Jacques Loeb Centre
for the History and Philosophy
of the Life Sciences
Prof. Ute Deichmann, Director

Ben-Gurion University of the Negev
P.O. Box 653, Beer-Sheva, 8410501, Israel
Building 39, Room -114 Phone: +972-8-6472258
Email: jloebcentre@post.bgu.ac.il Web: in.bgu.ac.il/en/loeb

Interview by Ute Deichmann with Howard Cedar, Hebrew University of Jerusalem, School of Medicine

First Interview - 19 June 2014

From insect physiology to the study of the genetics of pattern formation

From DNA to DNA methylation

UD: You received your B.A. at MIT and an M.A.and PhD at New York University. What did you study?

HC: At MIT I studied mathematics. Then I went to NYU - I arrived there in 1964. At that time, the United States had just started a new program to give fellowships to medical students to do a PhD. There are thousands of people that have gone through the program, but I was the first one to finish that program - the MD-PhD program. Igot to NYU and was studying medicine. The truth is that the reason why I went into the PhD program is because I felt sorry for my parents that they had to spend so much money on my education, so the fellowship was a good idea. But I quickly got very, very involved in the science, indicating more of a scientist and less of a doctor.

UD: On the homepage of the IMRIC you wrote that you began to start thinking about genetics and the genetic ‘book' when you entered NYU in 1964. What or who pointed you in the direction of DNA?

HC: It was Gary [Felsenfeld]. When I finished medical school and a PhD in 1970, I did an internship in pathology. It was 1970.The United States was still involved in Vietnam and everybody who had studied medicine had originally – when they studied medicine – to get an exemption from the army in order to study. And when we finished, the army wanted us. So 70% of my graduating class went to Vietnam and the Far East. I was

lucky because I got a position in the United States Public Health Service. They had a few positions at the NIH and I was lucky enough to get one. I went to Gary for two years.Before that I had done biochemistry and microbiology. But Gary introduced me to the world of DNA. And it was then that we started thinking about it.

When I was there, there was another post-doc who was in a similar situation - Richard Axel. Richard went on to get the Nobel Prize in medicine for his work on smell. We worked together and took upon ourselves - there were very few labs that worked with chromatin, and Gary had done a big service to the field by developing ways to use solubilized chromatin. So it wasn't just an aggregate. Everybody else had the isolated chromatin that was an aggregate. It was very difficult to do biochemistry and molecular biology on that. But Gary developed a way of solubilizing it. And Richard and I decided to see whether it is chromatin in general that restricts the use of genes.

The first part of this I did by myself, with Gary, of course. I just took chromatin and added RNA polymerase from bacteria. It showed that when you do that on a chromatin template, you get a lot less - a hundred-fold less - initiation of RNA polymerase. That suggested that chromatin sits on the DNA and blocks a lot of places so that only a certain number of small places on the DNA can serve as sites for RNA polymerase. It was a very crude experiment, but that was the first step. For the second step, Richard and I synthesized RNA on a chromatin template and, using hybridization, which was a new technique then, we asked what RNA is made. We showed, I think for the first time, that when you do that - if you take naked DNA, that RNA polymerase can make RNA all over the DNA. But if you take chromatin, the RNA that is made is enriched for globin. This suggested that RNA polymerase on the chromatin template is specifically binding to regions of globin, because those regions are open and accessible. In my opinion this was a really big step, and it was confirmed by the work of Howard Weintraub. He was not far away from us - we were in Washington, in Bethesda, and he was at Princeton. What he did was, instead of using

RNA polymerase as a probe, he used DNAse as a probe and showed basically the same thing.

UD: How was this work informed or influenced by the work of Allfrey and Mirsky?

HC: Allfrey was misunderstood. The problem was, he had no clear vision. He had described histone modification, but it wasn't clear from his studies what the role of it was, and whether it affects gene expression. It was just a finding with no real biology behind it. I feel sorry for him, because he made a big discovery, but - not that he lost his way, he just wasn't enough of a biologist.

