, who will be giving a scientific subcommittee talk, will talk all about this tomorrow morning. We've gone on in collaboration with him to look at notch loss because he's shown that notch is a tumor suppressant in a recent paper in Nature. Plus TET2 loss cause a fully transplantable, fully penetrant AML. No signaling mutation or activated signaling mutation whatsoever.

Slide 167. AML mutations and their effect on the epigenetic state

And so this suggests that mutations in the epigenetic state can clearly contribute to leukemia-genesis.

So in the last minute or two I just want to return to the question of what is the clinical relevance. And I'll just make two sort of I guess troubling comments and then I'll end, hopefully, on an interesting note.

Slide 168. Not so straightforward: divergent effects of IDH and TET2

The first is that IDH and TET are not the same thing and that's probably because IDH does many other things other than inhibiting TET function. The prognostic significance of IDH and TET could not be more different. So this concept that they're the same thing is very, very simplistic. They have different outcome in AML and this suggests that there's things that IDH is doing that TET's not doing and vice versa and we need to understand that better and that's something we're working very hard on.

And importantly, the TET2 mutant patients do very poorly and the IDH patients are equivocal to good.

Slide 169. Complications, part 2

And again this gets back to the IDH NPM1 thing, the concept being that it's not the TET or IDH that drive leukemia, but who they're playing with.

Slide 170. Newest Epigenetic Mutation: DNMT3A mutations in AML

What about the newest mutation? DNMT3A mutations. So two groups, one from Wash U, one from China, identified somatic mutations ___ DNMT3A. And they showed in non-clinical trial cohorts that this was associated with adverse outcome in AML and that got our attention. So we looked at our cohort and we found that DNMT mutations in our cohort, despite the fact that all the other cohorts that have been described, showed adverse outcome, didn't show adverse outcome. And that surprised us. So we wondered maybe the DNMT mutant patients, their outcomes being improved in this randomized trial by the higher dose of daunorubicin, that half the patients got, whereas all the other trials were using standard induction. And that turns out exactly to be the case.

Slide 171. DNMT Mutational Status Does not Affect Outcome

So DNMT3A mutant patients have a three-fold improvement in their overall survival, with just a doubling of daunorubicin dose. Who would have predicted that? And the wild type patients do not.

Slide 172. High Dose Daunorubicin

And there's a recent paper from ___ randomized trial by the French that idarubicin also is better than daunorubicin in mutant patients, with a doubling in overall survival. These patients need more anthracyclines, we would predict.

Are there other mutations that do this?

Slide 173. High Dose Daunorubicin

There are. NPM1 and MLL. And importantly all three of these mutations, in ours and other cohorts, are associated with ___ genes signatures. And this suggests that there is a set of mutations in leukemia patients that predict for benefit from anthracycline at higher doses and everyone else does not benefit. You can see there's really no difference in this randomized trial in 70% of the patients, ___ , but a dramatic benefit, with just a doubling of daunorubicin dose. So in this Phase III trial all the benefit is explained by three genes. And we think that's important, particularly in older patients who don't want to get cardiotoxic agents.

So I'll just summarize and say that we think by studying primary patient samples and model systems we can learn a lot and I think ultimately impact the clinical outcome.

Slide 174. Acknowledgements

My collaborators are on this slide. And I thank you very much for your attention, thanks.

Dr. Irv Bernstein:

I have one question while people are thinking. One of the thoughts also is the context that these mutations occur or achieve clonal dominance in. A little surprising that the poor outcome patient, you

mature progenitors, but that doesn't mean that the context where it's initiating is different. Do you have any thoughts?

Dr. Ross Levine:

That's a really important question and one I think we and others are testing. So we're looking in the TET deficient context, whether the initiating population might impact not just the phenotype, but the therapeutic response to daunorubicin and cytarabine. And things like transplantability. So we think that's a very important question, which would be even in the exact same ___ context, if you engineer the events in a different initiating population or in a different order, whether that would affect therapeutic response or outcome.

Audience:

The IDH1 versus 2 mutants, what's your current thought about the qualitative difference? Do the mutants do something qualitatively different in terms of their novel enzymatic activity or is it a quantitative difference?

Dr. Ross Levine:

The IDH mutants?

Audience:

Yes.

Dr. Ross Levine:

There's definitely quantitative differences. So the R140, which has the favorable outcome has a very significant reduction in the production of 2-hydroxy-glutarate compared to the R172 or the IDH1 mutation. About a two to three-fold less potent, making this ___ enzyme. Whether there are also qualitative differences I think remains to be seen. But there are clear quantitative reproducible differences in everybody's hands.

Audience:

So it may be that a different set of alpha-__-glutarate dependent enzymes may be inhibited in one disease versus another and that could be the difference.

Dr. Ross Levine:

And I think importantly, and this is something I've learned in the last year or two about prognosis, prognosis is like Robert Frost. It's all about the road you take versus the road you don't take. So you can't think that a gene is bad in AML, it's all about what its alternative is. Because if you get a certain mutation that then precludes the need for another bad prognosis ___, that's why they're good. So I think one thing we need to think about is things don't predict for good or bad outcome, it's all about which alternative the cell takes to transformation and ___ compares to other routes.

Dr. Irv Bernstein:

Do you have sense to what extent these mutations are in the entire population or are these drivers of subsets? So if you get a clonal analysis, single cell analysis of leukemias, for example, shown that FLT3 mutations can be in a mature precursor and only in subsets, ___, do you have any sense?

Dr. Ross Levine:

We don't yet. We're starting to do some of those analyses and Jerry has done really I think the best

single cell work in the field at your institution. We've started to look more with sorted subsets within patients, not single cell, but taking ___ progenitor cells from a leukemia and asking in the bulk of those population, which is a little different than the single cell, but we are interested in asking whether different phenotypic, immunophenotypically defined populations have different subsets. I think ___. I think it's an important question we need to understand better.

Slide 175. Epigenetic Therapy of Myeloid Malignancies

Dr. Irv Bernstein:

Great, thank you. This is an interesting time.

Dr. Jean-Pierre Issa. For those of you who don't know him, he's a Professor of Medicine and Director, he's now at the Fels Institute for Cancer Research in Molecular Biology at Temple, having moved there recently from M. D. Anderson. And he will speak on epigenetic therapy of hematologic malignancies.

Dr. Jean-Pierre Issa:

Thank you. It's a pleasure to be here, thanks for the invitation.

So I'm going to tell you a little bit about epigenetic therapy, focusing on myeloid malignancy, but also see where we're going with this in other diseases as well.

And it's really a pleasure to be talking after all these speakers because they've done much of the background already, so I don't have to spend much time about it. But if you're like me, I find myself a little confused because there's been so much information that's been thrown at us. So I did put in just a couple of slides just to remind you what we're doing and perhaps use my relatively simple ideas to explain epigenetic therapy.

Slide 176. Disclosure of Conflicts of Interest

So these are my disclosures here. And I will mention the use of the hypomethylating agents in other diseases.

Slide 177. Multiple Defects in the Leukemia Epigenome

So what we heard from both Steve and Ross, at least for leukemias, is that there are multiple defects in the leukemia epigenome. And one can look at it in two different ways. One can look at the signals and Steve eloquently discussed that. The signals appear to be abnormal, so there are very clear abnormalities in DNA methylation, histone modifications and something that really isn't discussed much in this session, but also micro RNAs.

And so looking at the end result we clearly see epigenetic differences between leukemias and their normal counterparts here. And what we heard from Ross is that the recent genome-wide studies are suggesting that there are also probably upstream signals, upstream defects, genetic defects in the regulators of epigenetics. And Ross showed you some examples of that here.

Now one of the important things I think Ross was trying to get at is we still don't understand how these two things are linked. We don't have very clear picture of how mutations actually affect the epigenetic signals and it remains really unclear whether most of the epigenomic abnormalities are primary or are downstream of other effects.

Now in AML, Ari Melnick's work and Ross's work has suggested that TET2 mutations are associated with a DNA hypermethylator phenotype or a CpG ___ methylator phenotype. I won't show you

the data, but our data in CMML, which is a disease that has the most TET2 mutations, find absolutely no effect on CpG ___ methylator phenotype there. So I don't think it is entirely clear right now that these mutations are just simply affecting globally methylation. There might be very specific defects or very pinpoint defects, but there's a lot of work that still needs to be done to figure out whether the genetic changes are actually changing epigenetics or whether these are just two co-occurring things with very little overlap between the two here.

The ___ in my lab as well have very similar data for DNMT3A mutations. Now one would like to think that a mutation in a DNA methyltransferase is going to have profound effects on DNA methylation, but so far that has not been the case, at least in acute myelogenous leukemia. So again, something that really needs to be looked at and thought of very carefully here.

Slide 178. DNA Methylation is Abnormal in Cancer

Nevertheless, this is where we are. And this is an example of the DNA methylation abnormalities one sees in leukemias, in this case in acute myelogenous leukemia case, where we have compared normal white blood cells on the axis with leukemia on the Y axis. And what you see is that there is an enormous scatter in the points, suggesting profound abnormalities in AML compared to white blood cells. And these are very stable. So if you look at this AML, say, several days apart or several weeks apart, those changes are going to persist. And if you look at normal blood and ask about how much variation there is, say, between people in this room, there's actually relatively little variation compared to the kind of thing we see when we compare normal to cancer.

One of the things that we keep glossing over is that it is not a simple process of increased methylation, which is what Steve mentioned or talked about. It's because one sees simultaneously decreases in methylation in these red dots in this case, which corresponds to non-CpG island DNA, primarily DNA that is ___ . And one sees marked hypermethylation in CpG islands which are primarily in promoters. And so this dual process is happening at the same time in the same leukemias, so that it is difficult to talk about a single defining event. There's just increased methylation. Well, there is increased methylation at some genes, but there is decreased methylation at other genes. And that perhaps is going to color a little bit the results of the clinical trials I will tell you about because we are assuming that the dominant effect is hypermethylation and that's why we're going to talk about inhibiting DNA methylation, but there might be instances where this is not wise or there might even be instances where inhibiting the inhibitor might be a therapy for leukemia and it's something that hasn't been explored much at the present time here.