UD: But Mirsky was.

HC: But they didn't figure it out. I must say that until today - did you know that histone acetylation and associating gene activity - there has been no proof that histoneacetylation affects transcription. Histone methylation is a little bit better understood because methylation at several of the sites seems to form heterochromatin. But even there, there has been no real proof as you see with DNA methylation.We did a really concrete experiment that left no doubt that DNA methylation can cause gene repression. And an experiment like that, in my opinion, has still not been done either for histone acetylation or for histone methylation.

Gary was really one of the first physical chemists in this field. There were others - von Hippel also was a physical chemist - and they made a big contribution. Gary had developed this way of solubilizing chromatin. And as a physical chemist he wanted to learn something about how chromatin looked like now that it was solubilized. The tools that we use today were not available. All he had was an ultracentrifuge. And he threw the chromatin into the ultracentrifuge.The ultracentrifuge is a wonderful instrument, but all it gives you is average weight, average sedimentation rate, average size, average viscosity. And so you get all these average things, and you can write it down, but it doesn't tell you anything.

UD: What made you depart from histones? Gary worked with histones in the end, and you started DNA methylation. How did this come about?

HC: I was with Gary for two years and then I came here - I moved to Israel with my family. I continued to do the same kind of work that I had been doing with Gary. And here I met somebody down the hall named AharonRazin, who was working on DNA methylation in bacteria. DNA methylation in bacteria is used as a restriction modification system. Every bacterium can methylate its DNA at a specific site, and it can cut the same DNA at the same site. These are restriction enzymes. Every bacterium has a different system of methylating a site and being able to cut that site. But when it's methylated, it can't cut. So the bacterium itself - of course it can methylate those sites - is protected from its own restriction enzyme. But it serves as a method of protection. Because if the bacterium is attacked by another bacterium or phage, theymay be methylated, but methylated at different sites.So when the phage goes into the bacterium, it gets cut by the restriction enzyme. That is the role of methylation in bacteria.

Now Aharon was working on methylation in a phage. They didn't really know what the role of it was. And through that, we started to develop the idea that maybe methylation might be involved in regulation in animal cells. It was already known that cytosine is methylated in the animal cell. But nothing else was known about it. We thought that maybe this has something to do with gene regulation. In 1977 I went on sabbatical and went to Richard Axel's lab. Richard has just developed the technique of transfection, which allowed you to put naked DNA into animal cells. It was there that I came up with the idea that this is the perfect system for studying methylation. And as soon as I came back from sabbatical in '78, we started working on it. At the beginning we hoped that it had something to do with regulation. There were reports from others, in particular Adrian Bird and Frank Grossveld that there was a correlation between the lack of methylation and gene activity.

So I came back here and we did what I think of as key experiments. First of all, we took naked DNA and methylated it artificially in-vitro. We

used these enzymes from bacteria and methylated DNA. So we could take a gene, unmethylated, and in-vitro methylate it. Then we transfected those into animal cells in culture.The first thing we asked was, "what happens to the methylation when you put it into the cell?" Because when you do transfection, it goes into one cell, gets incorporated in the DNA, and then the cells divide. And they keep making more copies – when the cells divide they are replicating this DNA. What happens to the methylation that you put on? And we showed that if you put in unmethylated DNA it remains unmethylated – even after 30, 40, 50, 60 generations. If you methylate it first in-vitro and put it in, it remains methylated. That meant that the animal cells have the ability to maintain the methylation pattern. That was the first time that we understood, for instance, that the normal methylation in the cell is not just haphazard; it's not just a biochemical thing that methyl groups go on and methyl groups come off. But that there is a pattern of methylation and that pattern is maintained from generation to generation.