Slide 179. MDS: Genetically *and* Epigenetically Heterogeneous

So one of the things that is very clear is that epigenetic patterns are also very prognostic. So you've heard about all of the prognostic impact of the mutations in AML and also in other diseases such as in MDS, but DNA methylation is also equally powerful in segregating patients into those who will have a good outcome and those who will have a poor outcome. In this particular slide I'm showing you a methylation profile of patients with myelodysplastic syndrome, where red is abnormal and green is normal. We're looking at ten genes here. And patients with very high levels of DNA methylation, in this case a true hypermethylator phenotype, actually have a very poor outcome. And patients who don't have this hypermethylator phenotype have a relatively good outcome in MDS. And this outcome in this case is entirely linked to how rapidly MDS progresses to AML. So that as we move forward in terms of profiling patients for therapy and as well as for prognostic purposes, I do think that it will make sense in the future to not just look at mutations, but also look at epigenetic abnormalities. And it would be my guess that most of these DNA methylation abnormalities are actually independent of mutations rather than simply

downstream of mutations. Because as Ross has already told you, we already know a lot of the mutations that happen in these diseases. And so far none of them has been very cleanly associated with DNA hypermethylation events that Steve talked about and that we find in my lab. So that probably the future of profiling cases for prognostic and predictive purposes is going to involve both looking at the marks and looking at the genetic lesions that might in part modulate these marks. And in this case it was independent of cytogenetics.

Slide 180. Epigenetic Therapy

So nevertheless, with all the uncertainties, what is very clear is that there are a lot of epigenetic abnormalities in the leukemia epigenome. And epigenetic therapy has been defined as therapeutic targeting of epigenetic modifiers to achieve some degree of reprogramming in vivo. Now we have known for many, many years, for more than three decades, we have known that epigenetic reprogramming, say, through embryogenesis, can reverse the malignant phenotype. Even in hematologic malignancies this was done in the early eighties by Leo S__ lab, who demonstrated that if you simply took acute myelogenous leukemia and forced it to go through embryogenesis, which reprograms the epigenome, then you erase the leukemic phenotype.

So that we've known for a while that modulation of epigenetics could have a therapeutic benefit in leukemias. And in fact there have been a number of drugs discovered through differentiation screens and ultimately differentiation is an epigenetic process. And indeed it is very likely that some of these drugs that work through modulating differentiation are actually epigenetic drugs and some of these drugs are helpful in the clinic in patients here.

So in this case, though, we have taken a more directed approach that started many years ago in work indeed in Steven's lab and Peter Jones's lab that said well, DNA methylation is abnormal, what we can really link to biology is increased methylation of tumor suppressor genes in cancer, so why not try to target DNA methylation to reduce methylation of these tumor suppressor genes and see whether that will have an epigenetic reprogramming and therapeutic effect in vivo here.

Slide 181. Hypomethylating Cytosine Analogues

So this work started with DNA methylation inhibitors, azacitidine and decitabine, which are old drugs made new, of course. These drugs discovered in the 1960s, developed as cytotoxic agents, and resurrected in the 1990s at much lower doses than were tested in the 1970s, because it was clear that at lower doses they were more effective hypomethylators. I'll show you one of those slides here. And now, of course, FDA approved in the treatment of MDS here.

These are relatively simple nucleocyte analogues that were initially synthesized as Ara-C analogues, but really they look like ___with simply a nitrogen instead of a carbon at the 5 position of the ring here.

And the way these drugs work actually has been – the best explanation for why they've had two lives, one life in the 1970s which was not very successful, and one life in the 1990s, which was much more successful. And the way these drugs work is that they incorporate into DNA and they incorporate in place of cytosine and actually azacitidine actually gets converted to decitabine triphosphate and so ultimately the mechanism of action of the DNA, at the DNA level of the two drugs, are exactly the same.

Slide 182. Mechanism of Action

So they incorporate into DNA and the DNA methyltransferases will scan the DNA, recognize cytosines that need to be methylated and try to methylate the 5 position of the ring and when it encounters

these analogues, it cannot methylate fully the 5 position because there's a nitrogen instead of a carbon, and as a result the enzymes get stuck onto DNA.

Now you recognize from the old days of developing standard chemotherapy, that having big proteins stuck on DNA is not good. These are what we refer to as bulky ___. So if you have enough of these big protein stuck on DNA, what this will do is it will stop DNA synthesis and kill the cells. This is classical cytotoxicity in that sense. So these drugs are cytotoxic drugs here.

However, if you do this and you use much lower doses of the drugs, the cell can cope with that. It will actually simply excise these adducts, degrade the methyltransferases and DNA synthesis will continue without the methyltransferases, and that's the targeted effect. So at high doses these drugs will kill the cells through a classical mechanism of action of DNA adducts. At low doses it will not kill the cells. It will lead to demethylation. And the idea is that it's the demethylation that's going to have a therapeutic effect and the reprogramming effect in that case here.

And this turned out to be correct because these drugs at high doses, as tested in the 1970s, were not particularly effective. In fact, azacitidine was in front of the FDA for leukemias in 1978 and it was turned down and then it came back. It came back about eight years ago at much lower doses and that's when the efficacy was demonstrated as a hypomethylator agent here.