Then we used the same exact system; we took an unmethylated gene and a methylated gene, and we put them into cells in culture. Now we knew that if you grew these cells in culture what you put in unmethylated remains unmethylated and what you put in methylated remains methylated. So we could ask, "what about its activity?" And what we found was that if you put in unmethylated DNA, you get transcription; if you put in methylated DNA, transcription is inhibited. This was the first proof that methylation is not only correlated with repression, but actually can cause repression. Because in this experiment, we did something in-vitro - artificially - we put on methyl groups. It's not something that happened in the cell- we did it. There was no question, then, that methylation causes repression.

DNA methylation and development (1)

UD: Which brings me to the question, what regulates the methylases?

HC: That's a complicated question. And for that, you have to understand development.

Some of these things we have done, and some of these things other people have done. In the early embryo, the very first step, the DNA that comes from the egg and the DNA that comes from the sperm have their own methylation patterns. When that first cell divides, it copies the methylation pattern. But then, there is a process of de-methylation. And basically, the early embryo, up until about the 64-cell (blastocyst) stage undergoes massive de-methylation. At the blastocyst stage there is massive undermethylation. Now, you can look at the whole genome and see what's methylated and what's not methylated. We know it's very low - something like 10% or 20% of what it would be in a somatic cell. Very low. And almost everything gets erased.

Then that embryo, in the next step, implants into the UDrus. And at that stage there is an increase in the de novo methylases. And basically the whole genome gets de novo methylated. But there are sequences, called CpGislands, that are protected. The reason they are protected is cis-acting sequences - sequences that protect them. So it's basically indiscriminate methylation, but certain regions are protected.

Then, after implantation, the next stage is called gastrulation. That starts the beginning of forming the germ layers of the embryo, and then the tissues of the embryo. After the genome has been methylated, the de novo methylases (there are two of them) get down-regulated, so that in future cell generations de novo methylases are very, very low. But the pattern of methylation that was generated is maintained by the maintenance methylase, called DNMT1.

Everything is methylated except for the islands.

UD: So this is an unspecific process.

HC: It's unspecific in the sense that everything can get methylated, but specific in the sense that islands are protected.

UD: But then they get removed depending on the cell type they are going to develop -

HC: Not yet! We're going to get to that.

That basic pattern - there are 30 million methylations in a cell - almost all of those (99.99% of those) maintain the original pattern that was generated at the time of implantation. And the changes that you're talking about are minor events - they're important events but they're minor, in terms of numbers.

UD: But the other ones are specific?

HC: Correct. So up until now we had a general methylation - like you said, non-specific - and that pattern is maintained in every cell division by the maintenance methylase.Its work is based on symmetry - chemical symmetry. Typical DNA looks like this - some sites are methylated and some sites are unmethylated. It's always at CG. A site that is methylated is always methylated on both strands of the DNA because if you read CG from this direction, then this other strand you read in the other direction - there's a CG here and a CG there - and they're both methylated. And this one isunmethylated. When you replicate, this strand gets copied, and that generates a hemimethylated site, and the maintenance methylase recognizes these sites. It can't de novo methylate, so it can't methylate this. It can only methylate sites where one strand is alreay methylated. And as a result, you reproduce the original pattern. Of course, the other strand does the same thing.

So there's no longer de novo methylaseany more. Most somatic cells have very, very low levels of de novo methylase. But the pattern that was generated at the time of implantation remains throughout our lifetime.

UD: But this pattern is unspecific.

HC: Unspecific in a sense. There is a functional package specificity that is built in. I always tell this to my students - using one of these high throughput methods to look at methylation, what you see here is a

whole list of places in the DNA. And you look at their methylation state in different tissues - brain, liver, muscle, colon- yellow means methylated. They're all methylated everywhere. The islands, which were protected, are unmethylated - and they remain unmethylated - every island. Now this assay is done in a 70 year old man like me. And this pattern that you see, which is the major pattern of the cell, was generated 70 years ago. It is maintained all the time. So there was a developmental decision to methylate at the time of implantation, and that decision was a one-time thing but it's then preserved or maintained by methylation. That's the role of methylation. It maintains previous decisions.

Basically, the whole genome looks like this, except for these islands. All these places in the genome have sites that could be expressed but they're not because they are methylated. A lot of these, for instance, are endogenous viruses. One of the ways that the organism - the animal organism - prevents activity of these viruses is DNA methylation at the beginning of development. So they remain methylated and inactive.

UD: And that's why the DNA can stay there.

HC: That's right. Exactly.On the other hand, many of the islands are promoters for housekeeping genes. So when the decision is made at implantation to methylate these and leave these unmethylated, it basically sets up the genome so that housekeeping genes are unmethylated always. That is why I said that the decision to methylate everything indiscriminately except for islands has some sort of functional specificity. Because it turns off or closes up everything you don't want but leaves open the things that you do want.

UD: How come the cells develop in a specific way?

HC: First of all I want to point out some things because these are things that are not understood by most people including scientists who work on methylation. I'm going to go back a step and point out that methylation patterns are not inherited from our parents, because

everything is erased. The first step after implantation is turning off pluripotency. At the time of implantation, cells are pluripotent – each cell can develop into anything. You have to turn that off if you want to make a differentiated organism. There are a number of genes that are involved in pluripotency, such as Oct-4 and Nanog and Sox, and you have to turn them off. How are they turned off?

First, the genes are turned off by a repressor – a simple protein repressor that recognizes the gene sequence. This is specific, – no more RNA is produced. At this point, these genes are still unmethylated and have an open chromatin structure.

The second step in this process is to close the chromatin. That is carried out by recruiting to the same site proteins and enzymes that can close the chromatin. These include enzymes that take off acetylation – deacetylates it, and enzymes that methylate histones.

The third step is that the same chromatin system also recruits the de novo methylase of DNA. And then these genes, pluripotency genes, undergo DNA methylation. What's important here is that the DNA methylation is not what's turning off these genes; they get turned off by a repressor. The DNA methylation comes after that; it doesn't turn off the gene because methylation is not an active repressor. But it is very important, because until those genes become DNA methylated, the repressor that inactivates them is reversible. You can take away the repressor. If you now just push those cells a little backwards, they'll go back to being pluripotent. But as soon as the pluripotency genes undergo DNA methylation the repression becomes irreversible; you can't turn them on again.

UD: How do those de novo methylases know where to go?

HC: They get there because the pluripotency gene is recognized by a factor, just like in bacteria when the repressor finds its gene and recognizes those genes. And in animal cells, those repressors are capable of recruiting all sorts of additional things – recruiting the machinery for changing chromatin, recruiting the machinery for

changing DNA methylation. So it is specific because there's a factor, a repressor, that recognizes those genes. And all the changes in methylation from the time of implantation on are specific changes and they all have to be directed by factors.

So now we've turned off the pluripotency genes, and the process of making layers and tissues starts. That is all directed by proteins that are called morphogens. They start making, say, a liver. So in the process of making the liver, there is a master gene that turns on a whole bunch of liver-specific genes. Again, that is because the sequences near those genes are recognized by the master gene. And only after the gene gets turned on does it undergo demethylation.

UD: Does it mean that the repression is removed before the methylation is taken away?

HC: That's right. Probably it's not at its full activity, but basically in almost every case, you first turn on transcription and then you get demethylation. The change in methylation is not meant to turn on the gene; it's meant to make the decision to turn on the gene permanently.

So from the point of view of the tissue, the master gene and its sub-master genes are trying to turn on all these liver-specific genes. And it's hard, because those genes start off in an off conformation. They have chromatin on them, they may be DNA methylated – it's hard. But these factors work and start turning it on. And as soon as these transcription factors touch down on the gene, they are also capable of bringing the machinery to open the chromatin and do demethylation. As soon as they succeed in doing that, the tissue sort of goes on automatic, because now it doesn't have to open these genes - the transcription factors have a much easier time now – because it's open and it's going to stay open. Most people misunderstand the role of methylation.