Slide 183. Azacitidine in MDS CALGB Study Group and Therapy

So let me go through some of these clinical data, since this is what I was asked to do here. Azacitidine was brought to MDS by Lou Silverman and the CALGB. And this was the pivotal study that led to its approval by the FDA in the U.S. This was a study that randomized patients to azacitidine or supportive care and had a crossover design in that those patients who failed supportive care could receive azacitidine.

Slide 184. Azacitidine – Phase III Overall Survival

And that study showed a significant survival advantage to patients randomized to azacitidine. And when I mean significant I mean significant to the eye, but it was not statistically significant with a P value of about .1 in that particular case here. But as in many things in oncology, we can have a sense whether this could be real or not and it appeared to be a real effect and indeed the drug was eventually approved by the FDA based on responses and quality of life and this trend for improved survival, which in that case, however, was not statistically significant.

One thing that is not often discussed or recognized is that in Lou's trial he actually had both low risk MDS patients and high risk MDS patients and in subset analyses the survival advantage was the same in low risk and high risk. And in fact it was even better in the lower risk patients than in the advanced risk patients. And for some reason this drug or this class of drugs in the U.S. tend to be given to more advanced risk MDS, although I really don't understand why that is. Because if you actually look at the trial that led to FDA approval of azacitidine there was significant benefit in patients with relatively lower risk MDS in that case here.

And one would imagine that if a drug works in the advanced setting, it might work even better in the early setting and that's probably something that hasn't been tested enough in MDS here.

Slide 185. Azacitidine Prolongs Survival in MDS

Now that trial had a follow-up trial which was run by Pierre Fenaux and was a multi-country trial, which had relatively similar design except for the crossover. Patients were randomized to best of care as decided by their doctors. This could be nothing, supportive care or chemotherapy and/or versus azacitidine

at the same dose used by the CALGB. Advanced MDS trial that however excluded secondary MDS. And that's an important comparator because secondary MDS patients have a much worse outcome than primary MDS patients and it turns out everybody in this trial had a better outcome than almost all the other trials in MDS ever done. And that's really in part because what they did here is they selected newly diagnosed patients, previously untreated, and they excluded secondary MDS and that's really the group of patients who generally does the best, even with supportive care.

Slide 186. Azacitidine vs CC in MDS

So in that trial there was responses to azacitidine that was higher than low dose Ara-C or best supportive care, but that was actually lower than chemotherapy. Despite that, the median survival was significantly better for azacitidine than for any of the other options. And this is something that is quite interesting because people often ask whether these drugs are really just cytotoxics and we're just talking about them as hypomethylators or something like that. I'll point out that in this trial and in a trial that I will show you later on, there is a direct comparison between the hypomethylator and very aggressive cytotoxic therapy, and the hypomethylators appear to win out over cytotoxics. So I think that it's not that these drugs are better cytotoxics, it's that they are truly different drugs that work on these epigenetic pathways here.

Slide 187. Survival With Azacitidine vs CC

And this was the Kaplan-Meier curve for that particular trial here.

Slide 188. Decitabine Dose vs. Methylation

What about decitabine? Well, decitabine lagged in its development, at least in the U.S., it was developed relatively more aggressively in Europe than in the U.S. But a few years ago at M. D. Anderson Kantarjian and myself started working on that in myeloid leukemias and one of the things that we did is we revisited the dose issue, based on papers published by Peter Jones in the early 80s, that suggested that there was a very close relationship between dose and response in terms of methylation, but somewhat paradoxical. And this paradoxical response is explained by the mechanism I told you about, whereby these drugs are cytotoxic at high doses. And what you see on this slide is if you follow DNA methylation with increasing dose of decitabine, what you see is DNA methylation decreases, then plateaus, and then at high doses you actually lose the hypomethylation effect. So it's very important to look at the dose that is being given to patients because depending on the patient and the dose you give, this could be a hypomethylator or it could be a cytotoxic drug.

Slide 189. Decitabine Reduced-Dose Schedule (100 mg/m^2/course)

And so at Anderson, which had a very long tradition of doing high dose everything, we actually did a paradoxical trial where we started with a much lower dose and we demonstrated that a much lower dose was more effective in patients with MDS, and AML for that matter, than a higher dose. And this led to this followup trial led again by ___ Kantarjian where we compared different ways of giving decitabine at five days, ten days or subcu, in a group of patients that were relatively advanced MDS and CMML.

Slide 190. 3-Arm Dosing Study Data Responses By Treatment Arm

And in this trial the total response rate was very impressive. We had a total response rate of about 74%, with the highest response rate being in the patients who received the drug daily for five days IV

every four weeks, and this is the most common way by which the drug is currently used in the USA here.

Slide 191. Decitabine vs. Intensive Chemotherapy in HR MDS

Now we did not do a randomized study, however, because of the strength of the leukemia database at Anderson, we were able to do a case controlled study, where we compared the outcome of patients who received decitabine to matched controls who received chemotherapy. In the modern era, this is, you know, after 2000. And you see that just like with azacitidine in our database it was very clear that the hypomethylator was more effective than very strong cytotoxic therapy in those patients. And that supported the idea that decitabine was also an effective drug in patients with MDS here.

Slide 192. Side-Effects

Now the side effects of both azacitidine and decitabine are globally similar. The main problem in patients with MDS and AML is myelosuppression, that happens early and that actually may not be entirely myelosuppression. There is a strong component out there of activity in that patients who have more myelosuppression actually respond better and in many cases this is apparent myelosuppression. What is really happening is simply a disappearance of an MDS clone and as you know MDS is a differentiated disease. Oftentimes in these patients neutrophils are MDS and all of the cells are MDS, so if you're really effective and MDS goes away, you're going to see counts dropping, which is what happens in some of these patients. And indeed those patients who don't drop their counts tend not to respond to this therapy. If you use the same doses of drugs in subsequent courses, you don't really see as much myelosuppression, which suggests that it is not simply myelosuppression, but in part activity and in part myelosuppression.

Still there is some neutropenia associated with the use of these drugs in solid tumors, so this is actually something that happens in those patients. There's also a paradoxical hyper-thrombocytosis, increased platelets, that also happens in solid tumor patients. And there is some evidence that both the neutropenia and the thrombocytosis are actually differentiation effects of the drugs rather than simply cytotoxic effects of the drugs here.

Slide 193. Histone Deacetylase Inhibitors

So what about HDAC inhibitors? As you know, there are two of them approved, vorinostat and
.

Slide 194. HDAC Inhibitors: Clinical Results

Both of them are approved for cutaneous T cell lymphomas and has activity in Hodgkin's lymphomas. Perhaps consistent with the fact that the effect of histone acetylation is much broader in terms of its function in normal cells. These actually have significantly more constitutional side effects than the DNA methylation inhibitors with things like fatigue, diarrhea, effects on the QT, etc. here.

Interestingly, the only disease where we have extensive experience with both HDAC inhibitors and DNA methylation inhibitors is myeloid leukemias, and in there it appears that the methylation inhibitors are substantially more active and whether that's simply a disease effect or whether it's related to the actual mechanism of action of the drugs is not known here.

Slide 195. Evidence for an Epigenetic Mechanism of Action

So I'm going to just spend a few minutes discussing with you the data that we have that suggests

that this is truly an epigenetic effect rather than a cytotoxic effect. And one could use data from different lines of evidence here. There are the response patterns, there are the pharmacodynamic data that I'm going to show you on some of the trials that we did at M. D. Anderson here.

Slide 196. Cytotoxic vs. Epigenetic Responses

The first thing I think that has been very convincing to people who actually use these drugs in the clinic is that the way they work is very different than the classical mechanism of action of drugs like Ara-C in myeloid leukemias. What you see typically when you treat, say, MDS or AML with standard dose Ara-C is that the disease burden immediately gets better, oftentimes the bone marrow empties within about two weeks of starting therapy. And if you're successful, the disease will stay low and the normal marrow will come back. That's what you usually expect with cytotoxic therapy.

Slide 197. Tumor-Suppressor Gene Hypomethylation After DAC

What you see with DNA methylation inhibitors is different pattern, where the disease pattern actually remains stable for somewhere between two and three months. And then the clone disappears. And this delayed disappearance of the clone is a remarkable phenomenon that's been documented molecularly, but that remains unexplained. The best hypothesis, of course, is that you're modulating the epigenome and that that takes a little bit of time to lead to disappearance of the clone. Now why that is remains a mystery. It could be perhaps activation of an immune response, which however so far no one has clearly documented. Or it could be effect on stem cell renewal, which is what Steve would favor, I suppose, based on his data. But that would be very consistent with an effect on stem cell renewal.

One also sees that some patients take a lot longer to actually have an effect, so sometimes we see this curve delayed and there are patients who remain stable for five or six months and then respond again. Very atypical for a cytotoxic. And we've even seen patients with very clear worsening early on. In AML you can measure that worsening by an increased number of blasts and we have patients where the blasts truly show marked acceleration for a few days or a couple of weeks and then disappear and the patients go in remission. And all of those things are very atypical for a cytotoxic drug. And at least for clinicians and clinical investigators' experience with these drugs, these appear to be different.

Slide 198. Tumor-Suppressor Gene Induction After DAC

What about molecularly? Well, molecularly we and others have measured tumor suppressor gene methylation in these patients and what I'm showing you here is a few patients with MDS who are methylated at the P15 cyclin-dependent kinase inhibitor. And virtually in all the patients we looked at methylation decreases very early on, within five to ten days of treatment, with these drugs. And interestingly, in some patients it stays low and in those patients they tend to respond and in other patients it comes back within the first four weeks and those patients tend to be resistant to therapy. This is not specific to P15, we have also seen it for another gene, micro-RNA here. And other people have described it for other genes. In fact, in our genome-wide studies we have not found that these drugs effect any genomic region preferentially over the other. And it appears that if you look quantitatively, every gene pretty much behaves the same way, everything decreases at the same rate, and then if it stays low, if tumor suppressor genes stay low, those patients go in remission.

Now this was methylation data. We've also looked at expression. And as expected, expression goes up and it goes back more in responders than in non-responders. So the pharmacodynamic data seem to be very consistent with an epigenetic mechanism of action of these drugs.