So all these specific changes have to be targeted by something that recognizes the DNA sequence. Again, in the big picture, thesechanges are minor. But, of course for those tissues, they are very important.

DNA methylation and environment

UD: In an article on the home page of the InstitUD for Medical Research Israel-Canada, Tim Spector from King's College relates your research to early 19th-century Lamarckism. I completely disagree with what he writes about Lamarck, Darwin and Kammerer because Lamarck didn't invent soft inheritance, Darwin was a Lamarckian, and Kammerer's experiment could never be repeated. So after everything you have said now, it seems to me that your research does not support soft inheritance.

HC: I'm going to tell you, in my terms, what I think. You're right – this has been – in fact, this is what people call epigenetics. So the concept is that – true, Darwin was correct that the environment doesn't change the DNA sequence. But the proponents of epigenetics say, "That's true, but the environment can change methylation, which affects expression." That's the general concept. So, first of all, we don't know if the environment affects DNA methylation or how it affects it. In general, we just don't know. But there are lots of problems with this idea. The biggest problem is the one of inheritance. The fact that methylation patterns are erased in the early embryo makes it very difficult to explain how an environmental effect could then be inherited to later generations.

UD: And be adaptive.

HC: And then it would also have to be in the germ cells. So in principle, there's a problematic element here. Forget about the inheritance and just ask about the possibility that environment affects us in a stable way through methylation. Let's just take that part of the story. If I can paraphrase it in terms of molecular biology, we know that the body is very flexible. The system, the molecular biology is very flexible. If cells are exposed to something; they are capable of changing their expression patterns. We know how these things work. There are substances in the environment, they impact on the cells, the cells now

down-regulate this or up-regulate this, or you get phosphorylation of something, and that affects the activity of genes. But if you take away that environmental factor, the changes that the factor makes are going to go away. Because all of those changes are based on protein-DNA interactions, which are reversible. A repressor binds to the DNA and represses – you take away the repressor and it doesn't repress any more. So a person is exposed, at the age of 12, to something in the environment; it changes the way his cells are working – the expression pattern in his cells. As soon as that environmental factor goes away, it will go back to normal again. Unless-

UD: Unless it's a mutation.

HC: But that's unlikely, because then it would occur in one cell. It might cause cancer, but it does not make a permanent change. Unless, as a result of the environmental stimulus, you also change its methylation. If you change its methylation pattern, you can now immortalize what that environmental factor did.

I'll give you an example. This is true in any animal that's ever been tested – any mammal that's been tested. If the female develops diabetes during pregnancy, the offspring come out normal, but 40 years later, 80% of them develop diabetes. This is true in rats, mice, dogs, cats, and humans. So here was an environmental change, but something remained. In other words, this child who came out normal has some sort of molecular memory that makes it get diabetes 40 years later.

UD: In spite of the erasure?

HC: There’s no erasure here, because they’re embryos, like in the 7th or 8th month of pregnancy. So perhaps that’s because of methylation. Nobody knows, by the way. That would be an example of environmental epigenetics.

UD: Which, in that case, is not adaptive.

HC: It's adaptive in the sense that these offspring have adapted to something that happened to them in early life as an embryo.

UD: Right, but does not make them better adapted to their environment.

HC: No, this had exactly the opposite effect.

So now we're in the area of speculation, but I think it is good speculation. I think that if the environment can do this - can affect methylation, it has to be targeted. In other words, it can't be random. This is for two reasons.

The major reason is that if the environment causes random changes in methylation - more methylation or less methylation - then it wouldn't be uniform. Every cell, and every gene in every cell would be going through something different. You can't get an effect out of that. The other argument is that to make a change in methylation you need machinery. You need either a de novo methylase or a demethylase. All of the changes that occur during development are targeted. So it doesn't make sense that in the case of the environment it would be random changes in methylation.