Slide 199. Epigenetic Therapy – Unknowns

There are many things we don't know yet. We don't know why some patients respond much better than others. So far DNA methylation profiling at baseline has not been predictive of response. And there's a lot of hope that perhaps mutation testing will be – there's some early data that will be presented at ASH. There's data from the Ohio State group that suggests that 3A mutations are associated with higher response to decitabine in AML. Those are based on very few patients, so it needs to be confirmed, but that's a really interesting observation. And there are data that have been published Pierre Fenaux that two mutations are associated with a higher response to decitabine in CMML, again, or in MDS, again something that needs to be confirmed in other studies here.

We don't really understand what happens after that initial period of epigenetic reprogramming, the differentiation stem cell effects and so on. It's something that still needs to be worked out. And there hasn't been much done about mechanisms of resistance to the drug. At least my lab has published some data to suggest that the primary mechanisms of resistance, the primary resistance might be pharmacologic in that patients may have less drug uptake and then these tend not to be responsive. While secondary resistance in our patients appear to be independent of DNA methylation pathways and we suggest that these patients are acquiring mutations and activating some pathway that is no longer dependent on DNA methylation for survival of leukemic cells here.

Slide 200. Epigenetic Therapy – Unknowns (cont.)

Long term side effects are not known, but of course many of these patients would love to live long enough to find out what the long term side effects are here.

And one of the things that really needs to be done is how active are these drugs in solid tumors. And there is this common perception that they are not active in solid tumors and it's an entirely wrong perception. It is based on the fact that all the trials in solid tumors were done in patients who were refractory to other therapies. If you use this approach in liquid tumors, these drugs also are not effective. They are most effective in patients in the front line setting. These trials have just not been done in solid tumors. And in fact Johns Hopkins group, Stand Up to Cancer, has just reported that in lung cancer the occasional patient can have a spectacular response to the drug and we just need to figure out whether there's something special about these patients or whether this will be true for all patients if we treat them early enough here.

One of the things that remains unresolved also is azacitidine versus decitabine. Both drugs are dissimilar pharmacologically and there's reason to think that some patients might be sensitive to one and resistant to the other. There's certainly some data that if you switch from one drug to the other you can rescue some patients who were losing their response and it's something that remains to be determined here.

Slide 201. The Next Generation

Now where are we going? I will very quickly mention these things. I guess the first thing in oncology that people think about are combinations and there have been a number of combinations of azacitidine, decitabine and other drugs, including things that will be presented at ASH here. Some interesting on these drugs plus HDAC inhibitors. But so far the randomized studies have not showed increased survival for this approach. Some interesting data for azacitidine combined with lenalidomide. Again, single arm studies, increased toxicity. So this is something that remains to be determined.

Slide 202. Clofarabine and Low-Dose Cytarabine Alternating with Decitabine

One of the approaches that we have taken at M. D. Anderson was rather than combine the drugs, is sequentially administer these drugs in the hope of overcoming resistance with the idea that resistance to cytotoxic therapy and to epigenetic therapy is by different mechanisms, we designed this trial that was run by colleague Stephan ___ at M. D. Anderson, where we induced the patients with cytotoxics, in this case clofarabine for a couple of cycles, and then switched them to full therapeutic dose of decitabine for three cycles and continue this approach as long as the patients are doing well. This was applied to patients with very high risk MDS or elderly AML.

Slide 203. Survival in AML

And so far this approach has given us the best survival in elderly AML that we have seen in 20 or 30 years of research on the topic at M. D. Anderson. And I would bank on sequential therapy rather than combination therapy as a way to improve outcomes of patients here. Identify non-cross-resistant therapies and administer to these patients in sequential cycles, the way it has been done very nicely in breast cancer. This would minimize the side effects of adding drugs one on top of each other here. And potentially prolong remissions in those patients here.

Slide 204. The Next Generation

Now of course the other approaches are better drugs for similar targets and there are a number of trials right now for this. For example, there's a new analogue of decitabine which combines the cytosine analogue with a nitrogen in the 5 position and marries to a guanisine, so SGI10 is now in Phase I studies, about 50 patients treated. It appears to be promising with some responses already observed, so this is something to follow up on.

There have been some trials on oral azacitidine with a study recently reported by __ from M. D. Anderson, showing some promise for this approach. Whether it's better than subcu azacitidine remains to be known.

And there are of course all the newer HDAC inhibitors here.

What about new drugs for new targets? I have a couple of slides here, but because my time is running short I will very quickly just mention them, because this has really been the topic of some of the previous talks here.

Slide 205. Up and Coming Epigenetic Targets

I think that perhaps the two most promising targets that are going to get into clinical trials in the next year or two, hopefully, based on some continued early development, one of them is EZH2. As you heard very nicely from previous talk, there are activating mutations in lymphoma and over-expression is a bad prognosis. There are now drugs that, at least two compounds, that seem to effectively inhibit EZH2 and hopefully some of these will enter the clinic soon.

And this hasn't been discussed here, but DOT1L is an H3K79 histone methyltransferase that appears to be absolutely required for the survival of MML mutated leukemias. And preclinical data with inhibitors of DOT1L are very promising . And this may introduce a new class of epigenetic drugs in the treatment of a devastating disease really, the MLL rearranged leukemias here.

Slide 206. Epigenetic Silencing Mechanisms

So in summary, there are many epigenetic silencing mechanisms and many of them are being targeted. You've heard about a bunch of them . The data we have so far are on methylation inhibitors and

HDAC inhibitors, but I think it would be very easy to predict that in five years this talk would be very different with a lot more targets and a lot of other things in development here.