I will not publicize this before it is published.

It's getting complicated, but now I'm going to take this a step further. I do think that the environment can affect methylation. And we do have evidence for this as well; I'm not just saying this. I'll give you an example of something that we've done, but you can't really write about this because we haven't published it yet. But once you hear it, you'll realize that it's probably correct. We did the following thing: we said that it's very hard to study how the environment affects methylation, because it's hard to put a mouse into a constant environment. And then you have the problem that mice behave differently. So you could maybe expose them to something in the environment, but you can't make them behave in exactly the same way. So we did a simpler experiment. We said, "Forget the external environment, let's look at the internal environment. What's running through the mouse's blood?" We asked

what the differences were between a male and a female mouse. Genetically, they're basically the same, if you use laboratory mice. But in a male mouse, most of its life it has testosterone, and in the female mouse there's no testosterone or low levels of testosterone. But she has estrogen. And so we took the liver and said, "Are there any differences between the male and the female?" And there are plenty of differences. And they are caused by testosterone. Testosterone in the male is only secreted after three weeks of life. Then these genes start undergoing demethylation.

UD: Genes in the liver?

HC: Yes, in the liver. In the male; it doesn't happen in the female. And this is, just like you said, it's adaptive. In other words, the male mouse gets used to, it's adapted, to being exposed all the time to testosterone. Because at the beginning of its life, when the testosterone first starts, these genes are off and methylated. And during a slow process they get demethylated and are easily on. They've adapted themselves to being male. And, in fact, you can take a female mouse and give her testosterone and she will do the same thing. You can fool her. All these are specific genes that undergo demethylation because they're targeted.

UD: That means the process is controlled by genes?

HC: Exactly. So this whole process of adaptation is built in to begin with.

The rise of 'epigenetics'

UD: One last question - a sociological one. I did a citation analysis to see the development of research in "epigenetics" or "epigenetic" It shows a strong increase of "epigenetic" in the title of articles from around 2000,but not before. Though epigenetic research started much earlier -

HC: I'll tell you why that happened. For the last couple of years there has been a lot of emphasis on high throughput analyses. Many geneticists

have said, “Let’s take a disease (it’s not important what this disease is - it could be type-2 diabetes, it could be heart disease, it could be schizophrenia - what’s important is that we don’t know what causes it) and take thousands of people and analyze their genomes and see if we can find something that’s common between all the people who have this particular heart disease. And then we’ll know what genes are involved in these diseases.” So if you do this, you get very disappointed. You take a disease like type-2 diabetes - it’s a plague today in Western countries - which you know is genetic - you know that your chances of having type-2 diabetes are five or tenfold higher if one of your parents had type-2 diabetes. So you do these analyses and you don’t find anything. You can find a few genes here and there but the correlation is not good and there are a lot of them. So people got discouraged. And I always joked, when I was a medical student, every time the professor didn’t know what caused a disease, he said, “it must be a virus.” Today, if you don’t know the cause, you say it’s epigenetic - that’s the explanation!

Now, with the more higher-resolution gene analyses, some of these diseases are beginning to pick up genes the way they should be. So it’s very unlikely that they’re so-called “epigenetic”. Although it might be a part of it.Type-2 diabetes is a good candidate for epigenetic changes because there’s definitely a genetic basis, but you might need something more than that. And it might need something that’s more stable, not transient.

UD: The papers I have read are so contradictory. In some of them epigenetics accounts for all basic biological or medical phenomena, whereas others say, this cannot be true, because they neglect basic facts of gene regulation, for example papers by Mark Ptashne.

HC: The problem with Mark Ptashne, by the way, is that he’s right! But he’s so anti everything except transcription factors that it’s very hard to take him seriously. He doesn’t believe in chromatin, he doesn’t believe in DNA methylation at all.