Slide 207. Summary

So I will just let you read my summary since I'm out of time here. And thank you very much for your attention.

Dr. Irv Bernstein:
We'll take a couple of questions.

Audience:
Question is, for chemotherapy combinations, you think that the sequential is a better approach, but do you have any insight or any thoughts about how to combine them with ___?

Dr. Jean-Pierre Issa:
Yes, so the combinations of these drugs with targeted therapy such as tyrosine kinase inhibitors are really interesting things to think about. One of the reasons that combinations of these drugs are problematic is that the epigenetic effects of these drugs are best seen in proliferating cells. If you shut down proliferation, these drugs will really not get incorporated and then they won't have the effect that you want to get. If you use high doses of the drug, then of course they become cytotoxics and in those cases you're just combining a cytotoxic with a TKI and things like that. So I think it is something that simply needs to be tested. If you have a TKI that very rapidly shuts down proliferation of the cells, then I don't think it makes sense to give a TKI with a DNA methylation inhibitor at the same time. And one could think of ways by which to sequentially use these drugs even in those settings. Now there could be some reason to think that giving the methylation inhibitors first might be good. There is some data that they sensitize to the action of chemotherapy, for example, and there might even be data they would sensitize to the action of targeted therapy, including TKIs, depending on the mechanisms of resistance to the TKIs. There is also some data to think that you could give those after TKIs. For example, if you look at the FLT3 inhibitors, there is this surge of the FLT3 ligand that seems to overcome the activity of the inhibitors and these drugs don't appear to influence FLT3 ligand, so that in that case it might be reasonable to sequentially use them. As you know, there has been this idea of using them sequentially after bone marrow transplant as well, and some of the results of that have been very promising so far. So one could make a rationale for both, giving them before or after, but I would personally stay away from giving them at the same time as tyrosine kinase inhibitors.

Audience:
Thank you for a wonderful talk. Do we really know how to use these drugs, the dose? Because the sense that we get, and it looks like the history tells that we started out with higher doses and slower and lower and the question is could we go much lower and longer because as you have suggested, the hypomethylating effect may be much better at lower dose. So what are your thoughts on that? And is anybody looking at that in trials?

Dr. Jean-Pierre Issa:
Yes. You're absolutely right. We don't know how low is low. However, you clearly can go too low because at some point you won't get any hypomethylation. So there is a trial that will be presented at ASH by I think ___, where he has tested much lower doses of decitabine in low risk MDS. And if you look at that trial, the results were interesting. There were really interesting effects with a lot of transfusion

independence, however, those patients did not achieve complete remissions. They were no longer transfusion-independent, but their hemoglobins didn't really normalize. And you know if you look at them by classical IWG criteria, they did not have CRs. While patients at the higher dose clearly have CRs. So I think that you can go too low. With azacitidine it's also not clear. There's been a trial that has compared five days to seven days. If anything the five days looks better with azacitidine than the seven days. So is it possible that going lower might improve things? It's certainly possible. However, keep in mind that the major problem really in the clinic is not so much the initial response, but resistance to these patients. And so I really think that there are a number of patients who show unequivocal clinical benefits, and then lose that benefit after a few months. So I think ultimately what we really need to do is see how we can prolong remissions and that will have more of an impact than simply changing a little bit the dose earlier. And it's possible that next generation drugs will have better pharmacodynamics and will overcome these dose effects and lead to better responses.

Audience:

Jean-Pierre, I'll ask a question not only to make sure Jay has more time to come in, but it was very interesting what you show about the difference between the cytotoxic versus the epigenetic response for these agents. And I'm wondering out loud, does that pattern persist for other classes of quote-unquote epigenetic agents, such as the histone deacetylase inhibitors and do you think that many of the trials that have been employing combinations of histone deacetylase inhibitors with cytotoxic agents, gearing towards what's a more potent cytotoxic effect, are actually not probing the epigenetic effects of the histone deacetylase inhibitors? For instance, the combinations of HDAC inhibitors with proteasome inhibitors in myeloma and other diseases.

Dr. Jean-Pierre Issa:

Right. So I will separate this question into two to prolong my response.

So there are two separate issues here. One of them is are HDAC inhibitors epigenetic drugs. And the second one is this idea of dose response applicable to other epigenetic drugs or other things.

The first one, you know, HDAC inhibitors clearly change the epigenome in terms of activating drugs. However, as I mentioned, first of all, they are not only histone deacetylase inhibitors, but they are protein deacetylase inhibitors, many other targets. It is very clear that almost all cells treated with these drugs will see a lowering of their apoptotic threshold. Now I don't think this is actually an epigenetic effect. It's probably some sort of signaling effect that results in the fact that these cells are more prone to apoptosis. And in fact, I don't know anything that is not synergistic with HDAC inhibitors. I think anything that has been tried so far, every single drug on the planet and radiation therapy, are synergistic with HDAC inhibitors. Again I think that's because HDAC inhibitors simply make cells more prone to death and therefore these drugs that kill essentially will kill better with HDAC inhibitors. So in that respect I think most of the combination trials done with HDAC inhibitors and chemotherapy haven't really been testing the epigenetic component, they're simply trying to put and shape the cytotoxic component.

In that setting it is actually reasonable to think that perhaps a higher dose of an HDAC inhibitor might be better. Because ultimately what you're trying to do is lower the apoptotic threshold as much as possible and this is something that hasn't been explored, but there is some reason to think that higher doses of HDAC inhibitors might be useful. And there's some data in Hodgkin's disease, for example, that higher dose leads to substantially better responses than lower doses. So in that setting with the HDAC inhibitors, we're talking about a different story.

Is this idea of a dual mechanism of action applicable to other drugs? Now that is, of course, true. In fact, one is hard pressed to find many drugs in oncology where there is clearly a dose response relationship. There is a dose response relationship to a certain level, but there are actually very few drugs where you just keep going and they keep responding more and more. There's almost always some degree

of saturation. And there might be some drugs where you actually lose the effect at high doses. And as oncologists we've simply ignored this, because we are trained based on 1950s ___ hypotheses, which are almost certainly not real in the real world. And we've all trained to think that higher is better in many circumstances, so that we've actually very rarely tested the idea that higher is better. And it may well be that for many classes of drugs, higher is not better. And so this concept probably will be true in other instances as well. And there's some data, for example, there's some data that topotecan and some of these drugs have activity in MDS. And in those cases, it's not clear that higher is better. There's some early data that perhaps lower doses might be better and maybe at lower doses, they're being used as differentiation agents rather than as cytotoxics. Again, something that needs to be done a little more carefully.

Audience:

I'd like to ask about these mutations. They might be some familial – for example, Hodgkin's disease, in some Hodgkin's disease, for example, ___.

Dr. Jean-Pierre Issa:

Yes, Hodgkin's disease and lymphomas in general have a lot of somatic mutations in epigenetic drugs. And this is true of leukemias as you just heard from Ross's talk. I don't think this has been described very clearly for familial cases yet. But I will tell you that the way the studies have been done so far, the genome-wide studies that have looked for mutations across the genome, have largely ignored familial mutations. So Ross would know much more about this than I would, but the TCGA, for example, which has described a lot of these mutations, is really paying most attention to somatic mutations. They're really looking for things that are not present in normal tissue. And the way these studies are done, you would completely miss a familial mutation because it would be present in both normal tissue and the disease. So I don't think it has been ruled out, that some cases of familial cancers are true to mutations in epigenetic regulators. I think it just hasn't been looked at extensively enough because of the way the genome-wide studies have been done so far.

Dr. Irv Bernstein:

So you identified genes with aberrant methylation and it may contribute to the leukemic process. Do you think this therapeutic approach is having on-target effects with respect to those genes or off-target effects, either making this synthetic lethal in a sense or disposing towards increased susceptibility to chemotherapy?

Dr. Jean-Pierre Issa:

Well, this therapy is very targeted in that the only known target of azacitidine, decitabine, doses, is really trapping methyltransferases. But trapping methyltransferases is very non-specific because as you would imagine, there would be effects throughout the genome. And in fact we have no reason to think that tumor suppressors are preferentially targeted by these drugs. So there is the potential for these drugs to be doing many, many things, not just affecting tumor suppressor genes. Now the data that Steve showed you on the low dose effect, affecting stem cell self-renewal, would suggest that these tumor suppressor genes are a target, but of course, how to relate this to what happens in the clinic is very difficult.

Dr. Irv Bernstein:

Great. Let's grab a few questions.

Audience:

Great talk. I was curious, one thing struck me in the solid tumor work that you mentioned so far, that it was 10%. Myc is so broadly up-regulated, so many, does that mean that many other things other

than BRD4 are subset of Myc target? How do you see that?

Dr. James Bradner:

I think that, hopefully you heard the question, it really is one of the new areas of discovery in our group, is to understand, number one, why a subset of cancers that all depend on Myc are sensitive to BRD4, not all of them. And secondarily, how do these cancers become resistant to this molecule, we're not curing them. And these things are linked. When we do genome-wide __ for BRD4, we identify it at enhancer sites. And so really BRD4 is an enhancer cofactor as we think about it, and in myeloma, sustaining Myc transcription by the positive reinforcement of, in this case, in this cell immunoglobulin heavy chain enhancer region, is then very BRD4 dependent. Where in other types of cancers, where Myc is an end-factor of the EGFR signaling pathway or the like, it is perhaps then less essential that these enhancer, dominant enhancers, are driving BRD4. This is our thinking. But that's ROI aim level speculation. What we can say is that the other flavors of Myc, like N-Myc, are also exquisitely sensitive to JQ1. So there is some sense of coactivator relationship. But I'd be the first to say that one of the take-home messages of all of the recipient labs of JQ1 that could care less about Myc in many cases is that other master regulatory transcription factors are also co-oped in BRD4, which makes this molecule block a adipogenesis, makes it block spermatogenesis. And so these tissues-specifying or phenotype-defining master regulatory proteins, if their transcription factor is a function through enhancers, BRD4 may well be important. But it is not a direct Myc inhibitor, so consequently other Myc-dependent cancers do not respond.

Dr. Irv Bernstein:

I think this has been great. Thanks again to all the speakers.

END